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Serotonergic System and Gait: Dorsal Raphe Nucleus as a Control System for Gait

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Neuroscience

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«SEROTONERGIC SYSTEM AND GAIT: DORSAL RAPHE NUCLEUS AS A
CONTROL SYSTEM FOR GAIT »

by

Nahal Farhani

Graduate Program in Neuroscience

A thesis submitted in partial fulfillment
of the requirements for the degree of
« Masters of Science »

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

In advanced stages of Parkinson Disease (PD), gait and postural abnormalities emerge. These symptoms are not prominent at early stages of PD despite significant dopaminergic neuronal loss. Gait abnormalities are largely not responsive to levodopa. Therefore, other types of neurons might be responsible for gait abnormalities of the PD.

Since the reticulospinal tract (RET) is mainly implicated in the control of axial muscles, the degeneration of this pathway or populations of neurons controlling this pathway might be responsible for axial symptoms. However, there is limited data about the neurons controlling the RET. Our aim in this study is to delineate these pathways.

We found that serotonergic projections from dorsal raphe nucleus (DRN) exert an important control on the RET. Inhibition of the DRN resulted in severe episodes of gait freezing in rats. Activation of 5HT_{1A} receptors in the gigantocellular neurons changes muscle tone in rats during gait. The DRN also sends projections to the mesencephalic locomotor region, which are implicated in controlling dynamic gait parameters. DRN neurons receive nigral dopaminergic projections and post-mortem studies in PD patients have identified significant loss of DRN neurons; therefore DRN neurons may play a role in the pathophysiology of gait abnormalities of PD.

Keywords

Gait and Posture, Serotonergic System, Dorsal Raphe Nucleus, Reticulospinal Tract, Mesencephalic Locomotor Region.

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Abbreviations

GiN	gigantocellular nucleus
DRN	dorsal raphe nucleus
MLR	mesencephalic locomotor region
CPGs	Central Pattern Generators
CTB	Cholera Toxin Beta Subunit
PPN	pedunculopontine nucleus
RET	reticulospinal tract
PD	Parkinson disease
5-HT	5-Hydroxytryptamine

Chapter 1

« General Introduction »

Parkinson disease (PD) is the second most common neurodegenerative disease after Alzheimer disease, affecting approximately seven million people worldwide. During the early stages of PD, more than 80% of dopaminergic neurons in the substantia nigra pars compacta are lost. This loss of dopamine leads to an array of motor symptoms, including tremor, rigidity, and bradykinesia (Berg et al., 2015; Racette & Willis, 2015).

In advanced stages of PD, gait dysfunction, balance abnormalities, and impaired control of complex movements emerge. These symptoms are not prominent in the early stages of PD despite significant dopaminergic neuronal loss. Although levodopa is the main therapeutic treatment to treat PD motor symptoms, gait abnormalities are largely not responsive to this drug. Therefore, the postural and gait abnormalities of PD are not believed to be isolated to dopaminergic cell loss (Sarter, Albin, Kucinski, & Lustig, 2014; Virmani, Moskowitz, Vonsattel, & Fahn, 2015; Vorovenci, Biundo, & Antonini, 2015).

The reticulospinal tract (RET) is a well-known pathway implicated in locomotion, postural adjustments, and reaching. This pathway regulates gross motor functions by controlling motoneurons and central pattern generators (CPGs) of proximal and axial muscles (Baker, 2011b; Schepens & Drew, 2004). Therefore, degeneration of this pathway or the population of the neurons controlling this pathway might be responsible for axial symptoms in advanced stages of PD.

Neuroanatomical studies have shown that the mesencephalic locomotor region (MLR) and specifically the pedunculopontine nucleus (PPN) send projections down to the gigantocellular nucleus (GiN), where the RET originates in the medulla (Skinner & Garcia-Rill, 1984; Smetana, Juvina, Dubuc, & Alford, 2010); However, no study has comprehensively investigated the connections between the PPN, other brainstem nuclei and the CPGs and motoneurons of the spinal cord. In addition, several studies have suggested that other populations of neurons including serotonergic neurons in the dorsal

raphe nucleus (DRN) have a significant role in controlling motoneurons and locomotor CPGs (Barreiro-Iglesias, Villar-Cerviño, Anadón, & Rodicio, 2008; Courtine et al., 2009; Ghosh & Pearse, 2015).

In the next section, the role of CPGs, the serotonergic system and DRN, MLR, and GiN in gait will be reviewed in detail.

1.1 « Central Pattern Generators »

There are two main states in all species of animals: 1) quiescence or sleep state and 2) active or awake state. During each of these states, there are specific groups of neurons that control basic repetitive behaviors such as respiration, swallowing, mastication, locomotion, escape, as well as other rhythmic behaviors essential to the animals' survival (Gillette & Brown, 2015)

To generate rhythmic motor activities, precise spatiotemporal activation and inhibition of neurons controlling these behaviors are needed. Such synchronization is the first important property of this system. This type of spatiotemporal synchronization is found mostly between neuronal networks in more primitive areas of the CNS including, the spinal cord and brainstem (S. T. Alford & Alpert, 2014; Cherniak, Etlin, Strauss, Anglister, & Lev-Tov, 2014b). Due to their intrinsic property to produce rhythmic activity, these neuronal networks are known as central pattern generators (CPGs)(Buchanan & Cohen, 1982).

CPGs for locomotion are one of the most established circuits of the human body. They are located in the ventral horn of the lumbosacral spinal cord and have a very high level of automaticity (Grillner, 1985; Kiehn, 2006).

In a successful gait cycle, while the muscles of one side of a body segment are contracted, the muscles of the other side are relaxed. Glutamatergic excitatory neurons of the ventral horn of the spinal cord play an important role in producing this pattern (Buchanan & Cohen, 1982; Kiehn, 2006). These glutamatergic neurons innervate alpha motoneurons bilaterally and inhibitory interneurons of the ipsilateral side, which in turn send axons to the opposite side of the spinal cord and innervate the contralateral alpha motor neurons. As a result, when glutamatergic interneurons become activated, they excite both the motor neurons and ipsilateral inhibitory interneurons, innervating the motor neurons of the opposite site. This leads to the excitation of the ipsilateral motoneurons, and inhibition of contralateral motoneurons (S. T. Alford & Alpert, 2014; S. Alford & Williams, 1989).

Automaticity is another essential property of the neurons in CPGs circuits (S. T. Alford & Alpert, 2014). Studies have shown that the presence of a gradient for the level of automaticity between upper and lower lumbar areas. This indicates that upper areas of the lumbar spinal cord reproduce more automaticity compared to that of the lower areas (Berkowitz, 2004).

In a recent study, optogenetic methods were employed in mice to specifically stimulate glutamatergic neurons in CPGs. The results indicated that stimulation of these neurons alone is enough to initiate and maintain perfect gait cycles in mice. In fact, these networks of neurons are able to keep their rhythmic activity without any input from the brainstem or sensory feedback from supra-spinal areas in CNS (Hägglund, Borgius, Dougherty, & Kiehn, 2010). Constant exposure of these neurons to an NMDA receptor agonist does not change their rhythmic activity. This phenomenon can be explained by numerous inhibitory interneurons inside the network (Wallén & Grillner, 1987). In addition, NMDA receptors on excitatory neurons in CPGs are Ca^{2+} dependent K^{+} receptors (Buchanan & Cohen, 1982). This means that a single neuron becomes activated when glutamate binds to its receptor, but a calcium influx will eventually cause opening of potassium channels, resulting in hyperpolarization of the cell (Hill, Matsushima, Schotland, & Grillner, 1992). Therefore, even when neurons are exposed to Tetrodotoxin (TTX), which prevents the synaptic communication between neurons, NMDA receptors

are able to induce rhythmic activity in excitatory neurons of CPGs (Wallén & Grillner, 1987). These findings suggest that automaticity is an intrinsic characteristic of CPG neurons and is not dependent on inputs from supra-spinal areas of the CNS (Buchanan & Cohen, 1982; Buchanan & Grillner, 1987; Wallén & Grillner, 1987). An interesting fact about the gait CPGs is that gait pattern can be changed without altering speed. This can be explained by the existence of different types of neurons inside the CPGs, which each control a different aspect of gait (Wilson et al., 2005).

The central nervous system receives many extrinsic and intrinsic sensorimotor stimuli continuously, but the brain's resources for responding to these stimuli are very limited. To overcome this problem, highly specialized structures, such as the basal ganglia and many other neuronal networks in the brainstem have developed during evolution (Stephenson-Jones, Samuelsson, Ericsson, Robertson, & Grillner, 2011). These structures are responsible for identifying the salient stimuli and providing an appropriate motor response through activation of relevant CPGs (Redgrave, Prescott, & Gurney, 1999; Stephenson-Jones et al., 2011).

In primitive animals such as invertebrates, the amount of incoming sensory information and number of motor responses are very limited. As a consequence, the networks controlling CPGs in these animals are significantly simpler compared to those of complex animals including mammals (Cherniak, Etlin, Strauss, Anglister, & Lev-Tov, 2014a).

Moreover, the upper brain regions regulating CPGs are also able to control the different aspects of gait. For instance, studies on lampreys suggest that the MLR is able to initiate locomotion by stimulating locomotion CPGs in the ventral lumbar spinal cord. The MLR is also able to control the speed of locomotion and muscle tone by sending commands to locomotion CPGs (Smetana et al., 2010).

Gait is a very complex behavior in mammals and as a result, many neurotransmitters and brain areas may control CPGs and motoneurons. To understand the gait mechanism, learning about these neurotransmitters and supra-spinal centers is essential. Here we will discuss the role of the most important neurotransmitters and anatomical areas involved in controlling different aspects of CPGs' function.

1.2 « Raphe Nuclei and Serotonergic System»

Many studies suggest that serotonergic neurons play an essential role in controlling CPGs in both invertebrates and vertebrates (Ghosh & Pearse, 2015; Veasey, Fornal, Metzler, & Jacobs, 1995b). The mollusk is an invertebrate that diverged about 600 million years ago; but their serotonergic system retains many similarities with vertebrates (Gillette, 2006). 5-Hydroxytryptamine (5-HT) neurons in these invertebrates are located in three main motor CPGs, including the feeding, escape, and locomotion networks. The main role of the serotonergic system in these three networks is the excitation of neurons, which leads to activation of the CPGs. The serotonergic system is also important in controlling different aspects of CPGs function such as, the footfall pattern in rodents (Gillette, 2006).

In general, the serotonergic system in both invertebrates and vertebrates facilitates the spinal reflexes (Kiehn, 2006), but there are some key anatomical and physiological differences across species.

One of the main differences in the serotonergic system of invertebrates and vertebrates is the location of the neurons. As mentioned above, in invertebrates, serotonergic neurons are located next to the motor CPG networks; however in all vertebrates (from lampreys to mammals), most of the serotonergic neurons are concentrated in the raphe nuclei in the midbrain (Barreiro-Iglesias et al., 2008). These 5-HT neurons mainly project to motor nuclei in the brainstem and motor CPGs in the spinal cord (Veasey, Fornal, Metzler, & Jacobs, 1995a). Electrophysiological studies show that these neurons increase their activity during spontaneous behaviors like locomotion, feeding, and increased respiratory tone following hypercapnia (Aghajanian & Vandermaelen, 1982; Veasey et al., 1995b). In addition, there are strong inhibitory interconnections between different raphe nuclei and even intrinsically within each of these nuclei. It is proposed that these inhibitory interconnections are able to inhibit the activity of inappropriate CPGs and enhance the activity of the relevant CPGs (Gillette, 2006). Interestingly, in almost all vertebrates, projections of serotonergic neurons from the hindbrain to motoneurons in the spinal cord happen at a very early stage of embryogenesis. This emphasizes the importance of this system in controlling locomotion (Barreiro-Iglesias et al., 2008).

Serotonergic neurons of the raphe nuclei demonstrate specific patterns of electrical activity, which allows them to efficiently control CPGs. 5-HT neurons in the raphe nuclei have a rhythmic pattern of activity. In these neurons, action potential duration is equal to or more than 2 milliseconds, followed by a significant stage of after hyperpolarization (Aghajanian & Vandermaelen, 1982; Miguelez, Grandoso, & Ugedo, 2011). In addition, these neurons show significant reduction in their activity during sleep, specifically in the period of REM. (Urbain, Creamer, & Debonnel, 2006). This reduction in activity during sleep can be explained by increased production of melanin concentrating hormone (MCH) and decreased level of orexin during sleep and specifically during REM. Both MCH and orexin are secreted from the respective populations of neurons in the lateral hypothalamus and it is proposed that they are important in controlling different aspects of the sleep-awake cycle (Veasey et al., 1995a).

After complete spinal cord transection, invertebrates and primitive vertebrates such as lampreys, recover from paralysis and are capable of moving normally again after a few months (Chiasson, Baker, & Croll, 1994; Cornide-Petronio, Ruiz, Barreiro-Iglesias, & Rodicio, 2011; Koert et al., 2001). Cornide-Petronio ME et al, (Cornide-Petronio et al., 2011), showed that spontaneous regeneration of serotonergic descending projections are mainly responsible for recovery of locomotion in lampreys. Using immunohistochemistry methods, these authors have demonstrated that axons from serotonergic neurons in the rhombencephalon or dorsal raphe regenerate to the levels below the transection.

Since CPGs are mainly controlled by the serotonergic system in more primitive animals like lampreys, regeneration of serotonergic neurons is enough to recover locomotion. However, in more complex vertebrates including mammals, the motor cortex and basal ganglia also send projections to the spinal cord through the corticospinal tract, which controls the voluntary aspect of locomotion (Murray et al., 2010).

Murray et al., (Murray et al., 2010) have also discovered that changes in post transcriptional editing of 5HT_{2c} receptor mRNA causes an over expression of new receptor isoform on motoneurons after spinal cord injury. This isoform for 5HT_{2c}

receptor is able to become activated in the absence of serotonin neurotransmitters in the spinal cord. This compensatory mechanism helps the motoneurons to regain their excitability, which leads to sustained muscle contractions. However, due to the lack of the regulatory commands from the brain, the rats developed muscle spasms for a few days after the spinal cord injury.

In conclusion, the serotonergic system is responsible for turning the locomotion CPGs on or off, however regulatory effect of other tracts such as reticulospinal tract, and corticospinal tract from the supra-spinal areas is required for a successful goal-oriented locomotion.

1.3 « Mesencephalic Locomotor Region »

Fifty years ago, Shik and colleagues identified the MLR for the first time. In a fascinating experiment, they demonstrated that electrical stimulation of a small region in the dorsal area of the mesopontine junction in unconscious decerebrated cats induced walking, running, and even galloping depending on the frequency of stimulation (Shik, Severin, & Orlovskii, 1966). These findings in cats and similar findings in different species, such as rodents and salamanders, suggest that the MLR is the gait initiation center (Cabelguen, Bourcier-Lucas, & Dubuc, 2003; Skinner & Garcia-Rill, 1984). The MLR is part of the reticular formation in the mesopontine junction and includes the cuneiform nucleus and pedunculopontine tegmental nucleus (PPN) (Child & Benarroch, 2013). There are numerous studies on the PPN and much research focuses specifically on the cholinergic neurons of the PPN. These studies were designed to show the role of these neurons in gait (Gut & Winn, 2015). However, the role of the cholinergic neurons in gait remains controversial and in fact, many of these studies suggested that PPN cholinergic neurons have no effect or a very minor effect on gait (Gut & Winn, 2015; Hernández-Chan et al., 2011). In addition, PPN electrical stimulation in Parkinson disease patients suffering from axial symptoms and freezing of gait were mostly unsuccessful (Ferraye et al., 2010).

More recent studies suggest a potential role of the PPN in gait because of its essential role in integration of sensory information. For instance MacLaren et al., showed that the PPN is not important in fixed gait speed and gait initiation in rats (MacLaren, Santini, Russell, Markovic, & Clark, 2014). However, in accelerated rotarod studies, bilateral PPN cholinergic neuronal loss resulted in severely impaired gait. Gait on a fixed speed rotarod is considered an automatic and reflexive behavior. However, maintaining successful gait on an accelerated speed rotarod requires the motor system to continuously adjust to changes from incoming sensory inputs. The PPN receives fast sensory input and sends projections to the substantia nigra pars compacta. These projections contain information about the value and salience of the sensory information (Hong & Hikosaka, 2014). Contrary to fixed speed performance, in accelerated speed gait, there is a continuous change in the sensory information enters the PPN. Therefore, it is proposed that the PPN is responsible for integration of sensorimotor information in complex motor tasks (Gut & Winn, 2015; Hong & Hikosaka, 2014; MacLaren et al., 2014).

In addition, two different studies involving single-unit electrode recording from the PPN in Parkinson disease patients while they were asked to imagine walking, showed significant changes in electrical activity of neurons during gait imagination compared to the resting state. These changes suggest that PPN is involved in planning gait (Lau et al., 2015; Tattersall et al., 2014).

Therefore, while the PPN and specifically cholinergic neurons of the PPN are one of the main suspects in controlling CPGs, well designed studies are needed to prove this claim.

1.4 « Medullary Reticular Formation »

The medial part of the rostral medulla has been implicated in the modulation of the axial motor activity. It is composed of two main parts: the gigantocellular nucleus (GiN) located dorsally, and the magnocellular nucleus (MN) located ventrally.

The neurons of the GiN send glutamatergic projections down to the spinal cord CPGs via the reticulospinal tract (Martin et al., 2011; Vetrivelan, Fuller, Tong, & Lu, 2009).

It is well known that electrical stimulation of the medial part of the rostral medulla suppresses the muscle tone in both decerebrated normal animals during sleep and wakefulness (Hajnik, Lai, & Siegel, 2000; Karlsson & Blumberg, 2005; Lai, Kodama, Schenkel, & Siegel, 2010). In addition, cell body specific lesions of the glutamatergic neurons in this area induces increased muscle tone and phasic muscle activity during both REM and non-REM sleep (Vetrivelan et al., 2009) .

It is believed that the MLR controls the CPGs in the spinal cord through the reticulospinal tract. The MLR sends bilateral and symmetric cholinergic projections to the medulla, (Le Ray et al., 2003) (Rolland, Karachi, Muriel, Hirsch, & François, 2011) and GiN in the medulla sends projections to the spinal cord CPGs via the RET. However, it is not clear whether medullary neurons receive afferent projections from MLR do send projections down to the spinal cord. Recent study by Sherman et al. (Sherman et al., 2015) identified that MLR neurons send projections directly down to the CPGs in the spinal cord. They also found that these neurons are not cholinergic neurons of the pedunculopontine nucleus, but they are the fast spiking glutamatergic neurons located anterior and posterior to the PPN.

In order to achieve successful goal oriented locomotion, appropriate body orientation and posture is also needed. Studies have shown that the vestibular system in the medullary reticular formation controls head orientation and balance by modulating the activity of the reticulospinal pathway (Kolkman, Moghadam, & du Lac, 2011).

In conclusion, while there is no doubt that GiN glutamatergic neurons are important in controlling muscle tone during both wakefulness and sleep, there is a big gap in our knowledge about the brainstem areas controlling the RET and specifically the GiN in the rostral medulla. Our aim from the review in this section is to:

- a) Delineate the pathways controlling the RET and to determine their importance in gait.

- b) Address these questions, by first performing tracer studies to locate the pathways.
- c) Then by pharmacological studies, manipulate these pathways in rats and
- d) Study their gait using the Catwalk XT apparatus.

Next, different methods for the rats' gait assessment will be discussed and the advantage of using the Catwalk apparatus over other methods will be explained.

1.5 « Methods for Gait Analysis in Rodents »

There are many available tests to analyze gait and locomotion in animals. The open field measurement testing is commonly used by researchers to observe spontaneous locomotion of an animal in an open field. This test is developed to measure general locomotor activity and exploratory behavior in rodents. However, this test does not provide detailed information about different phases of a gait cycle including stance phase duration, swing phase duration, stride length, the intensity at which each paw contacts the ground, and many other parameters (Mendes et al., 2015).

Another method commonly used to assess gait and locomotion is foot print analysis. In this method, the paws of animals are dipped in ink and then the animals walk across a strip of paper. This technique provides information regarding print area (length and width) and stride length, but it is very difficult or even impossible to measure other parameters of gait including the duration of each phase of the gait cycle or the footprint intensity. In addition, controlling animals to walk on papers for several times is time consuming and difficult (Neckel, 2015).

Catwalk XT is an automated gait analysis system, which consists of a long and enclosed walkway with a glass plate floor (100* 12* 0.6 cm). It is designed to allow animals traverse from one side of the walkway to the other freely with their natural speed. A green light is illuminated to the glass plate and while the animals' paws contact the glass, the light becomes scattered. This scattering of light by the animals' contacts is captured by a high speed color video camera mounted below the glass. Then, the recorded videos

are transformed into a series of digital images whereby each image is composed of numerous pixels. Each pixel contains information about the brightness of the image at a very small point and therefore reflects the intensity by which the animal's paw touched the glass plate at that point. As a result, this device is capable of measuring many indices, and several studies have suggested that Catwalk testing provides valid data about gait in animal models of spinal cord injury and neurodegenerative disorders as well as Parkinson disease (Chuang et al., 2010; Vandeputte et al., 2010; Zhou et al., 2015). The following tables (**Table 1, and 2**) summarize and define the static and dynamic gait parameters that were analyzed in our study.

Table2. Static Gait Parameters and Their Definition

Static Gait Parameters	Definition
Max Contact Area (cm ²)	Maximum area of a paw that comes into contact with the glass plate.
Print Area (cm ²)	The surface area of the complete print.
Mean Intensity	The mean pressure the animal applies with each paw on the glass.

Table3. Dynamic Gait Parameters and Their Definition

Dynamic Gait Parameters	Definition
Stand (s)	The duration in seconds of a contact of a paw to the glass plate.
Swing (s)	The duration in seconds of no contact of a paw with the glass plate.
Swing Speed (m/s)	The speed (Distance Unit/second) of the paw during Swing.
Stride Length (cm)	The distance (in Distance Units) between successive placements of the same paw.
Step Cycle(s)	The duration in seconds of stand + swing.
Duty Cycle (%)	Expressed Stand as a percentage of Step Cycle.
Body Speed (m/s)	The distance from one initial contact of one paw to the next by the time to travel that distance.

Body Speed Variation (%)

Dividing the absolute difference between the Body speed and the Average Speed of a run by the Average Speed.

Stand Index

A measure for the speed at which the paw loses contact with the glass plate.

1.6 « Hypotheses and objectives of This Study »

The reticulospinal pathway is a phylogenetically old descending pathway, which mainly controls the locomotion CPGs and motoneurons of axial and proximal muscles. It is believed that this pathway is responsible for automatic and spontaneous control of locomotion and body posture (Isa, Kinoshita, & Nishimura, 2013). However, pathways and types of neurons and neurotransmitters involved in controlling the glutamatergic neurons of this pathway are unknown. The main goal of this study is to investigate the area(s) of the brain controlling reticulospinal tract neurons and determining their role in gait. Based on the information we discussed above, we hypothesized that:

- A) Either or both of DRN and MLR are sending axons to GiN to control reticulospinal tract.
- B) Since GiN controls muscle tone, the same region of the GiN controlling the reticulospinal tract also controls muscle tone.

The objectives in this study are summarized here.

1. Identify brainstem area(s) controlling the reticulospinal pathway. This will be discussed in detail in chapter 2.
2. Determine the importance of the region controlling the reticulospinal pathway in gait. This part will be discussed in chapter 3.

3. Identify neurotransmitters and receptor types controlling the reticulospinal pathway at the rostral RF using both gait studies and immunohistochemistry. It will be discussed in detail in chapter 4.
4. Determine whether DRN is important in controlling the MLR through gait studies and immunohistochemistry. This will be discussed in detail in chapter 5.
5. Identify if 5HT1A autoreceptors on DRN serotonergic neurons are important in controlling gait through gait studies and immunohistochemistry. This part will be discussed in chapter 6.

Chapter 2

« Identifying the pathways controlling the reticulospinal tract using neural retrograde tracer Cholera Toxin Beta Subunit »

2.1 « Introduction »

Motoneurons in the spinal cord are controlled by two main groups of pathways: 1) the corticospinal tract for fine motor tasks and 2) the reticulospinal tract for gross tasks including locomotion, reaching, and posture (Baker, 2011a; Holstege & Kuypers, 1987). In other words, in the corticospinal pathway, different combinations of neural activity in the motor cortex leads to fine independent finger movements, while the reticulospinal pathway controls more axial muscles to maintain posture and balance during locomotion (Holstege & Kuypers, 1987; Lawrence & Kuypers, 1968).

The medial part of the rostral medulla has been implicated in the modulation of axial motor activity. It is composed of two main parts: 1) GiN located dorsally and 2) magnocellular nucleus (MN) located ventrally (Lawrence & Kuypers, 1968). Electrical stimulation of the medial part of the rostral medulla suppresses the muscle tone in both decerebrated and intact animals during sleep and wakefulness (Hajnik et al., 2000; Lai et al., 2010; Vetrivelan et al., 2009).

It is believed that the MLR controls spinal cord CPGs through the RET. The MLR sends bilateral and symmetric cholinergic projections down to the medulla in sea lampreys, (Rolland et al., 2011) and the GiN in the medulla sends projections down to the spinal CPGs via the reticulospinal tract (Martin et al., 2011). However, there is no study showing that the neurons in the medulla which receive the projections from MLR are the same ones projecting to the spinal cord. A recent study by Sherman et al. identified that MLR neurons send projections directly down to the CPGs in the spinal cord. They also found that these neurons are not cholinergic neurons of the pedunculopontine nucleus, but they are fast spiking glutamatergic neurons located anteriorly and posteriorly to the PPN. In addition, MLR neurons are not the only brainstem neurons controlling CPGs in

the lumbar spinal cord. Many different types of neurotransmitters including different subtypes of serotonergic receptors are identified on the surface of motoneurons (Ghosh & Pearse, 2015; Noga, Johnson, Riesgo, & Pinzon, 2009). The serotonergic projections arise mainly from the DRN (Barreiro-Iglesias et al., 2008), therefore other mesopontine areas other than the MLR might also be important in controlling gait.

In this study, our first goal was to identify groups of neurons controlling the RET with the use of a retrograde tracer, Cholera Toxin Beta subunit (CTB).

2.2 « Materials and Methods »

All animal procedures were approved by Animal Care and Veterinary services (ACVS) at Western University, London, Ontario, Canada. Five male Sprague-Dawley rats weighing 350-400 grams were used for the first part of the tracer study. Rats were individually housed in Plexiglas-walled cages after the surgery with free access to food and water. The temperature of the room was kept at 22 ± 1 °C on a 12 hour light-dark-cycle (lights on from 7 AM to 7 PM).

Surgical Procedure

For the first series of experiments, retrograde tracer Cholera Toxin beta subunit (CTB) was microinjected in the ventral horn of the lumbar spinal cord. The animals were anaesthetized with ketamine hydrochloride (Vetalar, initial dose 80 mg/kg and maintenance dose 40 mg/kg,) and xylazine (0.8 mg/kg,). Anaesthetized rats were placed on a Kopf stereotaxic frame with spinal attachment (Kopf Instruments, Tjunga, CA).

The retrograde tracer, Cholera Toxin Subunit β (CTB) (Life Technologies) conjugated with Alexa Fluor 594, was microinjected (0.5 μ l of 1.0 mg/ml solution per site) in to the

ventral horn of the lumbar spinal cord in 5 rats, using 10 μ l Hamilton syringe, mounted and held by a Kopf Micro injector. To inject in the ventral horn of the lumbar spinal (L2/3) cord, a small hole was drilled in the lamina close to the spinous process. Dura was split to expose spinal cord. The syringe needle was lowered through the hole and spinal cord vertically until it reached the stereotactic targets at the ventral horn of the spinal cord, and the tracer was slowly injected over 3 minutes. After the injection, the needle was kept in place for an additional 3 minutes. Surgical wound was closed with nylon and skin clips. The rats were sacrificed 4 days after the surgery.

In another group of 10 rats weighting 250-270 grams, 0.5 μ l of 1 mg/ml CTB solution was microinjected in the medial part of the rostral medulla using stereotaxic coordinates for the GiN (AP= -11.0, M=0, DV= -9.0; Paxinos and Watson, 2007). These rats were sacrificed 48 hours after surgery.

Histology

After completion of experiments, animals received an overdose of sodium pentobarbital and were perfused transcardially with 0.9% NaCl containing 1 ml/lit heparin followed by 4% formaldehyde solution (in 0.05 M PB). The brains and spinal cords were removed and kept in 4% formaldehyde solution for 48 hours and then 26% sucrose solution in PB until they sink. Coronal sections (40 μ m) through the entire length of the brainstem were cut using a freezing sliding microtome. Every fifth section was mounted and the sections were examined using Olympus FV1000 confocal microscope, and representative images were digitally recorded.

2.3 « Results »

CPGs are mainly controlled by three areas in the brainstem

A representative injection site is shown in **figure 1**. Based on previous studies, we predicted that the CPGs in the ventral part of the spinal cord receive projections from two main areas: 1) The medial zone of the rostral reticular formation where the GiN is located and 2) The DRN (**Fig2A, B**). In addition to these two locations, we also found that CPGs are also receiving direct projections from the MLR which is known as an important center for gait initiation (**Fig2c**).

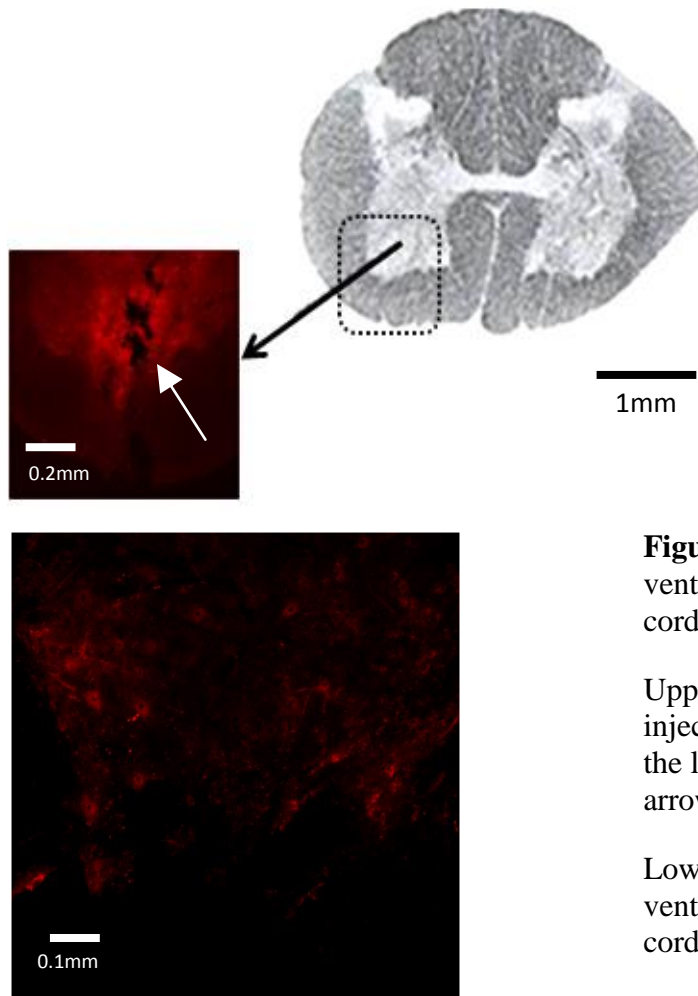


Figure1. CTB injection site at the ventral horn of the lumbar spinal cord.

Upper image shows the CTB injection site at the ventral horn of the lumbar spinal cord (white arrow).

Lower image shows a section of the ventral horn of the lumbar spinal cord rostral to the injection site.

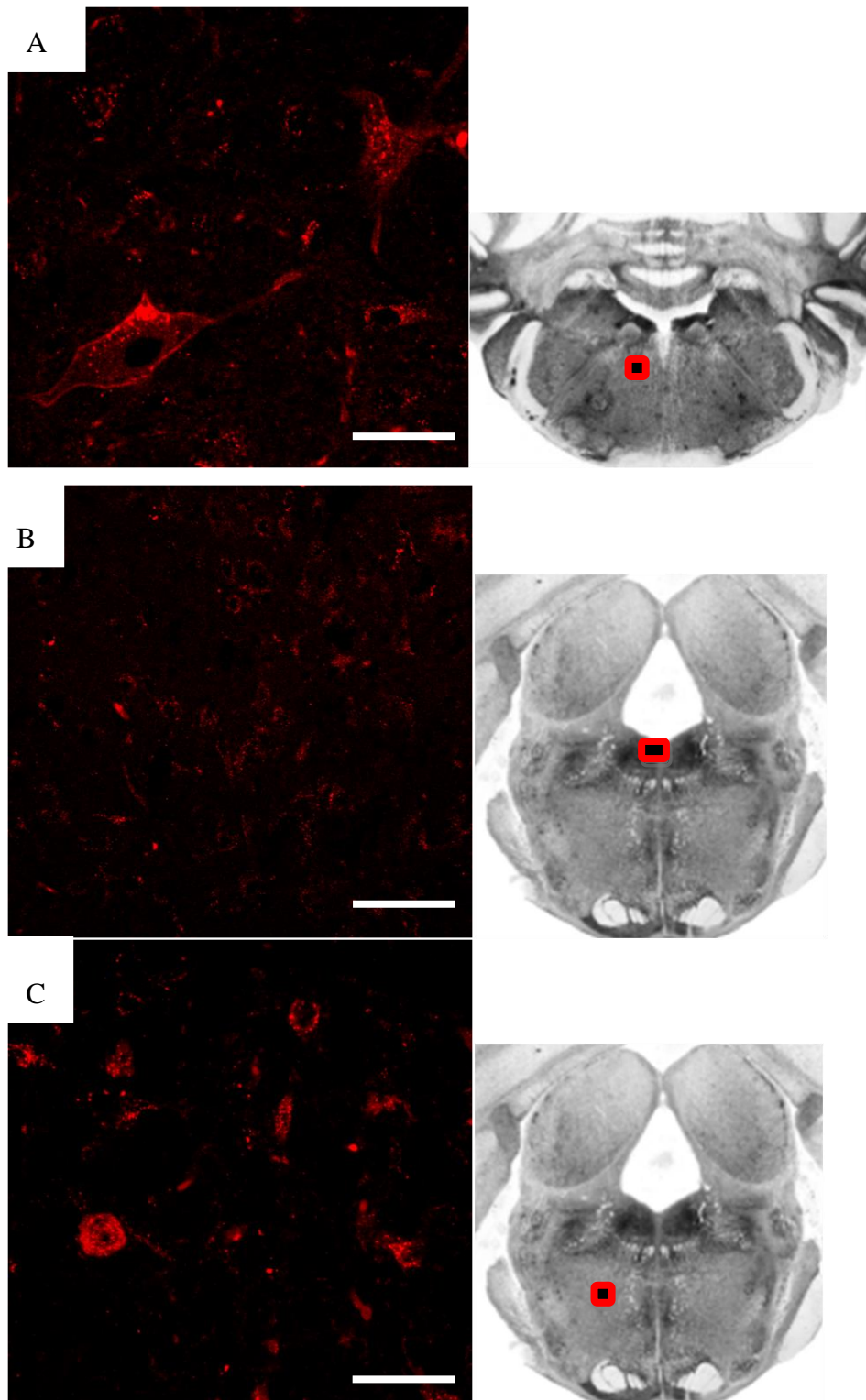


Figure2. Neurons in three areas of the brain stem picked up the CTB tracer GiN (A), DRN (B), and MLR (C). Scale bars = 50 μ m

The reticulospinal tract receives strong projections from the DRN and not the MLR

In contrast to a general consensus regarding the control of the reticulospinal tract by cholinergic neurons in the MLR, we found that the DRN sends projections down to the GiN (**Fig3**). In addition, we examined many sections under the microscope to find the neurons in the MLR area which would send projections down to the rostral reticular formation. However, we did not see any labeling in that area.

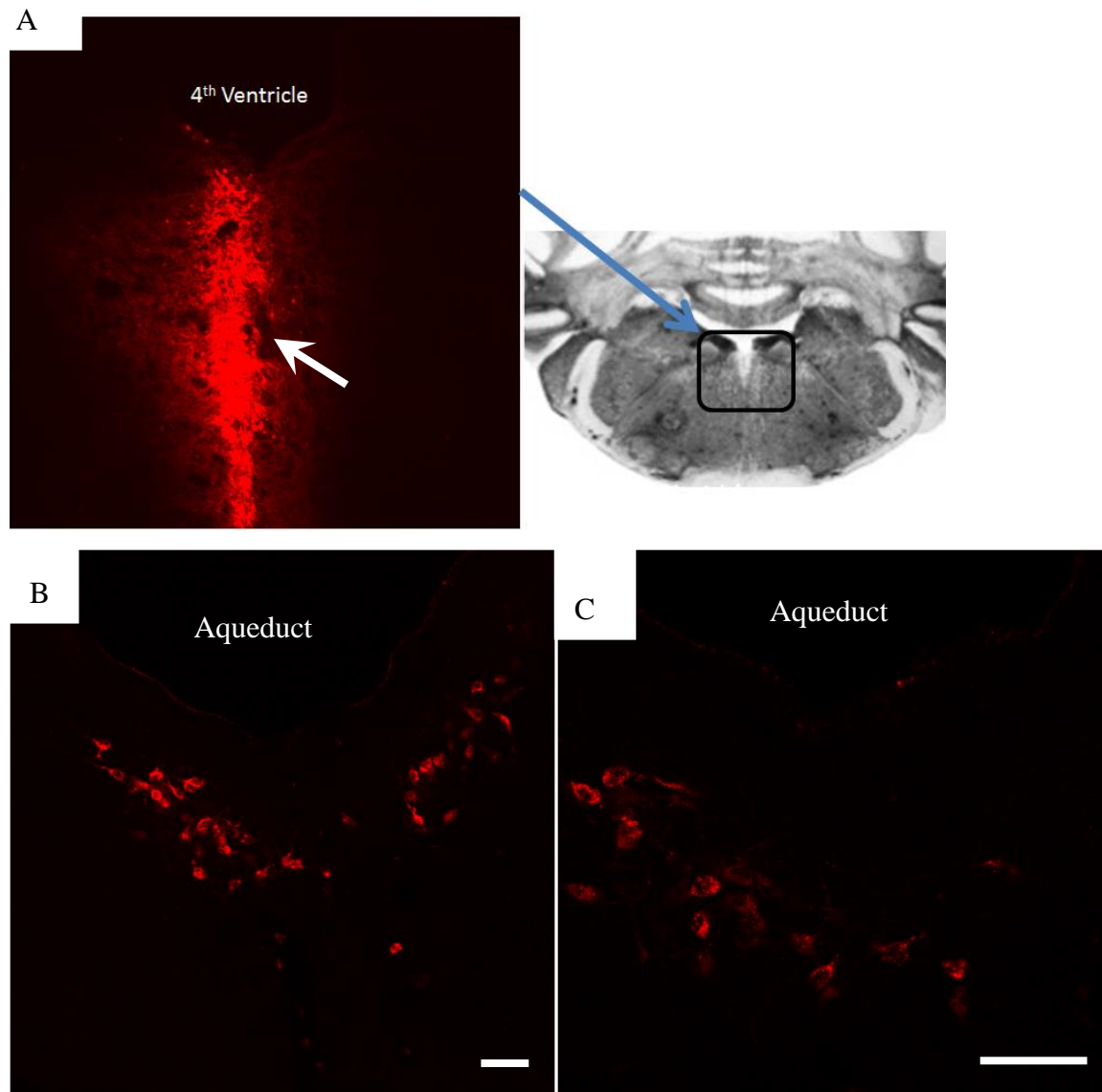


Figure3. CTB Injection Site at GiN. Injection site is presented at GiN (white arrow) (A). Neurons at DRN picked up the tracer: (B) shows 10 × magnification, and (C) shows 40 × magnification. Scale bars = 50μm

2.4 « Discussion »

In the present study, we injected the neural retrograde tracer CTB at the ventral horn of the lumbar spinal cord, where motoneurons and CPGs are located. Three main locomotion related areas in the brainstem were labeled for the tracer: DRN, MLR, and GiN. In a separate series of rats, we injected CTB in to the GiN. DRN neurons were labeled by CTB, but there was no signal for the tracer in areas anterolateral to the DRN where the MLR and PPN are located. In other words, we found evidence for three parallel pathways from the brainstem to the motoneurons and CPGs inside the spinal cord. The first pathway originates from the DRN directly, the second pathway is coming indirectly from the DRN through the GiN, and the third pathway arises from the MLR directly (**Figure 2, and 3**).

It is well known that many descending pathways including the corticospinal tract, vestibulospinal tract, and rubrospinal tract also project to the ventral horn of the spinal cord where lower motoneurons and CPGs are located (Isa et al., 2013), in the current study, while the tracer might also be found in other brain areas besides the MLR, DRN, and the GiN, we only focused on the brain stem structures which are proposed to have a role in controlling the reticulospinal tract.

The direct pathway from the DRN to CPGs is very well known, and spinal cord injury studies have shown that loss of serotonergic innervation in this pathway caused severe impairment in gait in almost all species (Courtine et al., 2009). On the other hand, stimulation of motoneurons by serotonin facilitates the gait and locomotion cycle which emphasizes the role of the DRN direct pathway in controlling motoneurons (Ghosh & Pearse, 2015; Noga et al., 2009; Veasey et al., 1995b).

Our findings on the other two pathways are against the prior literature about the MLR and PPN, which suggested that these areas are the main structures sending projections down to the GiN (Rye, Lee, Saper, & Wainer, 1988; Skinner & Garcia-Rill, 1984). In agreement with this, a recent study by Sherman et al. showed that the MLR in the mesopontine junction does not send projections to the medullary

reticular formation, rather does send direct projections down to the spinal cord motoneurons.

While prior studies have shown that PPN cholinergic neurons are sending projections down to the GiN, there are several recent studies suggesting that cholinergic neurons in the PPN mostly send projections to the ventrolateral thalamic nucleus, and dorsal striatum (Dautan et al., 2014; Wen et al., 2015).

Our current knowledge about the role of the MLR and PPN in gait stems mainly from electrical stimulation studies. Since cholinergic neurons in the PPN send projections up to the dorsal striatum and ventrolateral thalamic nucleus, the two subcortical structures which are important in motor function, it is difficult to interpret the effect of PPN stimulation on efferent pathways to the spinal cord, such as the RET. In recent studies, low frequency stimulation of the PPN has been shown to produce changes in neural activity in the ventrolateral thalamus, substantia nigra pars reticulata, and sub thalamic nucleus (Park, Song, Jang, & Kim, 2014; Wen et al., 2015). These three areas are affected in Parkinson disease and the studies suggested that the therapeutic effect of PPN stimulation on gait and posture might stem from the improvement in activity of these pathways.

On the other hand, deep brain stimulation of the PPN in PD patients does not always show satisfactory outcome, and many animal and human studies suggest that PPN stimulation is not improving gait and posture (Ferraye et al., 2010; Gut & Winn, 2015). In addition, studies have shown that the best area for deep brain stimulation is posterior or even outside of the PPN (Ferraye et al., 2010; Gut & Winn, 2015; Zrinzo, Zrinzo, & Hariz, 2007). Since the DRN is adjacent to the posterior part of the PPN, it is possible that by stimulation of this area, neural activities of the DRN neurons are also modified. This could be one of the mechanisms of gait improvement during stimulation of the posterior PPN.

In conclusion, tracer studies have shown that DRN is sending projections to both the GiN and motor neurons in the lumbar spinal cord. These findings imply that in addition

to the MLR, the DRN may also plays an important role in controlling gait and posture.

Chapter 3

3 « Role of the Dorsal Raphe Nucleus in Gait. »

3.1 « Introduction »

In the first part of the study, we showed that serotonergic neurons of the DRN send strong projections down to the GiN neurons of the medial part of the rostral reticular formation.

Serotonergic neurons in the raphe nuclei in most species have a spontaneous rhythmic activity. This pacemaker property is caused by intrinsic and gradual depolarizing ramps observed in these neurons. This gradual depolarization results in an action potential duration of 2 or more milliseconds, followed by a significant stage of after hyperpolarization (Aghajanian & Vandermaelen, 1982; Urbain et al., 2006). Another interesting property of these 5-HT neurons is their rate of firing during different states of activity. Most of these neurons have the high rate of tonic activity during wakefulness and show significant reduction in their activity during sleep. In fact, they are completely silent during REM sleep, when the voluntary muscles are paralyzed (Urbain et al., 2006). These properties make DRN serotonergic neurons good candidates for controlling locomotion related CPG neurons in the lumbar spinal cord. Many studies suggest that serotonergic projections to the motoneurons and CPGs play an essential role in controlling CPGs and making gait patterns in both invertebrates and vertebrates (Kiehn, 2006). These studies employed electrophysiological recording during pharmacological manipulation of motoneurons in *in vitro* slices of the spinal cord (Gackière & Vinay, 2014).

To the best of our knowledge, there are no studies analyzing gait parameters after inhibition of the DRN nucleus *in vivo* in awake behaving animals. In this part of the study, we inactivated DRN neurons by lidocaine and then analyzed the gait pattern while the rats were spontaneously walking on Catwalk. It was hypothesized that inactivation of

DRN projections to the motoneurons will result in abnormal foot fall pattern and abnormal coordination between limbs.

3.2 « Material and Methods »

Six male Sprague-Dawley rats (10-12 weeks old), weighing 250-270 grams were used. Rats were individually housed in Plexiglas cages, with free access to food and water. The room temperature was kept at 22 ± 1 °C on a 12 hour light-dark cycle (lights on from 7:00 am to 7:00 pm).

Surgical Procedures

Animals were anaesthetized with ketamine hydrochloride (Vetalar, initial dose 80 mg/kg and maintenance dose 40 mg/kg, i.m.) and xylazine (0.8 mg/kg, i.m.). Anaesthetized rats were fixed in stereotaxic frame (Kopf Instruments, Tadjunga, CA) and the incisor bar was adjusted until the anterior and posterior parts of skull were horizontal. Stereotaxic coordinates of Paxinos and Watson atlas (2007) were used and the reference point was bregma. Indwelling stainless steel 27-g guide cannulae were implanted 1.5 mm above the posterior border of the DRN. The coordinates are in millimeters: AP= -8.3, M=0, DV= -6.4 for DRN. Following surgery, rats were allowed to recover in their home cages for a period of 7 days.

Gait Studies

Gait parameters were measured using Catwalk XT (Noldus). While the animals are traversing the walkway with their natural speed, a green light illuminates the glass plate on the floor of the walkway. The light becomes scattered when the animals' paws contact the glass, and this will be recorded by a high-speed camera mounted below the glass. The glass dimension is 100 cm length x 12 cm width x 0.6 cm thickness, which lets the animals to complete at least 7 step cycles by walking up to 70 cm of the length. The

definition of the gait parameters we measured in this study are summarized on **Tables 3** and **4**.

One day prior to the above surgical procedure, all rats underwent Catwalk training to familiarize them to the walkway and to ensure their ability in conducting successful gait trails.

Table3. Static Gait Parameters and Their Definition

Static Gait Parameters	Definition
Max Contact Area (cm ²)	Maximum area of a paw that comes into contact with the glass plate.
Print Area (cm ²)	The surface area of the complete print.
Mean Intensity	The mean pressure the animal applies with each paw on the glass.

Table4. Dynamic Gait Parameters and Their Definition

Dynamic Gait Parameters	Definition
Stand (s)	The duration in seconds of a contact of a paw to the glass plate.
Swing (s)	The duration in seconds of no contact of a paw with the glass plate.
Swing Speed (m/s)	The speed (Distance Unit/second) of the paw during Swing.
Stride Length (cm)	The distance (in Distance Units) between successive placements of the same paw.
Step Cycle(s)	The duration in seconds of stand + swing.
Duty Cycle (%)	Expressed Stand as a percentage of Step Cycle.
Body Speed (m/s)	The distance from one initial contact of one paw to the next by the time to travel that distance.
Body Speed Variation (%)	Dividing the absolute difference between the Body speed and the Average Speed of a run by the Average Speed.
Stand Index	A measure for the speed at which the paw loses contact with the glass plate.

At the first day of the experiment, a tight-fitting 30-g injection cannulae were inserted into the guide cannula in such a way that protruded 1.5 mm from the tip of the guide cannulae to reach the DV coordinates of the DRN. All Infusions were carried out over 1 minute and the injection cannulae were kept in place for an additional 60 seconds to minimize back flow of the solution.

First, 0.5 μ l of sterile 0.9% saline was injected in DRN. Five minutes after the injection the animals were placed on catwalk walkway, where they were able to walk freely with their normal pace. For each vehicle or drug injection for each animal, three successful gait trials were recorded. A successful gait trial is when the animal starts walking from one side of the glass plate and finishes its gait to the other side of the glass without turning back.

An hour after the saline injection, 0.5 μ l of sterile lidocaine hydrochloride 4% (Lidodan 4%) was injected in the DRN (Prado & Faganello, 2000), and five minutes after the injection the rats were placed on catwalk and their gait was measured.

In the second day of the experiment, the same steps were repeated with administration of 1 μ l of lidocaine 4% in the DRN.

To verify the placement of the cannulae, 0.5 μ l of a 4% methylene blue solution was infused in the DRN and the extent of the dye spread after half an hour was considered as an indication of the presumed diffusion of the normal saline or Lidocaine which was given to each animal during the experiments.

Statistical Analysis

In this study, gait parameters of six animals in each group were measured, similar studies on Catwalk apparatus have shown that this number of the subjects provides a valid data set (Chuang et al., 2010; Mendes et al., 2015; Vandeputte et al., 2010; Zhou et al., 2015). All the recorded videos were double-checked to correct any possible errors that the Catwalk software might have made in labeling the paws. All walkway crossings were analyzed using the Catwalk software data. Each gait parameter is expressed as mean \pm S.E.M (Standard error of the mean). SPSS statistical software (version 19) was used to analyze all parameters. Statistical significance was evaluated by the Friedman Test and group differences were determined by the non-parametric Wilcoxon test. Since the

number of the subjects we used in each group of the animals was small (n=6), normalization of the data was not appropriate, therefore, we used Wilcoxon test that does not assume normality in the data. In this study, a P value lower than 0.05 was considered to be statistically significant.

3.3 « Results »

Verifying Cannulas Placement

Six male Sprague-Dawley rats underwent surgery and single cannulae were inserted in the cerebral aqueduct adjacent to the posterior border of the DRN. To verify the placement of the cannulae, 0.5 μ l of a 4% methylene blue solution was infused through the cannulae and half an hour later animals were sacrificed. Frozen sections through the brainstem were Nissl stained and the cannulae placement site was verified under the microscope **Figure4**.

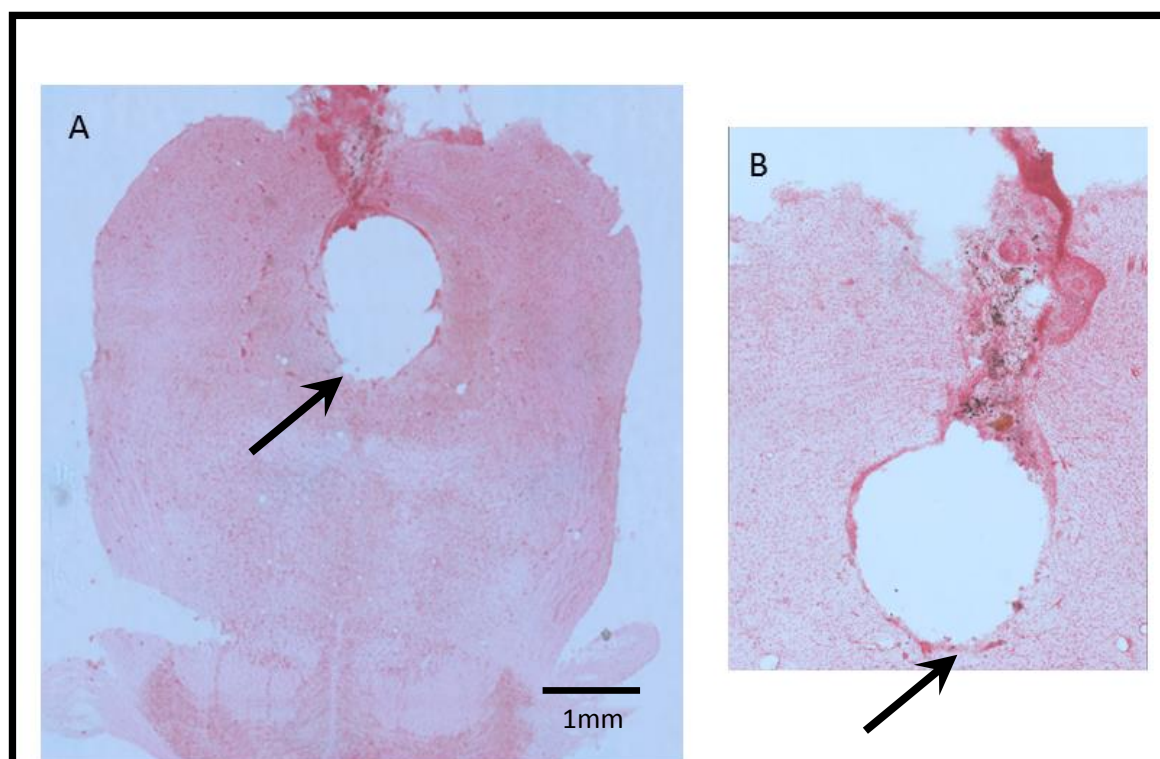


Figure4. Low (A), and high (B) magnification of cannula site at cerebral aqueduct, next to the dorsal border of DRN (black arrow) taken from two representative cases. Nissl stained sections showing the intended position of the cannula tip. Infusions were made just at the dorsal border of the DRN to prevent mechanical damage to the nucleus. In some cases, cannula tip has just penetrated the DRN (A).

Effect of DRN inhibition by 0.5µl Lidocaine 4% on gait:

The vehicle's effect on gait was tested by injection of 0.5 µl normal saline in the DRN and studying the rats' gait on Catwalk. Inhibition of DRN by 0.5µl Lidocaine 4% impaired both static (**Table 5**) and dynamic (**Table 6**) gait parameters significantly. Among static parameters, maximum contact area in forelimbs and print area in all limbs are significantly decreased. Although almost all other static parameters including maximum intensity and stand index are also decreased, the changes are not statistically significant (**Table 5**).

Table 5. Static Gait Parameters after Injection of Lidocaine: Mean \pm standard deviation is measured for each parameter in individual limbs. P values < 0.05 are considered significant and are highlighted with light green color.

Static Gait Parameters	Normal Saline	Lidocaine	P Value
MaxContactArea (cm ²) RF	4.2 \pm 0.99	2.99 \pm 0.44	0.046
MaxContactArea (cm ²) RH	3.6 \pm 1.29	3.1 \pm 0.84	0.345
MaxContactArea (cm ²) LF	4 \pm 0.78	3.08 \pm 0.30	0.046
MaxContactArea (cm ²) LH	3.96 \pm 1.6	3.13 \pm 0.89	0.463
PrintArea (cm ²) RF	5.88 \pm 1.64	3.7 \pm 0.53	0.046
PrintArea (cm ²) RH	4.86 \pm 1.68	4.07 \pm 0.9	0.028
PrintArea (cm ²) LF	5.9 \pm 1.65	3.9 \pm 0.36	0.046
PrintArea (cm ²) LH	5.2 \pm 1.96	3.9 \pm 0.91	0.028
Max Intensity RF	192.6 \pm 17.4	182.6 \pm 18.4	0.345
Max Intensity RH	190 \pm 21.6	170 \pm 31.4	0.249

Max Intensity LF	189.4±11.2	183±21.1	0.345
Max Intensity LH	179.5±31.2	177±22	0.6
Mean Intensity RF	71.74±3.6	73.27±6.46	0.463
Mean Intensity RH	78.3±11.4	74.4±11.9	0.6
Mean Intensity LF	71.3±5.1	73.6±7.6	0.6
Mean Intensity LH	78.1±14.2	77.3±13	0.753
Stand Index RF	4.2±0.75	3.2±0.87	0.075
Stand Index RH	2.2±0.4	1.5±0.3	0.046
Stand Index LF	4.4±0.92	3.4±0.82	0.116
Stand Index LH	2.2±0.44	2.1±0.5	0.463

In addition, most of the time dependent parameters or dynamic parameters significantly changed (**Table 6**). Each step cycle is made of two parts: stand and swing. The data shows that both of these two parameters were significantly longer in lidocaine treated rats. However, the duty cycle did not change, which suggests the ratio between stand and swing duration in each step cycle remain constant. While the rats were walking on Catwalk, several episodes of freezing happened during each trial after lidocaine injection. The freezing episodes are represented as a significant increase in body speed variation in all four limbs (p value= 0.028). This variation occurs because a gait freezing episode produces a dramatic reduction in body speed. The key findings in this part are also summarized in **Figure5 and 6**.

Table 6. Dynamic Gait Parameters after Injection of Lidocaine: Mean ± standard deviation is measured for each parameter in individual limbs. P values<0.05 are considered significant and are highlighted with light green color.

Dynamic Gait Parameters	Normal Saline	Lidocaine	P Value
Stand (s) RF	0.34±0.06	0.8±0.6	0.046
Stand (s) RH	0.46±0.09	1.22±1.1	0.028
Stand (s) LF	0.34±0.08	0.69±0.39	0.046
Stand (s) LH	0.44±0.12	1.3±0.9	0.028
Swing (s) RF	0.22±0.04	0.29±0.11	0.046
Swing (s) RH	0.125±0.02	0.14±0.03	0.028
Swing (s) LF	0.18±0.05	0.5±0.4	0.046
Swing (s) LH	0.135±0.04	0.17±0.05	0.028
Swing Speed (cm/s) RF	94.97±23.4	83.3±24.6	0.345
Swing Speed (cm/s) RH	130.2±17.1	110.5±31	0.116
Swing Speed (cm/s) LF	107.67±10.56	109.04±13.3	0.028
Swing Speed (cm/s) LH	129.07±7.74	149.39±35.9	0.249
Stride Length (cm) RF	14.28±1.8	12.4±1.3	0.116
Stride Length (cm) RH	15.6±1.45	13.7±0.9	0.046
Stride Length (cm) LF	13.2±2.5	13.1±1.4	0.917
Stride Length (cm)LH	14.9±1.8	13.3±1.8	0.345
Step Cycle (s)RF	0.59±0.12	1.01±0.4	0.046
Step Cycle (s) RH	0.6±0.12	1.1±0.6	0.028
Step Cycle (s) LF	0.53±0.1	1.25±0.9	0.028
Step Cycle (s)LH	0.6±0.14	1.25±0.7	0.028
Duty Cycle (%) RF	63.35±4.1	67.45±5.8	0.116
Duty Cycle (%)RH	74.98±3.89	78.4±3.79	0.075
Duty Cycle (%)LF	63.6±4.3	65.7±4.6	0.173
Duty Cycle (%)LH	74.36±3.9	73.4±2.00	0.917
Body Speed (cm/s) RF	25.4±3.85	19.6±5.88	0.075
Body Speed (cm/s) RH	26.1±3.8	21.02±4.8	0.028
Body Speed (cm/s) LF	25.7±3.5	19.65±5.4	0.116
Body Speed (cm/s) LH	26.6±4.3	20.5±5.4	0.249
Body Speed Variation (%) RF	41.8±10.4	86.3±30.3	0.028
Body Speed Variation (%) RH	42.4±13.5	88.6±32.4	0.028
Body Speed Variation (%) LF	45.1±12.4	81.5±17.8	0.028
Body Speed Variation (%) LH	41.0±10.3	85.2±28.6	0.028

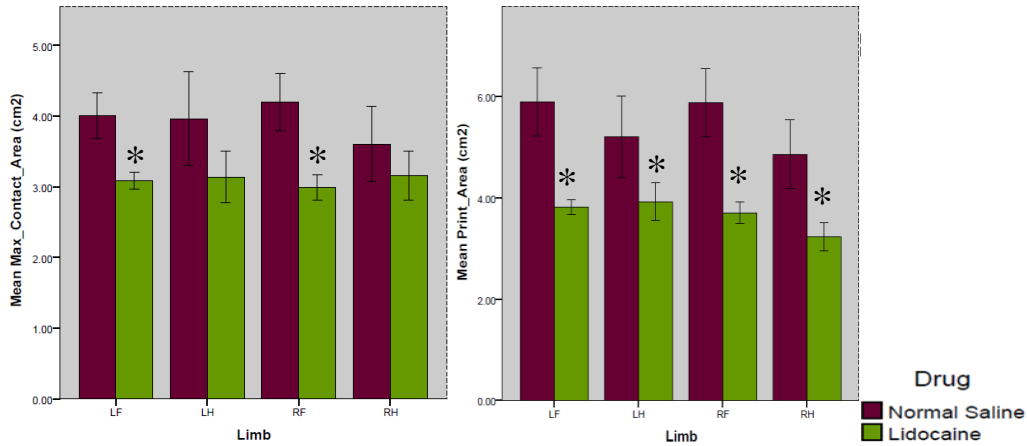


Figure 5. Key findings of the performance on the Catwalk for static gait parameters after injection of 0.5µl normal saline and after injection of 0.5µl Lidocaine 4% in DRN. Graphs show group means ± SEM of selected gait parameters. * p<0.05

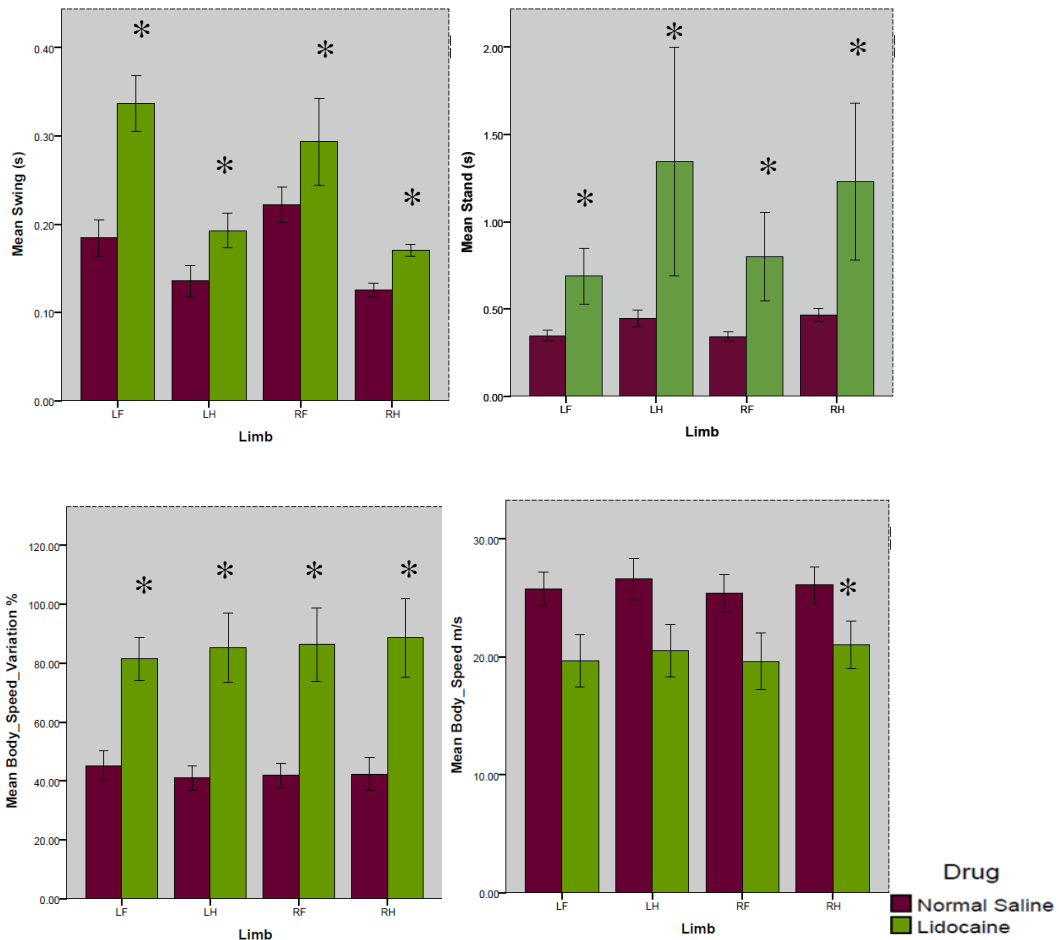


Figure6. Key findings of the performance on the Catwalk for dynamic gait parameters after injection of 0.5µl normal saline and after injection of 0.5µl Lidocaine 4% in DRN. Graphs show group means \pm SEM of selected gait parameters. * $p < 0.05$

One day after the first experiment 1µl of lidocaine was microinjected in DRN. Five minutes after the injection, rats were placed on Catwalk. While the rats were completely conscious and their reflexes intact, they experienced several episodes of severe freezing. Therefore, the Catwalk apparatus was not able to record their gait. In very rare experiments that rats were able to walk, their footfall patterns were totally irregular (**Figure 7**).

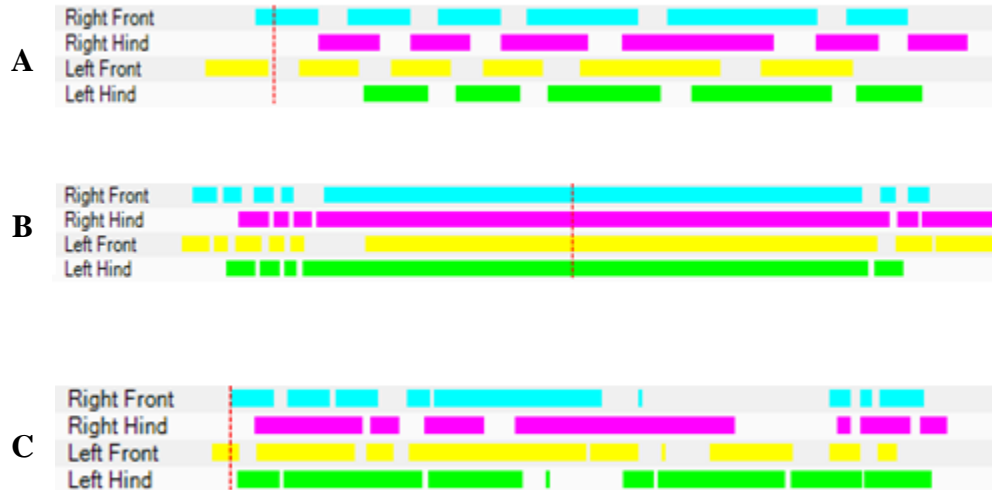


Figure7. Foot fall pattern after normal saline injection in DRN (A), 0.5µl Lidocaine 4% injection in DRN (B), and 1µl Lidocaine 4% injection in DRN (C). One episode of freezing of gait is seen in image B, but the foot fall pattern is preserved. Footfall pattern is completely irregular in image C.

3.4 « Discussion »

In this part of the study, we found that partial inhibition of the DRN in rats using 0.5 μ l of lidocaine results in significant impairment in static gait parameters including maximum contact area, and print area, as well as many dynamic gait parameters including stand, swing, step cycle, and body speed variation. We also injected 1 μ l of lidocaine in the DRN of rats and this time we were not able to record their gait due to severe freezing of gait in these animals.

In mammals, serotonergic projections from the DRN are among the very first axons to reach the motoneurons in the spinal cord (Barreiro-Iglesias et al., 2008; Vinay et al., 2002). Reduced 5HT secretion in early days after birth in rodents resulted in postural asymmetry and abnormal gait, which implies the importance of 5HT secretion from DRN projections in development of interconnections between CPGs (Pflieger, Clarac, & Vinay, 2002; Vinay et al., 2002). Injection of a 5HT₂ receptor agonist induced alternating air stepping in intact newborn rats (Brumley, Roberto, & Strain, 2012), and in vitro experiments on isolated spinal cord preparations of rats also suggested that activation of 5HT₂ receptors induce normal right/left and extensor/flexor pattern (Nakayama, Nishimaru, & Kudo, 2002). Since these studies all suggest that activation of 5HT receptors are playing an important role in inter and intra-limb coordination, many studies designed for transplantation of embryonic serotonergic neurons under the lesion site in spinal cord injury rodent models and surprisingly many of these studies found this treatment effective (Majczyński, Maleszak, Cabaj, & Sławińska, 2005; Sławińska et al., 2013).

Projection of DRN serotonergic neurons is not confined to motoneurons and CPGs. There are considerable number of 5HT_{1A} receptors on GABA interneurons and layer V pyramidal cells of motor cortex (Kruglikov & Rudy, 2008; Puig, Artigas, & Celada, 2005). In a study by Scullion et al., the 5,7 DHT neurotoxin was microinjected into the lateral ventricle which resulted in widespread depletion of serotonin specifically in cortex. The rats developed significant forelimb movement impairment in skilled reaching movements. They also demonstrated that 5HT_{1A}

receptor knock out mice had significantly smaller motor maps compared to wild type mice.

These studies suggest that DRN serotonergic projections to the motoneurons and motor cortex are essential in limb coordination and thus, animals' locomotion. However, no study has focused on the effect of direct DRN inhibition in gait parameters.

Based on the above studies, we were expecting to see significant changes in dynamic parameters and significant impairment in limb coordination. However, we also found that static parameters are also significantly impaired. Maximum contact area was decreased in forelimbs and print area was decreased in all four limbs. While changes of these two indices are dependent on muscle tone in limbs, these findings suggest that DRN neurons are also important in controlling the muscle tone during locomotion. Muscle tone is mainly controlled by the rostral medullary reticular formation or GiN. Therefore these findings are consistent with the tracer study findings in previous chapter, where we showed that the DRN sends projections to the GiN.

To prove this claim, we designed a study to manipulate neurons in the GiN by injection of different serotonergic receptors agonists and antagonists, while measuring the rats gait on Catwalk.

Chapter 4

«Dorsal Raphe Nucleus Controls Reticulospinal Tract Neurons In Gigantocellular Nucleus through 5HT_{1A} and 5HT₂ receptors. »

4.1 « Introduction »

Electrical stimulation of the medial part of the rostral medulla suppresses the muscle tone in both decerebrated and intact animals during sleep and wakefulness (Hajnik et al., 2000; Karlsson & Blumberg, 2005; Lai et al., 2010). There are several studies suggesting that the group of gigantocellular nucleus neurons send projections down to the spinal CPGs through the reticulospinal tract are glutamatergic (Martin et al., 2011; Vetrivelan et al., 2009). Cell body specific lesions of these glutamatergic neurons induces increased muscle tone and phasic muscle activity during both REM and non-REM sleep (Vetrivelan et al., 2009). However, there is not enough information about the detailed connectivity of these neurons and the type of the projections that these glutamatergic neurons are receiving.

In the past two chapters, it was shown that microinjection of the retrograde neural tracer CTB in the GiN of rats labeled serotonergic neurons of the dorsal raphe nucleus. Subsequently, to understand the role of these serotonergic neurons in gait, we injected Lidocaine into the DRN and studied rats' gait by Catwalk apparatus. We found a significant impairment in both static and dynamic parameters of gait in those rats. Moreover, with higher doses of the Lidocaine (1µl of Lidocaine 4%), the rats completely froze and recording their gait was almost impossible.

The next steps of this study were designed to uncover the subtypes of serotonergic receptors controlling reticulospinal neurons in the GiN, and their specific roles in gait.

Since there are 7 serotonin receptor classes and at least 13 known serotonin receptor subtypes in the brain (Raymond et al., 2001), it was difficult to study agonists and antagonists for all of these receptors in order to identify which receptors are located on GiN neurons. However, from previous studies, it is known that two types of receptors are abundantly found on the surface of motoneurons in the spinal cord, which are the 5HT_{1A} and 5HT₂ receptors (Courtine et al., 2009; Ghosh & Pearse, 2015; Murray et al., 2010). While the ligand (serotonin neurotransmitter) is the same for these two receptors, their activity is completely opposite. 5HT₂ receptors are excitatory and their excitation on motoneurons facilitates gait. In contrast, 5HT_{1A} receptors are inhibitory and their activation induces hyperpolarization in neurons (Scullion et al., 2013).

Here we hypothesized that injection of 5HT_{1A} and 5HT₂ agonists and antagonists respectively, will change the muscle tone in rats while they are walking on Catwalk.

4.2 « Materials and Methods »

Six male Sprague-Dawley rats (10-12 weeks old), weighing 250-270 grams were used. Rats were individually housed in Plexiglas cages, with free access to food and water. The room temperature was kept at 22±1 °C on a 12 hour light-dark cycle (lights on from 7:00 am to 7:00 pm).

Surgical Procedures

Animals were anaesthetized with ketamine hydrochloride (Vetalar, initial dose 80 mg/kg and maintenance dose 40 mg/kg, i.m.) and xylazine (0.8 mg/kg, i.m.). Anesthetized rats were fixed in stereotaxic frame (Kopf Instruments, Tadjunga, CA) and the incisor bar was adjusted until the anterior and posterior parts of skull were horizontal. Stereotaxic coordinates of Paxinos and Watson atlas (2007) were used and the reference point was bregma. Indwelling stainless steel 27-g guide cannulae were implanted 1.5 mm above the

GiN. The coordinates are in millimeters: F= -11, L=0, H= -9 for GiN. These rats were kept in their cages for a 7 days of recovery period.

Drugs

8OH-DPAT (8-HYDROXY-2-(DI-n-PROPYLAMINO)TETRALIN) and Ketanserin tartrate were purchased from Tocris Cookson (Ellisville, MO, USA).

Gait Studies

One day prior to surgery, all rats underwent Catwalk training to familiarize them to the walkway and to ensure their ability in conducting successful gait trails.

Tight fitting 30-g injection cannulae were inserted into the guides. They protruded 1.5 mm from the guide cannulae to reach the coordinates mentioned above for the GiN. Infusions were carried out over 1 minute and the injection cannulae were kept in place for another 60 seconds to minimize backflow.

On the first experimental day, 0.5 μ l of sterile 0.9% saline was injected in the GiN. Five minutes after the injection, the animals were placed on the Catwalk apparatus, where they were able to walk freely with their normal pace. For each vehicle or drug injection of each animal, at least three successful gait trials were recorded. A gait trial is considered successful if the animal starts walking from one side of the glass plate and completes its gait to the other side of the glass without turning back.

An hour after the saline injection, 10 μ M of sterile 8OH-DPAT (Pham-Le, Cockburn, Nowell, & Brown, 2011) was injected in the GiN. Five minutes after the injection, the rats were placed on Catwalk and their gait was measured. Few days later, the same steps were repeated with 0.5 μ g/0.5 μ l Ketanserin tartrate, a 5HT_{2A/2c} antagonist (McCool, Christian, Fetzter, & Chappell, 2014) instead of 8OH-DPAT.

Histology

Upon completion of experiments, animals received an overdose of sodium pentobarbital and were perfused transcardially with 0.9% NaCl (containing 1 ml/lit heparin) followed

by 4% formaldehyde solution (in 0.1 M phosphate buffer). The brains and spinal cords were removed and kept in 4% formaldehyde solution for 48 hours and then 26% glycerol solution until they sink.

To visualize colocalization of the CTB retrograde tracer (from animals in chapter 2, in which CTB was microinjected in CPGs) with 5HT_{1A} and 5HT₂ in the GiN, 40µm coronal sections were cut using a microtome and every fifth section was stained for 5HT_{1A} or 5HT₂ receptors.

A rabbit anti-5HT_{1A} primary antibody (SR-1A Antibody, Santa Cruz Biotechnology) and a Biotinylated Goat Anti-Rabbit IgG secondary antibody (Vector Laboratories) were used. The primary antibody for 5HT₂ receptors was a goat anti-5HT₂ antibody (SR-2c Antibody, Santa Cruz Biotechnology) and the secondary antibody was a Biotinylated rabbit Anti-Goat IGg Antibody (Vector Laboratories). Then, sections were studied under Olympus FV1000 microscope.

To verify the placement of the cannulae, 1µl of a 4% methylene blue solution was infused in the GiN and the extension of the dye after half an hour was considered as an indication of the presumed diffusion of the normal saline or the drugs which were given to each animal during the experiments.

Statistical Analysis

In this study, gait parameters of six animals in each group were measured, similar studies on Catwalk apparatus have shown that this number of the subjects provides a valid data set (Chuang et al., 2010; Mendes et al., 2015; Vandeputte et al., 2010; Zhou et al., 2015). All the recorded videos were double-checked to correct any possible errors that the Catwalk software might have made in labeling the paws. All walkway crossings were analyzed using the Catwalk software data. Each gait parameter is expressed as mean ± S.E.M (Standard error of the mean). SPSS statistical software (version 19) was used to analyze all parameters. Statistical significance was evaluated by the Friedman Test and group differences were determined by the non-parametric Wilcoxon test. Since the number of the subjects we used in each group of the animals was small (n=6), normalization of the data was not appropriate, therefore, we used Wilcoxon test which

does not assume normality in the data. In this study, a P value lower than 0.05 was considered to be statistically significant.

4.3 « Results »

Verifying Cannulas Placement

To verify the placement of the cannulae, 1 μ l of a 4% methylene blue solution was infused through the cannulae and half an hour later animals were sacrificed. Sections were prepared and the cannulae sites were verified under the microscope **Figure 8**.



Figure 8. Cannula site at GiN (black arrow). Methylene blue 4% was microinjected through cannulae. After making sections Nissl staining with neutral red color is done on the sections.

Effect of 5HT_{1A} agonist, 8OH-DPAT injection in the GiN on gait.

After injection of the vehicle (normal saline) the animal's baseline normal gait was recorded on the Catwalk. One hour later, 10 μ M of 8OH-DPAT, a 5HT_{1A} agonist (Pham-Le et al., 2011) was microinjected in the GiN and then the rats were placed on the Catwalk again. The findings showed significant decrease in static parameters including maximum contact area, print area, and maximum intensity, which suggest that the 5HT_{1A}

antagonist is affecting the muscle tone of limbs during gait. In contrast to static parameters, there was no significant change in any of the dynamic parameters including body speed variation (See **Table 7**).

Table 7. Selected dynamic and static gait parameters after injection of 8OH-DPAT in GiN. Mean \pm standard deviation is measured for each parameter in individual limbs. P values < 0.05 are considered significant and are highlighted with light green.

Gait Parameters	Normal Saline	8OH-DPAT	p value
Max Contact Area RF	2.44 \pm 0.35	1.95 \pm 0.66	0.249
Max Contact Area RH	2.13 \pm 0.49	1.4 \pm 0.49	0.075
Max Contact Area LF	2.69 \pm 0.36	2.01 \pm 0.4	0.046
Max Contact Area LH	2.09 \pm 0.6	1.2 \pm 0.5	0.028
Print Area RF	2.9 \pm 0.42	2.3 \pm 0.8	0.249
Print Area RH	2.77 \pm 0.58	1.74 \pm 0.57	0.046
Print Area LF	3.11 \pm 0.36	2.35 \pm 0.47	0.046
Print Area LH	2.65 \pm 0.7	1.5 \pm 0.6	0.028
Body Speed RF	32.9 \pm 4.9	29.2 \pm 8.6	0.345
Body Speed RH	31.5 \pm 5.1	28.6 \pm 8.8	0.249
Body Speed LF	31.1 \pm 5.58	27.3 \pm 8.8	0.249
Body Speed LH	31.7 \pm 5.4	28.7 \pm 9.2	0.345
Body Speed Variation RF	50.66 \pm 35.7	45.5 \pm 15.7	0.917
Body Speed Variation RH	55.01 \pm 40.6	46.9 \pm 17	0.753
Body Speed Variation LF	52.3 \pm 36.3	42.8 \pm 12.5	0.917
Body Speed Variation LH	52.26 \pm 30.2	47.9 \pm 16.9	0.6
Max Intensity RF	190.4 \pm 7.8	169.2 \pm 21.8	0.116
Max Intensity RH	165.8 \pm 20	134.2 \pm 32.5	0.046
Max Intensity LF	192.5 \pm 7.07	164.9 \pm 20.8	0.028
Max Intensity LH	158.4 \pm 28.4	121.6 \pm 26.2	0.028

Effect of 5HT₂ antagonist, Ketanserin Tartrate injection in GiN on gait.

In a subsequent experiment, the rats' baseline gait was recorded after 0.5 μ l normal saline. Subsequently, the same animals were microinjected in their GiN with 0.5 μ g/0.5 μ l Ketanserin tartrate, a 5HT_{2A/2C} antagonist (McCool et al., 2014) one hour later, and then their gait was studied (see **Table 8**). The findings suggest that the only gait parameter that

changed significantly was body speed variation. Therefore, in contrast to 5HT_{1A} receptors, 5HT_{2A/C} receptors were demonstrated as not being involved in controlling static parameters.

Table 8. Selected dynamic and static gait parameters. Mean \pm standard deviation is measured for each parameter in individual limbs. P values < 0.05 are considered significant and are highlighted with light green.

Gait Parameters	Normal Saline	Ketanserine	p value
MaxContactArea RF	3.1 \pm 0.5	3.27 \pm 0.66	0.463
MaxContactArea RH	2.7 \pm 0.66	2.9 \pm 0.9	0.249
MaxContactArea LF	3.25 \pm 0.5	3.21 \pm 0.5	0.917
MaxContactArea LH	2.94 \pm 0.9	3.08 \pm 0.8	0.6
PrintArea RF	3.8 \pm 0.6	3.88 \pm 0.7	0.345
PrintArea RH	3.6 \pm 0.7	3.8 \pm 1.09	0.249
PrintArea LF	3.95 \pm 0.7	3.8 \pm 0.5	0.6
PrintArea LH	3.84 \pm 1.3	4.01 \pm 1.25	0.6
Body Speed RF	37.8 \pm 10.9	39 \pm 9.3	
Body Speed RH	34.9 \pm 9.6	37.5 \pm 9.3	0.917
Body Speed LF	35.5 \pm 10	36 \pm 9.7	0.917
Body Speed LH	37.9 \pm 11.6	37.8 \pm 9.7	0.917
Body Speed Variation RF	49.3 \pm 18.4	33.7 \pm 10.3	0.046
Body Speed Variation RH	56.2 \pm 27.05	37.2 \pm 10.6	0.046
Body Speed Variation LF	47.2 \pm 16.7	33.5 \pm 10.6	0.046
Body Speed Variation LH	54.6 \pm 20.8	39.1 \pm 8.6	0.046
Max Intensity RF	200.7 \pm 3.5	203.4 \pm 3.4	0.116
Max Intensity RH	185 \pm 15.2	191 \pm 25	0.463
Max Intensity LF	200.2 \pm 7.8	202.7 \pm 4.8	0.463
Max Intensity LH	184.4 \pm 26.4	191.8 \pm 14.2	0.463

The key findings in this part of the experiment are summarized in **Figure 9**.

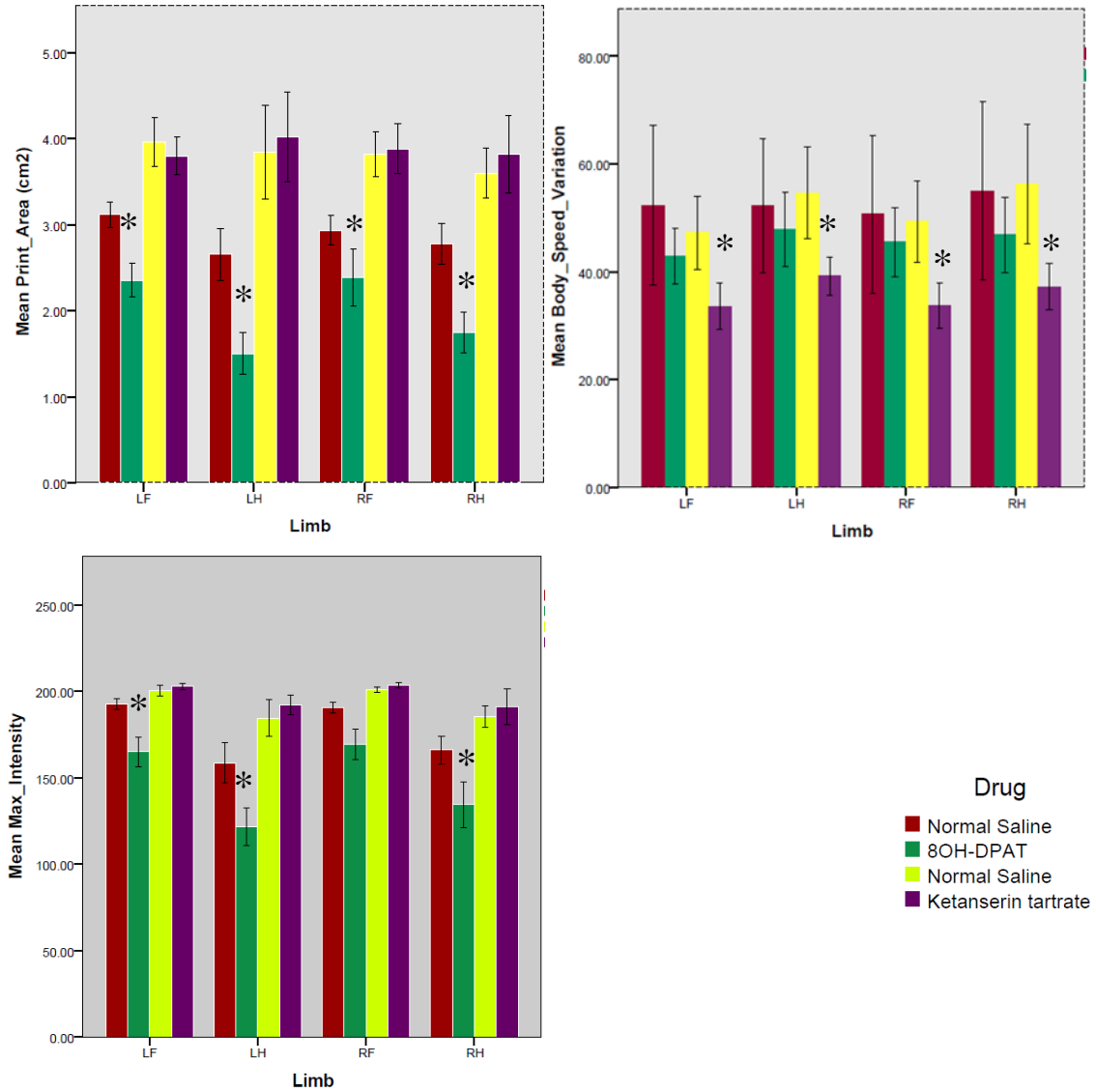


Figure 9. Key findings of the performance on the Catwalk for dynamic gait parameters after injection of normal saline, 5HT_{1A} agonist, and 5HT₂ antagonist. Graphs show group means \pm SEM of selected gait parameters. * $p < 0.05$

Immunohistochemistry Labeling for 5HT_{1A}, and 5HT₂ Receptors in GiN.

Here we found that both 5HT_{1A} and 5HT_{2c} receptors are present on the surface of the neurons which were previously labeled for CTB injected in CPGs in the lumbar spinal cord (Figure 10, and 11).

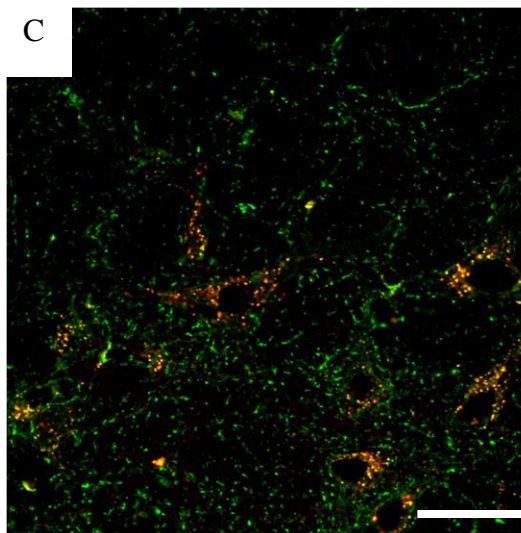
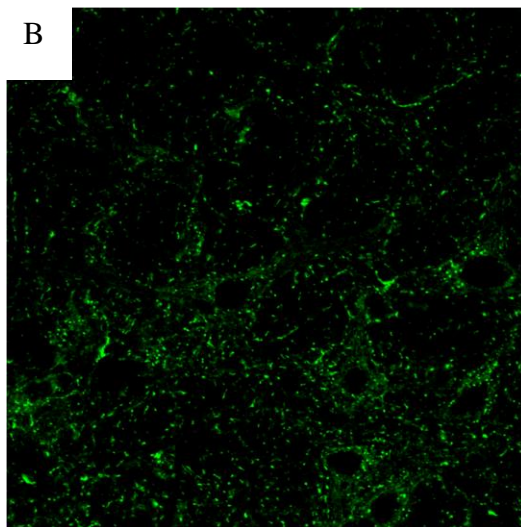
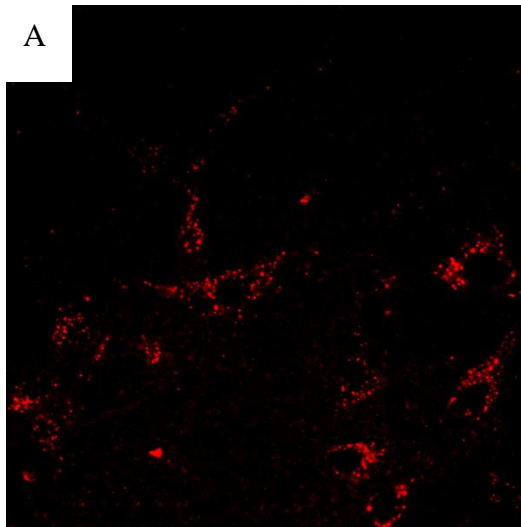


Figure 10. Double Labeling of GiN neurons for CTB tracer, and 5HT_{1A} receptor antibody. (A) CTB was injected in CPGs in the ventral horn of the spinal cord. CTB vesicles in GiN neurons axons and cell bodies are seen in this image. (B) GiN neurons stained for 5HT_{1A} receptor antibody. (C) Double staining of GiN neurons for CTB and 5HT_{1A} receptors antibody

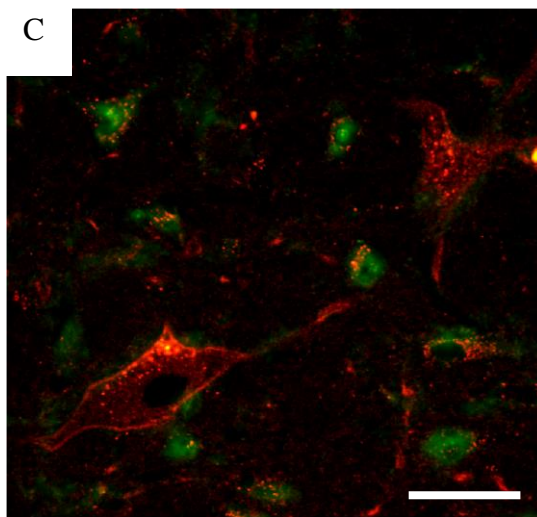
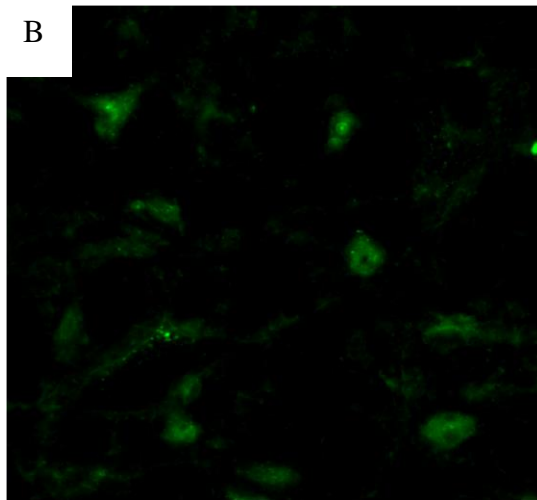
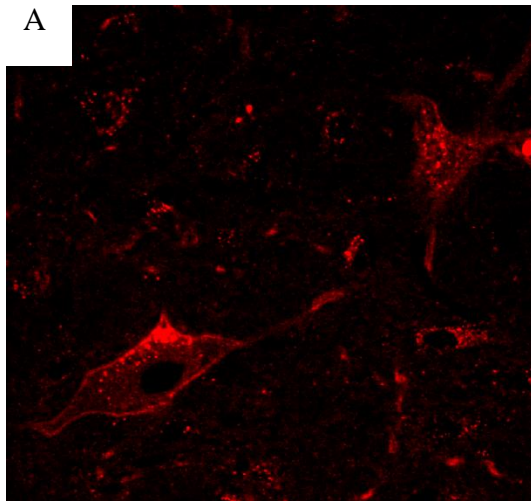


Figure 11. Double Labeling of GiN neurons for CTB tracer, and 5HT_{2C} receptor antibody. (A) CTB was injected in CPGs in ventral horn of the spinal cord. CTB vesicles in GiN neurons axons and cell bodies are seen in this image. (B) GiN neurons stained for 5HT_{2C} receptor antibody. (C) Double staining of GiN neurons for CTB and 5HT_{2c} receptors antibody. Scale bars = 50 μ m

4.4 « Discussion »

The present study provides evidence that GiN descending neurons are controlled by serotonergic projections. Among more than 30 known serotonergic receptor subtypes, we examined the activity of two main receptors: 5HT_{1A} and 5HT₂ family receptors. We found that 5HT_{1A} receptors stimulation caused significant decrease in maximum contact area, print area, and maximum intensity. Also, 5HT₂ receptors inhibition only decreased body speed variation significantly.

Our finding about the role of the GiN in controlling muscle tone is consistent with the findings in many previous studies (Hajnik et al., 2000; Karlsson & Blumberg, 2005; Lai et al., 2010; Martin et al., 2011; Vetrivelan et al., 2009). However, in almost all of these studies, the methods that were used involved electrical stimulation or inhibition of the neurons in the GiN. These studies suggested that the GiN causes atonia during REM sleep (Vetrivelan et al., 2009). Since the DRN changes its level of activity during different sleep cycles (Urbain et al., 2006) and during wakefulness, it can efficiently control GiN neurons.

In previous chapter, we showed that general inhibition of the DRN by lidocaine resulted in impairment of both dynamic and static gait parameters. The dynamic parameters were impaired because of the inhibition of DRN serotonergic projections to the motoneurons and CPGs in spinal cord. It is possible that the static parameters were impaired due to changes in activity of DRN projections to GiN neurons.

One of the biggest controversies about the MLR or PPN's role in controlling the reticulospinal tract is the lack of evidence demonstrating changes in muscle tone or body posture after stimulation or destruction of these structures in numerous studies, which were conducted to date (Gut & Winn, 2015; Hernández-Chan et al., 2011; MacLaren et al., 2014).

In a recent study by Gut et al, Catwalk was used to measure different gait parameters in the 6-OHDA model of PD rats with partial or complete PPN lesions. They only found significant changes in stride length, swing speed, and duty cycle of these rats. These

changes improved after posterior PPN deep brain stimulation and worsened with anterior PPN stimulation. However, they did not report any changes in any static gait parameters.

In conclusion, while review of literature about the role of the PPN and MLR in gait improvement and control of reticulospinal tract is inconclusive, we found that the DRN is a new target which plays an essential role in controlling both muscle tone and dynamic parameters of gait.

Chapter 5

«5HT_{1A} Receptors On Mesencephalic Locomotor Region Neurons Are Responsible for Dynamic Gait Parameters. »

5.1 « Introduction »

Shik and colleagues stimulated the dorsal area of the mesopontine junction in unconscious decerebrated cats by electrodes. Their research showed that with lower frequency stimulation of this area, the animals started to walk. With higher frequency stimulation, the walking pattern of movement changed to running and even galloping (Shik et al., 1966). Since that time, the dorsal area of the mesopontine junction has been identified as the mesencephalic locomotor region (MLR). These findings in cats and other similar findings in other species, like rodents and salamander, suggest that the MLR is the gait initiation center (Cabelguen et al., 2003; Skinner & Garcia-Rill, 1984). The MLR is part of the reticular formation in the mesopontine junction and includes the cuneiform nucleus and pedunculo pontine tegmental nucleus (PPN) (Child & Benarroch, 2013).

Previous studies suggest that serotonergic projections from the DRN have facilitatory effects on motoneurons and CPGs (Noga et al., 2009). In this study, we showed that the DRN play an essential role in controlling gait. In fact, inhibition of these neurons by lidocaine resulted in severe impairment in animals' gait (Chapter 3). We also demonstrated that the DRN may be responsible in controlling muscle tone in rats by sending axons down to the GiN. Therefore, it seems that the DRN is one of the main centers of controlling locomotion in vertebrates. Since the MLR is known as an important gait initiation center, we hypothesized that the DRN is controlling the MLR through 5HT_{1A} receptors. To test our hypothesis, we microinjected a 5HT_{1A} agonist (8OH_DPAT) in the MLR region bilaterally and then studied the animals' gait on Catwalk.

5.2 « Material and Methods »

Ten male Sprague-Dawley rats (10-12 weeks old), weighing 250-270 grams were used. Rats were individually housed in Plexiglas-walled cages with free access to food and water throughout the experiment. The temperature of the room was kept at 22 ± 1 °C on a 12 hour reversed light-dark cycle (lights on from 7 AM to 7PM).

Surgical Procedures

The animals were anaesthetized with ketamine hydrochloride (Vetalar, initial dose 80 mg/kg and maintenance dose 40 mg/kg, via intramuscular) and xylazine (0.8 mg/kg, via intramuscular). Anaesthetized rats were fixed in stereotaxic frame (Kopf Instruments, Tadjunga, CA) and the incisor bar was adjusted until the anterior and posterior parts of skull were at the same level. Stereotaxic coordinates of Paxinos and Watson atlas (2007) were used and the reference point was bregma. Indwelling stainless bilateral steel 27-g guide cannulae were implanted 1.5 mm above the MLR. The coordinates are in millimeters: AP= -8, M= ± 1.9 , DV= -7 for MLR. These rats were kept in their cages for a 7 days of recovery period.

Drugs

8OH-DPAT was purchased from Tocris Cookson (Ellisville, MO, USA).

Gait Studies

One day prior to surgery, all rats underwent Catwalk training to familiarize them to the walkway and to ensure their ability in conducting successful gait trails.

Tight fitting 30-g bilateral injection cannulae were inserted into the guides. They protruded 1.5 mm from the guide cannulae to reach the coordinates mentioned above for the MLR. Infusions were carried out over 1 minute and the injection cannulae were kept in place for another 60 seconds to minimize backflow.

On the first day of the experiment, 0.5 μ l of sterile 0.9% saline was injected in MLR. Five minutes after the injection the animals were placed on Catwalk glass, where they were able to walk freely with their normal pace. For each vehicle or drug injection for each

animal, at least three successful gait trials were recorded. A gait trial is considered successful if the animal starts walking from one side of the glass plate and finishes its gait to the other side of the glass without turning back.

Baseline gait parameters were recorded after saline injection. An hour after the saline injection, 5 μ M of sterile 8OH-DPAT (Pham-Le et al., 2011) was injected in the MLR. Five minutes after the injection the rats were placed on Catwalk and their gait was measured.

Histology

Upon completion of experiments, animals received an overdose of sodium pentobarbital and were perfused transcardially with 0.9% NaCl (containing 1 ml/lit heparin) followed by 4% formaldehyde solution (in 0.1 M phosphate buffer). The brains and spinal cords were removed and stored in 4% formaldehyde solution for 48 hours and then, 26% glycerol solution until they were settled.

To visualize colocalization of the CTB retrograde tracer (from animals in chapter 2, in which CTB was microinjected in CPGs) with 5HT_{1A} in the MLR, 40 μ m coronal sections were cut using a microtome and every fifth section was stained for 5HT_{1A} receptors.

A rabbit anti-5HT_{1A} primary antibody (SR-1A Antibody, Santa Cruz Biotechnology) and a Biotinylated Goat Anti-Rabbit IgG secondary Antibody (Vector Laboratories) were used. Then sections were studied under Olympus FV1000 microscope.

To verify the placement of the cannulae, 0.5 μ l of a 4% methylene blue solution was infused in the MLR. The extension of the dye after half an hour was considered as an indication of the presumed diffusion of the normal saline or 8OH-DPAT, which was given to each animal during the experiments. In this part of the experiment, 4 animals with an inaccurate cannulae site were excluded from our study.

Statistical Analysis

In this study, gait parameters of six animals in each group were measured, similar studies on Catwalk apparatus have shown that this number of the subjects provides a valid data

set (Chuang et al., 2010; Mendes et al., 2015; Vandeputte et al., 2010; Zhou et al., 2015). All the recorded videos were double-checked to correct any possible errors that the Catwalk software might have made in labeling the paws. All walkway crossings were analyzed using the Catwalk software data. Each gait parameter is expressed as mean \pm S.E.M (Standard error of the mean). SPSS statistical software (version 19) was used to analyze all parameters. Statistical significance was evaluated by the Friedman Test and group differences were determined by the non-parametric Wilcoxon test. Since the number of the subjects we used in each group of the animals was small (n=6), normalization of the data was not appropriate, therefore, we used Wilcoxon test which does not assume normality in the data. In this study, a P value lower than 0.05 was considered to be statistically significant.

5.3 « Results »

Verifying Cannulae Placement

Ten male Sprague-Dawley rats underwent surgery and double cannulas were inserted in the MLR (mainly in the PPN). To verify the placement of the cannulas, 1 μ l of a 4% methylene blue solution was infused through the cannulae and half an hour later animals were sacrificed. Sections were prepared and the cannulae sites were verified under the microscope (**Figure12**). Four of the animals were excluded due to an inaccurate cannulae injection site.

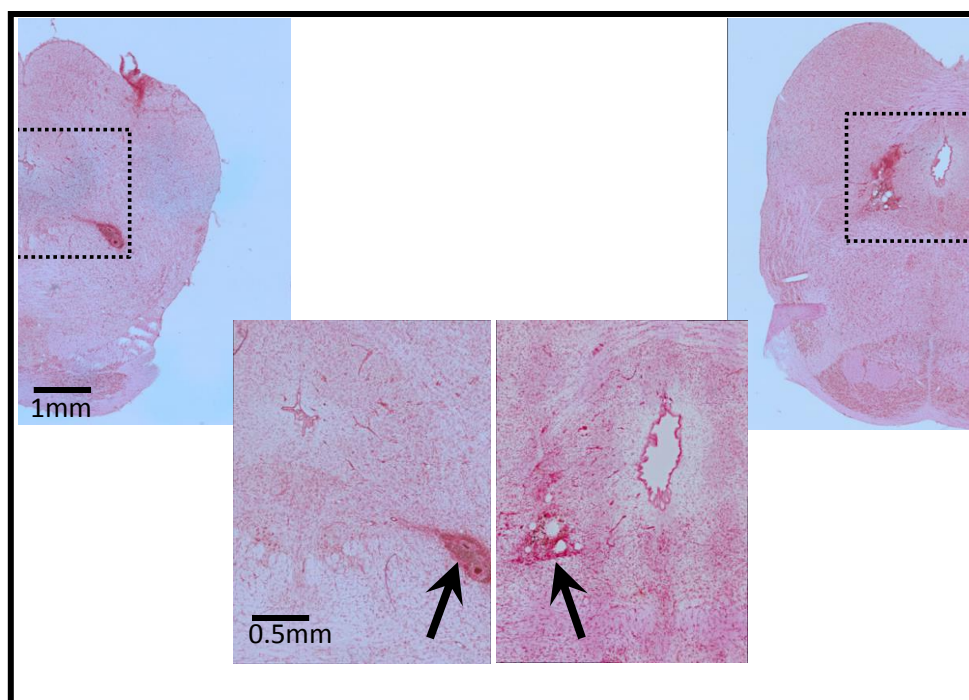


Figure12. Cannula site at the MLR. 0.5 μ l Methylene blue 4% was microinjected through cannula. After making sections Nissl Staining with neutral red color is done on the sections. Two upper sections are presenting cannula site at caudal part (Right upper image), and rostral part (left upper image) of the MLR in the same animal. Lower images are higher magnification of upper sections' injection site, black arrows show the injection site.

Effect of 5HT_{1A} agonist injection in MLR on gait.

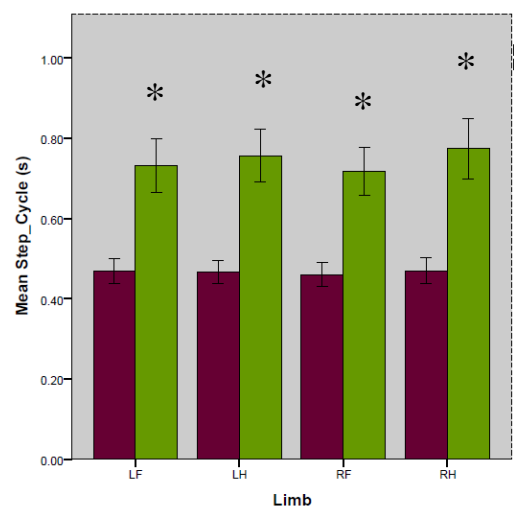
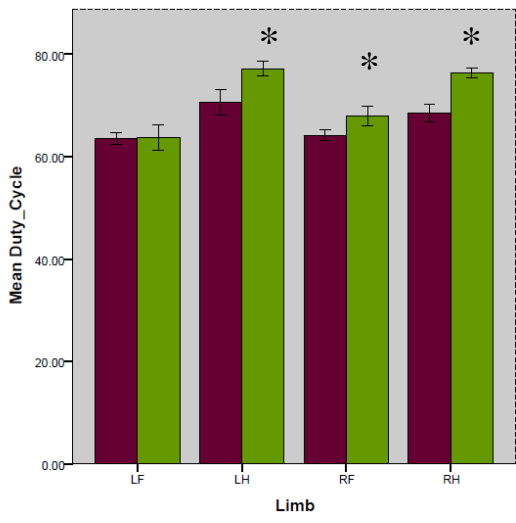
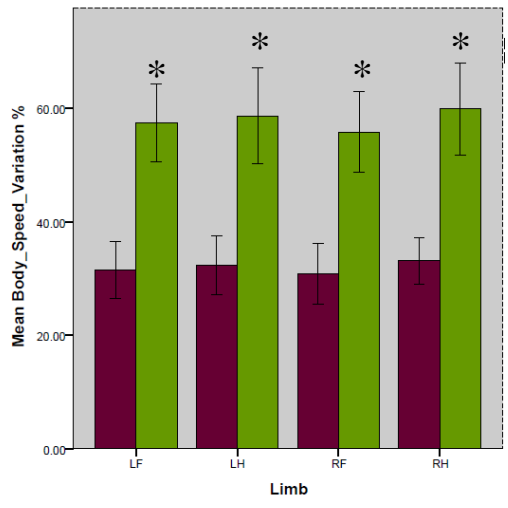
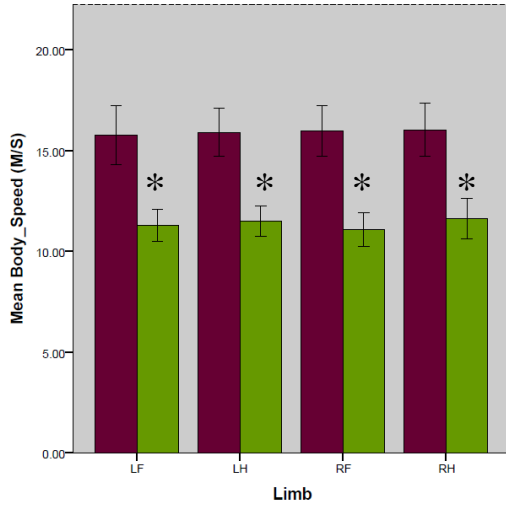
The vehicle's effect on gait was tested by bilateral injection of 0.5 μ l normal saline in the MLR and studying the rats' gait on Catwalk. One hour after vehicle injection, 5 μ M of 8OH-DPAT, a 5HT_{1A} receptor agonist was microinjected bilaterally (Pham-Le et al., 2011) and the rats were placed on Catwalk. While we tested the rats on Catwalk, we noticed several mild episodes of freezing of gait in rats. These freezing episodes caused significant increase in body speed variation in all four limbs, $P= 0.048$ (**Table9 and Figure 13**). Stand duration also increased but the swing phase duration did not change significantly, which resulted in significant increase in duty cycle, which Expresses Stand as a percentage of Step Cycle . In addition, there was a significant decrease in body

speeds and stride length (**Table 9**). The key findings in this part of the study are summarized in **Figure 13**.

Table9. Key Findings after injection of 5HT_{1A} in MLR. Mean \pm standard deviation is measured for each parameter in individual limbs. P values <0.05 are considered significant and

Dynamic Gait Parameters	Normal		P value
	Saline	8OH-DPAT	
Stand RF	0.3 \pm 0.05	0.51 \pm 0.14	0.046
Stand RH	0.35 \pm 0.07	0.62 \pm 0.14	0.046
Stand LF	0.3 \pm 0.05	0.5 \pm 0.17	0.046
Stand LH	0.3 \pm 0.05	0.5 \pm 0.17	0.046
Swing RF	0.16 \pm 0.02	0.19 \pm 0.03	0.075
Swing RH	0.13 \pm 0.009	0.13 \pm 0.009	0.345
Swing LF	0.16 \pm 0.01	0.22 \pm 0.03	0.028
Swing LH	0.12 \pm 0.02	0.127 \pm 0.001	0.917
Swing Speed RF	51 \pm 8.1	39.8 \pm 5.3	0.028
Swing Speed RH	57.8 \pm 7.4	52.3 \pm 8.05	0.173
Swing Speed LF	50.9 \pm 7.09	38.4 \pm 5.1	0.028
Swing Speed LH	62.1 \pm 9.6	54.1 \pm 5.3	0.116
Stride Length RF	7.46 \pm 0.67	6.5 \pm 0.73	0.028
Stride Length RH	7.35 \pm 0.63	6.75 \pm 0.63	0.028
Stride Length LF	7.48 \pm 0.75	6.45 \pm 0.49	0.028
Stride Length LH	7.5 \pm 0.51	6.7 \pm 0.55	0.028
Step Cycle RF	0.46 \pm 0.07	0.71 \pm 0.14	0.028
Step Cycle RH	0.46 \pm 0.07	0.77 \pm 0.18	0.028
Step Cycle LF	0.47 \pm 0.07	0.73 \pm 0.16	0.028
Step Cycle LH	0.46 \pm 0.06	0.75 \pm 0.15	0.028
Body Speed RF	15.96 \pm 3.07	11.07 \pm 2.04	0.028
Body Speed RH	16.02 \pm 3.18	11.6 \pm 2.4	0.028
Body Speed LF	15.76 \pm 3.5	11.3 \pm 1.9	0.028
Body Speed LH	15.9 \pm 2.6	11.5 \pm 1.7	0.028
Body Speed Variation RF	30.9 \pm 13.08	55.8 \pm 17.4	0.046
Body Speed Variation RH	33.09 \pm 9.35	59.9 \pm 18.09	0.046
Body Speed Variation LF	31.5 \pm 12.3	57.46 \pm 16.9	0.046
Body Speed Variation LH	32.39 \pm 12.7	58.68 \pm 20.7	0.046
Duty Cycle RF	64.1 \pm 2.66	67.9 \pm 4.78	0.046
Duty Cycle RH	68.5 \pm 4.25	76.4 \pm 2.38	0.028
Duty Cycle LF	63.5 \pm 2.88	63.7 \pm 6.03	0.753
Duty Cycle LH	70.6 \pm 6.06	77.1 \pm 3.5	0.028

Stand Index RF	5.08±0.77	3.01±1.01	0.028
Stand Index RH	2.94±1.1	2.07±0.5	0.116
Stand Index LF	5.32±1.46	3.19±0.66	0.028
Stand Index LH	2.46±0.63	1.67±0.36	0.028



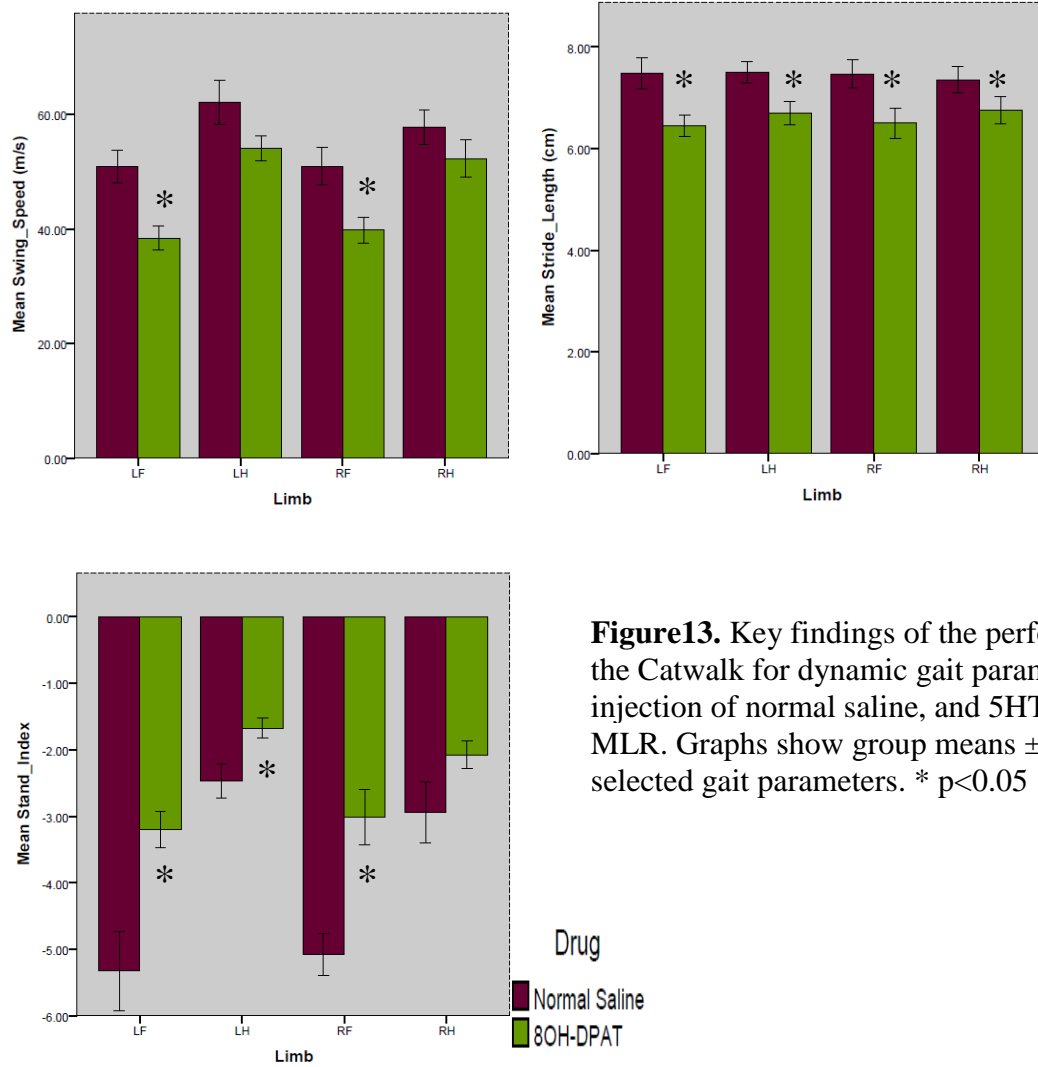


Figure 13. Key findings of the performance on the Catwalk for dynamic gait parameters after injection of normal saline, and 5HT_{1A} agonist in MLR. Graphs show group means \pm SEM of selected gait parameters. * $p < 0.05$

Immunohistochemistry Labeling for 5HT_{1A} in MLR.

After observing significant changes in gait by injection of 5HT_{1A} agonist in the MLR, we performed immunohistochemistry studies to find 5HT_{1A} receptors on MLR neurons.

These neurons were already labeled with CTB injected in CPGs of the lumbar spinal cord as described in chapter 2. We found a dense population of 5HT_{1A} receptors in the MLR.

The images are shown in **Figure 14**.

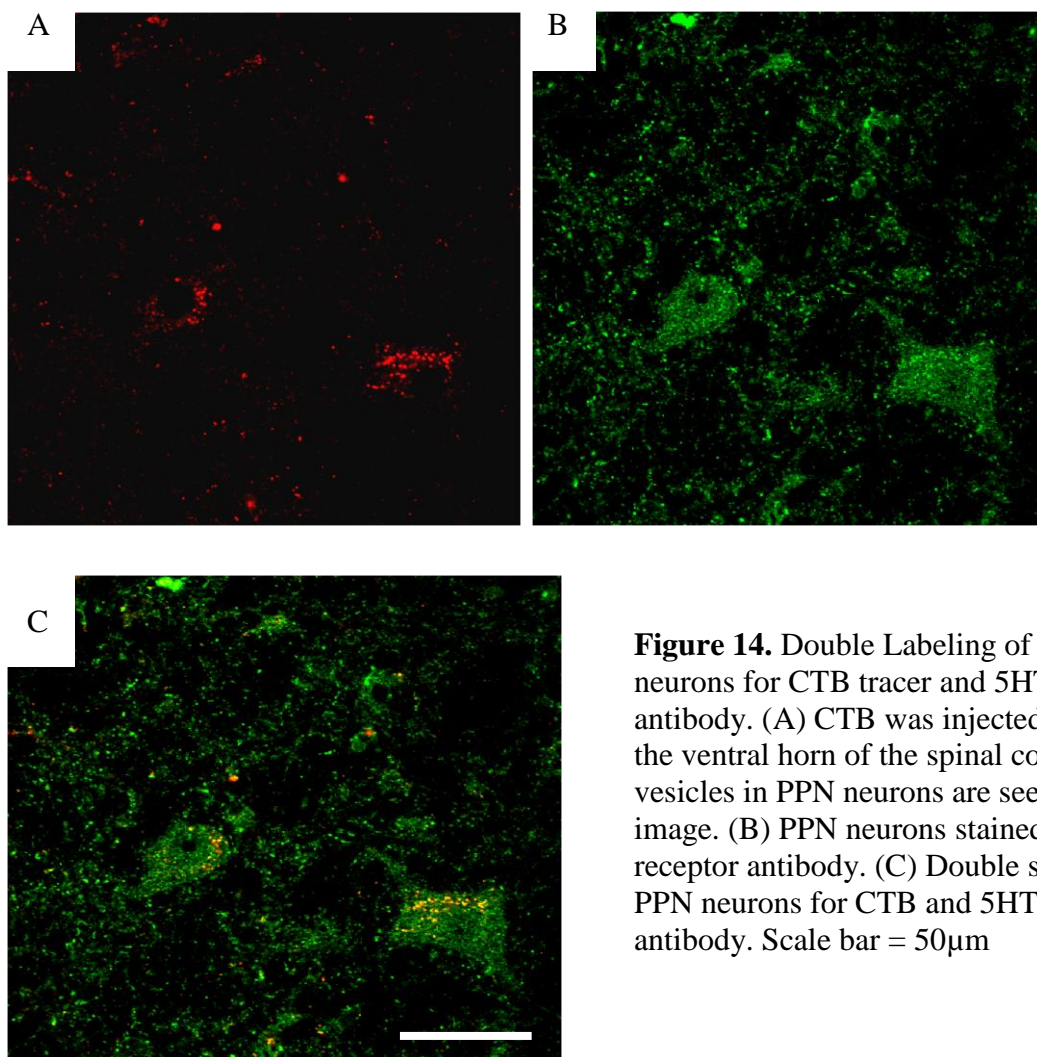


Figure 14. Double Labeling of the PPN neurons for CTB tracer and 5HT_{1A} receptor antibody. (A) CTB was injected in CPGs in the ventral horn of the spinal cord. CTB vesicles in PPN neurons are seen in this image. (B) PPN neurons stained for 5HT_{1A} receptor antibody. (C) Double staining of PPN neurons for CTB and 5HT_{1A} receptors antibody. Scale bar = 50µm

5.4 « Discussion »

In this part of the study, we found that 5HT_{1A} receptors on MLR neurons strictly control their gait features. Activation of these receptors by 5HT_{1A} agonist, 8OH-DPAT, resulted in significant changes in dynamic gait parameters, but none of the static parameters.

Comparing this data to the results of chapter 3, where we inhibited DRN with lidocaine, it seems that the patterns in which these two centers affect dynamic gait parameters are different. Inhibition of the DRN resulted in significant increase in duration of both stand

and swing phases, which lead to increase in step cycle duration. However, since the ratio between stand and swing phases remained constant, there was no significant change in duty cycle index, which measures stand as a percentage of step cycle. In contrast, injection of 5HT_{1A} agonist to the MLR resulted in significant increase in duty cycle due to the increase in stand phase without altering swing phase duration.

In 1966, when Shik and colleagues identified the MLR for the first time, they also emphasized a unique property of these neurons: increasing frequency of electrical stimulation in these neurons resulted in increased speed (Orlovskiĭ, Severin, & Shik, 1966; Shik et al., 1966). In other words, by increasing the frequency, these animals changed their gait pattern from walking to running, and eventually galloping. Biomechanical gait analysis in different speeds showed that an increase in gait speed leads to a decrease in stand duration, which decreases duty cycle. Moreover, by decreasing the gait speed, stand duration increases and thus, duty cycle increases (Hebenstreit et al., 2015). Therefore, biomechanical analysis of our rats gait after injection of 5HT_{1A} agonist in the MLR shows that the MLR determines the gait speed. One of the main neurotransmitters in controlling the gait speed is serotonin.

But what makes the MLR and its components (PPN and cuneiform nucleus) appropriate to control the gait speed? Recent studies suggest that the posterior part of the PPN receives a considerable amount of fast sensory input, which it projects to the substantia nigra pars compacta. These projections contain information about the value and salience of the incoming sensory information received by the PPN (Hong & Hikosaka, 2014). As a result, there is a potential role for PPN in gait due to its essential role in integration of sensory information. For instance, MacLaren et al. showed that the PPN is not important in fixed speed gait in rats. However, bilateral PPN cholinergic neuronal loss will result in severely impaired gait on the accelerated rotarod. Gait on a fixed speed rotarod is considered an automatic and reflexive behavior. However, a successful gait on an accelerated speed rotarod requires the motor system to continuously adjust to changes from incoming sensory inputs. In other words, one of the ways that the PPN adapts gait to incoming sensory changes is by altering gait speed, stopping gait and initiating gait.

In addition to a significant decrease in body speed, body speed variation increased and stride length decreased significantly. These three changes are the essential features of an abnormal gait in Parkinson disease patients. There are numerous postmortem studies which suggest that there is a significant decrease in both DRN and PPN neurons in patients in late stages of PD (Buddhala et al., 2015; Frisina, Haroutunian, & Libow, 2009; Pienaar et al., 2013). Therefore, two main gait centers in the brainstem show severe neurodegeneration by the time axial and gait symptoms start to emerge in PD patients.

To sum up, our studies and previous studies suggest that there are two main gait controlling centers in the brainstem, the DRN and MLR. The DRN shows the highest level of activity during wakefulness (specifically while the animal is moving) and lowest level of activity during sleep (specifically during REM sleep when muscles are completely atonic) (Aghajanian & Vandermaelen, 1982; Urbain et al., 2006). The DRN is also important in footfall pattern and according to the literature, it controls it through 5HT₂ receptors on the surface of motoneurons in the spinal cord (Aghajanian & Vandermaelen, 1982; Courtine et al., 2009). Finally, our studies suggest that through inhibition of the DRN with lidocaine, the regular footfall pattern disappears. Therefore, we conclude that the DRN mainly controls automatic aspects of gait, which are not dependent to dynamic changes of sensorimotor inputs. It also acts as a switch which turns on or turns off the CPGs in the lumbar spinal cord. This depends on the general state of animals in terms of mobility or immobility, as well as sleep and wakefulness.

On the other hand, the MLR is another brainstem center controlling gait. The MLR receives fast sensory inputs from tracks of the spinal cord, in addition to extensive inputs from basal ganglia structures such as the subthalamic nucleus and substantia nigra pars reticulata. Thus, it seems the MLR mainly controls the aspects of gait that need intact cognitive function, decision making, and goal orientation.

Chapter 6

« Investigating the Role of 5HT_{1A} receptors on Dorsal Raphe Nucleus Neurons in Gait. »

6.1 « Introduction »

Previous studies have established that 5HT_{1A} receptors are expressed on the surface of serotonergic neurons in the DRN (Biegon, Rainbow, & McEwen, 1982; Weissmann-Nanopoulos, Mach, Magre, Demassey, & Pujol, 1985). The number of 5HT_{1A} receptors in the anterior part of the DRN is significantly higher than the posterior part (Weissmann-Nanopoulos et al., 1985), this gradient potentially correlates to the different roles of these neurons. The DRN is known to be important in different disorders like depression, addiction to drugs and alcohol, sleep disorders, and stress-related disorders (Chang et al., 2011; Waselus, Valentino, & Van Bockstaele, 2011). Therefore, many studies are focusing on the role of 5HT_{1A} receptors in the pathophysiology of these disorders (Gannon & Millan, 2006; Koprowska, Krotewicz, Romaniuk, Strzelczuk, & Wiczorek, 2002; You et al., 2015).

In previous chapters, we demonstrated that the DRN is controlling two important gait centers in the brainstem: the MLR, and GiN. We hypothesized that 5HT_{1A} receptors are playing an important role in modulating the activity of DRN neurons in gait. To test our hypothesis, we microinjected 8OH-DPAT, a 5HT_{1A} agonist, in the DRN of rats and then studied their gait by Catwalk apparatus.

6.2 « Material and Methods »

Six male Sprague-Dawley rats (10-12 weeks old), weighing 250-270 grams were used. Rats were individually housed in Plexiglas-walled cages with free access to food and

water throughout the experiment. The temperature of the room was kept at 22 ± 1 °C on a 12 hour reversed dark-light cycle (lights on from 7 AM to 7PM).

Surgical Procedures

The animals were anaesthetized with ketamine hydrochloride (Vetalar, initial dose 80 mg/kg and maintenance dose 40 mg/kg, via intramuscular) and xylazine (0.8 mg/kg, via intramuscular). Anaesthetized rats were fixed in stereotaxic frame (Kopf Instruments, Tazunga, CA) and the incisor bar was adjusted until the anterior and posterior parts of skull were at the same level. Stereotaxic coordinates of Paxinos and Watson atlas (2007) were used and the reference point was bregma. Indwelling stainless steel 27-g guide cannulae were implanted 1.5 mm above the posterior border of DRN. The coordinates are in millimeters: AP= -8.3, M=0, DV= -6.4 for DRN. These rats were kept in their cages for a 7 days of recovery period.

Gait Studies

One day prior to surgery, all rats underwent Catwalk training to familiarize them to the walkway and to ensure their ability in conducting successful gait trails.

Tight fitting 30-g injection cannulae were inserted into the guides. They protruded 1.5 mm from the guide cannulae to reach the coordinates mentioned above for the DRN.

Infusions were carried out over 1 minute and the injection cannulae were kept in place for another 60 seconds to minimize backflow.

During the first day of the experiment, 0.5µl of sterile 0.9% saline was injected in the DRN. Five minutes after the injection, the animals were placed on Catwalk, where they were able to walk freely with their normal pace. For each vehicle or drug injection of each animal, at least three successful gait trials were recorded. A gait trial is considered successful if the animal starts walking from one side of the glass plate and finishes its gait to the other side of the glass without turning back.

The vehicle's effect on gait was tested by bilateral injection of 0.5 µl normal saline in the DRN and studying the rats' gait on Catwalk. An hour after the saline injection, 10µM of 8OH-DPAT, a 5HT_{1A} receptor agonist, was injected in the DRN. Five minutes after the injection, the rats were placed on Catwalk and their gait was measured.

Histology

Upon completion of experiments, animals received an overdose of sodium pentobarbital and were perfused transcardially with 0.9% NaCl (containing 1 ml/lit heparin) followed by 4% formaldehyde solution (in 0.1 M phosphate buffer). The brains and spinal cords were removed and kept in 4% formaldehyde solution for 48 hours and then, 26% glycerol solution until they were settled.

To visualize colocalization of the CTB retrograde tracer (from animals in chapter 2, in which CTB was microinjected in CPGs) with 5HT_{1A} autoreceptors in the DRN, 40µm coronal sections were cut using a microtome and every fifth section was stained for 5HT_{1A} receptors.

Rabbit anti-5HT_{1A} primary antibody (SR-1A Antibody, Santa Cruz Biotechnology) and a Biotinylated Goat Anti-Rabbit IgG secondary Antibody (Vector Laboratories) were used. Then sections were studied under Olympus FV1000 microscope.

To verify the placement of the cannulae, 0.5µl of a 4% methylene blue solution was infused in the DRN. The extension of the dye after half an hour was considered as an indication of the presumed diffusion of the normal saline or 8OH-DPAT, which was given to each animal during the experiments.

Statistical Analysis

In this study, gait parameters of six animals in each group were measured, similar studies on Catwalk apparatus have shown that this number of the subjects provides a valid data set (Chuang et al., 2010; Mendes et al., 2015; Vandeputte et al., 2010; Zhou et al., 2015). All the recorded videos were double-checked to correct any possible errors that the Catwalk software might have made in labeling the paws. All walkway crossings were analyzed using the Catwalk software data. Each gait parameter is expressed as mean ± S.E.M (Standard error of the mean). SPSS statistical software (version 19) was used to

analyze all parameters. Statistical significance was evaluated by the Friedman Test and group differences were determined by the non-parametric Wilcoxon test. Since the number of the subjects we used in each group of the animals was small (n=6), normalization of the data was not appropriate, therefore, we used Wilcoxon test which does not assume normality in the data. In this study, a P value lower than 0.05 was considered to be statistically significant.

6.3 « Results »

Effect of 5HT_{1A} agonist injection in DRN on gait.

The vehicle's effect on gait was tested by injection of 0.5 µl normal saline in the DRN and studying the rats' gait on Catwalk. After injection of 10µM 8OH-DPAT, the rats were placed on Catwalk and their gait was recorded. Statistical analysis of the data showed that swing phase significantly decreased. Additionally, the data demonstrated that swing speed increased in all limbs, but was only significant in the right hind and left front limbs. There was no significant change in stance phase; however the changes in swing and swing speed resulted in increased body speed while the forelimbs were actively moving. The key findings are summarized in **Table 10** and **Figure15**.

Table10. Selected gait parameters after injection of 8OH-DPAT in the DRN. Mean ± standard deviation is measured for each parameter in individual limbs. P values <0.05 are considered significant and are highlighted with light green color.

Gait Parameters	Normal		P Value
	Saline	8OH-DPAT	
Swing RF	0.271±0.08	0.209±0.04	0.046
Swing RH	0.187±0.04	0.158±.02	0.046
Swing LF	0.275±0.1	0.225±0.05	0.468
Swing LH	0.192±0.06	0.161±0.02	0.435

Swing Speed RF	96.27±28.06	107.86±28.7	0.075
Swing Speed RH	122.9±25	131.1±24.6	0.046
Swing Speed LF	90.4±34.9	112.6±31.9	0.028
Swing Speed LH	120.41±29.3	130.5±24.8	0.173
Body Speed RF	29.9±12.4	33.9±12.8	0.046
Body Speed RH	30.8±11.2	34.38±9.97	0.116
Body Speed LF	28.8±12.2	33.7±10.6	0.046
Body Speed LH	30.8±10.1	34.2±9.5	0.116

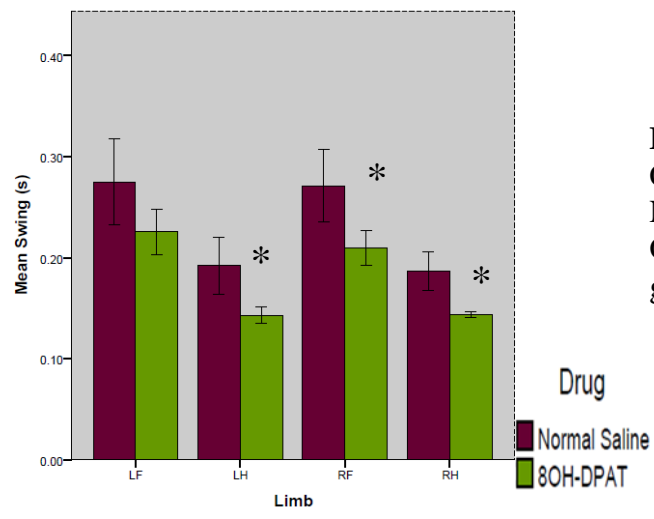
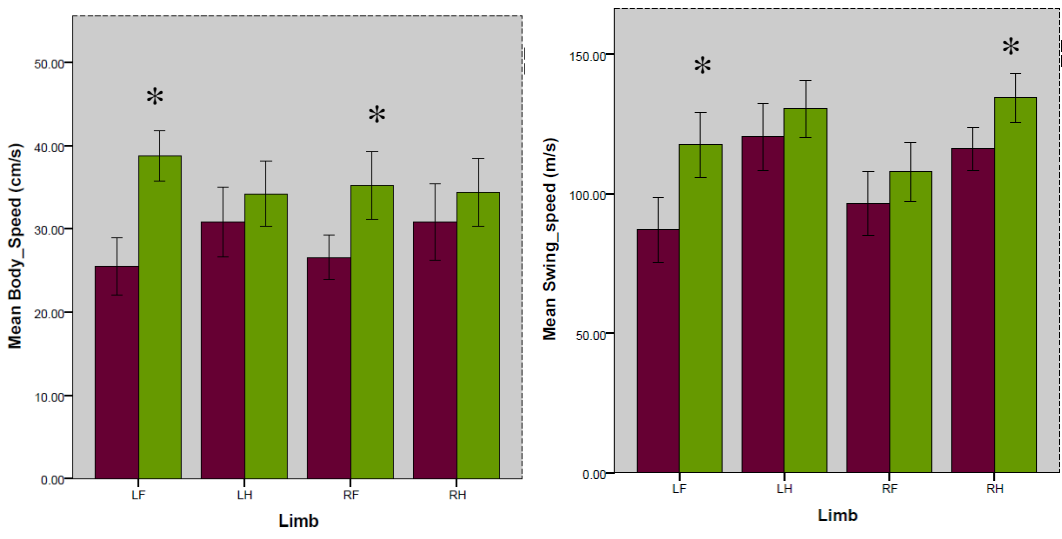
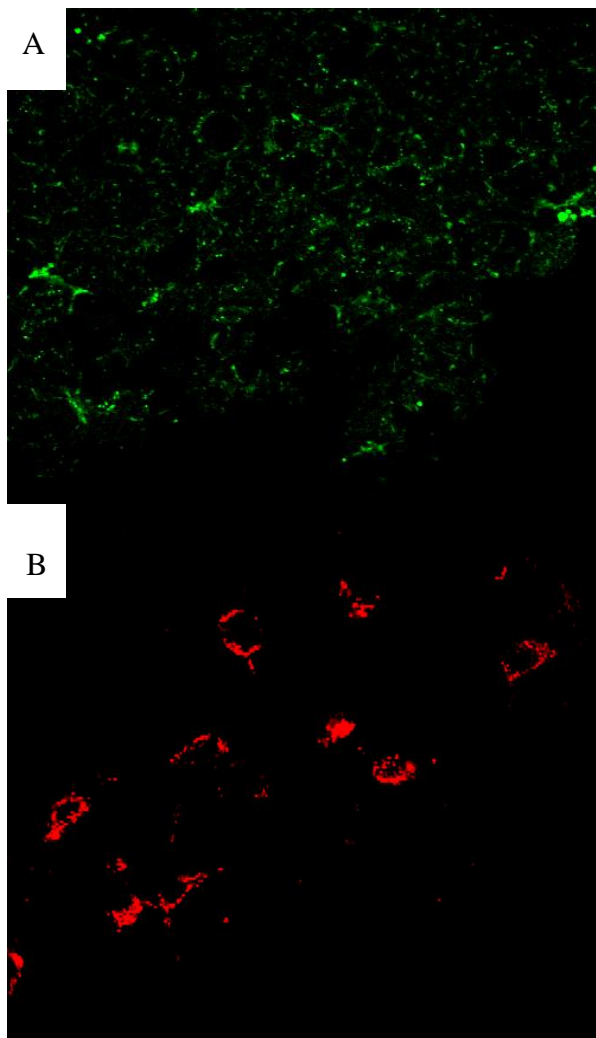


Figure15. Key Findings of the Performance on Catwalk for Gait Parameters after injection of Normal Saline, and 5HT1A Agonist in DRN. Graphs show group means ± SEM of selected gait parameters. * p<0.05

Immunohistochemistry labeling for 5HT_{1A} in MLR.

After significant changes in gait by injection of 5HT_{1A} agonist in the DRN were found, we performed immunohistochemistry studies to find 5HT_{1A} receptors on DRN neurons, which were already labeled with CTB injected in the GiN in the second chapter. We found 5HT_{1A} receptors on these neurons. The images are shown in **Figure 16**.



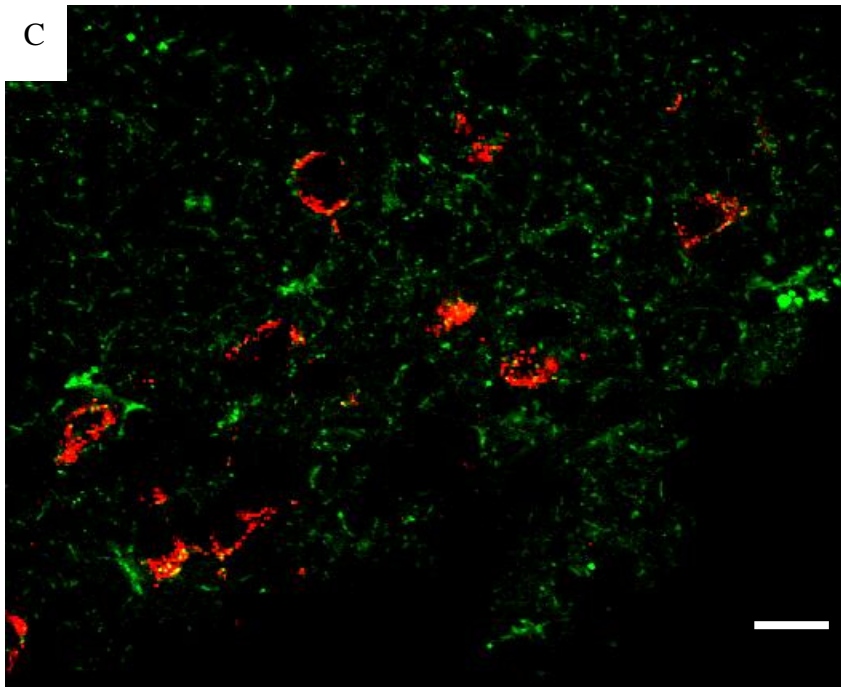


Figure16. Double Labeling of DRN neurons for CTB tracer, and 5HT_{1A} receptors antibody. (A) CTB was injected in CPGs in GiN. CTB vesicles in DRN neurons are seen in this image. (B) DRN neurons stained for 5HT_{1A} receptor antibody. (C) Double staining of DRN neurons for CTB and 5HT_{1A} receptors antibody. Scale bar = 20μm

6.4 « Discussion »

In this part of the study, we found that 5HT_{1A} receptors stimulation in the DRN changed swing phases in gait cycle. Swing duration in all limbs decreased, but it was not significant in the left front limb. Additionally, swing speed also increased, but they were only significant in two limbs: right hind and left front limbs. These changes result in increased body speed in anterior limbs while these limbs were actively moving. To avoid affecting the MLR which are located anterolateral to DRN, we inserted cannulas in the aqueduct canal and the needle tips of the injector cannulas were just touching the dorsal border of the DRN. Therefore, there is a possibility that the drug is not distributed to all

areas of the DRN and this fact might explain why significant changes in swing phase appeared only in some limbs but not all four limbs.

There is an anteroposterior gradient in the density of 5HT_{1A} receptors. The density is higher in the ventral part compared to the dorsal part of the DRN. In chapter two, we injected cholera toxin beta subunit, a neural retrograde tracer in the GiN, and we found that the neurons which project to the GiN are mostly located in the posterior part of the DRN. Therefore, a low density of 5HT_{1A} receptors on serotonergic neurons sending projections down to the GiN in our study is consistent with previous studies (Weissmann-Nanopoulos et al., 1985)

Since the DRN is known to play a crucial role in controlling sleep/wake cycles, mood, stress level, and appetite (Chang et al., 2011; Waselus et al., 2011), several studies are currently focusing on physiological mechanisms that control serotonergic neurons in the DRN. 5HT_{1A} receptors are important modulators of serotonergic activity in the DRN, and studies have shown that these receptors might play an important role in the pathophysiology of disorders like drug and alcohol abuse, sleep disorders, and depression (Gannon & Millan, 2006; Koprowska et al., 2002; You et al., 2015). Here we found that 5HT_{1A} receptors in the DRN are also important in controlling gait by affecting swing phase. However, due to the low density of these receptors on serotonergic neurons that control gait, they might not be as important in gait as they are in controlling other DRN functions.

Chapter 7

7 « General Discussion »

The main findings in this study are listed here: 1) The DRN, MLR, and GiN, project directly to the ventral horn of the lumbar spinal cord where lower motoneurons and CPGs for locomotion are located (Chapter 2). 2) The DRN also sends projections to the GiN, however, MLR does not send projections to the GiN (Chapter 2). 3) Inhibition of the neurons in the DRN with lidocaine resulted in significant changes in both static and dynamic gait parameters (Chapter 3). 3) The DRN affects static gait parameters through its projections to the GiN, and 5HT_{1A} receptors on GiN neurons are responsible for this effect (Chapter 4). 4) There is a dense population of 5HT_{1A} receptors on MLR, and activation of these receptors with 8OH-DPAT, a 5HT_{1A} specific agonist, resulted in significant changes in almost all dynamic gait parameters tested but none of the static parameters were changed (Chapter 5). 5) 5HT_{1A} receptors are also present on the surface of the neurons in the DRN, while their effect on gait was limited to the swing phase of the gait cycle (Chapter 6). The main findings of this study are summarized in **Figure 17**.

The effect of the direct projections from the DRN to the motoneurons in the ventral horn of the spinal cord are well studied (Barreiro-Iglesias et al., 2008; Ghosh & Pearce, 2015), and it has been proposed that this pathway might facilitate activation of the motoneurons and CPGs. Consequently, by inhibiting DRN neurons directly with lidocaine, we expected to see impairment in dynamic gait parameters; however, we found that both dynamic and static gait parameters were impaired.

In addition, DRN neurons projected directly to the lower motoneurons and locomotor CPGs in the ventral horn of the spinal cord. We identified a novel pathway from DRN to the GiN. The DRN neurons send projections to the medial part of the rostral medulla, where GiN is located. Prior literature has shown that electrical stimulation of the neurons at GiN, resulted in changes in muscle tone during both sleep, and awake state (Lai et al., 2010). The discovery of the pathway from the DRN to the GiN in the current study may

explain the changes we saw in static gait parameters after injections of the lidocaine in the DRN. To clarify the role of this new pathway in gait and to identify the types of the serotonergic receptors on GiN neurons, we have investigated the effect of agonists and antagonists for 5HT_{1A} and 5HT₂ receptors, respectively. The Catwalk study results suggested that 5HT_{1A} receptors are at least one of the serotonergic receptors controlling the gait parameters in the GiN when animals walk freely.

Prior studies have shown that PPN and specifically cholinergic neurons in the PPN send projections to the GiN (Bachmann et al., 2013; Rye et al., 1988; Skinner & Garcia-Rill, 1984). Previous studies of the PPN and MLR investigating gait, failed to find changes in muscle tone and static gait parameters following destruction, inhibition or stimulation of these structures (Bachmann et al., 2013; MacLaren et al., 2014; Smetana et al., 2010; Wen et al., 2015). In a recent study, Gut et al. (Gut & Winn, 2015) employed Catwalk method to measure different gait parameters in the 6-OHDA model of PD rats with partial or complete PPN lesions. They only found significant changes in stride length, swing speed, and duty cycle of these rats. These changes improved after posterior PPN deep brain stimulation and worsened with anterior PPN stimulation. However, they did not report any changes in any static gait parameters.

In this study, we injected the retrograde tracer CTB in the GiN, but only DRN neurons in the mesopontine junction were labeled for the tracer, and the number of the neurons in the MLR which were labeled from the tracer was negligible. Although we did not find a pathway from the MLR to the GiN, instead, we found that a considerable number of neurons in the MLR send direct projections to the lower motor neurons and the CPGs of the lumbar spinal cord. In agreement with this, a recent study by Sherman et al. (Sherman et al., 2015) showed that the MLR in the mesopontine junction does not send projections to the medullary reticular formation, rather sends direct projections down to the spinal cord motoneurons.

We then tested the effect of 5HT_{1A} agonist in the MLR while the animals were walking on the Catwalk. We found that the body speed and the stride length were significantly decreased. Meanwhile, the ratio between the stand phase duration and the step cycle

duration were changed, indicating changes in the gait pattern. These findings might be explained by the direct projections from the MLR to the lower motoneurons and CPGs of the lumbar spinal cord we found in the present study.

In addition, PPN also receives extensive inputs from basal ganglia structures such as the subthalamic nucleus and substantia nigra pars reticulata (Child & Benarroch, 2013; Sherman et al., 2015; Tattersall et al., 2014). As a result, there is a potential role for PPN in controlling the aspects of the gait that are dependent on intact decision making and cognitive function of the brain.

To sum up, our results along with previous findings indicate that there are two main gait controlling centers in the brainstem, namely, the DRN and MLR (**Figure 17**). The DRN shows the highest level of activity during wakefulness (specially, while the animal is moving) and lowest level of activity during sleep (specially, during REM sleep when muscles are atonic) (Aghajanian & Vandermaelen, 1982; Urbain et al., 2006). The DRN is also important in mediating the foot-fall pattern via 5HT₂ receptors on the surface of motoneurons in the spinal cord (Aghajanian & Vandermaelen, 1982; Courtine et al., 2009). Finally, our studies suggest that through inhibition of the DRN with lidocaine, the normal foot-fall pattern disappears. Therefore, we conclude that the DRN mainly controls automatic aspects of gait, which are not dependent to dynamic changes of sensorimotor inputs. In addition, it may act as a switch which turns on or turns off the CPGs in the lumbar spinal cord. This depends on the general state of animals in terms of mobility or immobility, as well as sleep and wakefulness.

On the other hand, the MLR is another brainstem center controlling gait. The MLR receives fast sensory inputs from the spinal cord, in addition to extensive inputs from basal ganglia structures such as the subthalamic nucleus and substantia nigra pars reticulata. Thus, it seems that the MLR mainly controls the aspects of gait that require intact cognitive function, decision making, and goal-orientated.

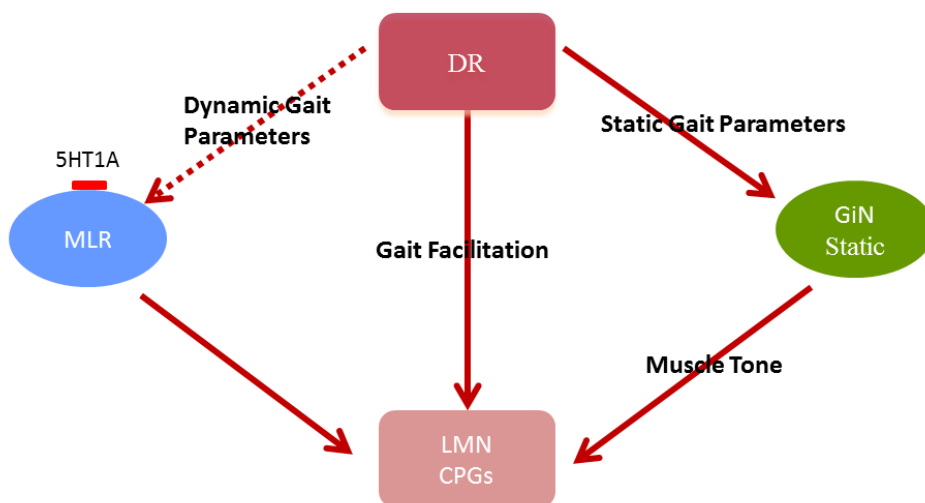


Figure17. A proposed circuitry controlling gait parameters. DRN sends projections to the GiN, ventral horn of the spinal cord and possibly to the MLR. DRN controls the static gait parameters through its projections to the GiN, and controls dynamic gait parameters through possible projections to the MLR. LMN: Lower Motor Neurons

Parkinsonian gait is characterized as slow gait with short steps. We found the similar gait pattern in our rats after the injection of 5HT_{1A} agonist, in the MLR: there was a significant decrease in body speed, and stride length, and a significant increase in body speed variation. Based on these findings, degeneration of the DRN or PPN or both of these structures will result in the abnormal gait pattern we saw after activation of 5HT_{1A} receptors in MLR. Post-mortem studies indicate a significant loss of both DRN and PPN neurons in late stages of PD (Buddhala et al., 2015; Frisina et al., 2009; Pienaar et al., 2013). Clinical trials suggest that there are a considerable number of patients who do not show improvement in gait and axial symptoms after the deep brain stimulation of the PPN. In these patients, degeneration of the DRN neurons might be responsible for these symptoms.

Limitations of the Study

The DRN is located dorsomedial to the MLR. To study the effect of the drugs on DRN without affecting the function of the MLR, cannulas targeting the DRN were inserted in the aqueduct canal and the tip of the injector cannulas were located near the dorsal border of the DRN. The animal's gait was measured only five minutes after the injection, but it is possible that the drugs leak through the aqueduct canal to the adjacent structures like the cerebellum.

While Catwalk apparatus measures many gait parameters, there is no parameter to measure the freezing episodes. The only parameter that indirectly indicates the freezing episodes is body speed variation which was increased significantly while animals had freezing. However, the Catwalk software is programmed to measure the gait parameters when the animals are actively moving, therefore, the effect of gait freezing episodes on other gait parameters is not substantial.

Conclusions

All these findings together suggest that the DRN serotonergic neurons are one of the main controllers of the gait. These neurons send projections to at least three areas: The GiN, the ventral horn of the spinal cord, and possibly to the MLR. While they facilitate the activity of motoneurons and CPGs through their direct projections to the ventral horn of the spinal cord, they modulate static gait parameters through their projections to the GiN, and also modulate the dynamic gait parameters through their possible projections to the MLR.

Chapter 8

8 « References »

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Chapter 9

9 « Curriculum Vitae »

NAHAL FARHANI, M.D.

EDUCATION

• **2013- present: M.Sc Candidate in Neuroscience.**

-Research Project: “Serotonergic System and Gait.”

Supervisors: Dr. Nagalingam Rajakumar, and Dr. Mandar Jog

Movement Disorder Clinic, University Hospital, University of Western Ontario

• **2003-2010: M.D.** Shiraz University of Medical Sciences, Shiraz, Iran

-Thesis: “Pulse Doppler tissue imaging and myocardial function in Thalassemia intermedia.”

Mentors: Dr. Hamid Amoozgar, and Dr. Mehran Karimi

Grade: A+ (19.5/20)

-Internship (2008-2010): Hospitals affiliated with Shiraz University of Medical Sciences

RESEARCH EXPERIENCE

• **2008- 2013: Research Assistant**

Cardiovascular Research Center, Shiraz University of Medical Sciences, Iran

My activities include writing proposals, collecting samples, collecting data, interpreting echocardiographic data and clinical laboratory results, analyzing data using statistical methods and writing scientific articles.

WORK EXPERIENCE

• **2010-July 2012: General Physician**

Emergency Department, Imam Hossein General Hospital, Malayer, Iran

Responsible for all aspects of patient management.

2013- Present: Teacher assistant

General Psychology 1000

Integrative Neuroscience

PUBLICATIONS

1. Amoozgar H, Barekati M, **Farhani N**, Pishva N. (2014) Effect of birth asphyxia on p wave dispersion. Indian J Pediatr. 81(3):238-42. PMID: 23640697

2. Amoozgar H, Saleh F, **Farhani N**, Rafiei M, Inaloo S, Asadipooya AA. (2013) Cardiac repolarization changes in the children with breath-holding spells. Iran J Pediatr. 23(6):687-92. PMID: 24910749

3. Amoozgar H., **Farhani N.**, Khodadadi N., Karimi M., Cheriki S. (2011) Comparative study of pulmonary circulation and myocardial function in patients with β -thalassemia intermedia with and without hydroxyurea, a case-control study. *Eur J Haematol.* 87(1):61-7. PMID: 21447009
4. Amoozgar H., **Farhani N.**, Karimi M. (2011) Early echocardiographic findings in β -thalassemia intermedia patients using standard and tissue Doppler methods. *Pediatr Cardiol.* 32(2):154-9. PMID: 21082174
5. Amoozgar H., **Farhani N.**, Karimi M. (2010) Risk factors for pulmonary hypertension in patients with thalassemia intermedia. *Eur J Haematol.* 85(6):549-51. PMID: 20698879

POSTER PRESENTATIONS

1. **Farhani N.**, Rajakumar R., Jog M. **Investigation of Neuronal Circuit responsible for Gait.** Society for Neuroscience (SfN) Annual Conference, Chicago, October 2015.
2. **Farhani N.**, Rajakumar R., Jog M. **Role of pedunculopontine cholinergic neurons in gait.** Society for Neuroscience (SfN) Annual Conference, Washington DC, November 2014.