University of Alberta

Single Cell Recordings During Intraspinal Microstimulation of the Feline Spinal Cord

by

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To my Father, who taught me to never stop dreaming

Abstract

Intraspinal microstimulation (ISMS) is a novel functional electrical stimulation technique for the restoration of locomotion after spinal cord injury. Through stimulation of the ventral gray matter of the lumbar spinal cord ISMS has elicited a series of functional results such as weight bearing stepping movements, near normal recruitment order of motoneurones and fatigue resistant standing. Furthermore, ISMS elicits activation of axons and fibres in passage at lower thresholds than motoneurones. Together these data suggest that ISMS relies on fibre in passage systems and not primarily on the activation of cell bodies directly. This project sought to investigate the fibre in passage systems involved in the effective spread of activation of ISMS, specifically comparing the contribution of afferent and propriospinal fibres to the effective spread of ISMS.

In the animals with intact afferents, 47% of the cells recorded from responded to ISMS. The majority of the cells (87%) exhibited an increase in firing rate in response to ISMS; 73% of the total within 3ms (short latency excitation). In the deafferented model, only excitation was observed. Given our stimulation parameters, the absolute current spread was approximately 0.5mm. However, cellular responses up to 10mm from the stimulation site were observed in the intact model and 6mm in the deafferented model. These results show that ISMS does not rely solely on the afferent projections for the effective spread of current but that propriospinal and descending projections play an active role. Furthermore, these data suggest that, as anticipated, ISMS works primarily through the fibre in passage systems for its functional outcomes.

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Chapter 1

Introduction

"The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory."

-Santiago Ramon y Cajal

Physiology or Medicine Nobel Prize 1906

This statement, made over a hundred years ago by one of the founders of modern neuroscience, seems just as valid today as it was at his time. Over the past century and a half we have gone from seeing the central nervous system as a single physiological unit to discovering and unravelling this marvellously efficient and complex structure. With the novel staining techniques developed by Camillo Golgi in the late 19th century, Ramon y Cajal discovered the intricate detail hidden below the surface of the brain and began the study of its individual cellular units: neurones. Enthralled by the brains structures, he created what is known as the "neurone doctrine", which stated that the nervous system is composed of billions of separate neurones that communicated with each other through physical contacts he called "synapses". He believed, and rightly so, that neurones receive input from their upper dendritic structures and transmit information through their projecting axonal branches. Today, with the development of modern staining, electrophysiological and imaging techniques, we have a much better understanding of how the central nervous system (CNS) is constructed and how its circuits are arranged. The understanding of how

these complex interactions are arranged is imperative for the development of technologies which are to interface with or control the CNS, such as neural prostheses.

Neural prostheses, such as intraspinal microstimulation (ISMS), seek to take advantage of these internal circuits, exploiting our understanding of them to restore function after injury. In the particular case of ISMS, microwires are implanted into the spinal cord targeting the area containing the motoneurones, called the ventral horn. By stimulating through these microwires, functional limb movements have been achieved, but the underlying mechanisms involved are poorly understood. The goal of this project is to identify the sites of action of intraspinal microstimulation in the lumbosacral spinal cord of the cat. With this, I hope to further advance this technique towards becoming a functional neural prosthesis.

1.1 Intraspinal microstimulation

Intraspinal microstimulation is a novel functional electrical stimulation technique for the restoration of stepping and standing after spinal cord injury.

1.1.1 Overview of the history of intraspinal microstimulation

Intraspinal microstimulation, originally used as a method to study the spinal circuitry, has been in use for over half a century and has provided invaluable insights into the organization of the spinal cord. Intraspinal microstimulation was first used by Renshaw to study the activation of motoneurones of the spinal cord (Renshaw, 1940). Later, in the 1970s, the technique was applied to the study of reflex circuitry and interneurones of the spinal cord (Jankowska and Roberts, 1972) and provided much of the data for what is considered one of the more comprehensive descriptions of the spinal interneuronal circuitry (Jankowska, 1992). The first clinical applications involving ISMS began with the investigation of bladder voiding after spinal cord injury (Nashold et al., 1971). This technique presented promising results in animals and was clinically implemented. However, the functional outcome of ISMS for bladder voiding was limited by the need for sphincterectomies in male patients (for a review on bladder prosthesis see (Gaunt and Prochazka, 2006)). The use of ISMS for the restoration of functional stepping and standing after spinal cord injury has been

effective in animals to date and has also demonstrated certain advantages over conventional functional electrical stimulation systems.

1.1.2 ISMS implantation technique

The technique of intraspinal microstimulation consists of the implantation of microwires 25-30μm in diameter into the lumbosacral region of the spinal cord (Fig. 1.1). For this the spinal cord is exposed, the dura mater opened and landmarks of the dorsal surface of the spinal cord are identified for the targeting of the ISMS microwires. The deinsulated tips of these microwires are then inserted into the cord through the dorsal surface, targeting the ventral horn of the gray matter based on previous mapping of the motoneurone pools (Vanderhorst and Holstege, 1997; Mushahwar and Horch, 2000). Stimulation through each of the implanted microwires is used to verify the proper placement and adjustments are made as necessary. The microwires are then adhered to the cord through the application of cyanoacrylate glue and further anchored to an adjacent spinous process with dental acrylic (Fig. 1.1). In chronic implants, the microwires are commonly tunnelled beneath the skin to the head where they terminate in a connector that is fixed to the skull with screws and dental acrylic.

1.1.3 Intraspinal microstimulation for locomotion

Intraspinal microstimulation through a single microwire can elicit the coordinated contraction of multiple muscles over multiple joints (multi-joint synergies) (Mushahwar et al., 2000; Saigal et al., 2004). Tonic, subthreshold stimulation through as few as 4 microwires in each side of the cord elicits stepping movements in the cat (Guevremont et al., 2006). This stimulation paradigm elicited coordinated, partial weight bearing, bilateral stepping that could be modulated by the speed of a treadmill in chronically spinal and decerebrate cats. This demonstrated that (a) tonic, sub-threshold ISMS can activate locomotor networks to produce rhythmic locomotor-like patterns, (b) ISMS does not impede afferent modulation of the locomotor networks of the spinal cord, and (c) afferent activity does not disrupt the functional output of ISMS.

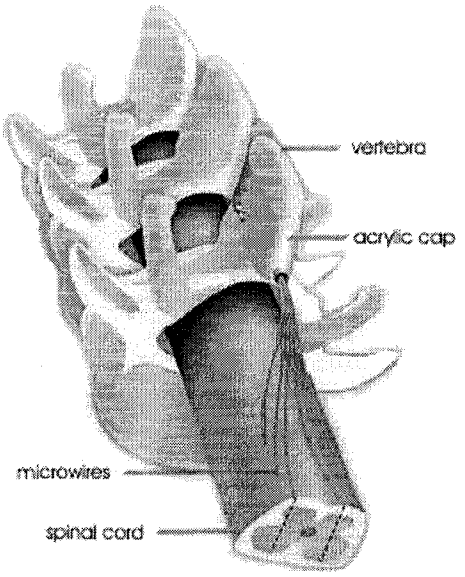

Figure 1.1: Example of ISMS implant. The exposed spinal cord is implanted with microwires deinsulated only at the tips. The microwires are Pt-Ir (platinum-iridium) or stainless steel with diameters of 25-30 μmeters. Stimulation through these wires, implanted to target the ventral horn, has been shown to produce multi-joint synergies and functional weight bearing stepping (see text).

ISMS also provides fatigue resistance in standing trials. Recent experiments demonstrated that up to 32 minutes of continuous weight bearing force can be obtained with ISMS using a closed-loop control system (Lau et al., 2006; Guevremont et al., 2007). When peripheral stimulation was used only 8.5 minutes of continuous standing was achieved. In part, the fatigue resistant component of ISMS can be explained by the near normal physiological recruitment order of the motoneurones, obtained in this mode of stimulation (Bamford et al., 2005). Due to the near normal recruitment order of motor units, ISMS produces graded force levels as the stimulation amplitude is increased, as opposed to the steep force build up seen with peripheral stimulation. This provides much more control of the generated force and, as seen in the standing experiments, considerably reduces muscle fatigue.

Together these observations give strong evidence that ISMS activated the motoneurones indirectly. This indirect activation may originate from the activation of various fibre in passage pathways including the backfiring of afferent projections, interneuronal networks and descending projections in the ventral horn of the spinal cord (Gustafsson and Jankowska,

1976; Gaunt et al., 2006). The importance of the afferent systems to ISMS is in question and the possible participation of other local pathways, such as propriospinal and interneuronal, remains to be answered. To understand the participation of the various spinal circuits and afferent projections within the spinal cord in spreading the effect of focally applied ISMS, I will review these circuits and their contributions to standing and locomotion, the two functions ISMS seeks to restore.

1.2 Study of CNS circuitry

The study of the connectivity in the CNS yields important information as to how its complex tasks, such as running or catching a ball, are acquired and executed. For such tasks it is necessary that groups of neurones, or structures, come together and coordinate an organized, functional output. For voluntary movements, such as locomotion, the higher centres provide the initiative but the stereotypical and rhythmic components of movement are generated in the spinal cord (Kiehn, 2006).

Since the nervous system communicates through electrical impulses, the identification and tracking of the central areas involved in specific tasks is possible by applying techniques such as extracellular and intracellular neural recordings. For the former, the electrical signal, or action potential, is recorded as it manifests itself by placing an electrode in the grey matter, where the cell bodies are located. With this procedure it is possible to record from and differentiate between individual neurones in the immediate vicinity. Intracellular recordings, on the other hand, are obtained by entering the cell itself and recording the internal potentials as they are expressed. If, in parallel to recording, we place stimulation electrodes into the CNS and excite specific structures, it is possible to verify the connectivity between the stimulated and recorded regions. To establish this, an analysis of the firing patterns of the recorded neurones in relation to the stimulation is performed to estimate the type of connection and potentially the number of neuronal links in the synaptic chain. This can then be used to create maps of the circuitry of the CNS which in turn can be used to identify functional linkages between different structures of the CNS.

The recording of extracellular activity, or local field potentials, is usually performed

either *in vivo* or after removing the tissue of interest and recording *in vitro*. Since the nervous system is intact and undamaged, the *in vivo* studies provide a more global view of the influences that different structures can have on each other. However, teasing out the effects of particular structures is more challenging in these studies due to the redundancy and high level of convergence in the CNS. Therefore, it is common to perform controlled lesion studies in which specific areas of the CNS are damaged and the new state of the CNS is compared to an intact control. This provides, through the process of elimination, an idea of the inputs a cell receives from predefined structures.

In contrast to the *in vivo* studies, the *in vitro* preparation allows for the investigation of local networks and projections. In this technique a tissue slice, partially containing an individual structure, is removed and placed in a dish and bathed in artificial cerebro-spinal fluid. Intracellular recordings are often used in these studies to assess the individual effects local neurones have onto one another or the effects of drugs applied to the bath on particular, predetermined cells.

For my particular project I chose to conduct *in vivo* extracellular recordings to identify the areas of the lumbosacral enlargement ISMS acts on. Through the analysis of neuronal firing rates during and in the absence of ISMS, the location of cells that respond to ISMS can be plotted, thereby identifying the sites of action of ISMS.

1.3 Spinal cord organization and circuits

The spinal cord contains both neurones and groups of axonal projections, or pathways. The grey matter, the location where the neuronal cell bodies reside, is a butterfly shaped area in the centre of the cord encapsulated by the local, ascending and descending pathways which make up the surrounding white matter. The spinal circuitry receives input from 3 main systems: the afferents, descending systems and local interneuronal circuits.

The spinal grey matter can be grossly grouped into 3 general regions in a dorsal-ventral fashion: (i) the dorsal horn; (ii) the intermediate zone; and (iii) the ventral horn. The dorsal horn is the target of most of the initial synapses made by afferent fibres as they enter the cord through the dorsal roots. The ventral horn contains the motoneuron pools which

give rise to the ventral roots that form the motor axons which innervate the muscles. These regions of the grey matter were further divided based on their cytoarchitecture by Rexed (Rexed, 1954) into 10 individual layers. Rexed mapped the variable sizes and shapes of these layers from the cervical to sacral spinal segments in the cat. These layers, known as the Rexed Laminae, are used for the localization and characterization of cells in the spinal cord. The laminae, which go from I-IX in an incremental dorsal-ventral sequence with the exception of X which is around the central canal, are distinguished by cellular characteristics and therefore are not necessarily grouped by function. Although lamina IX, for example, is known to contain the motoneuron pools it does not solely contain motoneurones but also a large quantity of interneurones. The same can be said of the functional cell types in other laminae.

1.3.1 The afferent system

The afferent system is composed of the peripheral information relayed to the spinal cord through the dorsal root ganglia. Divided into four main types, these bipolar cells convey individual types of peripheral stimuli depending on their terminal morphology and location. Of these four afferent types: touch, temperature, pain and limb proprioception (Kandel, 2000), I will focus on the last, for it is the prominent system involved in locomotion. The limb proprioceptors, or muscle and skeletal mechanoreceptors, are divided into groups I, II and III, each relaying different types of information to the cord at different conduction velocities.

Group I

The group I afferents originate from the muscle spindle primary terminals (Ia) and tendon organs (Ib) providing information on limb velocity and muscle force. These myelinated axons are largest in diameter and fastest conducting amongst the afferents with conduction velocities of 70-120m/s. Upon entering the cord both Ia and Ib branch, invading several adjacent segments (Burke and Glenn, 1996) and making extensive synapses onto interneurones. The target interneurones for these afferents are named Ia and Ib interneurones accordingly and have various effects. Ia afferents have a unique, monosynaptic, pathway onto motoneu-

rones and each afferent has been shown to synapse on up to 60% of a given motoneuronal pool (Kandel, 2000). This monosynaptic, excitatory pathway does not restrict itself to the homonymous MNs (of which it may synapse onto all MNs), but instead targets multiple agonistic muscles as well (Kandel, 2000). This extensive distribution of terminals serves the purpose of recruiting, or at least facilitating, the muscles participating in a synergistic movement. Also imposed in this recruitment is the Ia inhibitory interneurone which inhibits MNs of antagonistic muscles. So, together, these two pathways facilitate the contraction of synergists while removing the resistance that could be caused by its antagonists. This phenomenon, called reciprocal inhibition, was originally described by Sherrington in what he called reciprocal innervation, where the antagonistic muscles relaxed when a limb was stretched.

Group II afferents

The group II afferents originate in the muscle spindle secondary terminals and joint capsules. Together they convey muscle stretch and joint angle at conduction velocities of 36-72m/s. Interneurones receiving monosynaptic group II input are divided into 2 types: those residing in the dorsal horn (laminae IV-V) and those in the intermediate/ventral horn (laminae VII-VIII) (Edgley and Jankowska, 1987a). Since the latter group is a last order neuronal group, these are called group II interneurones while those present in the dorsal laminae are called the dorsal horn group II interneurones (Jankowska, 1992). The lumbosacral concentration of group II interneurones is highest in segments L4-L5 and send axons either rostrally or caudally through the ventrolateral funiculus. Their projections reach as far as S1 and target laminae VII-IX, including multiple motoneurone pools. They have been shown to be coactivated (up to 60%) by Ia and Ib as well as cutaneous afferents (Edgley and Jankowska, 1987b). The descending systems act on group II interneurones presynaptically and are predominantly inhibitory, and very selective. Localized application of noradrenergic and serotonergic agonists have been shown to have very specific actions on group II terminals in L4-L5 segments. Inhibition of the dorsal horn group II potentials was primarily achieved with serotonin whereas noradrenergic agonists primarily inhibited group II terminals of the intermediate zone (Bras et al., 1990). The Ia terminals were un-

affected by the aforementioned inhibitory effect, maintaining their actions on group II type interneurones. The group II interneurones also have inhibitory effects on each other, producing a selectivity in the afferent convergence to the MNs (Edgley and Jankowska, 1987a). The effects of group II interneurones onto other populations, both ipsilaterally (Edgley and Jankowska, 1987a) and contralaterally (Jankowska, 1992) may have important functional roles in locomotion, such as participating in crossed limb coordination and swing to stance transitions, which will be discussed below (sect. 1.2.5).

Flexor reflex afferents

Flexor reflex afferents (FRA) are the group II, III and cutaneous afferents that contribute to the production of the flexor withdrawal reflex. This reflex consists of the rapid retraction (flexion) from a noxious stimulus. To achieve this, these afferents produce polysynaptic effects on ipsilateral flexor MNs, while also inhibiting antagonist (extensor) MNs (Baldissera F, 1981). The speed and intensity of the corresponding withdrawal is directly proportional to the strength of the stimulus: the stronger the stimulus, the stronger the reflex. Group III afferents are stretch sensitive free endings which signal excess stretch or force being applied to a given area of the limb. They are the smallest of the afferents mentioned here and therefore the slowest conducting with velocities of 4-36m/s.

1.3.2 The descending input

The second system providing input to spinal circuitry is the descending drive. There are five main pathways providing what is known as the "descending drive" to the spinal cord and they are the: (i) corticospinal (CST); (ii) reticulospinal; (iii) vestubulospinal; (iv) rubrospinal; and (v) tectospinal tracts. The primary descending cortical control is through the corticospinal tract which is divided into the lateral and ventral projections. The lateral projects contralaterally onto medial spinal interneurones and motoneurones while the ventral CST targets the same areas, but ipsilaterally. The remaining four tracts originate in the brainstem and provide input from a variety of structures such as the red nucleus (rubrospinal), reticular formation (reticulospinal), tectum (tectospinal) and vestibular nucleus (vestibulospinal). The importance of the descending brainstem pathways was

reinforced upon the discovery that specific areas, when electrically stimulated, provoked locomotion in cats (Shik and Orlovsky, 1976). Even more interesting was that this centre, called the mesencephalic locomotor region (MLR), responds to different frequencies of stimulation in the decerebrate animal. Changes in stimulation frequency elicited walking, running or galloping when accompanied by the appropriate treadmill speeds. It was later shown, through the cfos staining technique, that the MLR is composed of various nuclei in the brainstem (Jordan, 1998), each contributing to locomotion in different behavioural contexts (defensive, exploratory, etc.).

Shik also described the effects of the brainstem's descending drive. At rest these tracts exert subthreshold facilitatory actions on different muscle groups. The vestibulospinal tract facilitates extensors, the CST and reticulospinal tract facilitate flexors while the rubrospinal tract (which projects contralaterally) facilitates flexors. This facilitation is actually reinforced during locomotion where stimulation of the vestibulospinal tract will significantly enhance extensor EMG activity during stance but elicits no EMG activity if stimulated during swing. These tracts are predominantly (and sometimes exclusively) active during specific phases of locomotion. The vestibulospinal cells were active predominantly during initiation of stance while the reticulo and rubro-spinal centres were maximally active during swing (Shik and Orlovsky, 1976). This evidence in conjunction with the descriptions of locomotion in spinalized animals led Shik and colleagues to conclude that the participation of the higher centres was limited to initiating and facilitating locomotion and not that of creating its rhythm. Currently the use of agonistic drugs that mimic descending activation (noradrenaline and serotonin) are used to study the spinal cord and its locomotor circuits (Hammar et al., 2004; Kjaerulff and Kiehn, 1996; Magnuson and Trinder, 1997). Through the application of neurotransmitters *in vitro* and stimulation of fibres, it has been shown that the locomotor descending systems travel through the ventrolateral funiculus in the rat (Magnuson and Trinder, 1997; Antonino-Green et al., 2002). This pathway targets the locomotor networks in the spinal cord and participates in the selection of patterns as well as their initiation and general control. As a whole, the descending systems provide cortical and subcortical control of the movements to be executed by the spinal cord. Voluntary or goal directed movements, such as running after a ball or reaching for a beer glass, require

cues and modulation that are beyond the scope of the local control networks of the spinal cord and, therefore must be provided by higher centres such as the cortex and brainstem. Their damage or removal, as will be discussed in Spinal Cord Injury (section 1.3), deprives the spinal cord of voluntary and goal directed movements of the limbs.

1.3.3 Local interneuronal input

The spinal circuits receive extensive input from the interneurones of the spinal cord, which in turn compose the spinal circuitry itself. Not surprisingly, there are many types of interneurones in the spinal cord (for a good review see (Jankowska, 1992)) which can be either excitatory or inhibitory. Jankowska describes all spinal neurones as being interneurones, even motoneurones, since these too have synapses onto other neurones. The discrimination amongst this population, therefore, must depend on another means. One such way is to group them by axonal projections, which gives us three groups. The first group contains those whose axons arborize within up to a few neighbouring segments and have localized actions, these are known as segmental interneurones. The second group are the propriospinal neurones, which project over several segments and coordinate activity in different parts of the cord. The final group are the ascending tract neurones which have axons that leave the spinal cord and provide sensory input to the higher centres. Unfortunately even this categorization is far from optimal since, through collaterals, each of these individual groups can act as another. For example, ascending tract cells commonly have collaterals that can also categorize them as segmental neurones. A further classification given to interneurones is their order, which indicates the level of convergence from afferents (Ia, Ib, II, etc.) an individual neurone may have. First order neurones are those which receive direct afferent projections while second, third or higher order interneurones are those removed by one, two or more synapses from an afferent projection. The classification by afferent order, such as a first order Ia interneurone or second order group II interneurone can be misleading and must be used with caution. There is such an extensive convergence of afferent input that, although an interneurone may have predominant Ia input, it is highly inaccurate to say it receives solely such input. This topic is addressed extensively by Edgley (Edgley, 2001) where he notes that most spinal interneurones receive input from multiple afferent systems,

directly or indirectly. Finally, the last order neurones are those that synapse directly onto motoneurones, helping coordinate or suppress muscle activation for different functional outcomes. The functionality and configuration of interneuronal populations will be discussed below.

1.3.4 The motor unit

Functionally, the main objective of the spinal cord is to produce patterns of muscle activation so as to generate functional movements. This is achieved by activating the motor units, which are composed of a motoneurone and the muscle fibres it innervates. The motoneurones, as mentioned previously, are found in the ventral horn or more specifically in lamina IX of the grey matter. Due to the distribution of muscles innervated by each spinal segment, there are two areas of the cord that are larger than the rest. These enlargements are due to the increased number of local motoneuronal groups, called pools, for the upper limb muscles in the cervical enlargement and the lower limb muscles in the lumbosacral enlargement. Each motoneurone pool innervates an individual muscle and, in the cat, they can be 8-9mm long stretching out rostro-caudally, in a cigar shaped fashion. The pools are composed of two main types of motoneurones: the α-motoneurones, those that innervate contractile muscle fibres, and γ-motoneurones, those that innervate the muscle spindles. The α-motoneurones are further sub-divided by the contractile properties of the motor unit. These properties, and therefore motor unit types, are: fast fatiguable (FF); fast fatigue resistant (FR); and slow fatigue resistant (S). Each individual type of motor unit has differentiating properties in both the motoneurone as well as the muscle fibres. One of the main properties of the motoneurone is its size which is directly related to its activation pattern. Due to difference in the input resistance of motoneurones, given the same stimulus, smaller fatigue resistant motor units will be activated before large fast fatigue ones. Therefore, when a muscle is activated its motor units are recruited in an orderly fashion from slow, the smaller neurones with low fatigue fibres, to fast fatigue resistant and finally fast fatiguable as more and more force is required. Since the most commonly used fibres are S fibres, these tend to be the smallest motoneurones and therefore the easiest to activate, but their small diameter axons have the slowest conduction velocities and their fibres produce

the least force (as little as 1% of that produced by a FF motor unit (Kandel, 2000)). On the opposite side of the scale the fast fatiguable fibres are the largest neurones with the largest number of innervated muscle fibres, produce large levels of force and have the fastest conduction velocities since they have the largest axons, but fail to be able to produce force for extended periods of time. The distribution of fibre types in a muscle is proportional to how that muscle is used. Postural muscles, for example, have more slow fibres so as to sustain force for long periods of time without fatigue. The proportion of fibre types in a muscle can be changed with constant exercise, or lack thereof, which changes the demands on the muscle and therefore the size and type of the fibres it contains. After spinal cord injury, with the loss of functions like locomotion, the fibre types transform to predominantly the FF type and atrophy begins, leading to smaller muscles with less sustainable force.

1.3.5 Locomotion

Locomotion is executed in two separate facets: the generation and the modification of patterns. These patterns, such as hopping, running or walking, consist in the smooth coordination of neuromuscular activity for the given functional outcome. As for how the body generates and controls these patterns, there is more than one theory (for reviews see (Kiehn, 2006; MacKay-Lyons, 2002; Bizzi et al., 2000)). However an independent spinal circuit that controls locomotion is widely accepted. This circuit resides in the spinal cord and has been often referred to as the central pattern generator.

Central pattern generators

The central pattern generator, or CPG, was originally proposed by Brown (Brown, 1911). At the time, this proposal contradicted Sherrington's suggestion that locomotion was a chain of reflexes coordinated by the spinal cord (Sherrington, 1910, 1913). Brown's experiments on spinalized and deafferented cats led him to note that they express a "balance" between extension and flexion even after both lesions. What he observed was that, when one movement was present (flexion or extension), the other was inhibited. It was these intrinsic factors of the spinal cord that were the basis of what he called the "half centre oscillator". Over the past century pattern generation theories have been intensively investigated and it

has since been shown that the spinal cord contains regions with rhythmic properties theorized to be part of the CPG (Kjaerulff et al., 1994). More recent investigations include various models such as the lamprey (Grillner et al., 1998), tadpole (Roberts et al., 1998), frog (Kargo et al., 1998), turtle (Stein et al., 1998), chick (Wenner and O'Donovan, 2001), rat (Kjaerulff and Kiehn, 1996), cat (Langlet et al., 2005), monkey (Fedirchuk et al., 1998) and human (Dimitrijevic et al., 1998; Lam et al., 2003). Through these investigations it has been shown that the spinal cord contains the basic elements necessary for the production of complex rhythmic patterns such as locomotion, even in human infants too young to walk (Lam et al., 2003). This function, presumably performed by the CPG, can be divided into two parts: the generation of rhythm and pattern formation.

CPG rhythmogenesis

Locating the CPG in vertebrates is an idea that has been investigated extensively over the past 30 years. Brown's original theory proposed a tight knit control centre that oscillated to produce flexion or extension. Later this theory was expanded and identification of the actual segmental localization of the CPG was pursued. The main idea was that, if there is a centralized structure in charge of rhythmic behaviour, it should be able to be located and studied as was the case for the breathing rhythms generated by the pre-Bötzinger complex in the brainstem. This appeared not to be the case with spinal rhythm generators, as Grillner states: "The term 'central pattern generator' refers to function, not a circumscribed anatomical entity: The individual neurones that constitute the CPG may in principle be located in widely separate parts of the central nervous system." (Grillner and Wallen, 1985). The pursuit of the cells that perform this function, and their location, has pressed on nonetheless.

Early research with DOPA (L-3,4-dihydroxyphenylalanine) in cats led to the targeting of the interneuronal networks controlling FRA transmission to the MNs (located primarily in lamina VII) as participating in the half-centre model for locomotion (Jankowska et al., 1967b,a). Later studies (Grillner and Zangger, 1979) showed that, in the spinal cat, rhythmic behaviours could be seen in caudal segments (L6-S1) after separating these from rostral segments (L3-L5). However, Grillner reported that the drug-induced (DOPA) rhythm was

not normal, it was slower with less definite transitions between flexor and extensor bursts, but presented the general fingerprint of locomotor activity. He also reported the possibility of different segments to produce rhythm and suggested that the rhythmogenesis could be distributed throughout the lumbar segments. In contrast to these findings, there exists evidence that rhythmogenesis may be concentrated in the rostral segments (Cazalets et al., 1995). Studies *in vitro* of the rat spinal cord have shown that, when baths are isolated, NMA/5-HT induced locomotion only occurs if the drugs are applied to the L1-L2 segments. When the same drugs were applied only to the caudal segments they failed to evoke locomotor bursting patterns. This led to the conclusion that rhythmogenesis is a rostrally evoked phenomenon. Later studies in spinalized cats subjected to serial acute spinal transections showed that removal of the rostral segments (L3-L4 in the cat) from the caudal ones abolished clonidine evoked locomotion, further strengthening the hypothesis that the CPG is rostrally located (Cazalets and Bertrand, 2000; Marcoux and Rossignol, 2000; Bertrand and Cazalets, 2002).

In contrast to these findings, it has also been shown that the rhythmic capabilities of the cord may be segmental and distributed throughout the lumbar enlargement (Grillner and Wallen, 1985; Kiehn and Kjaerulff, 1998; Dai et al., 2005). To evaluate this, a hemisection lesion study was performed that removed the dorsal half of the cord from L2-L5 (rat) and rhythmic activity persisted in the L5 ventral root (Kiehn and Kjaerulff, 1998). This led the authors to conclude that there exists a rhythmic capability distributed throughout the enlargement, which they propose to be graded from the rostral to the caudal segments. Further investigation through cfos staining during treadmill locomotion in cats indicates active neurones following a graded column (more dense rostral and tapering caudal) that stretched as far as the sacral segments of the lumbosacral enlargement (Jordan, 1998). Subsequent evidence of the distributed CPG can be seen through the actions of 5-HT on the cord. It was seen that pharmacological block of 5-HT_7 receptors rostrally (above L3) or 5-HT_{2A} receptors caudally (below L3) eliminates locomotor rhythmogenesis in the fictive MLR preparation *in vitro* (Jordan and Schmidt, 2002). In addition, by combining c-fos with 5-HT_7 staining in rats, it was seen that 80% of c-fos positive cells in the caudal thoracic segments were co-stained for 5-HT_7 during treadmill evoked locomotion. These neurones

are suggested as being part of the CPG (Jordan and Schmidt, 2002).

Organization of distributed CPGs

Grillner performed a series of studies on deafferented and spinalized cats (Grillner and Zangger, 1979) to better characterize the locomotor CPG. He proposed (as did Brown) that there is at least one CPG per limb and that multi-limb coordination is through the interactions of these independent rhythm generators. These distributed rhythmic centres are what he called "unit burst generators". Later (Grillner and Wallen, 1985) suggested that these units may be distributed segmentally, each giving rise to that segments rhythmic output. These units are connected to each other in both a rostral and caudal fashion and can come together to coordinate each other in patterns such as locomotion. These units can create movement through phase delay, as shown in the lamprey, in which a domino effect produces the undulatory movement of the body for propulsion. For this the signal of initiation travels from the first segment down to the last, for forward movement. A time delay, from propagating from segment to segment, leads to a phase shift as it progresses, giving rise to the undulatory motion that courses throughout the lamprey's body. He also noted that the lamprey can perform the same motion in an inverse fashion and propel itself backwards. He theorized that this shows how units can be recruited in more than one fashion, changing the output pattern as necessary. This is the main advantage of Grillner's theory, the individual units control the flexor-extensor activation of their segment, thus reducing the complexity of the overall pattern.

Much later, while performing microstimulation in the spinal cord of spinalized frogs, it was discovered that stimulation of specific regions of the cord produced a consistent predefined movement. The direction and force of these individual movements break down into a small number of force fields coined "motor primitives" (Saltiel et al., 1998) which coordinate the multi-muscle activation in synergies. It was further noted that simultaneous stimulation of multiple regions appeared to produce a linear summation of both individual movements. The motor primitives theory suggested that the spinal cord is composed of a set of neural networks that generate directional movements; any complex movement produced is therefore no more than a summation of one or more of these spatially independent

networks. Through these primitives, activation of synergies by the CPG may be facilitated and therefore the complexity of stereotypical movements can be reduced.

The coordination of the locomotor centres, independently of their configuration and distribution, requires information to travel from one centre to another. This information is relayed through the propriospinal interneurones. For the coordination of upper and lower limbs these projections must travel from the cervical to the lumbar enlargement, as can be seen by the long propriospinals that travel in the ventral lateral funiculus (VLF) of the cat (Miller et al., 1973). It was soon after reported that hind inter-limb coordination was maintained after a lumbar hemisection retaining solely the ventral cord (Shik and Orlovsky, 1976), but upper-lower coordination was lost. After the VLF was discovered to initiate locomotion in vitro (Magnuson and Trinder, 1997) an investigation of its projections in the rat revealed that it targets primarily the ipsilateral L1-3 and contralateral L2-3 segment, where there were more fibres innervating the contralateral side (Magnuson et al., 1998). Further investigation revealed that dye injected into the cervical enlargement was transported as far as S1 both contra- and ipsilaterally (Reed et al., 2006). The same procedure in the lumbar enlargement showed the ascending projections to the cervical enlargement, which tended to cross in the L1-3 segments before ascending within the VLF. These pathways may provide not only sensory information for the cervical and lumbar CPGs but probably also provide inter-CPG modulation for proper coordination of upper and lower limb rhythm and movement.

In a similar fashion to fore and hindlimb coordination, the left-right limb coordination also requires inter-limb cues. This has been shown to be through neurones that project through the ventral commissure and across to the opposite side of the cord, called commissural interneurones (CIN) (Kjaerulff and Kiehn, 1996; Jankowska et al., 2005). These neurones target motoneurones as well as other interneurones and have been shown to be of various types and even genetic lineages. Electrophysiologically, they can be divided into two main groups that are monosynaptically excited by: (i) group II fibres or (ii) reticulospinal neurones (Jankowska et al., 2005). Aside from these apparently mutually exclusive inputs, these populations have a large convergence of group I afferents as well as di/polysynaptic excitation and inhibition from various other descending and afferent systems. The func-

tional relevance of these two groups can be seen as the two pathways for limb control during rhythmogenesis. The descending command from the MLR can be relayed through the reticulospinal tract while local sensory information can be signalled by the group II afferents. The location of the origin of these fibres also coincides with the rhythmogenic segments identified for the CPG (see CPG rhythmogenesis). Retrograde labelling studies have shown that, in the neonatal rat, the ascending and descending commissural interneurones have short and few collaterals with cell bodies in the ventromedial grey matter (Eide et al., 1999) in accordance to the suggested location of the CPG by previous studies (Kjaerulff and Kiehn, 1996). Spanning from T10-L5 (in the neonatal rat) these neurones were found to be essential in the coordination of left-right rhythmicity (Kjaerulff and Kiehn, 1996; Butt and Kiehn, 2003). Intracellular and extracellular recordings revealed excitatory and inhibitory effects leading to four functional groups of CIN: (i) extensor CIN that monosynaptically excite contralateral flexors; (ii) flexor CINs that monosynaptically excite contralateral extensors; (iii) flexor polysynaptic inhibition of contralateral flexors; and (iv) extensor polysynaptic inhibition of contralateral extensors (Butt and Kiehn, 2003). While applying drugs to the bath it was discovered that some cells, called switch cells, are polysynaptic inhibitory except in the presence of NMDA+5HT, upon which they switch to monosynaptic excitatory. Other studies confirm that CINs can be either glutamatergic (excitatory) or glycinergic (inhibitory) and that they contact both MNs and interneurones contralaterally (Bannatyne et al., 2003). These cells could play a role in augmenting the selectivity of the descending systems during different locomotor patterns or the selective activation of synergies (Hammar et al., 2004). The mutually exclusive CIN groups which receive reticulospinal projection or group II afferents also shows the importance of both the descending drive and the afferent systems for coordinating locomotion and goal directed movements.

The role of afferent input in locomotion

As has been known for more than a century, the afferent system is not essential for the production of rhythmic behaviours (Brown, 1911). Deafferentation studies (Murray and Goldberger, 1986; Goldberger, 1988b,a; Sanner et al., 1993) have shown that rhythmic output can be attained in the absence of afferents. In some cases, when a root is spared,

locomotion can even be regained after training (Goldberger, 1988b,a). But that does not mean that afferent input is of no importance to locomotion nor that it is not necessary for the proper functioning of the CPG. The sensory information provided to the CPG during locomotion is of great importance and can influence it in many ways. For example, a cat on a treadmill will adjust its gait according to the speed of the belt; as that speed increases, so does the frequency of the gait cycle (Lovely et al., 1990). Similarly, artificial loading of the hindlimb (signalled through the stretch of ankle extensors) during bouts of locomotor activity have been shown to cease the locomotor pattern, leading to prolonged stance phases until the loading had been terminated (Duysens and Pearson, 1980). The appendage of weight to the legs of human infants also has an immediate effect on the gait cycle, causing increased flexion to compensate for the additional weight (Lam et al., 2003). Immediately after the removal of the weight, these infants commonly showed high stepping (overcompensation). The overcorrection subsided as afferent signals informed the CPG of the lack of weighting, leading to a return to baseline within a few steps. Proprioceptors of the hip also play an important role in initiating the transition between phases of the gait cycle (Grillner and Rossignol, 1978; McVea et al., 2005). Early studies in spinal cats in which a hind paw was suspended and slowly moved backwards, extending the hip, saw a marked moment in which the leg would flex and resume a locomotor pattern (Grillner and Rossignol, 1978). This pattern was either walking or galloping, depending on the state of the contralateral limb when the hip reached its transition point. The angle of the hip during which the transition would manifest itself was similar to the angle at which the stance-to-swing transition initiated during normal walking. It has also been shown that, in the decerebrate mesencephalic cat, passive movement of the hip during flexion (swing) prematurely terminates hip flexor activity and advances the onset of hip extensors (stance) to compensate for the perturbation (McVea et al., 2005). Human infants, when their walking patterns are disturbed by flexion of the hip also showed a delayed, or complete lack of, initiation of swing phase (Pang and Yang, 2002). In these cases swing would only initiate if the hip was then extended. If the infants were walking sideways, hip extension had little effect on initiating swing but adduction became the prominent cue for the stance to swing transition. Early studies of the necessity of cutaneous afferents in the modification

of locomotor patterns was investigated by tapping the forelimb of a cat whilst walking on a treadmill (Drew and Rossignol, 1987). The reaction was a complex coordination of muscle activity that smoothly led the paw to avoid the obstacle. This same situation, with the skin under local anaesthesia, failed to evoke a response. The same lack of response was observed if the paw was tapped while planted on the belt during stance phase. This shows not only that it is specifically the cutaneous afferents that participate in the modification of the rhythm, but also how dependant the response is to the phase of the step cycle.

In spinalized animals, where the descending drive exerts no role, the cutaneous afferents have been shown to play a fundamental role in locomotor recovery. Spinalized cats in which the foot was previously deafferented, showed no recovery of proper plantar placement nor weight bearing of the hindlimbs even after 35 days of treadmill training (Bouyer and Rossignol, 2003b). Full afferentation was not necessary for recovery of treadmill stepping with training. In one case a partial cutaneous deafferentation was performed followed by spinalization. The cat was trained and began to recover locomotion with proper foot placement on a treadmill, at which point the remaining afferents were cut leading to a loss of proper foot placement even after 71 days of subsequent training. Originally, Sherington (Sherrington, 1910) suggested that there was little effect of cutaneous deafferentation on locomotion but Bouyer and Rossingol recently showed this not to be the case. Although in level overground walking the cutaneously deafferented cats showed minimal deficits, their performance on ladder walking was inadequate (Bouyer and Rossignol, 2003a). Over a period of 3-7 weeks the animals were trained and a complete recovery of treadmill kinematics accompanied by the recovery of ladder walking was observed. Stimulation of cutaneous afferents alone is enough to elicit the stumble reflex in the fictive preparation in cats (McCrea et al., 1998). These studies emphasize the importance of cutaneous afferents to signal the proper state of the limb for locomotion in intact animals as well as their indispensable participation in the retraining of the spinal cord following injury.

The primary afferents, as mentioned previously, branch extensively once entering the cord and make widespread synapses. Their influence on descending systems, the local CPG and the motoneurones is summarized by (Pearson, 1995) in 4 pathways based on evidence in spinal, decerebrate and fictive locomotion cat preparations with supporting

evidence from invertebrates such as crayfish and the stick insect. The four primary afferent pathways are summarized as: 1) the monosynaptic Ia pathway onto motoneurones; 2) the disynaptic inhibitory Ib pathway; 3) the disynaptic Ia excitatory pathway and; 4) the slow excitatory pathways for Ia and Ib. Through these pathways the primary afferents can signal the states necessary for the proper execution of locomotion or, when necessary, evoke the necessary response to an untimely stimulus or load (McCrea, 1998). As with cutaneous afferents, the response is dependant on the current state of the pattern generator and can be contrastingly different for different states. Activation of extensor Ib afferents in the absence of CPG activity produces inhibition of the extensor motoneurones (Pearson, 1995; Pearson et al., 1998). This same activation, if performed during locomotor stance, produces large EMG responses in the extensor muscles, demonstrating the importance the extensor load signalled by extensor Ib afferents. This load leads to the prolonging of stance and delaying of swing, until the leg is unloaded (Pearson et al., 1998).

1.4 Spinal Cord Injury

As shown above, spinal cord injury (SCI) has devastating effects on the function and behaviour produced by the spinal cord. The general process of spinal cord injury is composed of two phases: primary and secondary injury. The primary injury refers to the lesion caused by the insult itself while the secondary injury is a cascade of physiological effects that, not uncommonly, leads to more extensive damage than created by the original insult. Deafferentation is a spinal injury but its implications in primary and secondary injuries are restricted. Given the scope of my project, which involves spinal deafferentation, only the aspects of SCI pertinent to deafferentation will be addressed. A complete overview of SCI is provided by Dumont (Dumont et al., 2001).

1.4.1 Spinal deafferentation

Deafferentation is a technique long used to study the necessity of sensory information to motor output (Sherrington, 1910; Brown, 1911; Goldberger, 1988a; Goldberger et al., 1993). By severing the dorsal roots (extradurally) or rootlets (intradurally), the cord is deprived

of all sensory information provided by these fibres. In the following days and weeks there is a cascade of events that can led to, depending on the extent of deafferentation, an almost full recovery of function. To understand the implications that deafferentation has on the spinal cord it is necessary to review the physiological effects it causes. It is important to note that the physiological effects of deafferentation in particular have very contrasting time frames between the peripheral and central nervous systems, even though the overall process is essentially the same (George and Griffin, 1994).

Since the deafferentation causes no mechanical damage to the spinal cord, there is no rupture of the blood brain barrier and no mechanical insult. Although initial edema may result in the initial hours post surgery, inflammation of the cord is minimal and the Wallerian degeneration (WD) of the axons is the first local cue of injury. A very marked difference is seen between the time courses of the subsequent physiological responses between the rootlets (peripheral nervous system PNS) and the white/grey matter of the cord (CNS). Centrally, the degeneration of the axons occurs at a pace of 3mm/h and within 72hr there is almost complete granular disintegration of the cytoskeleton in the adult rat (George and Griffin, 1994). The axoplasmic clearance in the CNS is extremely delayed and axonal debris can be identified up to 21 days postoperatively, neurofilaments being present up to day 90. In contrast, the PNS where there had been a disruption of the blood nerve barrier, macrophages invade the tissue by the second day, where they persist until the 21st postoperative day. Rounded macrophages, present from day 2 and persisting until day 21, perform axoplasmic clearance (George and Griffin, 1994; Liu et al., 2000). Clearance of both axonal debris and myelin is complete by 14-21 days. In the CNS, the microglia transform and begin phagocytic cleanup on the 18th day. Their action is slow and long lasting, axonal debris being present up to 90 days postoperatively, neurofilaments (myelin debris) for even longer than that (George and Griffin, 1994). In parallel to this the astrocytes' response to the lesion proliferates as soon as 2 days postoperatively (Liu et al., 2000). Although it is unclear whether the astrocytes participate in phagocytosis, they are recruited to the site of WD and are shown to be metabolically active (Liu et al., 2000). It is noted that centrally this entire process occurs in the absence of complement factors, normally present to recruit phagocytic cells in a region of damage, and that this may be one of the main reasons for

the slow and delayed actions of microglia and astrocytes (Liu et al., 2000).

Although the degeneration of the terminals made by the segmented afferents happens in a time course similar to axonal degeneration, an examination of the quantity of terminals in the cat within weeks post lesion reveals no decrease in the number of terminals of the dorsal horn cells (Goldberger and Murray, 1988). Goldberger also reports that the grey matter, contrary to the white, shows only minor shrinkage and little alteration in the laminar architecture of the cord. Functional recovery, in general, can be divided into two stages: (i) a recovery of locomotion and (ii) the recovery of accurate control of the limb. The first stage may be due to the subsiding of spinal shock, with an onset of 1-2 days postoperatively. The second stage, during the second postoperative week, may be due to the repopulation of the denervated terminals by adjacent axons through sprouting. The source of these axons may be descending systems, predominantly due to the increase in 5-HT and substance P (SP) seen after complete rhizotomy but not observed in spared root preparations (Goldberger and Murray, 1988). Comparably, the spared root preparation causes no decrease in terminal number. In this case it is shown to be through the overextension of spared afferents and not descending systems since a subsequent spinal transection does not eliminate the recovered function (Goldberger and Murray, 1988; Goldberger, 1988b). These studies suggest that the functional recovery seen after dorsal rhizotomy is due to the "enhancement of unimpaired spinal mechanisms rather than the restitution of those initially lost" (Goldberger and Murray, 1988).

1.5 Neural Prostheses

Neural prostheses use the technique of functional electrical stimulation (FES) to activate the nervous system and elicit functional muscle contractions. The term FES, coined by Moe and Post (Moe and Post, 1962), originally was used to refer to stimulators for foot drop in hemiplegic patients. Since then the field of neural prostheses has expanded and includes a multitude of devices that apply current to the nervous system to restore lost function after injury or disease (Prochazka et al., 2001). Currently, there are two general types of neural prostheses: surface and implantable.

1.5.1 Surface stimulation

The simplest, and oldest, type of neural prosthesis is the surface stimulators. Current is applied through an electrode placed on the surface of the skin to activate the underlying nerve or motor point (where the nerve enters the muscle). These systems require a tedious procedure of placement (donning) and removal (doffing) for each use and can only specifically activate superficial muscles. Since the current must act transdermically it is also common for the patients to experience pain or discomfort with these systems and, when not properly controlled, can cause burns due to the elevated current necessary to penetrate the skin. These drawbacks aside, their non-invasiveness and facility of use makes them highly appealing for many applications. Currently, applications of surface stimulation include foot drop (Dai et al., 1996) as well as for various rehabilitation strategies after CNS injury such as FES rowing machines and FES bicycles (Sterr et al., 2006). The therapeutic advantages of surface stimulation have been shown in their improvement in functional outcome and recovery after CNS injury as well as providing the possibility of a physical workout for such patients (Btefisch et al., 1995; Sterr et al., 2006).

1.5.2 Implanted systems

Contrary to surface stimulators, implanted systems require an invasive surgical procedure. The largest benefit of implantable systems is their specificity. For example, implanted neuromuscular FES systems can target specific muscles or muscle groups, auditory systems can target the specific auditory structures (House and Berliner, 1982), deep brain stimulation (DBS) (Benabid et al., 1991) can target individual subcortical nuclei and so on. Beginning with the cardiac pacemakers in the 1960s, implantable systems have grown in popularity and today many routine surgical interventions use devices that require little or no maintenance. The main advantage of implantable neural prostheses is the reduced power requirements due to lower current amplitudes that are needed to evoke functional muscle contractions. Currently, implantable electrodes are available in various types and sizes depending on the target structure, each having its advantages and disadvantages. Given the focus of the current project, only motor related devices will be discussed.

Peripheral stimulation

Peripheral nerve stimulation has traditionally been achieved through nerve-cuff electrodes (Naples et al., 1988) which have the benefit of targeting specific nerves and, therefore, activate muscles selectively. However, targeting specific muscles requires that the nerves be dissected at their entry to the muscle and the surgical implantation of one nerve cuff per muscle, with wires (leads) connecting all the cuff electrodes to a central stimulator / controller. These leads, often threaded long distances and passing over joints, commonly suffer from breakage and the cuff electrodes commonly occasionally cause nerve damage which jeopardizes their feasibility for long term implantation (Loeb and Peck, 1996). Other electrodes with similar properties, such as epimysial electrodes (Waters et al., 1988), intramuscular electrodes (Shimada et al., 1996) and flat interface nerve electrodes (Leventhal and Durand, 2003) suffer from comparable issues. To solve these difficulties, a recent wireless alternative was designed, the BION™(Loeb et al., 2001). This miniature encapsulated electrode can be injected directly into the desired muscle, near the motor point, and is powered by external inductive coils. Although still in clinical trials (Loeb et al., 2006; Weber et al., 2005), this novel technology is a promising replacement for the complexity of implanted electrodes and wiring for the stimulation of peripheral nerves and muscles. Due to the properties of peripheral stimulation the muscle fibres activated first are the FF followed by FR and finally S. This leads to a series of drawbacks including rapid muscle fatigue and, over time, a change in fibre type to predominantly S leading to more fatigue resistant muscles that produce less force. In a hope to overcome these shortcomings, central stimulation with intraspinal microstimulation is being actively pursued as an alternative technique for restoring neuromuscular function (Bamford et al., 2005; Prochazka et al., 2001; Mushahwar et al., 2000).

Central stimulation

To avoid the complications of a system with distributed electrodes requiring multiple invasive surgeries and long leads that can break, much research has been focused on the development of centrally implanted neural prostheses (Prochazka et al., 2001). In most

cases the central nervous system retains the ability to execute the functions lost by injury, but lacks the capability to initiate these functions voluntarily. Neural prostheses that, for example, target the central mechanisms of locomotion or goal directed movements, such as ISMS and epidural stimulation, seek to take advantage of these intact networks. Other systems, such as deep brain stimulation (DBS) (Benabid et al., 1991), seek to activate latent structures damaged or dwindling by neuropathies such as Parkinson's disease (Hamani et al., 2006) as well as psychiatric disorders (Aouizerate et al., 2004; Kopell et al., 2004).

Deep brain stimulation

Although stimulation of the brain has long been used in research as a localization technique for selective lesioning, it was first used for clinical purposes to treat chronic pain (Ray and Burton, 1980). Later, Benabid targeted the thalamic nucleus to suppress tremor (Benabid et al., 1991) and since then investigations have led to the implantation of DBS systems for essential tremor, Parkinson's disease, dystonia, Tourette syndrome, intractable pain, depression and obsessive compulsive disorder (Perlmutter and Mink, 2006). Over the past 25 years, although there has been much speculation, the mechanisms of DBS are still not understood. Recently experiments were conducted to ascertain the claim that DBS preferentially activated the structures (nucleus) in which it was implanted. Actually fibres in passage and axon hillocks, not cell bodies, were preferentially activated at the stimulation parameters tested (Nowak and Bullier, 1998b,a). Caution is therefore suggested in the interpretation of results of electrophysiological stimulation of such nuclei, since much more than just the somas in the immediate vicinity are being activated. The use of high frequency stimulation in DBS has been shown to have similar effects to that of lesion studies and it has been suggested that these parameters interrupt the abnormal transmission passing through the region being stimulated (Brown et al., 2004), fundamentally acting as an artificial lesion.

Epidural stimulation

For central stimulation to restore locomotion, two main techniques are being investigated: intraspinal microstimulation and epidural stimulation (ES). Although less invasive, epidural stimulation is also less specific since it consists in stimulating the dorsal surface of the

cord to elicit locomotion as shown by studies in the early 1990s (Iwahara et al., 1992). These studies showed that ES to the midline (above the dorsal columns), through plate electrodes, elicits bilateral coordinated locomotor like rhythm in the mesencephalic decerebrate cat, spinal decerebrate cat and fictive decerebrate preparation. When ES is performed on the cervical enlargement, coordinated locomotor like activity can be seen in all limbs and it was noted as being indistinguishable from MLR induced locomotion (in non spinal animals). Stimulation of solely the lumbosacral enlargement was able to elicit coordinated hindlimb locomotor rhythm, in the spinal and intact preparation, but failed to initiate four limbed locomotion in the intact spinal cord. In humans it was demonstrated that ES could elicit stepping movements after complete SCI (Dimitrijevic et al., 1998). The use of ES in spasticity treatment has also been investigated and appears to be a viable alternative to pharmacological treatment (Pinter et al., 2000). As is the case with ISMS, the underlying mechanisms of action of this technique are poorly understood. Although, it has been suggested that the functional outcome of ES is through the activation of large afferent fibres (Hunter and Ashby, 1994). Since the epidural electrode sits over the dorsal columns, the closest and largest fibres should be activated first, namely the Ia afferents. In humans, responses coinciding with monosynaptic motoneuronal activation have been seen, as well as polysynaptic inhibition and a late excitatory phase (Hunter and Ashby, 1994). This shows that ES acts through multiple pathways not restricted to the Ia fibres although specifically which pathways has yet to be experimentally shown (Dimitrijevic et al., 1998; Edgerton et al., 2006).

1.6 Sites of action of ISMS

Although its benefits and advantages are well established, a comprehensive study of the central mechanisms involved in ISMS has not been performed. Such an understanding is imperative to the proper development of ISMS into a functional neural prosthesis as well as to maximize its benefits and functionality. Once identified, the sites activated by ISMS can be better targeted and better controlled, increasing the specificity of its functional results.

For such a study there are various experimental techniques that can be used to identify

the sites of action of ISMS in the spinal cord. Staining techniques, such as cfos (Dai et al., 2005), can be used to identify the neurones most active during ISMS, thus determining the regional distribution of cells activated by ISMS. Another technique that can be applied is the recording of extracellular potentials during ISMS so as to locate spinal regions activated based on measurements of the changes in firing rate of the cells being recorded. These single cell recordings can yield important information about the changes in firing pattern invoked by ISMS, while onset latencies of cell firing may provide estimates of the number of synapses between the stimulated neuron and the recorded one. Finally, these techniques can be combined with others such as lesion studies, to identify the role and participation of different pathways in mediating the effects of ISMS.

This project sought to identify the sites in the lumbosacral spinal cord activated by ISMS through the study of extracellular recordings. These recordings were performed on both intact and unilaterally rhizotomized animals. Together, these two sets of experiments provided valuable insight into the effects of afferent and propriospinal fibres in coordinating the effect of ISMS in different regions of the spinal cord.

1.7 Hypothesis

Intraspinal microstimulation activates fibres in passage that are not solely composed of afferent projections but also axons of propriospinal and other interneurones. To test this hypothesis I investigated the locations of cells that show a change in firing rate elicited by ISMS in both intact and deafferented spinal cords. Through the analysis of firing rate and post stimulus spike latency I was able to identify, quantify and categorize cells that responded to ISMS.

Chapter 2

Materials and Methods

Two sets of experiments were conducted. The first set consisted of a series of acute, terminal experiments on adult cats. In these experiments ISMS was applied in the ventral horn of the lumbosacral enlargement and local field potentials were simultaneously recorded throughout the grey matter. In the second set, a chronic unilateral dorsal rhizotomy of the L4-S3 roots was performed and the experiments were repeated. The sites of activation in both sets of experiments were then compared to identify the role of afferent and propriospinal projections in distributing the effect of the focally applied ISMS. All experimental protocols were approved by the University of Alberta Animal Welfare Committee.

2.1 Surgery

A total of 11 male adult cats (4.2-6.7kg) were used (intact n=6; deafferented n=5). Under isoflurane anaesthesia, pairs of intramuscular electromyography (EMG) electrodes were implanted into the major hip, knee and ankle flexor and extensor muscles of the hind limbs. The electrodes were made of insulated stainless-steel wire (Cooner AS632, Chatsworth, CA) insulated except for the tip and inserted into the muscle with an inter-electrode spacing of 5-7 mm. Nerve cuff electrodes (bipolar, pairs of deinsulated cooner wire with 2-4mm inter-electrode spacing, mounted in heat shrink tubing) were implanted around the sciatic and femoral nerves in some experiments (n=9), or around individual nerve branches producing selective hip, knee or ankle flexion or extension movements when stimulated in other

experiments (n=2). The animal was then placed in a stereotaxic frame (Kopf spinal unit). Blood pressure, heart rate and respiration rate were monitored at all times.

The ISMS microwires were implanted according to techniques described elsewhere (Mushahwar et al., 2000). Briefly, a laminectomy was performed and an array of 6-10 microwires [30μm stainless-steel (California Fine Wire, Gover City, CA), 30-70μm tip exposure, impedance 10-30 kΩ] were implanted in the lumbosacral enlargement. Inter-microwire distances were 1mm rostrally-caudally in the intact animals and 2mm rostrally-caudally in the deafferented animals. All microwire tips targeted the ventral horn. The exposed cord was covered with warm mineral oil to provide electrical isolation.

The animals were then decerebrated at the intercollicular level and anaesthesia was removed. Methylprednisolone was administered (i.v., 30 mg/kg) to reduce swelling and a 5% dextran solution was administered if necessary to stabilize blood pressure following decerbration. When necessary, i.e. if the spinal cord was hypoexcitable and spontaneous cellular activity was minimal, 5-hydroxytryptophan (5-HTP 0.5 mg/kg i.v.) was administered systemically to increase overall excitability (n=2). Due to excessive movement artefacts, in 2 animals the competitive acetylcholine (ACh) antagonist gallamine triethiodide was administered (i.v., 10 mg/kg), to block the neuromuscular junction.

For the deafferented animals, unilateral dorsal root rhizotomy was conducted approximately 4 weeks prior to the terminal experiments described above. Under isoflurane anaesthesia, a laminectomy was performed to expose the L4-S3 roots which were cleared towards their ganglia, bound using 6.0 silk suture and severed at the distal side of the suture. The proximal stump was later used for stimulation during the terminal experiment to verify that no intact sensory fibres remained and that all proximal projections of the afferent fibres had degenerated.

2.1.1 Recording

A sharpened tungsten microelectrode (impedance 1-4 MΩ) was used for recording unitary extracellular activity. The cellular activity was bandpass filtered (300-10,000 Hz), amplified 10,000 times and digitized at a rate of 17,000-20,000 samples/sec (Fig. 2.1 (b)) using a CED Power 1401 analog-to-digital board and Spike2 software (Cambridge Electronic Design,

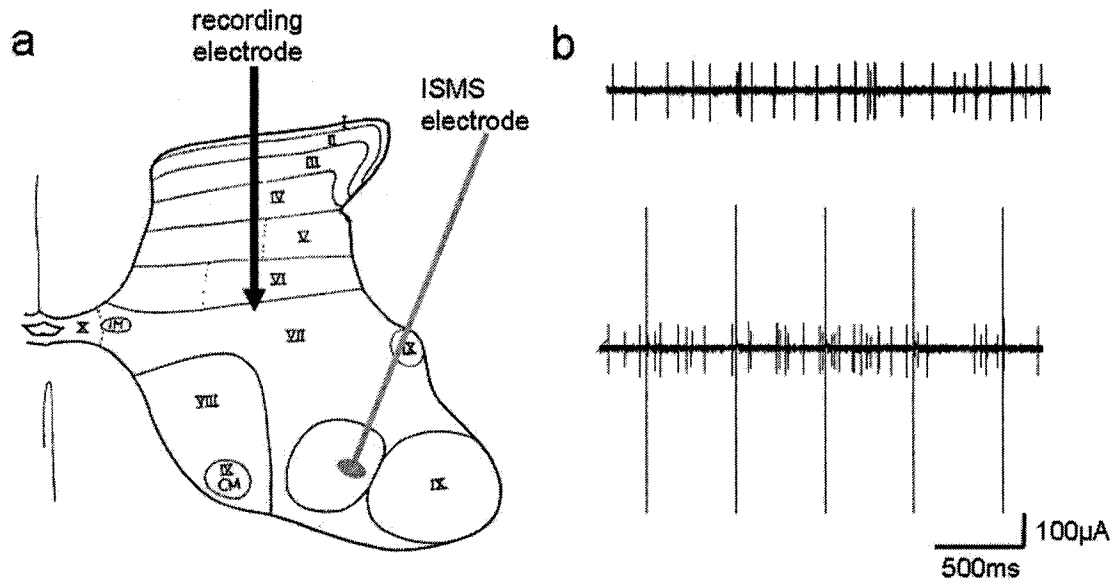

Figure 2.1: (a)Diagram of Rexed laminae indicating how the ISMS microwires targeted the ventral horn. The recording electrode travelled in a dorsal to ventral trajectory, stopping upon detection of spontaneous cellular activity. (b) Raw traces of extracellular recordings of spontaneous activity (top) and during an ISMS trial (bottom).

Cambridge, UK). The EMG recordings were bandpass filtered (30-1000 Hz), amplified 1000 times and digitized at a rate of 3000-5000 samples/s.

The recording microelectrode was inserted at a 90° angle to the exposed dorsal surface of the spinal cord (Fig. 2.1 (a)) at varying rostral-caudal and medial-lateral positions relative to the ISMS microwires. Dorsal-ventral depth was controlled with a microdrive (Newport motion controller PMC100 with an 850B actuator) and the unitary activity was displayed on an oscilloscope while also connected to a speaker for auditory feedback. Recordings of spontaneously firing cells were obtained (Fig. 2.1 (b top)) and their location within the spinal cord was noted. Intraspinal microstimulation through individual microwires [monophasic, 100 μs cathodic pulses, 2 pulses/sec, 30-100 μA (Neurolog, Digitimer, Letchworth Garden City, UK)] was then conducted and its effect on the firing rate of these cells was recorded (Fig. 2.1 (b bottom)).

Upon completion of the experiments animals were perfused through the heart with a 4% formaldehyde fixative solution, the area of interest of the spinal cord was carefully removed and preserved in the same solution. Post-mortem dissection of the spinal cord was used

to identify the locations of the microwire tips. Spinal cord dimensions were obtained and the readings on the microelectrode microdrive were used to determine the recording sites of unitary activity. Taken collectively, ISMS microwires were implanted throughout the lumbosacral enlargement and unitary recordings were obtained from locations spanning the entire length of the enlargement in both experimental series.

2.2 Analysis

For data files containing spontaneous unitary extracellular recordings, single cell discrimination was conducted using principal components analysis (Getspike; custom-written software, Dr. S. N. Baker, Newcastle, UK). The interspike interval (ISI) was then calculated for each discriminated cell to determine its preferred firing rate. Spike-triggered-averaging (STA) of EMG activity was subsequently calculated to determine the cell's connectivity with the sampled hind limb muscles.

For data files with ISMS, stimulus artefacts were removed using template matching methods prior to single cell discrimination. Post-stimulus time histograms (PSTHs) were then constructed to quantify the effect of ISMS on the firing rate of the discriminated cells. Raster plots were also generated to provide further visual confirmation of the responses seen in the PSTH. Cells exhibiting a change within the first 6ms of ISMS were further analyzed. All analyzes were conducted using custom-written programs in Matlab (The Mathworks, Natick, MA)

2.2.1 Removal of stimulus artefacts

For the removal of ISMS stimulus artefacts a method of template subtraction was used, as applied in deep brain stimulation (Hashimoto et al., 2002). This process consisted of the creation of a template and subtracting it from the original data. The template was composed of the average of all sweeps containing the stimulus, aligned to the stimulus onset. This average was then subtracted from each sweep and the remaining raw data trace was saved (Fig. 2.2 (a)). The raw files, from both ISMS stimulus and spontaneous firing trials, were then used for discrimination.

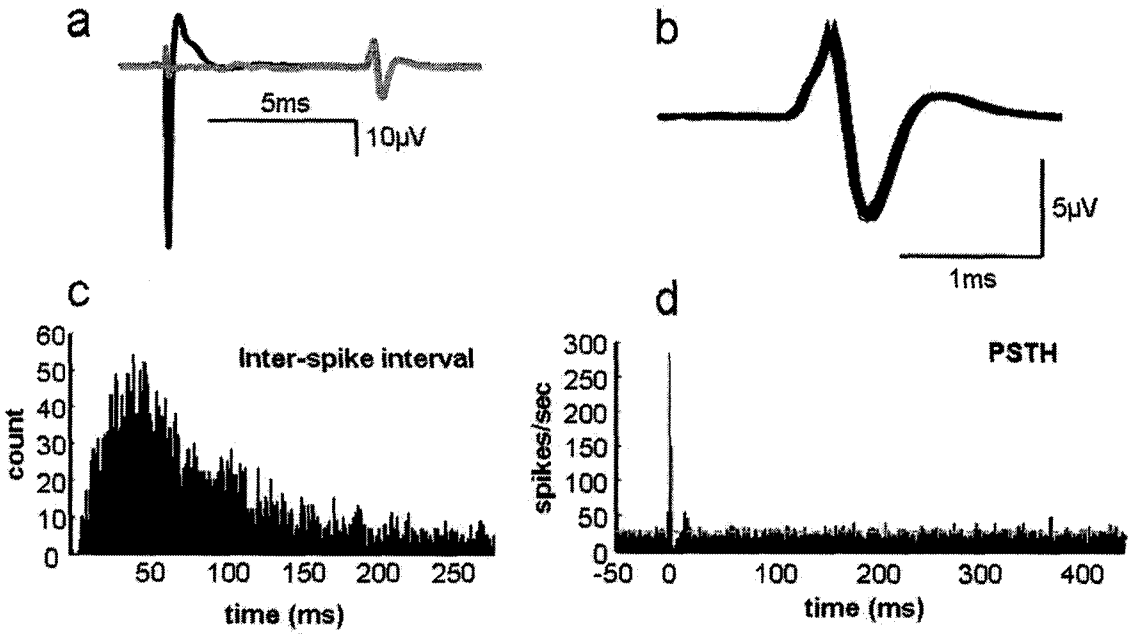

Figure 2.2: Analysis of one cell. (a) Example of raw traces before (black) and after (gray) artefact subtraction. Note how the subtraction reduced the artefact both in amplitude and duration but left unaffected the local field potential occurring afterwards. (b) Overlain local field potentials selected through principal components analysis. Inter-spike interval of cells firing in the absence of ISMS (c) and its excitatory response to ISMS in the post stimulus time histogram (d). Note that in the PSTH (each bar represents a 1ms bin) the response contained one predominant bin (significance shown by horizontal gray line) with a jitter of 1ms on either side.

2.2.2 Single cell discrimination

Principal component analysis of the local field potential (LFP) waveform was applied. This technique uses the variability of the dataset (the waveform in this case) and plots the most variable characteristics. The principal components were plotted against each other and the resulting clustering of similar waveforms that was visually identified. Individual clusters containing the LFPs of a single cell were selected and the consistency of the waveforms was visually verified (Fig. 2.2 (b)). The time of onset of each spike (LFP) for a single cell was then recorded.

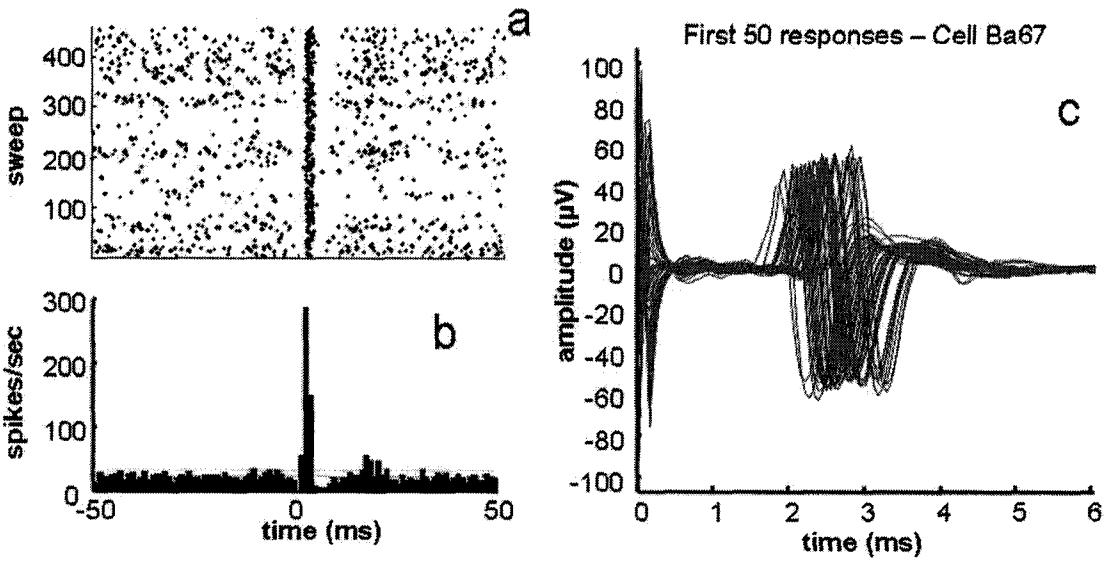

Figure 2.3: Raster plot (a), PSTH (b) and raw overlain spikes were used to verify each response. This case of short latency excitation shows the alignment of spikes in the raster plot (each horizontal line represents one sweep, each point one spike) with the peak in activity above significance of the PSTH (2*std shown as gray horizontal dotted line). These responses are verified as all being from the same cell by the raw overlain local field potentials which all have the same waveform. Note the jitter in all three plots: wide vertical bar in the raster plots, 3 significant bins in the PSTH and the variability of onset time in the raw traces. The case shown here is an atypical case in which cell response to stimuli was over 90% as discussed in text (sec. 3).

2.2.3 Verification of stimulus elicited responses

To construct the PSTH, LFP onset times were grouped into 500ms windows corresponding to the time between ISMS pulses, quantified and plotted from -50 to 450ms in 1ms bins, considering the stimulation onset at 0 (Fig. 2.2 (d)). Given that the immediate effects of ISMS would no longer be present after 250ms, each cell's mean firing rate and standard deviation were calculated based on the 200ms after this time (250-450ms). Any increase or decrease in activity that passed two standard deviations (±2*std) in the PSTH records was considered to be significant. The significant results were first grouped by lamina (dorsal-ventral location) then by response time (type of response). For the laminar analysis, placement was determined based on the post-mortem dissection parameters and the laminar drawings by Rexed (1954) (Fig. 2.1).

This project sought to consider solely the direct effects of ISMS in the spinal cord and,

therefore, I focused on the first 6ms post stimulus. Any response initiating after this time frame was discarded since it could not be distinguished from reflexive activation. The characterization of ISMS-induced cellular responses was divided into 2 time related criteria: a short latency response ($t \leq 3ms$), and a delayed response ($3ms < t \leq 6ms$). These were then separated into excitatory and inhibitory responses, creating a total of 4 different response types: i) short latency excitation (SLE); ii) delayed excitation (DE); iii) short latency inhibition (SLI); and iv) delayed inhibition (DI). Each individual response was verified through visual comparison of PSTH, raster and raw data plots (Fig. 2.3).

2.2.4 EMG correlations

The sampled EMG activity was analyzed depending on the presence or not of ISMS during the trials. For those data acquired during ISMS, the EMG activity was averaged based on the onset of each stimulus pulse. For the trials acquired during spontaneous cellular activity, spike triggered averaging was performed to verify the possible correlation of cell firing and EMG activity.

Stimulus evoked EMG activity

Verification and quantification of the rectified EMG activity in response to ISMS was performed for each microwire. Microwires with tips in the white matter and trials acquired under the effects of Flaxedil ®(pharmacological neuromuscular block) were discarded. All trials used for comparison contained responses to ISMS at 100 μA through a single microwire. Averages were created and plotted from -40 to +60ms post stimulus and the level of significance was based on $\pm 2^*$ standard deviations of the mean activity for the 40ms pre stimulus. To be considered significant, EMG activity also had to have a minimum duration above baseline of 6ms. Onset latency was considered to be the time of first crossing of EMG of baseline level. The maximum EMG activity elicited by ISMS in each muscle was quantified and used to compare the muscular responses to ISMS through a given microwire.

Spike triggered averaging

For trials not containing ISMS, the EMG data were correlated with the single cell activity through spike triggered averaging (STA). For each cell the ongoing EMG activity from -40 to +60ms was aligned to the onset of each spike and averaged. Ongoing EMG activity was considered to be present if in any sweep the peak activity was 2+ times the rectified noise band for a given muscle. Only sweeps containing ongoing EMG activity were included in the averaging. Through the STA MNs and last order interneurones of the muscles sampled could be identified.

2.2.5 Antidromic activation

The presence of antidromic potentials was investigated for all stimulus trials. For each stimulation file the individual traces were aligned to the stimulus onset and overlapped. Consistent potentials in the waveform of the stimulus artefact were considered to be ISMS induced antidromic potentials.

Chapter 3

Results

The objective of this study was to identify the areas of the lumbosacral enlargement that are activated by ISMS. The distributed effects of single point stimulation with ISMS, such as multi-joint synergies, lead us to believe that ISMS acts transsynaptically through fibres in passage that activate cells beyond the immediate vicinity of the microwire tip. This study was aimed at documenting the possible fibres in passage involved, the effect of ISMS on the firing rate of these cells and the location of these cells relative to the microwire tip.

3.1 ISMS in the intact model

Recordings in animals with intact afferent input yielded 2 populations of results: 1) recordings from cell bodies and 2) recordings from axons. Local field potentials with durations $\leq$0.5ms were considered to be originating from axons (Loeser and Ward, 1967). The subtraction of stimulus artefacts from each of trial yielded a period of saturation of the amplifiers known as a "blanking period". Examples of the best and worst case scenarios show (Fig. 3.1 a) that the longest blanking period lasted 3ms. Such long blanking was atypical and caused by very proximal (<1mm) stimulation and recording sites. Overall, 70% of the artefacts had blanking periods of 1ms or less (Fig. 3.1 b).

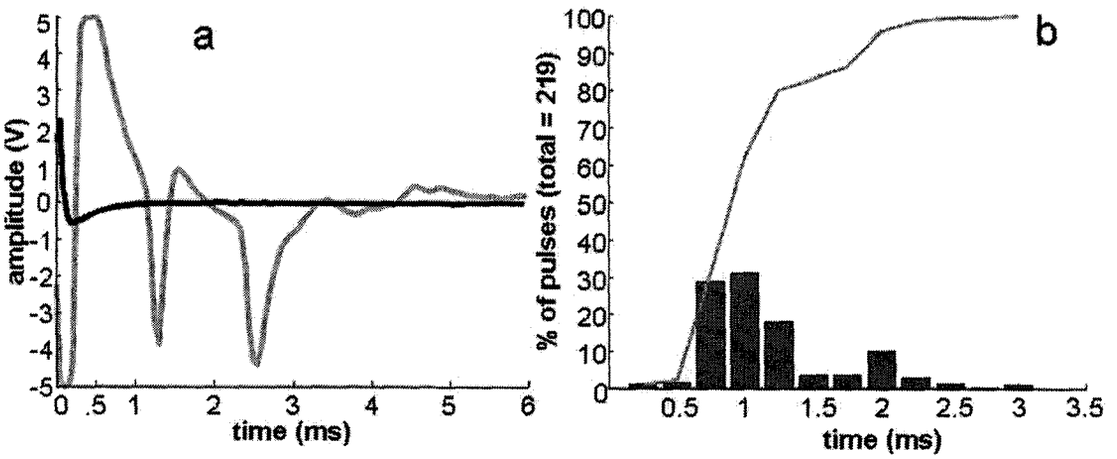

Figure 3.1: a) Overlain are best case (black) and worst case (gray) scenarios of stimulus artefact. The period of saturation of the amplifiers is a non-recoverable region called the "blanking period". b) The distribution of blanked periods in our recordings (bar plot) and their cumulative sum (gray line). Note that 70% of the artefacts were $\leq$1ms long.

3.1.1 Cell responses to ISMS

Post mortem dissection revealed that the microwire tips were located in laminae VII-IX. Results from microwire tips located in the white matter were removed from the analysis (n=9). Since previous studies (Mushahwar et al., 2000; Guevremont et al., 2006) demonstrated that ISMS in the ventral horn (laminae VII-IX) induces synergistic and stepping movements, we concentrated our cellular recordings in this area of the cord, as seen in Fig.3.2. Of the 278 total recording trials, 78 contained responses to ISMS (100-150μA) of which the majority (73%) were located in the ventral horn (Fig. 3.2 (a). Lamina IX, where the MNs reside, had the highest percentage of responses to ISMS, followed by lamina VII which is known to have interneurones that participate in the CPG (see introduction). Between all 6 cats, the whole length of the lumbosacral enlargement was stimulated and recorded from.

Recordings were obtained from a total of 106 cells, of which 50 cells responded to ISMS (47%) within the first 6ms of the stimulus onset. The distribution of cells that responded to ISMS relative to the cells recorded per lamina is shown in Fig. 3.2 b. Sixty eight percent of the cells that responded to ISMS resided in the ventral horn, and 50% of the cells recorded

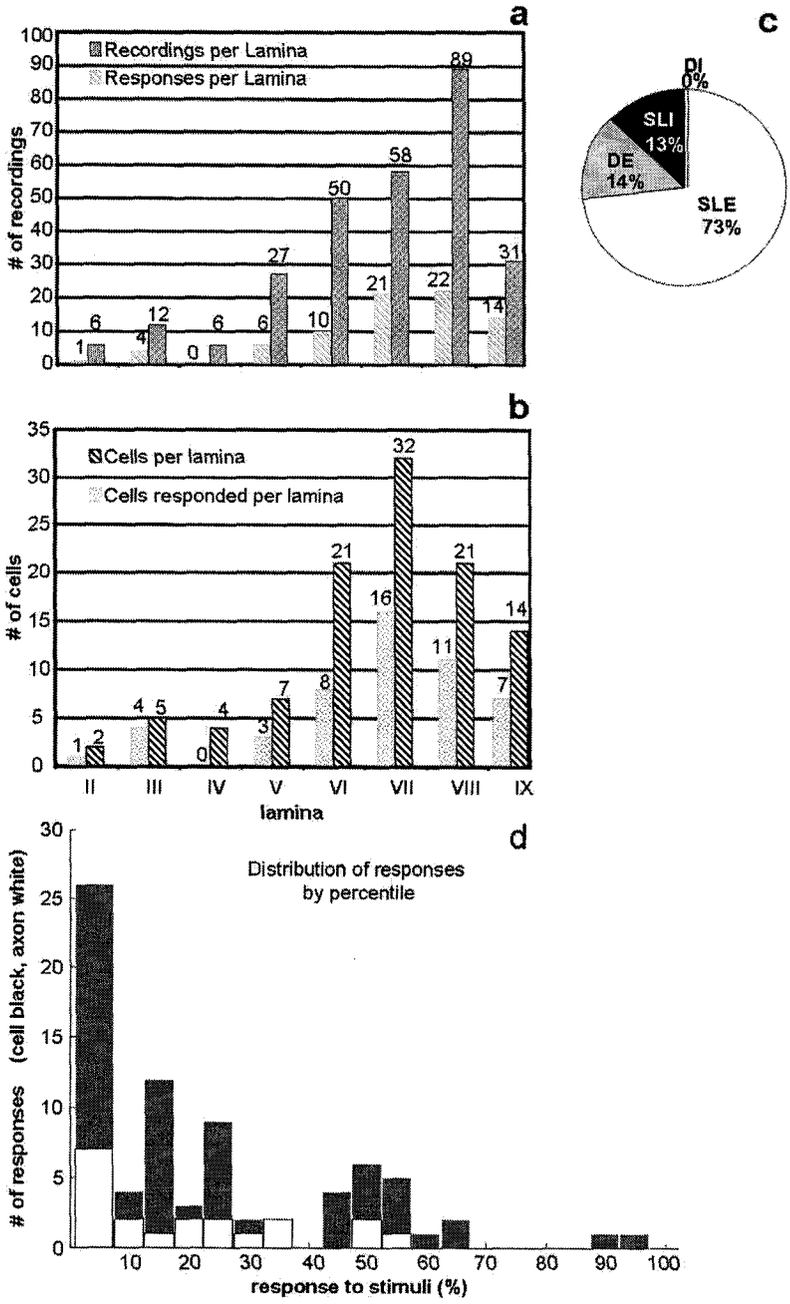

Figure 3.2: Overall distribution of cellular responses to ISMS. a) The number of recordings and responses per lamina show that the recordings were predominantly obtained from the ventral horn. The highest proportion of responses to ISMS was seen in lamina IX (45%). b) The distribution of cells responding to ISMS, with laminae VII-IX showing the highest proportional responses to ISMS. c) Distribution of the 78 responses by type, excitation accounted for 87% of the total. d) Indicates the probability of firing of cells (gray) and axons (white) to ISMS. Note that the majority responded to less than 50% of the stimuli in both cases.

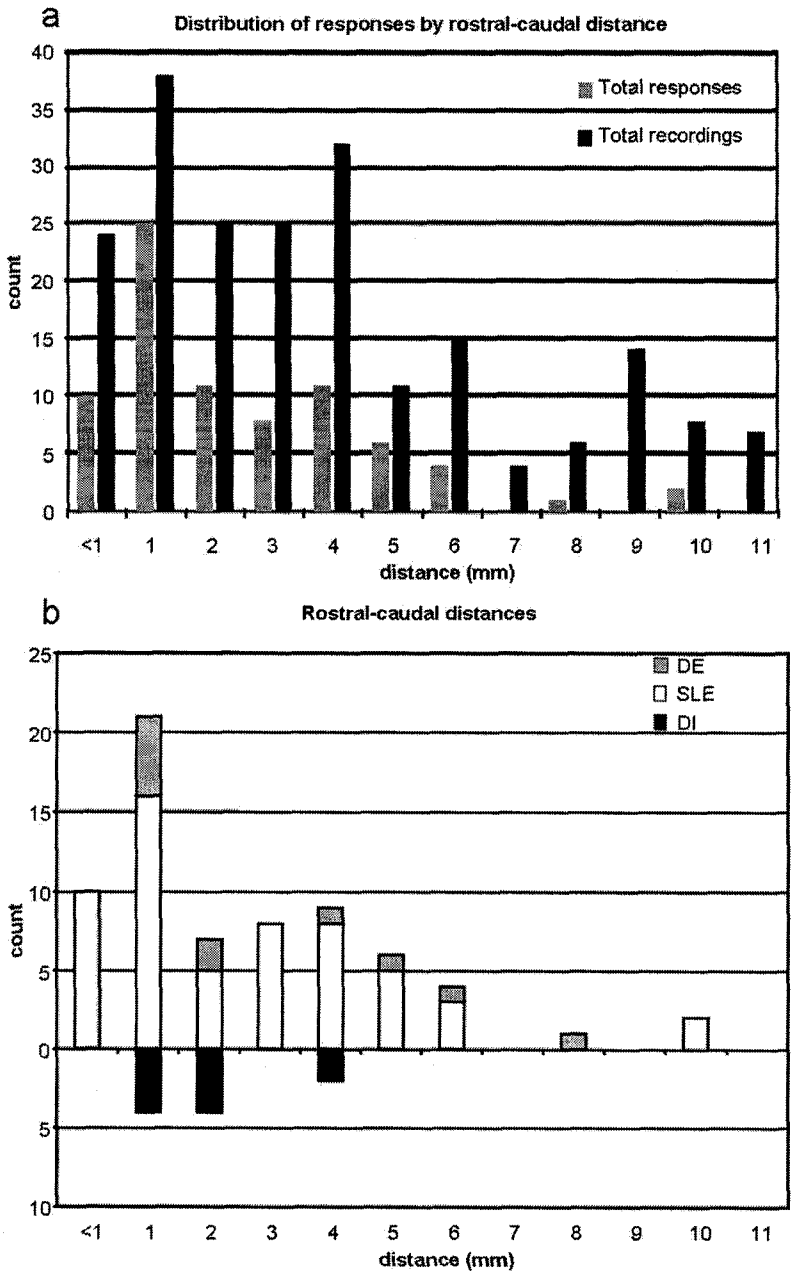

Figure 3.3: a) Rostro-caudal distribution of recordings per distance (black) and responses observed per distance (grey). b) Rostro-caudal distribution of the effects of ISMS (stacked). Cells located <1mm and up to 10mm from the stimulation site were seen to be activated by ISMS. A peak in activity was observed at 1mm where 16 cases of SLE, 5 DE and 4 SLI were obtained. Excitation was seen at both proximal and distal locations up to 10mm from the ISMS site while inhibition was restricted to the first 5mm.

from in laminae VII-IX responded to ISMS. The responses to ISMS were further divided by type based on the changes in firing rate within the first 6ms of stimulus onset (Fig. 3.2 c). Excitation comprised 87% of the responses (73% SLE, 14% DE). The remaining 13% was inhibitory in nature and exclusively SLI. Delayed inhibition, an onset of >3ms, was not observed. It was very common for one cell to respond to multiple microwires, hence 78 total responses were obtained from 50 cells. Curiously, there were three cells (6%) that not only responded to multiple microwires, but responded differentially to different microwires (data not shown). Of these three cells, two alternated between short and delayed excitation while the third alternated between short latency excitation and inhibition.

The stimulation-to-recording electrode distance shown in Fig. 3.3 portrays the effective spread achieved by ISMS. Cells up to 10mm away were excited, while those inhibited were restricted to 5mm from the ISMS microwire. No antidromic activation of any of the cells responding to ISMS was noted. Considering that the absolute current spread induced by ISMS is approximately 0.5mm, all responses at distances >1mm can be considered transsynaptic, which is confirmed by the jitter in the cells' response times to ISMS (e.g. Fig. 2.3). Therefore, to activate multiple synergistic motoneurone pools, the focally applied stimulation of ISMS must spread to these regions transsynaptically. The transsynaptic nature of ISMS is further shown by the failure of the cells to respond to all stimuli. Most cells responding to ISMS demonstrated <50% probability of firing (Fig. 3.2 c), the example used in Fig. 2.3 being an atypically responsive cell that fired in response to 93% of the ISMS stimulus pulses.

Excitation accounted for the majority of responses, which is not surprising given the excitatory nature of the stimulus. The dorso-ventral distribution of cells excited by ISMS (Fig. 3.4 a-b) shows that these cells were predominantly grouped in the ventral horn. Cells inhibited by ISMS, save one case, was also grouped ventrally. The PSTH for SLE records in Fig. 3.4 a-b, show that the peak changes in firing rate usually occurred 2-3ms after the ISMS pulse. This variability in the onset of changes in firing rate implies synaptic delay and not antidromic activation, since antidromic activation would produce changes in firing rate at a consistent post stimulus time. The inhibition observed in the PSTH was mostly sudden, resembling more a dropout than a gradual inhibitory effect (Fig. 3.4), although a few cells

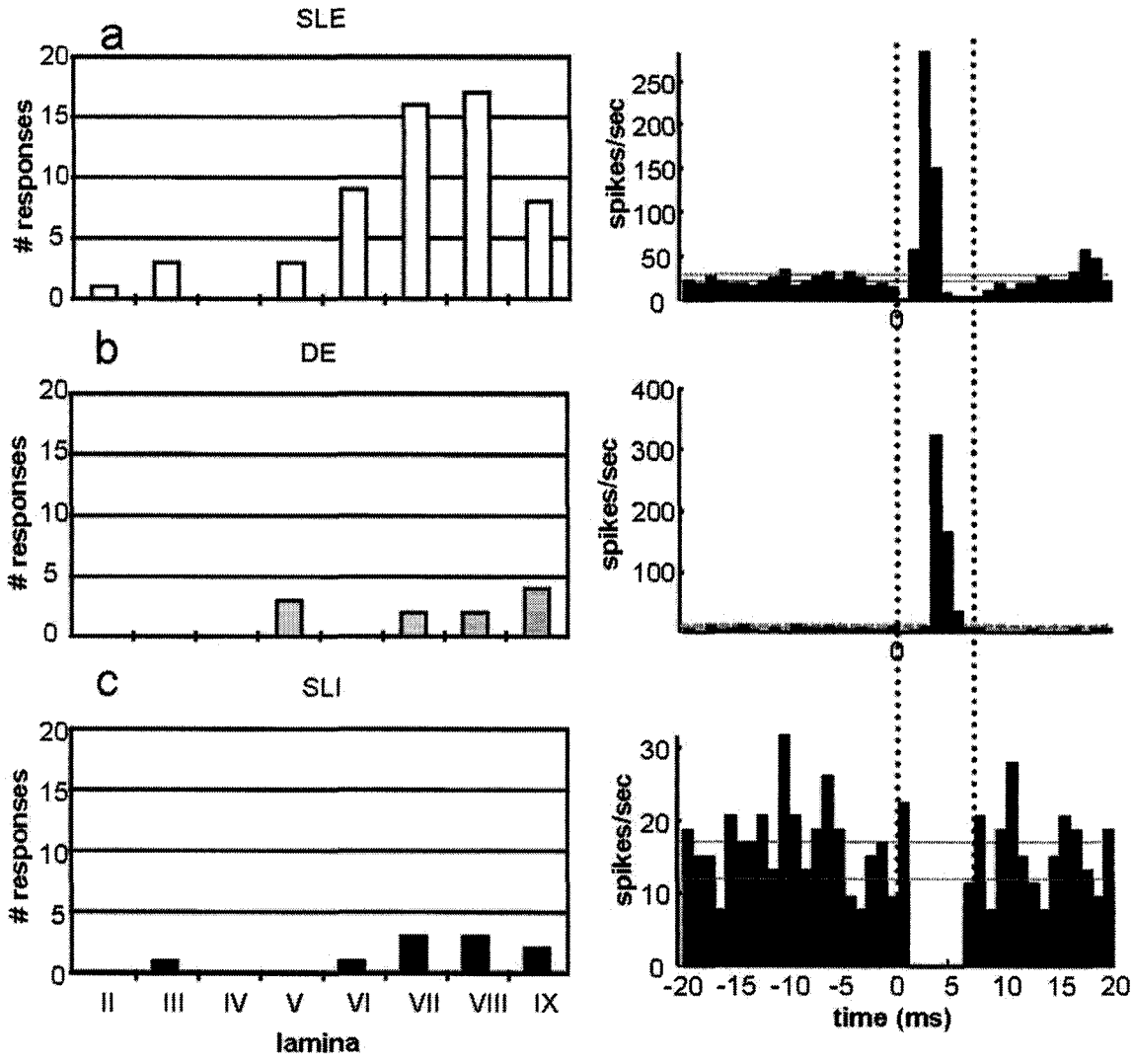

Figure 3.4: Each chart represents the distribution of responses divided by type and grouped by lamina. Plots on the right are PSTH examples of the corresponding response type charted to the left. These responses are: (a) short latency excitation (SLE); (b) delayed excitation (DE); and (c) short latency inhibition (SLI). The dotted vertical lines on the PSTH delimit the 6ms window analysed, the dashed horizontal lines indicate 2*standard deviation.

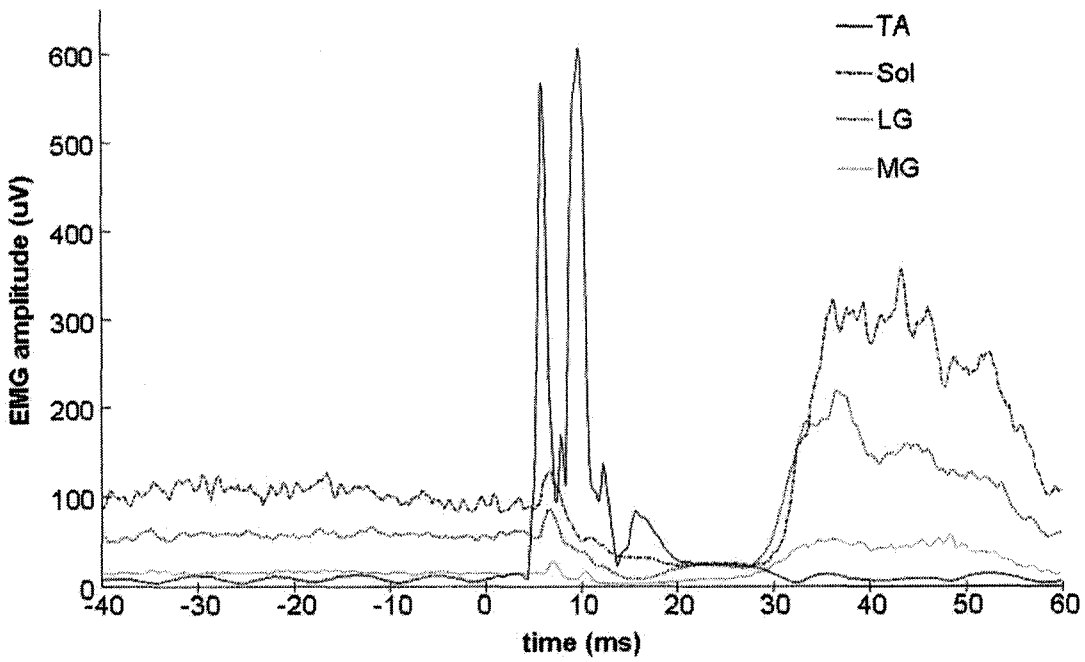

Figure 3.5: An example of EMG activity averaged over 205 ISMS stimuli. Stimulus onset is at 0. For clarification, only 4 muscles are shown. The ISMS pulse elicited activation of TA and inhibition of its antagonists (the triceps surae) was observed 2ms later. The other muscles were activated in similar time frames to TA, but peaked at amplitudes of least 4 times that of TA and, for scaling purposes, the responses from these muscles are not shown.

did present an inverse bell shape of graded inhibition that lasted between 20-30ms (data not shown). Another trend common to almost all inhibition was post inhibitory facilitation (rebound), resulting in an excitatory response shortly after the cessation of inhibition (data not shown).

Figure 3.5 shows overlain plots of averaged EMG activity elicited by ISMS. This case shows 4 muscles: tibialis anterior (TA, ankle flexor) and its antagonists, the triceps surae (ankle extensors: soleus, medial and lateral gastrocnemius), averaged over 205 stimulus pulses. Note that ISMS activates TA and, 2-3ms after the onset of activity in TA, its antagonists are inhibited. In this case, all muscles were activated to some degree, but the rest were omitted for clarity (Fig. 3.6).

In total there were 60 microwires implanted in these experiments and, in general, there was a large variability in the onset latencies and amplitudes of the EMG activity by ISMS (Fig. 3.6). Of the implanted microwires, 39 were analyzed and the rest were not included

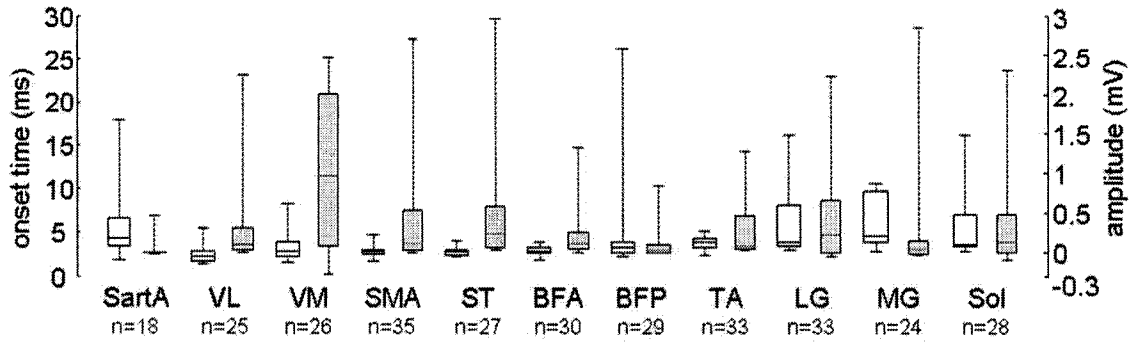

Figure 3.6: Whisker plot of pooled onset times in milliseconds and amplitude in millivolts of EMG activity elicited by ISMS (100μA). Not all muscles responded to stimulation through every microwire. The number of microwires through which ISMS elicited a response in each muscle is shown. The negative values of EMG amplitude, such as in VM and Sol, is due to cases where there was inhibition without prior excitation. The tendency of onset times to increase from left to right in this plot is due to the distance of the muscles from the spinal cord, which increases from left to right. The onset of EMG activity in the more proximal muscles, such as VL and VM, was <2ms. More distal muscles, such as Sol, were in general activated later, usually >3ms. Abbreviations: sartorious anterior (SartA); vastus lateralis (VL) and medialis (VM); semimembranosis anterior (SMA); semitendinosus (ST); biceps femorus anterior(BFA) and posterior (BFP); tibialis anterior (TA); lateral (LG) and medial gastrocnemius (MG); soleus (SOL).

because they either were not used for stimulation or they were used after administration of the neuromuscular blocker, Flaxedil ®. Overall, the EMG was of varying amplitudes and onset times, depending on microwire placement. Electromyographic activity was elicited in at least one muscle during all ISMS trials and some muscles were unanimously recruited (SMA). In other cases where background activity was present, as in Fig. 3.5, inhibition was induced after the activation of antagonistic muscles creating synergistic (activation of agonists with inhibition of antagonists) EMG patterns. The inhibition of antagonists emerged 2+ms after the onset of agonists and presented a similar pattern in all experiments.

In contrast to Fig. 3.6, spike triggered averaging of spontaneously firing cells revealed no EMG activity in any of the sampled muscles (Fig. 3.7). This suggested that none of the cells recorded from had direct connectivity with the sampled muscles. Had any cellular recordings been obtained from MNs, an obvious peak in the STA records would have been seen (as in TA Fig. 3.5) indicating the direct correlation between cell firing and EMG activity. In the case of a last order interneurone, there would also be a spike, albeit smaller

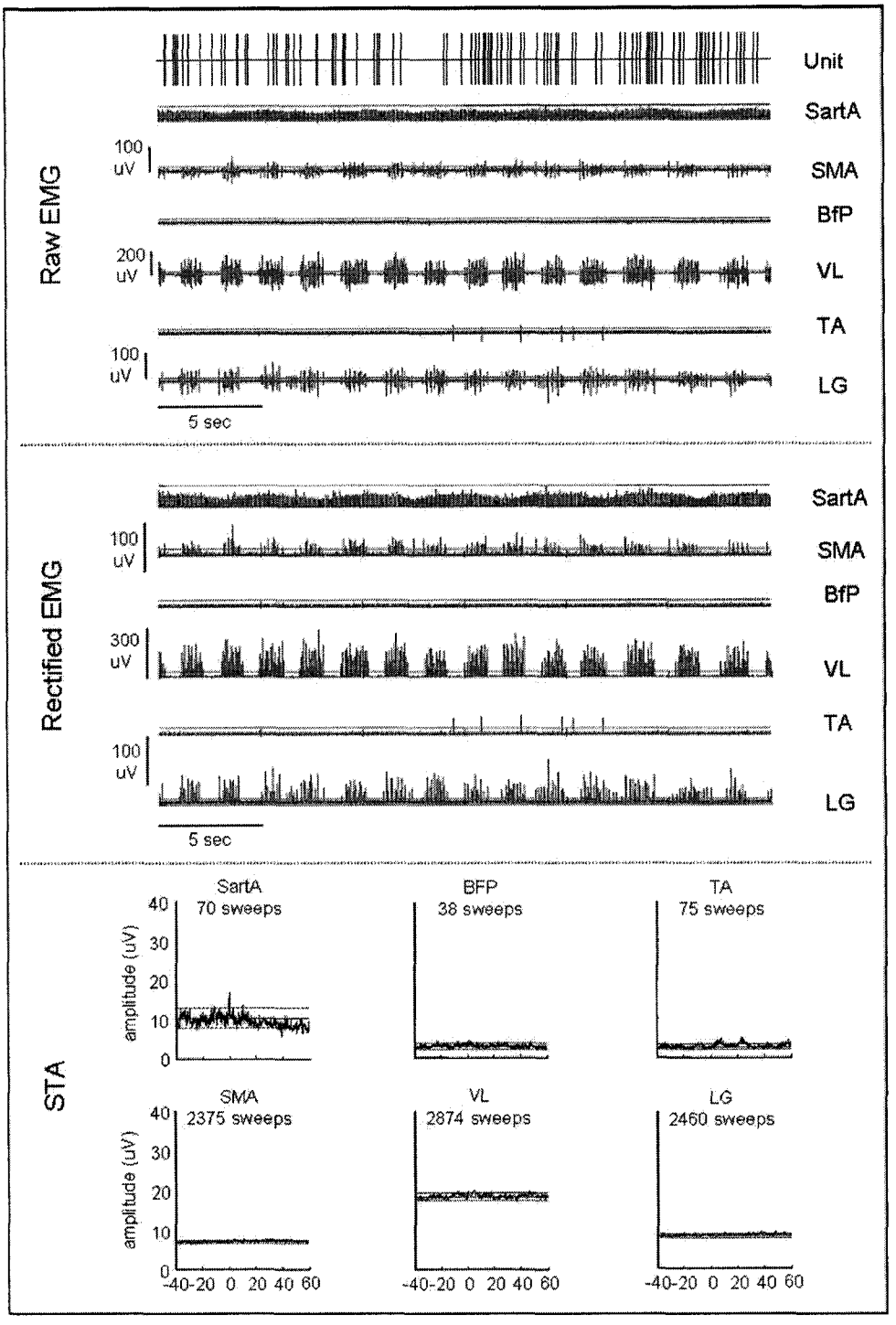

Figure 3.7: Spike triggered averaging of EMG activity for a single cell. Top block shows a 30s clip containing the time of spike activity and raw EMG for each muscle. The horizontal line in the EMG activity denotes the threshold level above which activity was considered to be present. The middle block shows the raw EMG rectified, before STA processing (also shows the thresholds). Bottom block is the final STA for this cell. No correlation between this cells' activity and the recorded EMG activity was present. This was the case for all cells, which suggests that neither motoneurones nor last order interneurones were represented in our recordings. The discriminated cell had 4800+ spikes in this record.

and with jitter. Given the fact that we saw no such cases in the muscles sampled in this study, it is assumed that none of the cells were MNs or last order interneurones. Note that, as can be seen by the various levels of EMG activity above "0", only motor activity (i.e. tonic drive or phasic activity) was present in the STA records. The absence of responses in the STA records is therefore not due to the lack of facilitation in the motoneurone pools nor the dominance of inactive periods in records of bursting EMG activity.

3.1.2 Antidromic potentials

Antidromic potentials such as those seen in Fig. 3.8 (top) were observed at various distances, ranging from 1-11mm from the stimulation site. The antidromic potentials were characterized by potentials (present in almost every sweep) that were not part of the stimulus artefact but still within 6ms. No effort was made to differentiate between antidromic action potentials and field potentials. However, a few cases the antidromic potential was absent from some fo the sweeps, suggesting that antidromic action potentials were evoked by ISMS. The decrease in number of potentials with distance (Fig 3.8 bottom left) is possibly due to slower conduction velocities combined with longer distances. This could lead some potentials to arrive after 6ms and therefore not be considered as antidromic in nature. Visual verification of the waveforms of all potentials revealed that none correlated to the cells that were spontaneously active prior to the initiation of ISMS trials, therefore no further analyzes on these potentials were performed.

3.1.3 Response from axons

A total of 25 axons were recorded from. The differentiation between axon and cell body was based on the duration of the local field potential, axons being $\leq$0.5ms as described by Loeser and Ward (1967). Of the 25 axons, 21% showed a response to ISMS (Fig. 3.9 (a). The axonal responses were predominantly excitatory, SLE (80%) and DE (10%), and followed a laminar distribution (Fig. 3.9 b) very similar to the cellular responses reported above (Fig. 3.2). The remaining 10% was comprised of SLI. Similar to the cellular responses, axonal PSTH of excitation (Fig. 3.10) presented prominent peaks with 2-3ms jitter. Although

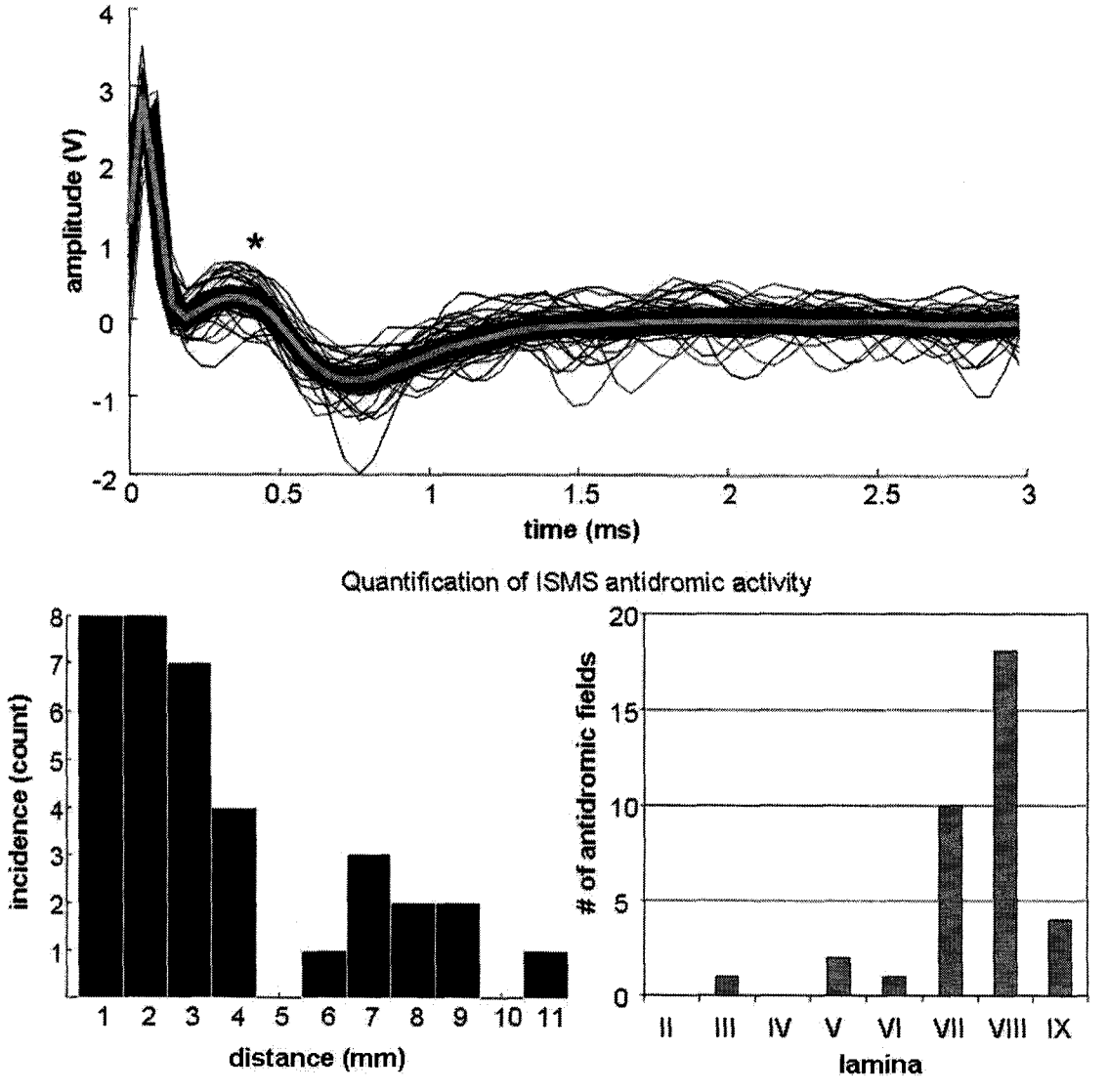

Figure 3.8: Overlain sweeps (top) containing an antidromic potential (marked by *) immediately following the stimulus artefact. Gray line indicates average of all 500+ sweeps. Distribution of antidromic potentials by rostral-caudal distance from site of ISMS (bottom left). Distribution of antidromic potentials by Rexed lamina (bottom right).

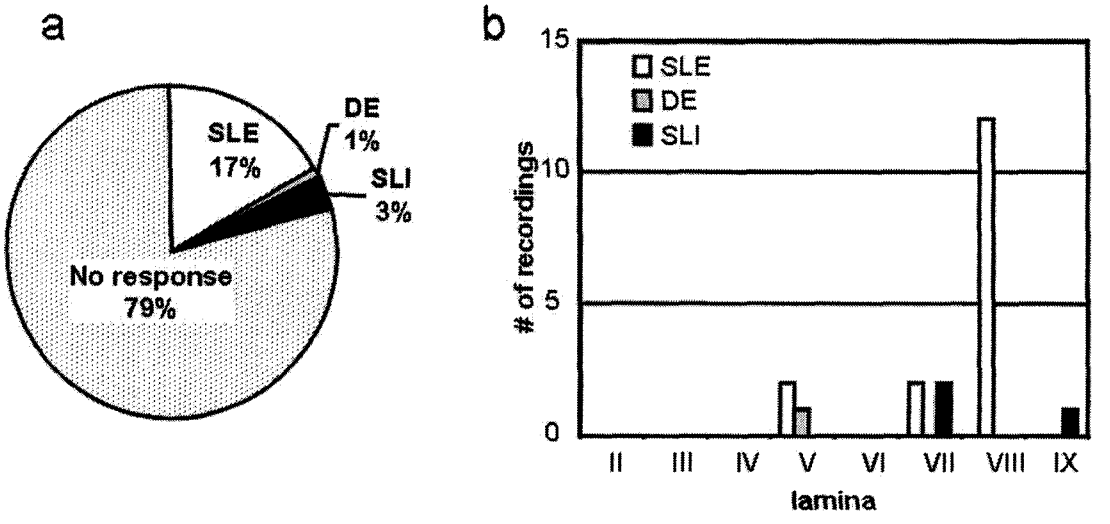

Figure 3.9: Results from axonal recordings. (a)Percentage of axonal responses by type, 21% of the axons responded to ISMS. b) Distribution of axonal responses by lamina.

the cases of excitation could be easily explained by the activation of fibres in passage close to the ISMS electrode, inhibition indicates the effects of ISMS on a cell body located elsewhere. Furthermore, since no antidromic activation was observed and jitter is present in all responses, it is assumed that the cell bodies were activated transsynaptically by ISMS. Finally, the distance between the recording and stimulation sites was predominantly long with 80% of the total axons (n=16) located at distances $\geq$4mm; the remaining axons (n=4) were all within 1mm of the stimulation site (data not shown).

3.2 ISMS in the deafferented model

In order to address the differential role of afferent fibres and propriospinal pathways in mediating the effects of ISMS, animals with complete unilateral deafferentation of the lumbosacral enlargement were used. Preliminary results from 5 cats are reported. All animals, except one, demonstrated significant disorders in the deafferented hindlimb. These disorders, as expected, consisted in treating the deafferented limb as if it were paralysed and rarely, if ever, using it for locomotion of postural control. The exception, which was later attributed to the preservation of some L4 rootlets, was the only animal that regained proper

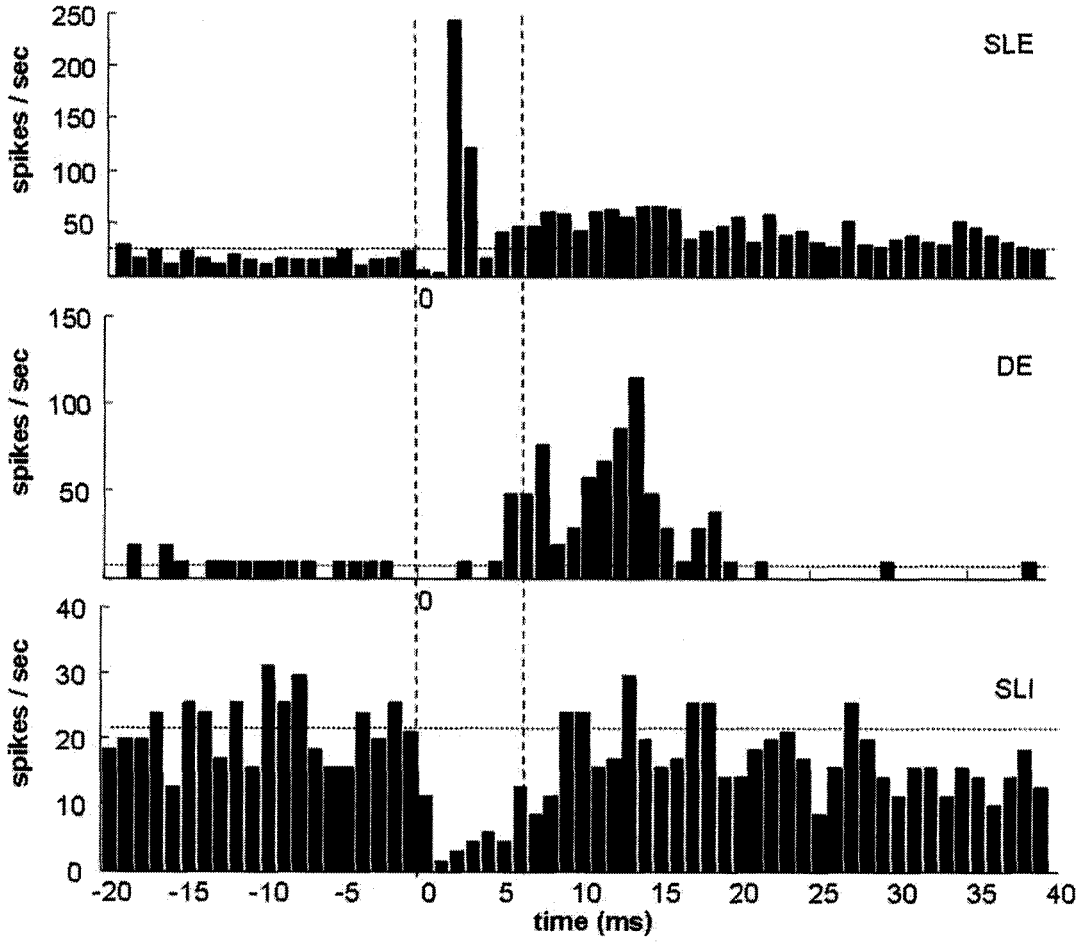

Figure 3.10: Results from axonal recordings. Post stimulus histogram (PSTH) examples of each response type: SLE (top); DE (centre); and SLI (bottom), horizontal dashed lines indicate significance (2*std). Vertical lines indicate 6ms window of interest. Note how the excitatory responses had a prominent peak. Also note the short, tight window of inhibition. Inhibitory response was composed of 7500+ spikes.

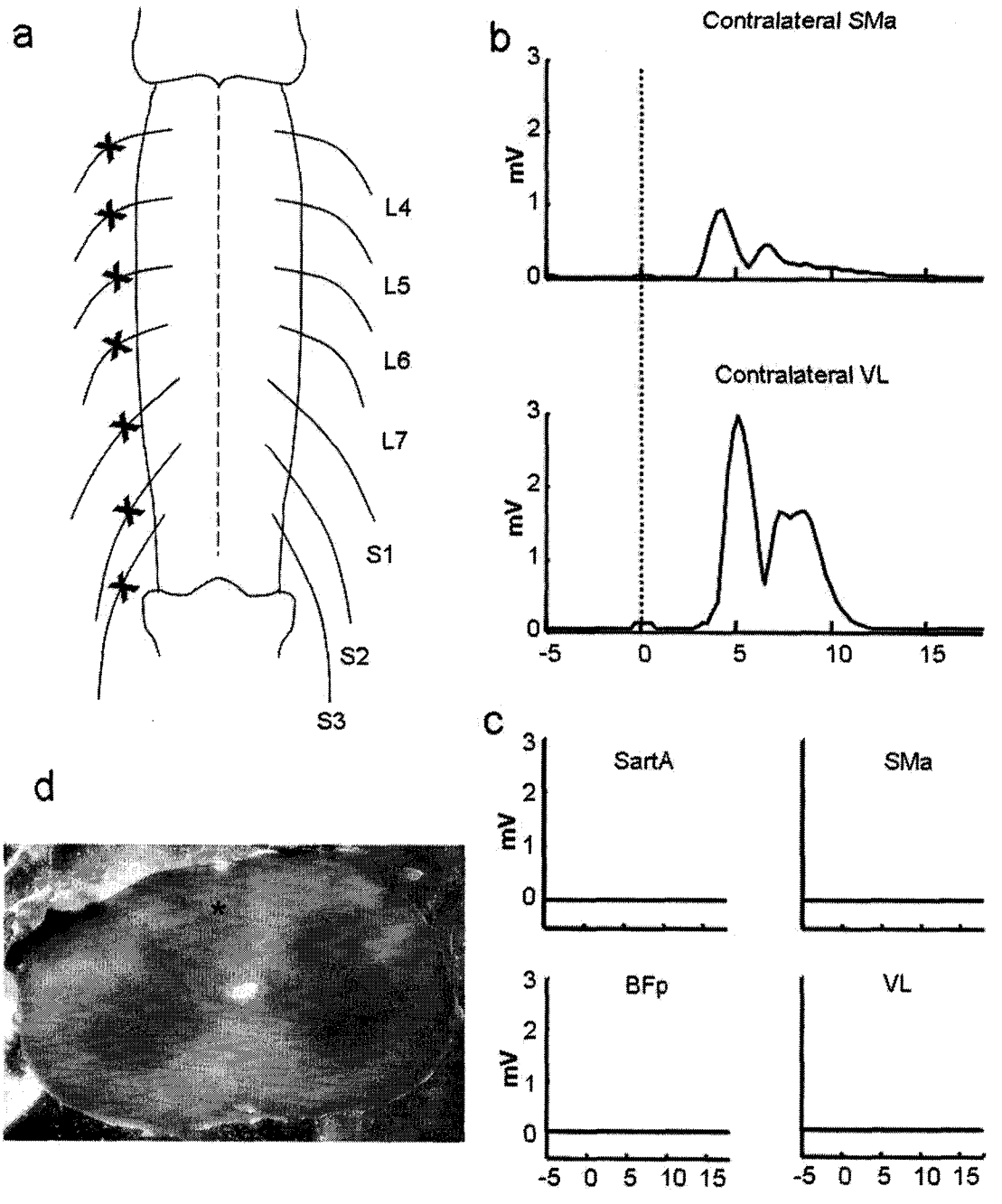

Figure 3.11: a) Dorsal rhizotomy consisted of the exposing, bundling and severing of the dorsal rootlets. 30-40 days post surgery recording sessions were performed. Contralateral EMG traces (b) during stimulation of the intact contralateral L7-S1 rootlets at 4mA. (c) Stimulation of the chronically transected L7-S1 dorsal rootlet bundle at 5 mA. As can be observed, contralateral stimulation at lower amplitudes elicited strong EMG activity while stimulation at higher amplitudes ipsilaterally elicited no EMG responses. (d) Grayscale photograph of the L6 segment of one animal. Note the discolouration of the ipsilateral dorsal column due to scarring (asterisk).

paw placement in the ~30 day period post deafferentation. None of the deafferented animals received locomotor training, and any recovery was based on the normal daily activity of the animal. Two animals presented solely three-legged gait, in which the deafferented limb was either maintained flexed or extended and dragged behind the animal as described in the early stages of rhizotomy by Goldberger and Murray (1988). The remaining two exhibited moderate recovery of function, using the limb for partial weight support during standing and walking. The invariably incorrect paw placement led to skin abrasions of the dorsal surface of the paw and, in one cat, abrasions of the knee as well. Due to technical difficulties (n=1) and premature death after decerebration (n=2) single cell recordings were obtained from only 2 animals. Post mortem dissection revealed a marked scarring of the dorsal columns (Fig. 3.11 asterisk in d) but little change in the profile of the grey matter, in accordance with the descriptions by Goldberger and Murray (1988).

In total I obtained 8 cells from these 2 animals. To verify the degeneration of the dorsal rootlets, stimulation of the proximal stump of the dorsal roots at various amplitudes (up to 5mA) was performed on 4 of the cats and no ipsilateral EMG responses were evoked (Fig. 3.11 shows EMG of 5mA stimulation). Conversely, stimulation of the intact contralateral roots at 4mA gave rise to contralateral EMG activity (Fig. 3.11).

As was the case in the intact animals, the majority of the blanking periods in the stimulus artefacts (60%) in the deafferented animals were 1ms or less in duration. Response probability to ISMS was also less than 50%, as was seen in the intact animals.

Recording from the deafferented animals was more challenging than those with intact afferent input since there was a marked decrease in overall excitability of the cord, leading to very few spontaneously active cells, and 5-HTP was frequently administered to increase excitability. Of the 8 cells recorded from, 2 responded to ISMS (Fig. 3.12).

The cellular responses were short latency excitation (n=2) and delayed excitation (n=1) (Fig. 3.12 (d)). The dorsal-ventral distribution of these results were from lamina VII and VIII (Fig. 3.11 (d)). Similar to the results from the intact animals, spike triggered averaging revealed that the cells recorded from were not MNs nor last order interneurones of the muscles sampled (Fig. 3.14 (d)). EMG activity in these experiments also showed a selectivity of ISMS-induced muscle activation (Fig. 3.15). It is also important to note

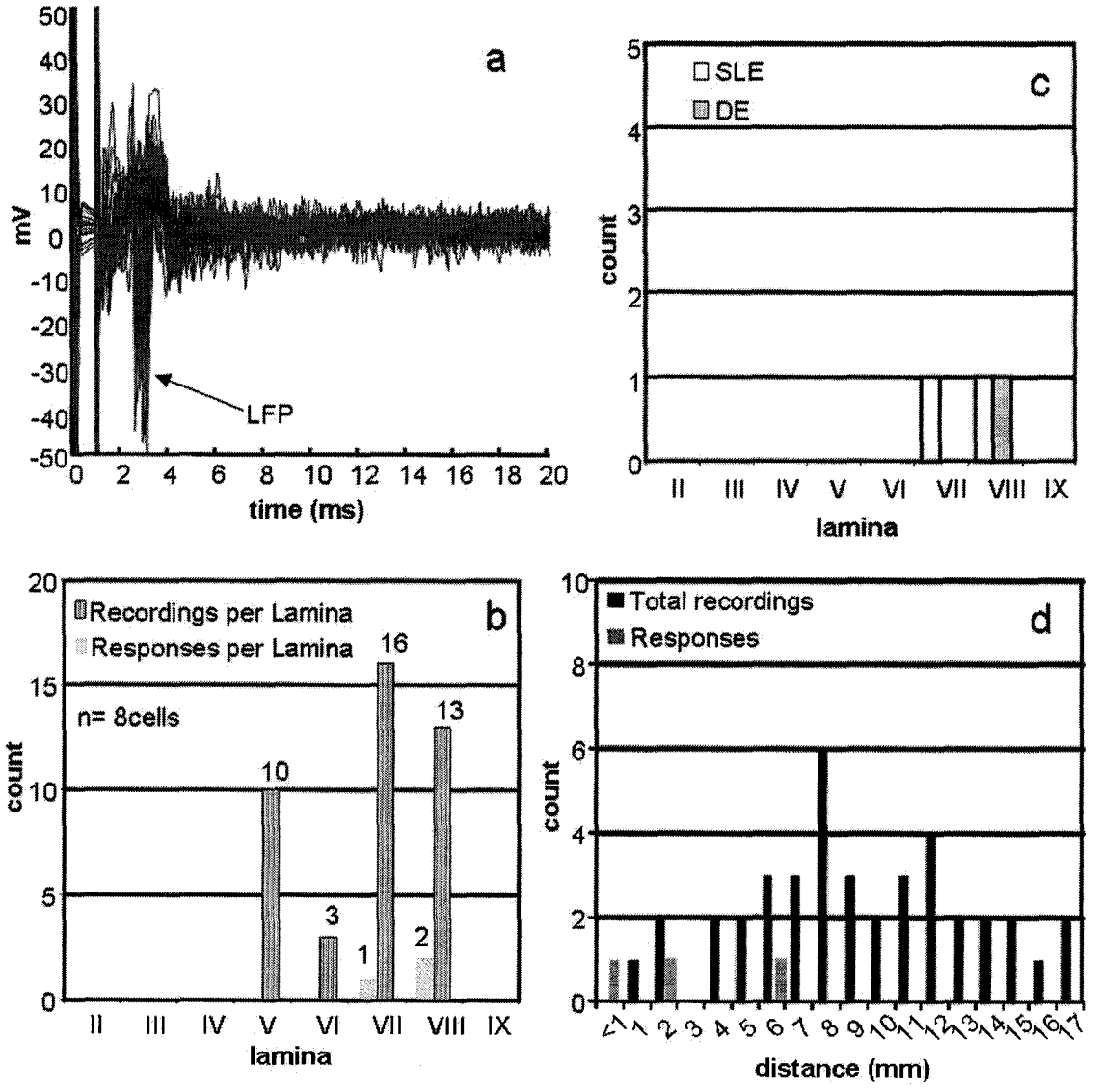

Figure 3.12: The preliminary results of the deafferentation experiments are shown as (a) raw traces LFP indicated by arrow; b) grouped showing the number of recordings (dotted fill) and responses (angled line fill) per lamina; c) response type laminae and; d) number of recordings performed (black) and the number of responses observed (gray) at each rostral-caudal distance.

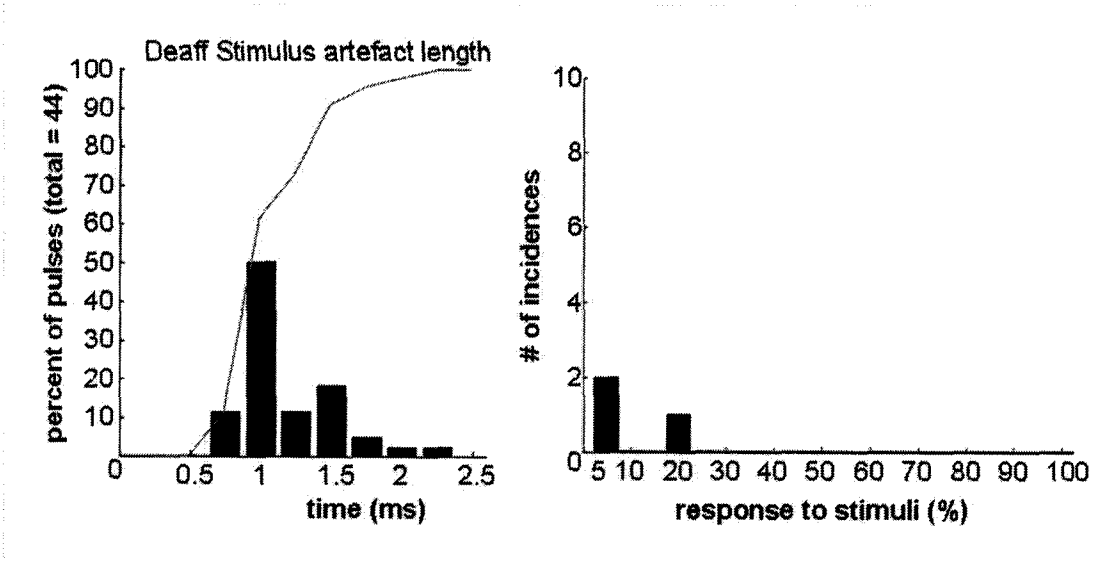

Figure 3.13: Distribution of durations of blanking periods in the stimulus artefact (left) and probability of firing in response to ISMS (right).

that, as in the intact model, responses were distributed in a rostral-caudal manner, reaching up to 6mm in the deafferented cats (Fig.3.12 (c)). The more distributed responses (up to 10mm seen in intact cords) are not, therefore, due to solely afferent backfiring.

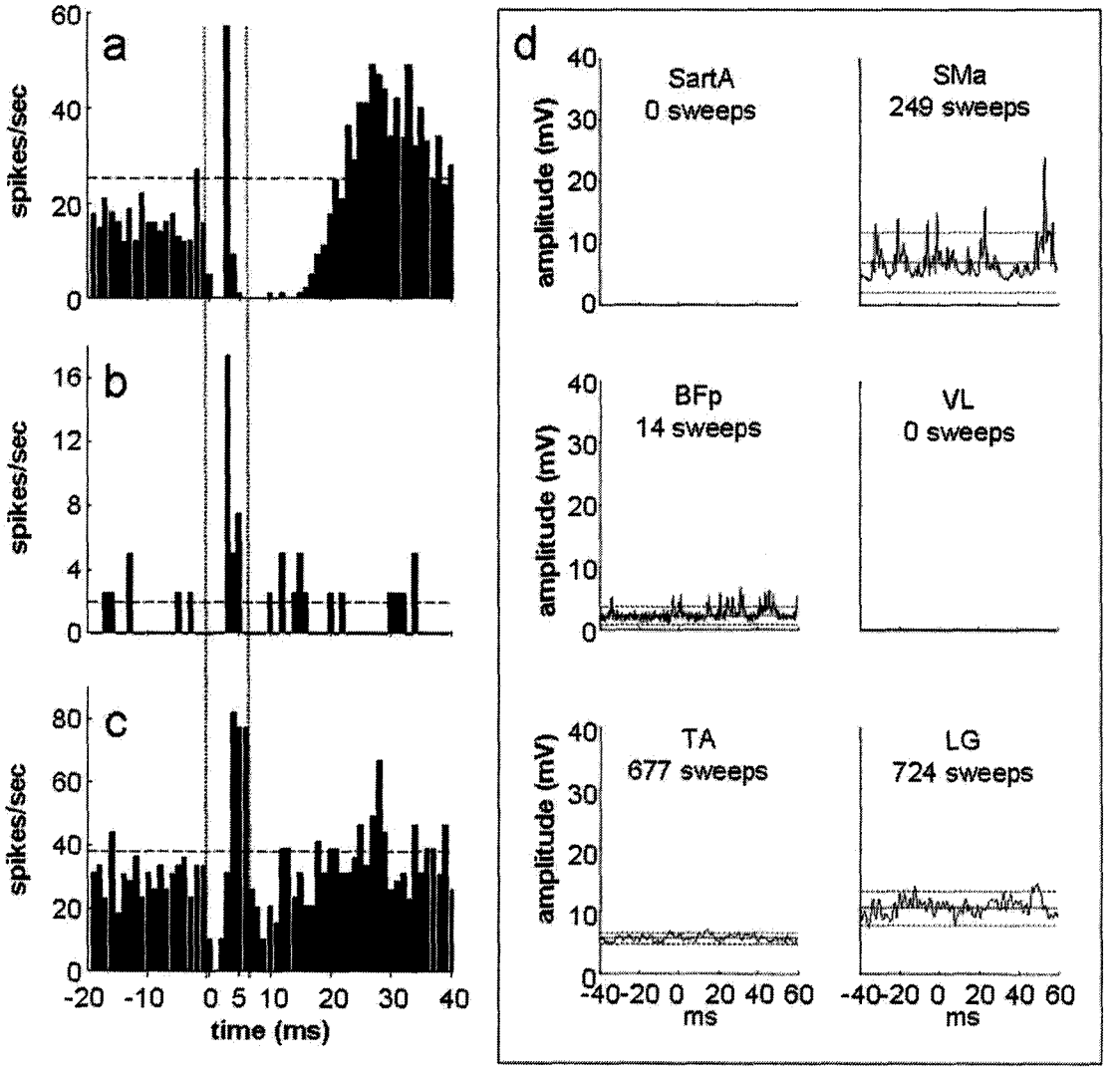

Figure 3.14: PSTH records of cellular responses to ISMS in the deafferented cord. Vertical dotted line delineates 6ms window in which short latency excitation (a-b) and delayed excitation (c) are elicited. Rostro-caudal distances between microwire and recording electrode were 2mm, 6mm and <1mm, in a-c respectively. d) Spike triggered averaging of 1000+ spikes of spontaneous firing from cell (a). Similar to the results in (d), no STA activity was observed in the 60ms analyzed of any of the cells recorded.

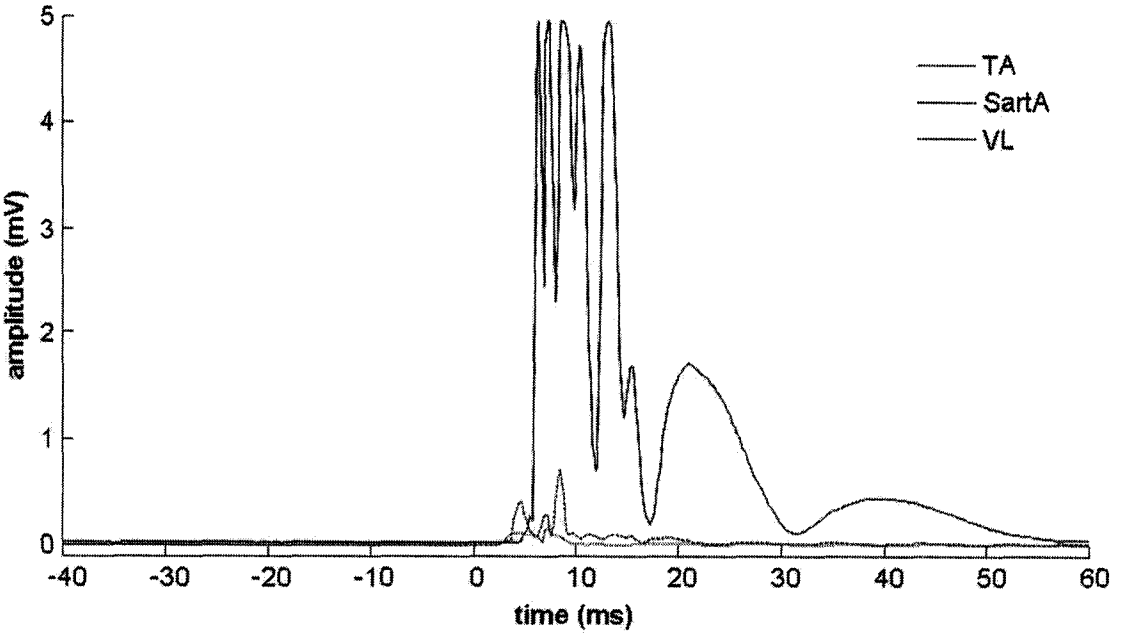

Figure 3.15: Overlain EMG traces from muscle activation responding to ISMS. This stimulus average is composed of 1000 stimuli and corresponds to the trial in Fig. 3.14 (a). Note that while TA was highly activated by the stimulation there was no correlation between the spontaneously firing cell and the TA EMG (TA STAin Fig. 3.14).

Chapter 4

Discussion and Future Directions

4.1 Discussion

The goal of this study was to investigate the sites of action of ISMS and determine the effect of ISMS on the firing rate of cells located throughout the lumbosacral enlargement. The main finding in this study was that ISMS elicits cellular responses beyond the area of electrical activation, even in the absence of afferent fibres.

4.1.1 Activation of fibres in passage

Recent work investigating the mechanisms of action of deep brain stimulation (DBS) revealed that it predominantly recruits fibres in passage as opposed to activating the cell bodies in the vicinity of the stimulation electrode (Nowak and Bullier, 1998a,b). The data presented here, in conjunction with previous studies (Gaunt et al., 2006), show that this is also the case with ISMS.

Transsynaptic activation of motoneurones by ISMS

All the cells responding to ISMS appeared to be activated transsynaptically by the stimulus. No antidromic activation was seen and significant jitter was present in all the PSTH records. In general, the excitatory cellular responses had a prominent peak in the PSTH records with a variability of $\pm$1ms. This shows that ISMS acts on these cells in one of two ways: (i) ISMS

preferentially activates fibres with very strong synaptic connections onto these cells; or (ii) ISMS activates multiple fibre types that simultaneously synapse onto these cells, creating enough overall postsynaptic drive to cause firing. Although either mechanism could have lead to the responses observed, it most probably was a combination of both that produced the overall responses to ISMS.

Participation of afferents projections in ISMS responses

A previous study with ISMS (Gaunt et al., 2006) raised the question of the dependence of ISMS on the afferent fibres for its distributed activation (effective spread). It was suggested that, since the afferents were activated at lower thresholds than MNs, the activation of fibres in passage (i.e., backfiring of afferent fibres) is more common than the direct activation of MNs. It was also suggested that activation of afferent projections alone accounts for the functional responses elicited by ISMS.

The current study investigated the differential activation of the afferent projections and the propriospinal systems by ISMS. Intraspinal microstimulation evoked similar cellular responses in both intact and deafferented models. The deafferented cords were less excitable and yielded less overall results but, even in the absence of afferent projections, ISMS elicited cellular responses at distances beyond the absolute spread of current (up to 6mm). These responses were elicited at latencies comparable to those seen in the intact animals and shared the same characteristic jitter of transsynaptic activation. Furthermore, the cellular responses observed in the deafferented spinal cord were of more than one type (SLE and DE), as in the intact animals.

Interestingly, the cellular response times to ISMS in the deafferented animals were consistent with those seen in the intact animals. Since this project considered solely the direct effects of ISMS and not the reflexive effects, only the first 6ms after the stimulus onset were considered. For the deafferented model we opened this "window" up to 20ms, since there would be no reflexive activation, but observed no difference in the results. The onset of all the cellular responses seen from the preliminary experiments was still within the first 6ms. This indicates that ISMS could be activating some of the same networks in both the intact and deafferented spinal cords. We suggest these networks include the propriospinal

networks of the lumbosacral enlargement.

Direct activation of the motoneurones

Undoubtedly, ISMS directly activates motoneurones in the proximity of the microwire tip as seen by the short latency EMG responses. Motoneurones activated beyond the absolute spread of current, as was the case in the present experiments, would have to be activated through other means. A recent study of the motor units activated by ISMS showed a predominant recruitment of type I and IIa muscle fibres. This suggested a recruitment pattern of MNs similar to the physiological recruitment order obtained during natural activation. The results of this study demonstrate that ISMS activates both afferent and propriospinal projections in the ventral horn which in turn activate MNs transsynaptically, thus recruiting them according to their size.

The activation of multi-joint synergistic movements also cannot be explained through the direct activation of adjacent MNs. For these types of movements activation of afferents or local propriospinal networks would be necessary. Although for our study specific functional responses were not targeted, the distal cellular responses during the deafferented experiments show that ISMS activates networks even in the absence of afferents projections.

4.1.2 Specificity of ISMS

A previous study (Gaunt et al., 2006) noted that ISMS appears to elicit nonselective muscle activation, commonly resulting in co-contraction. This comes in contrast to the data presented here and could be, at least in part, due to the placement of the microwires. Our experiments targeted the ventral horn, a region previously shown to elicit functional responses, and saw selectivity in both the EMG activity (not all muscles were activated Fig. 3.5) and in the cellular responses (53% did not respond to ISMS Fig. 3.2). Also, ISMS elicited inhibition of EMG activity in some muscles while evoking excitation in others, as seen in previous studies (Prochazka et al., 2002). Together these data show that, although ISMS may backfire the afferent system, it does not elicit, non-selective excitation of spinal neurones.

Furthermore, since all the cells that responded to ISMS were transsynaptically activated, ISMS may act as much through the backfiring of propriospinal and descending projections (in the intact animal) as it may through the activation of afferent fibres. Which fibres are being activated, therefore, would be defined by the density of projections within the range of the stimulus and fibre size, not the type of fibre. Any fibre, afferent, propriospinal or other, would be indifferently activated. What would make a difference would be the extent and destination of the activated terminals.

Previous studies demonstrated that subthreshold tonic ISMS in caudal segments of the lumbosacral enlargement (segments L7-S1) can induce rhythmic locomotor-like patterns of the hind limbs in the spinal and decerebrate cat (Guevremont et al., 2006). However, these rhythms could not be easily elicited after a spinal transection between the L4 and L5 segments. This suggests that ISMS in the L7-S1 segments stimulated fibre in passage which in turn activated the L4 segment. Given the evidence presented in the current study, activation of L4 from the L7-S1 segments (distances of 15+ mm) would be through transsynaptic activation and could be composed of activation of either the afferent or propriospinal systems. However, considering the role of the propriospinal interneurones in the CPG, it is very probable that the action of ISMS on these fibres is the main cause for the results seen.

Intraspinal microstimulation has also been applied in animals with chronic low-thoracic spinal transections and produced functional movements of the hind limbs (Saigal et al., 2004; Guevremont, 2007). Not only was muscle selectivity obtained, but the responses stabilized and remained functional over time. The successful outcome of these ISMS trial demonstrates that ISMS does not rely on activation of the descending systems. Given the current experimental model it is probable that descending projections were activated. Although they may have participated in the overall responses to ISMS, they are not required for the proper functioning of ISMS after spinal cord injury.

The preliminary data from the deafferented animals shed light on the importance of the propriospinal systems in mediating the effects of ISMS. The fact that ISMS-induced cellular activity was seen in the deafferented preparation suggests that fibres in passage other than the afferent projections are participating in the functional results obtained in intact and spinal animals. Given the current experimental data, it is difficult to identify which

propriospinal systems in particular are being activated and how much they contribute to the functional responses of ISMS. Taken collectively, the responses reported for spinal, intact and deafferented animals demonstrate that ISMS does not act solely through the descending, afferent or propriospinal systems. The present study verifies that propriospinal projections are activated by ISMS. Due to their contributions to locomotion (see introduction) it is likely that both the commissural propriospinal pathways as well as those participating in the flexor-extensor half-centre-oscillator are activated by ISMS. These, in combination with afferent backfiring, could produce the coordinated synergies and locomotor like rhythms seen in prior experiments. The inhibition of ongoing EMG activity in the current study suggest that reciprocal inhibition or Ia inhibitory interneurones are among the pathways activated by ISMS.

In general, the role each individual fibre in passage system may play in mediating the effects of ISMS remains unclear. The functional results previously obtained with ISMS are best ascribed to the activation of both the propriospinal and the afferent networks within the lumbosacral region of the spinal cord. Only through further experiments can these questions be addressed.

4.2 Limitations

A number of experimental limitations might have affected the results of this study. First, although analysis of extracellular activity provided valuable insight into the effect of ISMS on the firing rate of cells throughout the lumbosacral enlargement, no attempts were made to identify these cells. Second, of the spontaneously firing cells that were observed in the animals, only those which appeared to respond to ISMS (through auditory feedback) were recorded and subsequently analyzed. Therefore, the total number of cells responding to ISMS may be exaggerated. Third, during stimulation, the artefact recorded varied in size depending on the proximity of the recording electrode to the microwire and the amplitude of stimulation. Therefore, it is possible that antidromic activation was present but was masked during the saturation of the amplifiers. Fourth, in the deafferented animals, although the dorsal roots were transected, afferent fibres occasionally invade the ventral

roots (Hildebrand et al., 1997; Radim and Dubovy, 2006; Wee et al., 1985). Although it is still unclear whether these fibres actually enter the gray matter or end blindly in the ventral roots, it is assumed that these fibres did not participate in the results of this study. Finally, in these experiments, careful placement of the microwire tips in regions eliciting functional standing and stepping responses was not conducted. Therefore, the selectivity of activation of specific muscle groups could not be guaranteed. That notwithstanding, some selectivity of muscle activation was still seen. Furthermore, the cellular responses to ISMS would still be similar to those expected from careful placement of the microwire tips in the ventral horn.

4.3 Conclusion

This study investigated the effects of ISMS on the firing rate of single cells in both intact and deafferented cats. Intraspinal microstimulation elicited both excitation and inhibition in the spinal cord. Furthermore, ISMS did not produce generalized facilitation of all the cells in the lumbosacral enlargement. In the intact spinal cord, ISMS transsynaptically elicited changes in firing rate of cells up to 10mm from the stimulation site. Similarly, ISMS produced excitatory effects on cells up to at least 6mm away after chronic deafferentation. Therefore, the functional outcomes of ISMS could be attributed to the activation of both afferent and propriospinal projections in the ventral horn.

4.4 Future directions

The immediate future direction of this study is to increase the number of cellular recordings in the deafferented animals. This will be achieved through the addition of one more experimental animal to the dataset. Although it is not expected to change the results, it will provide more substantial conclusions. These experiments can then be taken one step further and cfos immunohistochemistry methods can be used to identify populations of neurones activated by ISMS and their locations in the spinal cord.

This study incites many questions about the underlying mechanisms of action of ISMS. What propriospinal pathways are involved? What networks elicit the inhibitory effects seen

with ISMS? What are the effects of ISMS on contralateral EMG activity?

These questions, as well as many others, can only be answered through the continued study of ISMS. I believe that the next logical step is to study the deafferented cord with one extra lesion: chronic thoracic spinalization. This model will help specifically understand the participation of the propriospinal and segmental interneuronal networks in the functional outcomes of ISMS. These experiments would require not only the multiple lesions but also specific targeting of the microwire tips to study the cellular responses during specific synergistic or single muscle recruitments. If, during these experiments, contralateral EMG is also monitored, it will be possible to also study the commissural effects of ISMS.

To investigate the activation of long propriospinal neurones, a different series of experiments in intact animals could be performed. In these, EMG activity of the ipsilateral and contralateral forelimb muscles could be monitored and recruitment of long propriospinal or inter-enlargement pathways could be investigated and quantified. If these networks are activated, ISMS could be shown to have an effective spread, with specific targets, that extends the whole length of the cord.

Finally, for the further development of ISMS as a neural prothesis, it would be necessary to verify that the human spinal cord responds in a similar fashion to the experimental models used.

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