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Plasticity following spinalization and step-training in the cat

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Cette thèse intitulée :
Plasticity following spinalization and step-training in the cat

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RÉSUMÉ

L'entraînement locomoteur est fréquemment intégré au programme de réadaptation chez les blessés médullaires (SCI) afin de maximiser leurs capacités locomotrices résiduelles. Cette stratégie est directement inspirée de travaux effectués au laboratoire chez le chat spinal, un modèle pour lequel les voies réflexes et les réseaux locomoteurs sont bien décrits. En l'absence des voies supraspinales, la moelle épinière a la capacité de générer des patrons locomoteurs suite à une stimulation sensorielle répétée procurée par l'entraînement sur tapis roulant. Cependant, les mécanismes impliqués dans cette récupération sont peu connus.

Projet I. On assume fréquemment que les changements plastiques dans les réseaux locomoteurs spinaux sont responsables de cette récupération. Cependant, la stimulation sensorielle répétée liée à l'entraînement pourrait aussi modifier la transmission dans les voies réflexes qui contribuent aussi à l'activité musculaire durant la marche. Dans ce projet, la transmission réflexe est évaluée par la mesure de réponses intra-motoneuronales de muscles fléchisseurs et extenseurs qui innervent la cheville, le genou et la hanche évoquées par la stimulation d'un nerf musculaire ou cutané de la patte postérieure lors d'une expérience en aigu chez le chat décérébré. Les modifications possibles sont déterminées par une comparaison statistique des résultats provenant de deux groupes de chats dont la moelle a été complètement sectionnée à T13, dont un seul groupe est entraîné à la marche sur tapis roulant. Les résultats montrent que la transmission dans les voies réflexes musculaires et cutanées est modifiée par l'entraînement locomoteur. L'excitation monosynaptique dans les extenseurs est diminuée suite à l'entraînement et la modulation phasique normalement observée est récupérée. Aussi, l'inhibition de groupe Ib est diminuée suite à l'entraînement et à l'injection de clonidine, un agoniste noradrénergique utile à la locomotion. De plus, il a été observé que la plasticité dans les voies cutanées est spécifique : elle n'est présente que dans quelques voies dans lesquelles la transmission est le plus souvent diminuée et les voies cutanées activées par la plante du pied sont particulièrement modifiées. L'ensemble de ces données suggère que l'entraînement locomoteur diminue l'hyperexcitabilité réflexe observée chez les SCIs et qu'il facilite le recrutement des extenseurs importants pour le support de poids.

Projet II. Plusieurs études illustrent que la plasticité des circuits spinaux est affectée par divers mécanismes moléculaires qui dépendent de l'activité physique et qui influencent la

capacité à récupérer les mouvements locomoteurs et à les maintenir. Plusieurs recherches récentes s'intéressent à des molécules impliquées dans la formation de la potentialisation à long-terme (LTP) afin de déterminer si les mécanismes responsables de l'apprentissage dans l'hippocampe sont similaires lors d'acquisition ou de la modulation de réflexes dans la moelle épinière. La protéine ERK joue un rôle reconnu lors de la plasticité synaptique et pour l'intégration des signaux de la surface cellulaire jusqu'aux facteurs de transcription. Elle apparaît donc comme un candidat idéal pour véhiculer les effets bénéfiques de l'entraînement et participer dans les événements synaptiques associés à la récupération de la marche suite à l'entraînement. Dans ce projet, des western blots ont été effectués pour mesurer l'expression de ERK et de sa forme activée, pERK, dans la moelle épinière de 3 groupes de chats : intacts, spinaux, spinaux avec entraînement locomoteur. Les résultats montrent que l'activation de ERK est augmentée dans la majorité des segments lombaires chez les spinaux et qu'elle est spécifiquement diminuée au niveau L5 suite à l'entraînement locomoteur. Nos résultats suggèrent que la SCI peut augmenter l'activation de ERK et ceci, pendant plusieurs semaines et que l'activation de ERK est potentiellement nuisible à la récupération locomotrice si elle est présente dans certains segments spinaux.

***Mots clés**: CPG, CREB, entraînement sur tapis roulant, enregistrement intracellulaire, ERK, lésion de la moelle épinière, locomotion, mise-en-charge (support de poids), plasticité spinale, réflexes musculaires, réflexes cutanés, western blot.*

ABSTRACT

Locomotor training has gained in popularity and is more and more integrated in rehabilitative strategies to enhance stepping recovery in spinal cord injured (SCI) individuals. This strategy is directly inspired from several decades of work performed in the laboratory taking advantage of a spinal cat model in which reflex and locomotor pathways are exhaustively described. Completely isolated from supraspinal influences, the spinal cord has the capacity to recover stepping movements when given repetitive and appropriate sensory feedback related to step-training on a treadmill. However, the underlying mechanisms for recuperating the appropriate motor patterns are still poorly understood and are the scope of this study.

Project I. It is generally assumed that plasticity in spinal locomotor circuits is responsible for the stepping recovery. However, the repetitive sensory stimulation related to step-training could also modify transmission in reflex pathways, which are also known to contribute significantly to the level of muscle activity during stepping. In this project, transmission in reflex pathways was evaluated by measuring responses evoked by a stimulation of a cutaneous or muscle nerve of the hindpaw and recorded intracellularly in motoneurons from extensor and flexor muscles involved in ankle, knee and hip joint movements during an acute experiment in decerebrate cats. Possible modifications in reflex transmission were determined by the statistical comparison of responses between 2 groups of spinal cats (complete transection at T13), but only one was assigned to a step-training regimen. Results showed that the synaptic transmission in both group I muscle reflex pathways from extensors and cutaneous pathways were modified following one month of step-training. The monosynaptic excitation was decreased after step-training and a normal pattern of modulation was recovered during locomotion. Moreover, group Ib inhibition and polysynaptic group I excitation of extensors were respectively decreased and increased after step-training and clonidine injection, a noradrenergic agonist useful for central pattern generation. It was further observed that plasticity in cutaneous pathways was highly specific: only certain pathways were modulated (mostly depressed). Transmission of cutaneous input originating from the sole of the foot was particularly modified. Overall, step-training is suggested to both decrease the hyperexcitability observed in reflex pathways after SCI and to facilitate the recruitment of antigravity muscles to assist recovery and weight-bearing.

Project II. There is now strong evidence that spinal circuits can be affected by activity-dependent biochemical processes that influence its ability to recover, perform and maintain an adequate locomotor pattern. Investigations have recently been oriented toward molecules involved in LTP to determine if similar mechanisms are both implicated in hippocampal learning and *spinal motor learning.* Given the preponderant effect of ERK on synaptic plasticity and function and its role in integrating signals from the cell surface to transcription factors, ERK appears to be a potential candidate for mediating the beneficial effects of step-training and may participate in the synaptic events associated with locomotor recovery after SCI. Protein expression was compared between 3 groups of cats (intact, SCI, SCI and step-trained) using western blot analysis of homogenates of spinal cord segments. The study focussed on assessing relative levels of ERK and pERK proteins. Results showed that ERK activation is up-regulated in a majority of lumbar segments following SCI and is specifically down-regulated in L5 by step-training. These results suggest that ERK activation is involved in long-term plasticity following SCI and that it may be detrimental to locomotor generation, at least in specific spinal segments.

Keywords: *cutaneous reflex pathways, CPG, CREB, ERK, intracellular recording, locomotion, muscle reflex pathways, treadmill training, spinal cord injury, spinal plasticity, weight-bearing, western blot.*

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5-HT:	5-hydroxytryptamine (serotonin)
AHP:	Afterhyperpolarization
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-5:	2-amino-5-phosphonovaleric acid
APV:	2-amino-5-phosphonopentanoic acid
BDNF:	Brain-derived neurotrophic factor
CaMKII:	Calcium/calmodulin-dependent kinase II
cAMP:	Cyclic adenosine monophosphate
Caspase:	Cystein aspartate-specific protease
CCS:	Caudal cutaneous sural
CDP:	Cord dorsum potential
CNQX:	6-cyano-7-nitroquinoxaline-2-3-dione
CNS:	Central nervous system
CPA:	Canadian Paraplegic Association
CPG:	Central pattern generator
CREB:	Cyclic AMP response element binding
CSPG:	Chondroitin sulfate proteoglycan
DOPA:	β-3,4-dihydroxyphenylalanine
DRG:	Dorsal root ganglion
EDL:	Extensor digitorum longus
ENG:	Electroneurogram
EPSP:	Excitatory postsynaptic potential
ERK:	Extracellular signal-regulated kinase
FDL:	Flexor digitorum longus
FHL:	Flexor hallucis longus
FRA:	Flexor reflex afferents
GABA:	Gamma-aminobutyric acid
GAD:	Glutamic acid decarboxylase
Grb2-SOS:	Growth factor receptor-binding protein 2 - the son of sevenless
GS:	Gastrocnemii - Soleus
H-reflex:	Hoffman reflex
Ip:	Iliopsoas
IPSP:	Inhibitory postsynaptic potential
LDP:	Locomotor drive potential
LGS:	Lateral gastrocnemius - soleus
LTP:	Long-term potentiation
MAG:	Myelin-associated glycoprotein
MAPK:	Mitogen-activated protein kinase
MG:	Medial gastrocnemius
MPL:	Medial plantar
NA:	Noradrenalin/noradrenergic
NMDA:	N-methyl-D-aspartate
NT:	Neurotrophins
OMgp:	Oligodendrocyte myelin glycoprotein
PI3K:	Phosphatidylinositol-3-kinase
PIC	Persistent inward current
PKB:	Protein kinase B
PKC:	Protein kinase C
PBSt:	Posterior biceps - semitendinosus
Pl:	Plantaris
PLCγ:	Phospholipase Cγ
SCI:	Spinal cord injury/injured
SmAB:	Semimenbranosus - anterior biceps
SOL:	Soleus
SP:	Superficial peroneal
Srt:	Sartorius
St:	Semitendinosus
SynP:	Synaptophysin
TA:	Tibialis anterior

Laura, Anne-Marie, Thomas-Xavier, Tristan,

Vos sourires, bisous et calins
sans cesse me rappellent
que l'essentiel
est de s'émerveiller d'un rien

Experience has shown, and a true philosophy will always show, that a vast, perhaps the larger portion of the truth arises from the seemingly irrelevant.

- Edgar Allan Poe

The great tragedy of science - the slaying of a beautiful hypothesis by an ugly fact.

- Thomas Huxley

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1. Spinal cord injury

1.1 General facts and problematic

Spinal cord injury (SCI) is a widespread condition which primarily affects young adult males between 15 and 34 years old. According to the *Canadian Paraplegic Association* (CPA)[1], 36000 Canadians live with SCI excluding non-deficit or fatal injuries. Approximately 1050 new injuries occur every year (35 individuals/million) resulting in some level of permanent paralysis or neurological deficit. In Canada, car and motorcycle accidents are a leading cause of SCI, followed by falls, medical conditions, diving and sports. Approximately 80% of SCI occurs under the age of 30 and many of these individuals will live a normal lifetime generating important societal costs in terms of medical, surgical and rehabilitative care. Hence, the financial care requirements, over this period, could vary from 1,25 million for a low thoracic paraplegic to 25 million Canadian dollars for a high cervical quadriplegic, such as Christopher Reeve, who required continuous ventilator support and 24/7 care[2]. Moreover, recent data collected by CPA suggests that there are a growing number of older adults being paralyzed as a result of disease and other medical conditions. Given these facts and knowing that the population is ageing, that physical and psychological consequences of the paralysis have a devastating effect on the quality of life of individuals and that cost will necessarily increase in the next years, research in the SCI field has gained support and popularity within the last ten years.

1.2 Recent advances in SCI research

Traumatic insults to the spinal cord induce both immediate mechanical damage and subsequent tissue degeneration. Hence, the outcome of SCI depends not only on the initial tissue injury at the time of the trauma, but also on secondary injury processes that may extend for hours, days, and even months. Incredible progress has been made in SCI research over the last decade. Major improvements affecting the quality of life of SCI individuals including chronic pain and bladder function management were inconceivable only a few years ago. For example, paraplegic SCI individuals present a syndrome in which the posture of the legs as well as voluntary and locomotor movements are exten-

1- Canadian Paraplegic Association (http://www.canparaplegic.org)
2- International Collaboration On Repair Discoveries (http://www.icord.org)

sively impaired. Many approaches have evolved to promote the recovery of function. Noteworthy, combining multiple strategies to enhance functional improvements in an effort to reach a *satisfactory* daily life is thought to have a positive effect. Several reviews were published recently and summarize the recent advances in SCI research (Fouad et al 2001, Kwon et al 2002, David & Lacroix 2003, Dobkin 2004, Fouad & Pearson 2004, Hall & Springer 2004, Klussmann & Martin-Villalba 2005). Years of fundamental and clinical research led to these conclusions and a detailed acknowledgment of achievements will be found in these reviews. The following section is solely aimed at drawing a succinct portrait.

From pre-clinical models to clinical application, therapeutic strategies are commonly divided in 4 subcategories: protection, regeneration, substitution, and management of sublesional networks. These 4 categories are somehow intermingled; for example neuroprotection or regeneration can actually be achieved via a substitutive process.

Neuroprotection is the first-step strategy targeting secondary injury mechanisms and intending to limit neuronal loss and inflammation soon after the injury onset. The main rationale is to block secondary biochemical and cellular cascades initiated by tissue damage due to glutamate excitotoxicity, ischemia, oedema, Ca^{2+} overload and oxidative stress. In animals, various pharmalocogical agents have been tested to prevent post-traumatic secondary lesions and decrease lesion extent. Among them are antagonists of opioid receptors (naloxone) or gangliosides, non-competitive antagonists of NMDA receptors (phencyclidine and ketamine) or massive doses of steroids such as methylprednisolone. Free radical scavengers have also been shown to preserve white and grey matter and to enhance motor performance. In human SCI, only methylprednisolone has been administered routinely. Trials are currently being held for other drugs but undesirable side effects often prevented therapeutic use.

The second type of intervention, regeneration, is aimed at re-establishing ascending and descending pathways. Promoting axonal regeneration and reconnection is currently a mainstream research field and has been the most dynamic in the last ten years. The majority of these interventions target elements that prevent axonal regeneration and accumulate in the myelin (arretinin, CSPG, Nogo-A, MAG, OMgp) or around the glial scar (CSPG, collagen-IV, tenascin, class-3-semaphorin, Eph3B) to neutralize them (see Kwon et al 2002, David & Lacroix 2003). Finally, a substitutive strategy can also be used to re-express some factors that are absent or decreased in the sublesional part of the spinal cord. This can be achieved by various means: trophic factors and graft transplantation

such as stem cells, Schwann cells, embryonic raphe neurons, olfactory ensheathing cells, etc (Ribotta et al 2002, Bunge & Pearse 2003, Fouad & Pearson 2004). Recently, new alternatives to neuronal-cell grafts have started to draw attention. Among them figure non-neuronal cells transfected to express a gene coding for tyrosine hydroxylase in order to express serotonin (astrocytes, fibroblasts), non-neuronal stem cells (muscle or bone marrow), NT-2 human neuron (testicular tumoral cells) treated not to be tumoral (allows 30% of differentiated serotoninergic cells).

Contrary to previous interventions aimed at trying to restore the *before-SCI* neural milieu, the 4th strategy is based on a different approach that is exclusively directed toward maximizing the residual function of the spinal cord. It takes advantage of the intrinsic capabilities of the spinal cord through the activation of the sublesional neural networks either with pharmacological intervention (reviewed in Rossignol et al 2001), transplantation of neurons or neural tissue (Ribotta et al 2000, Slawinska et al 2000) or with a specific motor training regimen. This thesis is especially interested in the latter case and further details will be given in the following chapters.

2. The ABC of locomotion

Over the years, the study of rhythmic movements has covered a large range of behaviors existing all over the animal kingdom. These stereotyped rhythmic patterns are part of very primitive behaviors necessary to live in the wildlife such as breathing (respiration rhythm), eating (mastication and swallowing rhythm), escaping from predators or joining a companion for reproduction (locomotor rhythm as flying, stepping, swimming, etc). A given behavior is produced by networks of interacting cells involving fine-tuning of molecule/gene activation and interaction within the cell, synapse and neural network. From invertebrate to human investigation, incredible progress has been made in understanding the neural control of such behaviors in the last 100 years. In this thesis, emphasis will be given to stepping and its control in the cat. This section is a glance at early studies of locomotion and will deal with basic concepts and their evolution.

2.1 The early description of the locomotor cycle

Locomotion results from the sequential activation of numerous muscles. Their activation patterns are nearly similar across individuals and to a lesser extent across vertebrates (Grillner 1981). Nowadays, the motor pattern of the stepping limbs can be described using 3 different parameters: kinetics (force), kinematics (movements) and electromyographic activity (EMG). Pioneer experiments investigating locomotion relied solely on anatomical studies of the legs. The improvements in photography and in the development of motion pictures in the late 19th century allowed analysis of these images of animate motion for the first time. Scientists and artists such as Marey and Muybridge were early explorers of human and animal motion in images and image sequences. These pioneers first captured the sequential positions during gait and could then take precise measurements of the leg in motion. A few years later, Marey's microphotographs were used by

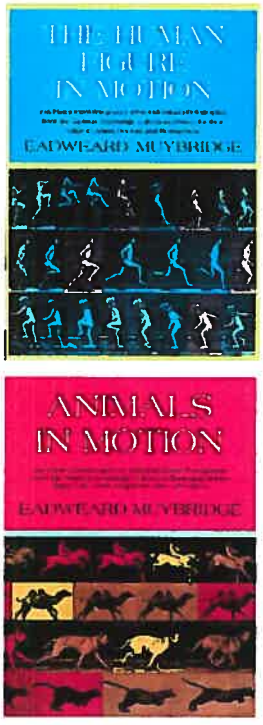

Figure 1: Bookcover from Eadweard Muybridge

The Human Figure in Motion (1955) and Animals in Motion (1957) report a collection of pictures taken by E. Muybridge at the beginning of the 19th century.

Philippson (1905) to precisely describe the step cycle, which he divided into 4 distinct portions. The first portion is a flexion of all joints (flexion phase), followed by an extension of the ankle and knee while the hip continues flexion (early extension phase). The third portion takes place at the very onset of the foot contact with the ground: knee and ankle joints are passively flexed (weight acceptation). The step-cycle ends with an extension of all joints to propel the body forward (propulsion phase). The extension phase is thus divided in 3 subcomponents: early extension (E_1), weight acceptation (E_2) and propulsion (E_3). This nomenclature is still commonly used to describe the step-cycle and a detailed analysis of the motor pattern of the hindlimbs of the cat has been performed by a number of investigators (reviewed in Rossignol 1996). When a detailed description is not required, the locomotor cycle is commonly defined as the period between two successive foot contacts and consists of two principal parts: stance (support) and swing phase (transfer). The swing phase starts when the limb reaches the posterior extreme position in relation to the body. The limb is then lifted above the ground, moves forward until it reaches the anterior extreme position and the paw is placed in contact with the ground. Then, the limb (still in contact with the ground) moves backward in relation to the body until it again reaches the posterior extreme position. During bipedal stepping, each limb alternatively performs a cycle starting with foot contact with the ground (stance or extension phase) followed by a lift-off directed in front of the body (swing or flexion phase).

Although the activation of each muscle has a specific temporal relationship with the step-cycle, of a general point of view, two functional groups of muscles are alternatively activated during stepping: extensors and flexors. Extensor muscles are generally active during stance and flexor muscles during the swing phase of locomotion. Extensor muscles have a very similar pattern of activity (Pratt et al 1991) and are activated 20 to 80 ms before paw contact with the ground (Halbertsma 1983). However, they can have a different profile of activation. For example, vastus lateralis peaks in E_3 whereas both gastrocnemii (MG and LG) have an abrupt onset and peak in E_2. The activation pattern of flexor muscles is not as homogeneous as for extensors. Many of the muscles related to the swing phase are biarticular (eg semitendinosus or St, sartorius or Srt) and may have two bursts of EMG activity per step-cycle under some conditions (Engberg & Lundberg 1969, Perret & Cabelguen 1980). A detailed description of EMG activity during locomotion has been written by Rossignol (1996).

2.2 Spinal locomotor networks in history

Philippson (1905), Sherrington (1910), and Brown (1911) were pioneers in elucidating some basic features of motor control and locomotion more specifically. Their experiments suggested that the spinal cord in itself is responsible for the genesis of an alternate pattern of muscle activation similar to locomotion. From these early experiments emerged two basic concepts: the *half-center hypothesis* and the *central pattern generator* (CPG). The following 2 sections describe these concepts and their development over the years.

2.2.1 Half-center hypothesis

Brown's half-center hypothesis emerges from experiments demonstrating that the locomotor pattern could still be expressed after a rhizotomy in spinal animals suggesting that the locomotor pattern is generated centrally (Brown 1911). His model assumes that each limb is controlled by an independent interneuronal spinal circuit composed of two half-centers driving either flexor or extensor muscles. Moreover, simultaneous activity in each half-center is prevented by mutual inhibitory connections. Strong evidence in favor of this hypothesis were later obtained by Lundberg and colleagues: the activation of the spinal networks with L-DOPA generated long-duration bursts alternating between flexors and extensors which were suggested to correspond to the half-center hypothesis (see section 3.3.3). According to the half-center hypothesis, rhythmicity would arise from a decrease in activity of one half-center due to a fatigue process, ie *refractoriness* (synaptic fatigue, spike frequency adaptation) until the other half-center is released from the opposing half-center inhibition and takes over. The process repeats and the system oscillates.

Brown's theory is also supported by experiments in which a detailed fictive locomotor pattern with a characteristic temporal organization is present and not fundamentally modified in decerebrate and acute spinal cats injected with curare (ie all afferent feedback is removed) and L-DOPA (Grillner & Zangger 1979, Perret & Cabelguen 1980, Fleshman et al 1984) and in chronic spinal cats injected with curare and clonidine (Pearson & Rossignol 1991). Eventually, the half-center hypothesis became unsatisfactory to explain the complexity of the locomotor pattern observed in these reduced preparations. The similarity of the pattern observed as compared to intact animals and the preservation of complex features, such as double bursting within a single step-cycle or delayed temporal

activation (or phase shift) of a single motor pool could not be explained without modifications to the original version of the model (Grillner & Zangger 1979, 1984).

The term half-center is still in use today; not in its original and strict sense, rather to refer to the *approximative* alternance between flexor and extensor muscles during locomotion. However, one needs to keep in mind that a precise timing does exist for every single muscle during the step-cycle.

The original version of the half-center hypothesis led to the concept of the central pattern generator that is described in the following chapter.

2.2.2 Central pattern generator

From early studies of various rhythmic movements, a common characteristic emerges: the presence of an interneuronal network located within the spinal cord and responsible for the basic locomotor commands, the so-called *central pattern generator* (CPG; Grillner 1981). This network is able to generate a given motor rhythmic behavior and to model the timing and amplitude of the output generated by motoneurons (Grillner 1981, Rossignol 1996, Pearson 2000, Rossignol et al 2006). During locomotion, for example, the CPG is responsible for the basic alternate activity between extensor and flexor muscles and for the very detailed muscle-specific temporal pattern (see section 2.1). Several excellent reviews with reference to the CPG and locomotion have been written recently (Grillner & Wallen 2002, Dietz 2003, Grillner 2002, 2003, Kiehn & Butt 2003, Kiehn 2006).

Here, the term CPG will refer exclusively to the CPG for locomotion unless mentioned

A fascinating finding from studies of locomotion is the remarkable similarity in the neural solutions across species from fish to mammals (Grillner 1981, Prochazka 1996, Orlovsky et al 1999, Duysens et al 2000). Not only does this interneuronal network exist in invertebrates, primitive vertebrates and mammals but also in non-human primates (Fedirchuk et al 1998). Yet other evidence suggests the presence of a CPG in humans (Calancie et al 1994, Harkema et al 1997, Dimitrijevic et al 1998, Gerasimenko et al 2002). This is supported by the presence and characteristic features of the locomotor pattern found in young infants (immature descending control) and anencephalic babies (reviewed

in Yang et al 2004). However, irrefutable and conclusive reports are difficult to obtain given the impossibility to completely discard the influence of peripheral and supraspinal input to spinal cord networks. It is believed that the locomotor pattern is innate as it is expressed in infants and spinalized kittens (Forssberg et al 1980ab, Yang et al 2004).

Modular organization of the CPG. Several hypotheses evolved concerning the organization of the CPG and a variety of conceptual models has been advanced (reviewed in Grillner 1981, Orlovsky et al 1999). In the 30's, Von Holst expressed the idea that each limb might be driven by an independent *limb controller* in quadrupeds (Orlovsky et al 1999). This hypothesis was later supported by experiments in which the hindlimbs of spinal animals (Forssberg et al 1980b, Halbertsma 1983) and infants (Yang et al 2005) walking on a treadmill with split belts moving at different speeds exhibit appropriate rhythmic activity on each side. Noteworthy, this divergence is not abolished following a longitudinal split of the lumbosacral enlargement suggesting that the controller for each limb is located within the ipsilateral half (Kato 1990). The Von Holst model was later refined and the *Unit burst generator* concept was proposed by Grillner (1981). This concept assumes that several interrelated unit burst generators interact together, ie either one for each limb, for each joint or each group of close synergists acting around a joint. In this model, each unit is independent but the global output is generated by the combined activity of a series of individual, but coupled unit generators (see also Stein 2005). This allows several types of output to be generated by the same networks from changes in excitability of a single or a set of unit burst generators. The different units can be recombined to achieve different left-right or forelimb-hindlimb coordination to generate different gaits (walk, trot and gallop for animals or walk and run for humans) or to walk with a different speed for each limb. This model implies that the interneurons constituting the CPG are responsible for the generation and timing of muscle activity and for the excitatory drive to motoneurons. The latest evidence suggests that the organization of the CPG must include a separation of the network for pattern-formation and rhythm generation (Lennard & Hermanson 1985, Burke et al 2001, Lafreniere-Roula & McCrea 2005). The two half-centers implied one level of control whereas the proposed organization involves two interdependent levels of control for motoneuron activation (*pattern formation*) and step-cycle timing (*rhythm generation*). This could explain, for example, an error in pattern formation (eg deletion of a burst) without any effect on the timing of the subsequent bursts (Lafreniere-Roula & McCrea 2005).

Constitutive elements of the CPG. What is the constitution of the CPG? Among vertebrates, the CPG was first characterized in the lamprey, a primitive specie in which the spinal interneuronal networks are simpler to study because there is a simple right-left alternation and a restricted number of neurons in each segment. In this preparation, the circuitry and neurotransmitters underlying locomotor activity have been well described and serve as building blocks for unraveling the complex mammalian CPG structure (Grillner & Wallen 2002, Grillner 2003). The CPG is controlled by both the reticulospinal pathways activated by various areas of the brainstem (section 3.1) and by monoaminergic pathways (section 4.1.3.2). Moreover, glutamate and glycine are essential neurotransmitters for CPGs in virtually all vertebrates whereas noradrenalin (NA) and serotonin (5-HT) are modulators of the basic locomotor pattern (Grillner 2003).

In mammals, considerable efforts have been made to identify fundamental elements of the CPG using a wide array of methods (lesions, intra- or extracellular recordings, labeling of locomotor-activated cells with markers; reviewed in Kiehn 2006). Still, very little is known about the identity, characteristics and organization of the interneurons forming the CPG. Indeed, it is quite complex to 1) avoid non specific labeling of cells, 2) distinguish cells labeled because of sensory feedback of those responsible for locomotion and 3) to discard cells specifically activated by supraspinal centers. However, a distinct population of commissural inhibitory interneurons has been identified and is suggested to constitute part of the rhythm coordinating networks in the neonatal rat spinal cord (Kiehn & Butt 2003). The growing popularity of the transgenic mice preparation and availability of various genetic markers has enabled further investigations to precisely identify and characterize interneurons that might constitute a functional component of the CPG. Recently, $EphA4^{+}$ (Kullander et al 2003, Butt et al 2005) and $HB9^{+}/GFP^{+}$ excitatory interneurons (Hinckley et al 2005, Wilson et al 2005) have been shown to be rhythmically active during locomotion and also suggested to be an integral component of the CPG in the mouse.

Segregation or distribution? Whether the neural networks responsible for generating locomotion are segregated in a specific area of the spinal cord or distributed along several spinal segments is still controversial. As first shown in the lamprey (Grillner 1981, 2003), there is evidence of a distribution of rhythm generating elements along several spinal segments in higher vertebrates (Deliagina et al 1983, Kremer & Lev-Tov 1997, Kiehn & Kjaerulff 1998). However, other work favors the concentration of these elements in the rostral segments of the lumbosacral enlargement (Cazalets et al 1995, Bertrand &

Cazalets 2002). From these experiments, it is believed that the rhythmogenic capacity of the mammalian hindlimb locomotor CPG is distributed along the lumbar spinal cord but with a rostrocaudal excitability gradient. The rostral segments (L1-L3 in rodents, L3-L5 in cats) have a greater capacity to generate rhythmic locomotor output than caudal segments. Recently, midlumbar segments (L3-L4) were shown to provide essential input to organize the locomotor pattern and their integrity is critical to sustain locomotor activity in the cat (Marcoux & Rossignol 2000, Langlet et al 2005) and to induce locomotion by intraspinal microstimulation or dorsal root stimulation at L5 to S1 in spinal cats (Barthélemy et al 2007). These results suggest that those segments may contain interneurons strongly involved in stepping generation in the cat. Noteworthy, a more caudal location of those interneurons in the cat might be expected given that hindlimb motoneurons are contained within L4-S1 segments (Vanderhorst & Holstege 1997) whereas these neurons are located in L1-L6 in the rat (Nicolopoulos-Stournaras & Iles 1983).

As illustrated in Figure 2, locomotor control relies on complex interactions between the CPG (blue) and supraspinal, spinal and multimodal sensory feedback to produce an appropriate temporal response and generate a highly adaptable motor pattern (reviewed in Armstrong 1986, Zehr & Stein 1999, Rossignol et al 2006). This regulation can be performed via actions on motoneurons, interneurons or primary afferents by means of presynaptic inhibition (yellow). Presynaptic inhibition may act on primary afferents, but also at other selected areas of the central nervous system (CNS). Moreover, the motor output may depend on specific motoneuronal properties (green) emerging during locomotion. The following sections describe the different levels of control during locomotion: supraspinal descending commands, spinal interneurons, sensory feedback and motoneurons. The focus is essentially directed toward the description of muscle and cutaneous reflex pathways (section 3.3) because they constitute the basis of our investigation.

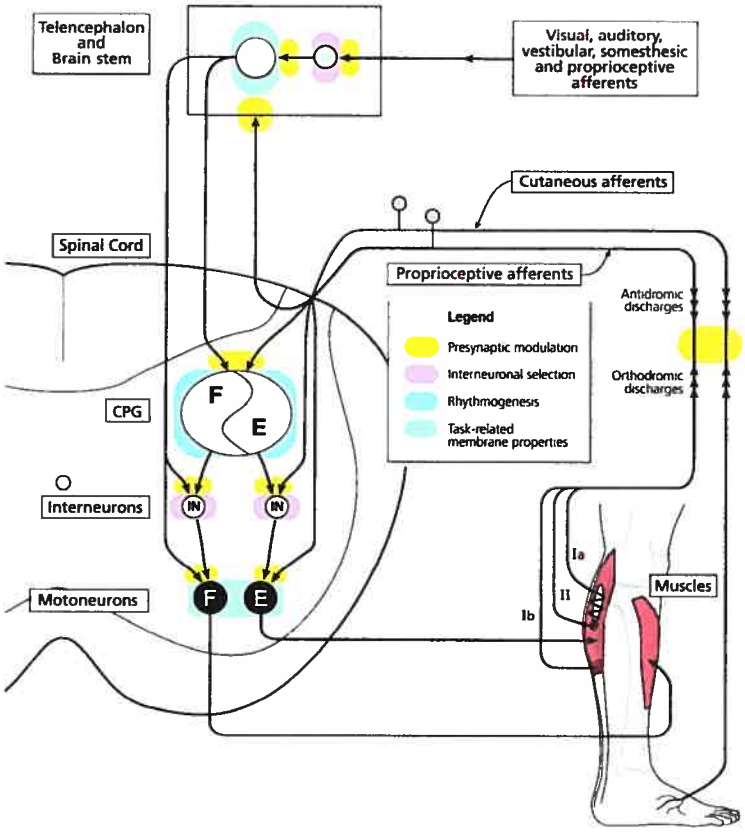

Figure 2: Neuronal organization of the mammalian locomotor system and dynamic sensory integration during stepping.

During stepping, multimodal inputs (supraspinal, cutaneous, muscular and joints afferents) reach both the brainstem and the spinal cord. In the spinal cord, some of these afferents directly contact motoneurons, but most of them synapse onto interneurons. The activity of primary afferents can be modulated by presynaptic inhibition before to reach a spinal target (yellow). This allows to activate or close some pathways, or to reverse the sign of the response (inhibition vs excitation) in different phases of the step-cycle (phase-dependency). Phasic presynaptic inhibition occurs at various levels of the spinal cord including just before the afferents contact the CPG (blue), interneurons (pink) and motoneurons (green). Moreover, membrane properties of motoneurons (green) are modulated during locomotion and may change the gain of the response to a given sensory input. From Rossignol et al 2006.

3.1 Supraspinal descending commands

During movement, descending pathways can exert a direct control (monosynaptic) onto motoneurons (eg control of fine movements of the hand by corticospinal pathways) or an indirect control (polysynaptic) via interneurons that project to motoneurons (eg postural regulation). Several supraspinal pathways including the reticulospinal, vestibulospinal, rubrospinal and corticospinal tracts are involved in the control of locomotion and their role has been well documented (reviewed in Armstrong 1986, Rossignol 1996, Jordan 1998, Orlovsky et al 1999, Drew et al 2002, 2004, Rossignol et al 2006). Most of the supraspinal inputs passes through a set of interneurons to ensure their integration with the basic locomotor pattern generated by the CPG and leads to an adequate context-related locomotor response (Baldissera et al 1981, Jankowska 1992, McCrea 1996, Orlovsky et al 1999, Drew et al 2004).

Our model does not involve supraspinal inputs to spinal pathways because a complete spinal cord transection has been performed. For that reason, the role of supraspinal pathways in the control of locomotion will be succinctly described and the functional consequences of disrupting these pathways will be emphasized.

Supraspinal pathways have extremely complex effects and interactions during locomotion. This paragraph is a general and simplified summary of their role during stepping and will not deal with specificities and exceptions. The motor cortex exerts a powerful influence on locomotion and has been shown to be crucial during precise visually guided walking (Beloozerova & Sirota 1993, Armstrong & Marple-Horvat 1996, Drew et al 2002, 2004). In the cat, the corticospinal pathway appears to contribute to the fine control and volitional positioning of the limbs in a locomotor context requiring accurate and precise foot placement such as gait modification to step over an obstacle or walking on a narrow beam or the rungs of a horizontal ladder (*skilled* locomotion). In the hindlimbs, the lateral corticospinal tract was shown to excite flexor and inhibit extensor motoneurons. Transcranial magnetic stimulation in human subjects showed that corticospinal inputs provide part of the drive to activate muscles for walking (Capaday et al 1999, Petersen et al 2001). The rubrospinal tract has been shown to control hindlimb flexion during swing when it evokes a facilitatory response in most flexor muscles (Orlovsky 1972, Rho et al 1999) and the vestibulospinal tract controls hindlimb extension during the stance phase of locomotion (Orlovsky 1972). Finally, beside its role in initiating locomotion (detailed in the

next paragraph), the reticulospinal tract may modify the activity in both flexor and extensor muscles tending to reinforce activity in muscles that are already active (Orlovsky 1972, Drew & Rossignol 1984, Drew 1991, Perreault et al 1994). This pathway is also suggested to be involved, together with the corticospinal pathway, in the control of posture when locomotion is disturbed (Drew et al 2004). These results confirm that the transmission in descending pathways to the spinal cord is modulated in a phase-dependent manner. Noteworthy, many supraspinal structures are also capable of resetting the locomotor rhythm. This suggests that they may act through interneurons that are part of the CPG (Orlovsky 1972, Perreault et al 1994, Rho et al 1999, Leblond et al 2000, 2001).

The *spontaneous* initiation of stepping, ie without drugs or electrical stimulation, requires the integrity of supraspinal structures. Decerebrated animals are only capable of spontaneous locomotion if the brainstem is not transected below a specific level. For example, if the decerebration is performed between the rostral border of the superior colliculus dorsally and rostral to the mammillary bodies ventrally (referred to as pre-mammillary cat), cats will preserve the ability to step spontaneously. However, if the transection terminates caudally to the mammilary bodies ventrally (post-mammilary or mesencephalic cat), a 2 weeks period is necessary for the recovery of locomotor movements. No recovery has been reported with more caudal transections. Over years, several areas of the brain and brainstem have been identified as being able to induce locomotion in decerebrated or intact animals (reviewed in Armstrong 1986, Jordan 1998, Orlovsky et al 1999): the mesencephalic locomotor region (MLR), the subthalamic locomotor region (SLR), the pontine locomotor region (PLR) and the cerebellar locomotor region (CLR, Mori et al 1999). All these areas converge on, and excite, reticulospinal neurons in the brainstem, which in turn exert their control in the lumbar spinal cord onto the CPG to initiate locomotion. The specific spinal targets of the reticulospinal neurons have not been clearly identified. However, maximal field potential following MLR stimulation occurs in the dorsomedial area of the spinal gray matter (laminae V-VII) suggesting the presence of a concentration of interneurons receiving reticulospinal input in this area (Noga et al 1995).

A lesion of the spinal cord interrupts (complete) or compromises (incomplete transection, contusion, etc) supraspinal descending tracts and propriospinal pathways to lumbosacral segments. The observed deficits during stepping can mainly be attributed to the disruption of the control previously provided by these pathways as revealed by partial spinal lesion studies (Gorska et al 1993, Jiang & Drew 1996, Brustein & Rossignol 1998, Rossignol et al

1999). The response of the spared pathways to the lack of supraspinal input determines both the extent of the recovery and the specific functions that may recover (Helgren & Goldberger 1993, Bregman et al 2002). Moreover, the amount of fibers preserved in the ventral and lateral funiculi of the spinal cord, particularly the white matter associated with the reticulospinal tract, was shown to be directly related to locomotor performance after SCI (Schucht et al 2002). However, locomotion has been reported in the absence of ventral and ventrolateral quadrant in the cat (Brustein & Rossignol 1998). Depending on the pathways disrupted (corticospinal, rubrospinal, reticulospinal, vestibulospinal), serious deficits impairing locomotion may be observed: incapacity to voluntary initiate stepping, lack of voluntary and anticipatory adjustment of locomotion (eg avoiding obstacles), impaired weight support, lateral stability and interlimb coordination (fore- vs hindpaw). Another functional consequence of lacking supraspinal control is the paw drag that is frequently observed at the onset of the swing phase in spinal cats. This behavior seems to be associated with an inappropriate timing of flexion movements in the hip, knee and ankle at the beginning of the swing phase. For example, the activation delay between St and Srt is absent so that the knee and hip joints flex simultaneously instead of one after the other to clear the foot before hip flexion onset (Rossignol et al 2004). This could be due to a lack of corticospinal and rubrospinal control, which are required for proper intralimb coordination (Jiang & Drew 1996). Indeed, paw dragging during stepping has been observed in cats with a restricted lesion to dorsolateral quadrants of the spinal cord (Jiang & Drew 1996, Rossignol et al 1999).

The disruption of descending input interferes with the ability to walk in a voluntary and controlled manner. After a *complete* spinal cord transection, all these deficits are observed. Indeed, the hindlimbs are flaccid and can barely perform weak and uncoordinated movements when placed over a treadmill belt. Ground contact will be performed on the dorsal surface of the paw. Moreover, the EMG is more clonic and cycle length is generally shorter for a given walking speed (Rossignol et al 2004).

Incomplete SCI may stimulate the reorganization of synaptic connections such as increasing collateral branching or shifting the representation of the hindlimbs in the motor cortex. Here, a complete transection model was chosen in order to assess the plastic potential of the spinal cord excluding any plasticity driven by supraspinal pathways.

Notably, there is a persistent hyperexcitability of several reflexes following partial or complete SCI because of the removal of inhibitory descending input from the brainstem (Holmqvist & Lundberg 1961, Lundberg 1964, Hultborn & Malmsten 1983, Malmsten 1983). This will be discussed in the appropriate section.

3.2 Spinal interneurons

Some afferent inputs may directly contact motoneurons. However, most of them will first transit through the interneuronal networks of the spinal cord. Interneurons are classified in 2 broad categories: segmental interneurons whose axons reside within the gray matter in the same or few nearby segments and propriospinal interneurons whose axons pass through the white matter to re-enter gray matter in distant spinal segments. The latter are meant to coordinate activity across spinal cord segments. There are also interneurons specialized in relaying spinal and sensory information to the brain. Classically, spinal interneurons in the cat have been functionally identified according to their dominant synaptic input, intrinsic properties, target neurons and role in motor activity (reviewed in Jankowska 1992, 2001). A recent review illustrates that the properties and organization of the spinal interneuronal networks share several similarities in cats and humans (Jankowska & Hammar 2002).

Spinal interneurons are involved in mediating both simple reflexes and complex movements. Descending and peripheral input were assumed to travel along independent pathways to reach the motoneurons. This was denied by an elegant series of studies conducted by Lundberg and colleagues showing that the spinal interneuronal networks are *integrative centers*, ie supraspinal and primary afferents of various modalities converge on common spinal interneurons before they reach the motoneurons (reviewed in Lundberg 1979, Baldissera et al 1981, Jankowska 1992). These interneurons then project in a divergent manner onto motoneurons, onto other spinal interneurons, and onto neurons projecting back to the supraspinal centers. This is a highly flexible network which includes mechanisms to select reflex pathways and allow the interaction between interneuronal populations. This results in the reconfiguration of the networks and provides a multifunctional character to a given set of interneurons. Spinal interneurons are crucial players involved both in the modulation of reflexes by supraspinal commands and in modulating the supraspinal command by sensory feedback before reaching the

motoneurons (Jankowska 1992). The integrated information is projected back to supraspinal centers where multiple loops project back downward to control the spinal circuitry (Armstrong 1986). This allows a rapid adaptation of motoneuron activity to the central command and environmental constraints. During locomotion, the activity of interneurons will result from the mix of convergent input from the CPG, sensory feedback, descending commands and intrinsic membrane properties of the cells.

Obviously, none of the spinal interneurons are interposed in a pathway with input from a single type of afferents, but their name is meant to identify the dominant input.

Given the diversity of spinal interneurons (anatomical, functional, molecular, source of input and target neuron) and for the text to be intelligible, their description is located in the appropriate section according to the reflex pathway in which they are involved

3.3 Sensory feedback

A reflex is a stereotyped motor response generated by the CNS in reaction to a particular sensory stimulus. Following a given peripheral afferent stimulation, a reproducible response is evoked (under similar conditions). Spinal reflexes were shown to be a great experimental tool to explore the organization of the CNS (Burke 1999) and are widely described in the literature (Baldissera et al 1981, Jankowska 1992, Zehr & Stein 1999, McCrea 2001). During movement, spinal segmental reflexes are highly flexible adjusting to the type, intensity and localization of the stimulus and also to the context. It can also be modulated in a task-dependent and phase-dependent manner. Recent reviews have been written to describe the spinal reflexes and their dynamic control during locomotion (Hultborn 2006, Rossignol et al 2006).

Sensory inputs are not required to generate a basic locomotor pattern but does substantially contributes to the motor output and adapts the central activation to environmental constraints. This feedback can have a global influence in allowing, preventing and selecting motor patterns. Whether of muscular (Duysens & Pearson 1980, Pearson 1995, Dietz & Duysens 2000), cutaneous (Duysens & Pearson 1976) or articular (Grillner & Rossignol 1978) origin, this dynamic sensorimotor interaction powerfully influences the basic motor output acting directly or indirectly on the CPG (Grillner 1981,

Gossard & Hultborn 1991, Pearson et al 1998, Rossignol et al 2006). This process is performed in the spinal cord and can modify the frequency, amplitude and structure of the motor output, which is crucial for establishing the final stepping pattern. This is illustrated by the capacity of spinal animals (Forssberg et al 1980ab, Grillner 1981, Lovely et al 1986, Pearson 2000, Leblond et al 2003) and babies with immature descending tracts (Yang et al 1998) to adapt their locomotor pace to the treadmill speed. When the locomotor pace is increased, the duration of extensor activity is decreased while the duration of flexor activity is relatively constant (Halbertsma 1983, Yang et al 1998, Orlovsky et al 1999). Flexor bursts vary little with change in step-cycle length as compared to extension and this basic feature of walking conserved in reduced preparation (Grillner & Zangger 1979, however see Yakovenko et al 2005). Well-coordinated locomotion depends heavily on sensory inputs signaling limb kinematics and loading (Pearson 1996, Rossignol 1996, Duysens et al 2000).

Anatomical and behavioral evidence suggest that sensory feedback plays a crucial role in the recovery of function after SCI in humans and animals to compensate for the loss of supraspinal input to spinal circuits. This is well illustrated by the ability to regain rhythmic locomotor movements after repetitive sensory stimulation provided by step-training (section 4.1.3.1).

Here, we will emphasize the description of spinal reflexes under investigation, ie reflexes evoked by muscle group Ia-Ib afferents and by specific cutaneous afferents. Reflexes will both be described in the absence of locomotion and when the spinal cord circuitry is *configured* for locomotion.

3.3.1 Muscle reflex pathways

Group I afferents, large diameter and high conduction velocity fibers, carry information originating from muscle receptors. Group Ia fibers innervate muscle spindles and transmit information concerning the extent and velocity of muscle stretch. The lengthening of the intrafusal fibers increases Ia afferents firing frequency (together with group II, see section 3.2.1.3). Group Ib fibers innervate Golgi tendon organs and carry information related to changes in tension applied to a given muscle. As shown in Figure 3, muscle afferents synapse either directly on motoneurons in the ventral horn (pathway 1) or on interneurons

in the intermediate zone of the ventral horn gray matter. In the latter case, the motoneuron is either contacted via a single interneuron (disynaptic pathway; Fig.3 pathways 2-3) or a chain of interneurons (polysynaptic pathway; Fig.3 pathways 4-5).

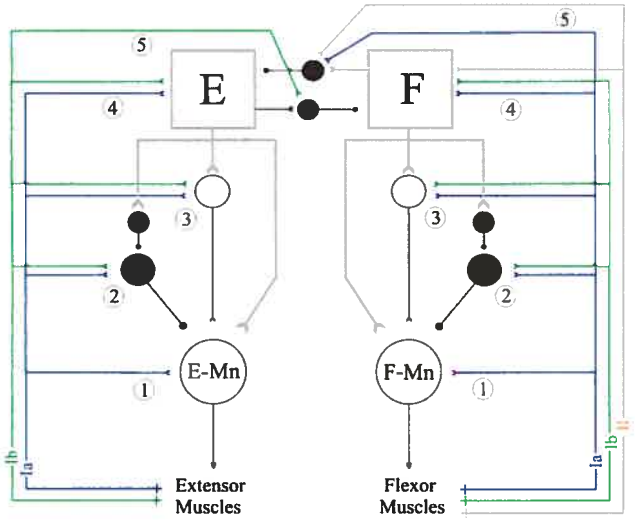

Figure 3: Proprioceptive pathways from extensors and flexors during locomotion

Left: 4 pathways project to extensor motoneurons (E-Mn): monosynaptic pathway from Ia afferents (1), disynaptic group Ia+Ib inhibitory pathway (2), the disynaptic group Ia+Ib excitatory pathway (3), polysynaptic excitatory pathway (4). Group Ib afferents input are able to interrupt flexion (5).

Right: Pathways to flexors are similarly organized to the pathways to extensors (left, pathways 1-4). However, most group I and II afferents from flexors can end the extension and reset the rhythm to flexion (5) while only a few can prolong the flexion phase (4).

Black circle, inhibitory interneuron; empty circle, excitatory interneuron; E-Mn, extensor motoneuron; F-Mn, flexor motoneuron; E, extensor generator; F, flexor generator; Ia afferents, blue; Ib afferents, green; group II afferents, brown. From Rossignol et al 2006.

3.3.1.1 Pathways from group Ia muscle afferents

The monosynaptic reflex. The activation of muscle spindles mainly evokes a monosynaptic EPSP in homonymous and synergistic α-motoneurons (simplified to motoneuron in the text unless mentioned) acting at the same joint (Eccles et al 1957ab). According to Mendell and Henneman (1971), each Ia afferent from a given muscle directly projects to every motoneuron innervating the same muscle; conversely, each motoneuron is contacted by every Ia homonymous fiber (Fig.3, pathway 1) representing 1-5% of all afferent terminals on motoneurons. During muscle stretch, Ia fibers discharge and excite motoneurons producing contraction of the muscle to counter its own stretch. The monosynaptic stretch reflex is thought to make a major contribution to the level of EMG activity during stepping in cats walking on the treadmill (Stein et al 2000). However, recent evidence in humans suggests otherwise: Ia afferent feedback generated during normal walking seems to make only a minor contribution to the SOL activity but would contribute significantly when the muscle is unexpectedly lengthened during walking (discussed in

Sinkjaer et al 2000, see also Yang et al 1991b, Sinkjaer et al 1996). It is suggested that the ongoing Ia feedback is gated by presynaptic inhibition whereas unexpected Ia signals trigger reflex activity to adjust the movement.

Task- and phase-dependency. The input-output relationship of the monosynaptic reflex is rather flexible and can be modulated to adapt to functional requirements of motor activity. The gain of the reflex is either increased to facilitate the motor task or reduced to ensure that the task is not compromised. In cats, the gain of the tricep surae stretch reflex is lower during stepping as compared to tonic contraction (*state-dependent*). Although gamma bias could contribute to modifying the gain of this reflex (Bennett et al 1996), intracellular recordings of lumbar motoneurons have shown a tonic reduction in Ia-EPSP amplitude during MLR-evoked fictive locomotion that was ascribed to an increased presynaptic inhibition of Ia afferents (Gosgnach et al 2000). Similarly, the H-reflex was first shown to be maximal during standing, decreased during walking and even more during running in humans (Capaday & Stein 1986, 1987, Edamura et al 1991) because of presynaptic inhibition of Ia afferents (Faist et al 1996). However, this was recently denied by Simonsen & Dyhre-Poulsen (1999). They demonstrated that there is no modulation of the H-reflex during various speed of running as compared to walking and that discrepancies with previous investigations arise from inadequacy of the stimulus intensity (Simonsen & Dyhre-Poulsen 1999).

Additionally, the monosynaptic reflex is modulated according to the phase in which it occurs during rhythmic behaviors. For example, the stretch reflex is modulated with a maximal amplitude during the stance phase of locomotion in SOL, when the motoneuronal pool is depolarized and the muscle active (Akazawa et al 1982, Capaday & Stein 1986, Crenna & Frigo 1987, Simonsen & Dyhre-Poulsen 1999). This is suggested to reinforce extensor activity during the ongoing stance phase (Guertin et al 1995). Intracellular recordings also provided evidence for a phase-dependent modulation of Ia-EPSPs in hindlimb motoneurons during fictive locomotion in spinal, decerebrate or decorticated cats. The presence of modulation in reduced preparation suggests that this cannot be solely attributed to the absence of reafference and depends, at least partially, on spinal mechanisms (Schomburg & Behrends 1978b, Perret & Cabelguen 1980, Shefchyk et al 1984, Gossard 1996, Ménard et al 1999, 2003). The gain of this reflex is believed to depend on cyclic changes in the excitability of motoneurons (section 3.4) and strength of synaptic transmission by means of presynaptic inhibition (section 3.3.4.3).

Ia interneurons and Renshaw cells: last-order inhibitory interneurons. Not only does Ia afferents activation evokes an excitatory response in agonist motoneurons, but also an inhibition in antagonists, the so-called *reciprocal inhibition* (Lloyd 1946). This reciprocal inhibition is mediated by a group of glycinergic interneurons, referred to as Ia interneurons, that innervates motoneurons within a spinal segment or adjacent segments (Eccles et al 1956, Baldissera et al 1981, Jankowska 1992). Ia interneurons are characterized by a strong activation by Ia afferents and the ability to discharge at high frequency (Hultborn et al 1971). Indeed, most of Ia interneurons respond to a synchronous volley in Ia afferents with a single discharge; however, high frequency trains of action potentials can be evoked by the stimulation of other peripheral afferents such as FRA (section 3.3.3) or during walking (Hultborn et al 1971, McCrea et al 1980).

As exhaustively reviewed by Jankowska (1992), Ia interneurons receive multiple converging inputs from supraspinal (pyramidal tracts, corticospinal, rubrospinal, reticulospinal and vestibulospinal), spinal (propriospinal, CPG interneurons) and peripheral (cutaneous, muscle, joint, FRA) afferents. Ia interneurons have been shown to contribute to the inhibition of motoneurons of antagonists during muscle stretch, the crossed extensor reflex, postural reflex, centrally induced locomotion and voluntary movements. In humans, reciprocal inhibition can be evaluated by means of the H-reflex: an inhibition of the H-reflex of a given muscle is observed following a conditioning activation of the antagonistic motor nerve (Pierrot-Deseilligny et al 1981b). Ia-IPSPs are also modulated in a phase-dependent manner with a maximum occurring during the hyperpolarized phase of fictive locomotion (Pratt & Jordan 1987, Degtyarenko et al 1998). Accordingly, reciprocal inhibition of extensors is maximal during swing in humans (Petersen et al 1999).

Notably, Ia interneurons are also contacted by Renshaw cells associated with the agonist motoneuron and from Ia interneurons associated with the antagonist (Baldissera et al 1981). Most Renshaw cells are glycinergic and respond with a train of high frequency discharges by recurrent collaterals originating from motoneurons. The activation of Renshaw cells leads to the inhibition of surrounding synergistic α- and γ-motoneurons, other Renshaw cells and Ia interneurons to antagonistic motor nuclei (Hultborn et al 1971, Baldissera et al 1981, Jankowska 1992). They also receive information originating from cutaneous afferents, group II-III muscle afferents and descending pathways. Renshaw cells have been found to adjust the excitability of Ia inhibitory interneurons and α-motoneurons to regulate the gain of motoneuron output. A strong stimulation of Renshaw cells both decreases the activation of the agonist muscle and the inhibition of the

antagonist to facilitate coactivation whereas a weaker stimulation leads to a selective activation of agonist muscle. This system is organized in order for the agonist motoneuron and antagonist Ia interneuron to be under the control of the same afferent input. This organization leads to a closely linked activation of the agonist to the inhibition of the antagonist during movement. Reciprocal and recurrent inhibitions have indissociable functions and are both involved in the control of motoneuron activity during various types of reflex and rhythmic movements such as stepping.

Ia inhibitory interneurons and Renshaw cells are rhythmically active during fictive locomotion respectively during the inactive and active period of the target motoneuron (McCrea et al 1980, Pratt & Jordan 1987). When strychnine is added to the system to block glycinergic transmission, fictive locomotion still occurs suggesting that Ia interneurons and Renshaw cells are not essential to locomotion (Pratt & Jordan 1987). Moreover, their rhythmic activity cannot be attributed to the phasic activation of peripheral receptors as shown during fictive locomotion (McCrea et al 1980, Pratt & Jordan 1987). Although not essential, reciprocal inhibition is thought to contribute to the generation of the basic locomotor pattern and recurrent inhibition is believed to help terminate the activity of motoneurons and Ia interneurons and help for the transition to the antagonist (McCrea et al 1980, Pratt & Jordan 1987).

Monosynaptic reflex and SCI. Inconsistencies arise as to the effect of SCI on the monosynaptic reflex and Ia-EPSPs. Some studies report that synaptic transmission is increased by a complete spinal transection enhancing the amplitude of Ia-EPSPs and projection frequency and efficacy of group Ia fibers (Cope et al 1988). Hence, both homonymous and heteronymous Ia-EPSPs were reported to be larger and have a faster rise time after a complete acute or chronic SCI (Cope et al 1986, 1988, Munson et al 1986, Hochman & McCrea 1994ab). On the other hand, homonymous Ia-EPSP amplitude has also been reported to be unchanged after a complete SCI (Mayer et al 1984). In fact, changes in Ia-EPSP amplitude seem to be a very specific process. For example, Hochman and McCrea (1994a) observed a general increase in homonymous Ia-EPSP amplitude in the triceps surae motoneurons. When PSPs were grouped according to motoneuronal pools, LG-EPSPs were of larger amplitude whereas MG-EPSPs were not modified in spinal animals as compared to intacts. Unchanged homonymous EPSPs in MG motoneurons of chronic spinal cats were also reported by others (Nelson & Mendell 1979, Mayer et al 1984, Munson et al 1986, Hochman & McCrea 1994a). Moreover, Munson and

colleagues (1986) did not report a difference in homonymous Ia-EPSPs amplitude in MG whereas they observed a transient increase in heteronymous EPSPs up to three months after SCI which was back to normal within 7 months. Finally, this increase in heteronymous Ia-EPSP amplitude was present only if the spinal transection was performed at L4-L5 level and not at L1 or L3 (Munson et al 1986). Thus, it seems that the transection level, the time post-injury and the selected motor pool have an important impact on variations of Ia-EPSP amplitude and can affect differentially homonymous and heteronymous Ia-EPSPs. This may prevent a conclusive comparison between studies. It was further shown that Ia-EPSP amplitude modulation could not solely result from alterations in motoneurons passive properties that would affect both heteronymous and homonymous similarly (Hochman & McCrea 1994b). Moreover, Ia-EPSP amplitude varies between different motor unit types, which were shown to be modified following SCI (Mayer et al 1984, Cope et al 1986, Munson et al 1986, Hochman & McCrea 1994c).

Facilitation of the monosynaptic reflexes after a spinal cord injury has been reported in SCI rats (Malmsten 1983, Lavrov et al 2006), cats (Hultborn & Malmsten 1983) and humans (Hiersemenzel et al 2000). In humans, the contributing factors associated with the increase in H-reflex amplitude have been suggested to include decreased presynaptic inhibition resulting from the disruption of supraspinal pathways (Calancie et al 1993, Faist et al 1994) and altered reciprocal inhibition (Trimble et al 2001). Moreover, load-related afferent input and changes in hip kinematics were shown to decrease the H-reflex in complete SCI suggesting that afferent input can modulate the H-reflex at the spinal level (Knikou & Conway 2001, Knikou & Rymer 2002). A lack or absence of phase-dependent modulation of the H-reflex and stretch reflex has also been reported during stepping and bicycling in SCI individuals (Yang et al 1991a, Boorman et al 1992, Fung & Barbeau 1994). However, it can be recovered with functional electrical stimulation with a conditioning stimulus to the medial plantar surface of the foot in incomplete SCI (Fung & Barbeau 1994). Locomotor treadmill training has also been shown to decrease and improve the gating of Ia reflexes in SCI individuals (Trimble et al 1998) and reinstate phase-dependent modulation of the H-reflex in complete SCI humans (Beres-Jones et al 2004). This suggests that the appropriate sensory input can be used to normalize such reflex output of the spinal cord that is disrupted after SCI.

3.2.1.2 Pathways from group Ib muscle afferents

Group Ib autogenetic inhibition. Laporte & Lloyd (1952) reported that stimulating muscle afferents at a greater intensity than that required to activate Ia fibers inhibited homonymous and synergistic motoneurons and excited the antagonists. This reflex pathway activated by Ib afferents is referred to as *non-reciprocal inhibition* or *group Ib autogenetic inhibition* (Jankowska et al 1981ab). This inhibitory action is performed through one or two interneurons, Ib interneurons, positioned between Ib afferents and motoneurons because central latencies of Ib IPSPs are distributed from 1.3 to 3.5 ms (Eccles et al 1957ab, Baldissera et al 1981). Stimulating Ib fibers has a widespread effect that can involve all the muscles of a limb. For example, the activation of Ib afferents from an extensor evokes an IPSP in every motoneuron innervating extensor muscles (Fig.3, left pathway 2, green) together with an EPSP in several motoneurons innervating flexor muscles of the hindlimb (Eccles et al 1957a). This reflex provides a negative feedback loop in order to regulate muscle tension. Ib inhibition can be studied by means of the H-reflex in humans. A sustained inhibition of the H-reflex in the quadriceps and SOL is observed following a conditioning stimulus to the SOL nerve suggesting a similar organization of this circuit in cats and humans (Pierrot-Deseilligny et al 1981ab). Ib volley from flexors is usually without much effect (Fig.3, right pathway 2, green; Eccles et al 1957a, Jankowska 1992).

Inhibitory and excitatory Ib interneurons. Ib interneurons are contacted by Ib fibers from several muscles acting at a same joint and on different joints (Eccles et al 1957a). Although Ib afferents are the main source of input to Ib interneurons, it is not exclusive: 30-50% also receive Ia afferent inputs which can be sufficient to evoke non-reciprocal inhibition by itself (Jankowska & McCrea 1983). The convergence of the Ia and Ib signal allows to increase the sensitivity of Ib interneurons during the dynamic phase of muscle stretch because the reflex is influenced by muscle length and the fusimotor system (Lundberg & Malmgren 1988). In addition to Ia and Ib afferents, Ib interneurons are contacted by converging input of supraspinal (corticospinal, rubrospinal and reticulospinal), spinal (propriospinal, group Ib and II interneurons) and peripheral (low threshold cutaneous and joint) afferents either directly or via interneurons (Pierrot-Deseilligny et al 1981ab, Jankowska 1992). Ib inhibitory interneurons, contact α- and γ-motoneurons of extensor muscles while Ib excitatory interneurons contact motoneurons to flexor muscles.

Each Ib interneuron projects to several motor nuclei and every motoneuron receives input from numerous Ib interneurons (Eccles et al 1957a). The pattern of activity of Ib interneurons shows a dominant inhibition in extensors and Ib excitation in flexors both in cat and humans (Eccles et al 1957a, Pierrot-Deseilligny et al 1981ab). Not that all the patterns described above were observed in non-locomoting preparations or subjects.

State-dependent transmission in group Ib reflex pathway. The Ib autogenetic inhibitory pathway is deeply reconfigured during locomotion (Fig.3, pathway 4): it is replaced by an excitation in extensors that reinforces weight-bearing during the stance phase (Conway et al 1987, Gossard et al 1994, Pearson 1995, Guertin et al 1995, Prochazka 1996, Pearson et al 1998, Dietz & Duysens 2000). It was shown that the administration of nialamide and L-DOPA rapidly reverses disynaptic Ib inhibition to a polysynaptic excitation in extensor motoneurons in spinal cats (Gossard et al 1994). During this reversal, the disynaptic IPSP evoked by Ib afferents of extensors disappears (McCrea et al 1995, Quevedo et al 2000, McCrea 2001) because of a locomotor-related tonic inhibition of Ib inhibitory interneurons located in the intermediate nucleus in L6-L7 that hardly respond to a stimulation during MLR-induced locomotion (Angel et al 2005). The polysynaptic excitation is suggested to be mediated by another set of interneurons (Jankowska 1992) and may be part of the CPG network (Gossard et al 1994). This positive feedback loop would enhance extensor muscle activity and control the tension level during the loading of the stance phase and decrease the activity in flexors during swing (Conway et al 1987, Gossard et al 1994, Pearson 1995, Guertin et al 1995, Prochazka 1996, Pearson et al 1998, Dietz & Duysens 2000). Indeed, Golgi tendon organs continuously monitor muscle tension during movements and could control extensor yield and muscle tension during walking (Eccles & Lundberg 1959). Moreover, it may regulate the transition from stance to swing to assure it is not initiated before unloading (see section 3.3.4.1).

In humans, it was found that there is a significant decrease in Ib inhibition during locomotion but it is not necessarily reversed to excitation (Stephens & Yang 1996). Further experiments suggest that this decrease in Ib inhibition does not require locomotion but loading of the limb; however, the reversal into excitation is expressed only during stepping episodes (Faist et al 2006). After SCI, Ib inhibition does not differ from intact subjects at rest (Downes et al 1995, Morita et al 2006, however see Delwaide & Oliver 1988) but a lack of modulation of Ib inhibition during tonic antagonist contraction in patients with

spasticity is reported (Morita et al 2006). More details regarding load signals conveyed by Ib afferents and SCI will be reported in section 3.3.4.2.

Disynaptic Ib excitation. During locomotion, group Ia-Ib afferents from extensors can also evoke an additional disynaptic excitatory component with a central latency of about 1.5 ms (Fig.3, pathway 3) superimposed on the monosynaptic EPSP in homonymous and synergistic extensor motoneurons. This EPSP reaches a maximal amplitude during the stance phase of MLR-induced locomotion in decerebrate cats (Schefchyk et al 1984, McCrea et al 1995, Angel et al 1996, McCrea 1998, Quevedo et al 2000) and nialamide and L-DOPA induced locomotion in acute spinal cats (Schomburg & Behrends 1978b). This action is recorded only in extensor motoneurons during the extension phase of walking. The latency of this response indicates that a single interneuron is intercalated in this pathway. This population of interneurons has been identified and localized in the intermediate nucleus in lamina V-VI in mid and caudal L7 (McCrea 1998, Quevedo et al 2000, Angel et al 2005). These candidates excitatory Ib interneurons cannot be activated at rest but become responsive and also fire spontaneously during the extensor phase of fictive locomotion. Although this excitatory disynaptic pathway is exclusively observed during locomotion, it is not believed that transmission in this pathway passes through the CPG but rather that it is strongly under its influence. This pathway needs further investigation to understand why it is absent during fictive locomotion evoked by clonidine (McCrea et al 1995, McCrea 1998) or DOPA in spinal cats (Gossard et al 1994). Note that the measurement of monosynaptic EPSP peak amplitude in previous studies ignored a possible disynaptic component.

3.2.1.3 Pathways from other muscle afferents

Group II afferents and interneurons. Group II fibers innervate muscle spindles and carry information concerning muscle length through a chain of spinal interneurons (group II interneurons). These interneurons may respond to group II afferent input either by a train or a single action potential (Schefchyk et al 1990). Group II interneurons are divided in 2 subpopulations according to their localization: ventral horn (lamina VI-VIII) or dorsal horn (lamina IV-V) (Jankowska 1992). Here, only the ventral group will be described because of its possible involvement in locomotion.

This population of interneurons not only receives excitatory input from group II afferents originating from several synergists and a number of antagonists but also from supraspinal (corticospinal, rubrospinal, reticulospinal, vestibulospinal) and other peripheral afferents (group Ia-Ib, cutaneous, joints, high threshold afferents) (Edgley & Jankowska 1987, Jankowska 1992). Moreover, they can be activated by a stimulation of the MLR and by propriospinal neurons (Shefchyk et al 1990, Jankowska 1992). Their main source of inhibition is the monoaminergic pathway although other group II interneurons, Ib interneurons and reticulospinal fibers can also inhibit them. Group II interneurons terminate on α-motoneurons (excite flexors, inhibit extensors) and γ-motoneurons (Edgley & Jankowska 1987). Similar to Ia and Ib interneurons, group II interneurons are under the control of presynaptic inhibition resulting from cutaneous, joint and muscles afferents.

Group II interneurons are involved in reflexes involving flexor muscle activation and extensor muscles inhibition (Eccles & Lundberg 1959) and participate in the coordination of muscle activity of a limb particularly during postural reactions and locomotion. In the cat, flexor group II afferents have been found to project to a group of mid-lumbar interneurons, which are closely integrated into the spinal neural locomotor circuitry (Edgley et al 1988, Perreault et al 1995). There is a population of group II interneurons that is localized around L3-L5 (Edgley & Jankowska 1987, Shefchyk et al 1990, Jankowska 1992) and part of this population is rhythmically active during swing and during the transition from the stance to swing phase of locomotion in MLR-induced locomotion (Schefchyk et al 1990). These interneurons respond in a phase-dependent manner to group I-II and cutaneous afferents stimulation, but only during the swing phase. During locomotion, group II interneurons are thought to carry information concerning hip position, a signal known to be important during the transition from stance to swing. Group II muscle afferents from knee and ankle extensors evoke a flexion reflex in spinal cats injected with L-DOPA and are considered to be included in the flexor reflex afferents (FRA, see section 3.3.3).

Group III and IV afferents. Group III and IV afferents originate from thermoreceptor, chemoreceptor, mechanoreceptor, nociceptors and free endings. Very few studies have described the individual activity of these high threshold afferents during locomotion. Preliminary results showed that the response of dorsal horn cells receiving group III and IV muscle afferent input in the lumbar spinal cord is greatly depressed by a central motor command originating from the MLR in paralyzed decerebrate cats (Degtyarenko & Kaufman 2000). Further experiments showed that biccuculine or strychnine suppresses

the inhibition in dorsal horn cells receiving group III efferents and suggest a locomotor-related release of GABA and glycine to gate group III afferents transmission (Degtyarenko & Kaufman 2003). The authors speculated that this might be a mechanism to prevent vasoconstriction and the associated decrease in blood flow to muscles or to decrease nociceptive inputs from working muscles. Note that group III and IV muscle afferents are also part of the FRAs (see section 3.3.3).

3.3.2 Pathways from cutaneous afferents

It was found that stimulation of skin areas overlying an extensor muscle tended to facilitate monosynaptic reflexes of that muscle, whereas stimulation of other skin areas tended to depress them (Engberg 1964). On the other hand, a flexor muscle tended to be excited by stimulation of the skin of the entire limb and inhibited by the skin over the extensor antagonist (Hagbarth 1952). The majority of the work for assessing the role of cutaneous information during locomotion was performed using neurectomies, anesthesia or mechanical stimulation of the skin or cutaneous nerves in different phases of the step-cycle. The hindleg of the cat is innervated by 5 different cutanous nerves: tibial, superficial peroneal, deep peroneal, saphenous and sural.

In this project, 3 cutaneous nerves were stimulated to evoke responses in motoneurons: superficial peroneal, caudal cutaneous sural and medial plantar.

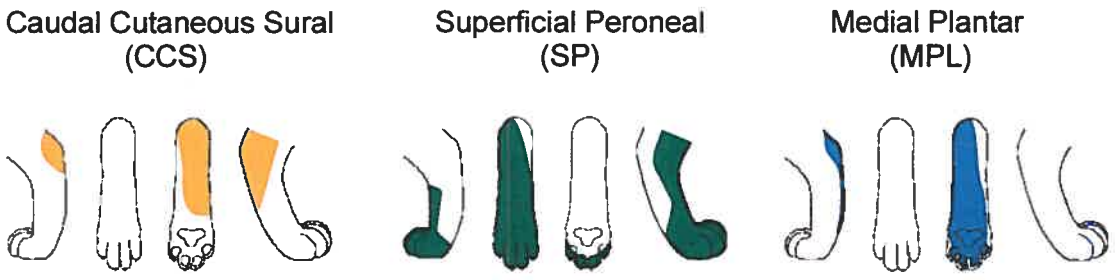

Figure 4: Cutaneous territories of the cat hindpaw
(modified from Bernard et al 2000).

As illustrated in figure 4, SP innervates the dorsal and distal surface of the hindpaw and toes, MPL (a branch of tibial) innervates the ventral surface of the foot and the central plantar pad and CCS (a branch of sural) innervates the lateral margin of the foot.

At the beginning of the 20th century, low threshold cutaneous stimulation of the distal hindlimb was found to produce extensor reflexes at rest (Philippson 1905, Sherrington 1910, see also Engberg 1964). During locomotion, a similar role has been proposed as to reinforce extension during the stance phase of locomotion. The idea was later discarded because the removal of cutaneous input of the hindpaw (denervation, local anesthesia, taking off the skin) did not prevent stepping and yielded only small deficits even after spinalization (Sherrington 1910, Engberg 1964, Forssberg et al 1977, Duysens & Stein 1978, Prochazka et al 1978). Therefore, cutaneous inputs were thought to be useful but not critical to locomotion. In a series of experiments, it was recently shown that after an extensive cutaneous neurectomy, cats could walk almost normally over the treadmill belt but had deficits during precision walking such as stepping on the rungs of a horizontal ladder or on inclines suggesting that cutaneous input provides input necessary to adjust walking (Bouyer & Rossignol 2003a).

The role of cutaneous afferents in adapting the motor response to perturbations during stepping is now obvious. Later experiments using other methods such as stimulation of cutaneous afferents shed some light on the nature of cutaneous contribution to locomotion. It was shown that the simulation of the dorsal surface of the foot in spinal cats resulted in phase-dependent reversal of the reflex: enhancement of flexion during swing and a shorter but more pronounced extension during stance (Forssberg et al 1975). During swing, a coordinated reflex is evoked: the stumbling corrective reaction. The contact of the limb with an obstacle (or electrical stimulation of the dorsum of the foot) leads to an additional and sequential activation of flexors at all joints to allow the perturbed limb to step over the obstacle (Prochazka et al 1978, Forssberg 1979, Wand et al 1980). The same stimulus applied during the stance phase enhances the ongoing activation of extensors in cats and humans (Forssberg et al 1975, 1977, Duysens & Pearson 1976, Yang et al 2004). The stimulation of the pad or the plantar surface of the foot also prolongs the ongoing extensor activity during stance and delay the following flexion (Duysens & Pearson 1976). Notably, the excitatory response following a cutaneous stimulation is preceded by a short latency inhibition in the intact cat (Abraham et al 1985, Bélanger et al 1986, Duysens & Loeb 1980, Forssberg et al 1977, Forssberg 1979). This short-patency inhibition is less pronounced in spinal cats or replaced by a short-latency excitation (Forssberg 1979, Abraham et al 1985)

These reflexes seem to be useful in adapting the gait to unpredictable terrain. If an unexpected loading of the foot occurs, cutaneous feedback will induce an increase in

extensor activity to counteract the applied load and delay the onset of swing. Cutaneous afferents thus evoke a powerful corrective response to overcome a perturbation during gait by maintaining the ongoing locomotion as unperturbed as possible (McCrea 1980). Although slightly different, reflexes evoked by cutaneous afferents in the human are reminiscent of the stumbling corrective response (Forssberg 1979) and meant to avoid a destabilizing stumble (Bélanger & Patla 1987, Van Wezel et al 1997, Zehr et al 1997). The common synergy of flexors during swing and extensors during stance can be observed independent of the location of the stimulus (Duysens & Stein 1978, Duysens & Loeb 1980, Abraham et al 1985), However, nerve-specific responses are also observed during locomotion in cats (Abraham et al 1985, Moschovakis et al 1991, Pratt et al 1991, LaBella et al 1992, Degtyarenko et al 1996) and humans (Van Wezel et al 1997, Zehr et al 1997) to provide location-specific information from the skin of the foot. In some cases, the anatomical location of the nerve seems crucial in determining the functional nature and sign (inhibitory or excitatory) of any reflex response, particularly those evoked to clear an obstacle.

In the cat, latencies of cutaneous responses are minimally trisynaptic (Lundberg et al 1977, Baker & Chandler 1987b, Fleshman et al 1988, LaBella et al 1989, LaBella & McCrea 1990) although exceptional disynaptic linkage in specific cutaneous pathways during the depolarized phase of stepping has been observed (Burke 1999). Some studies have described short-latency excitatory pathways from cutaneous afferents to extensor motoneurons in decerebrate and acute spinal cats (Hagbarth 1952, Fleshman et al 1984, LaBella et al 1989, LaBella & McCrea 1990). Cutaneous stimulation produces a differential distribution of early PSPs within the 3 extensor motor pools comprising the triceps surae in decerebrate cats (LaBella et al 1989), spinal cats (LaBella et al 1992) and also in humans (Duysens et al 1996). CCS preferentially evokes an excitation in MG whereas SP rather facilitates transmission to LG (LaBella et al 1989, 1992). The dominant excitatory effect on extensor muscles from the foot may participate in the regulation of stance together with muscle proprioceptors (Duysens & Pearson 1976).

Cutaneous reflexes are phase-dependent. As mentioned in the preceding paragraph, the amplitude of cutaneous reflexes is powerfully modulated during locomotion in intact cats (Prochazka et al 1978, Forssberg 1979, Duysens & Loeb 1980, Wand et al 1980, Drew & Rossignol 1987), during fictive locomotion in decerebrate (Duysens & Pearson

1976) and spinal cats (Forssberg et al 1975, 1977, Andersson et al 1978, LaBella et al 1992) and in humans (Van Wezel et al 1997, Zehr et al 1997).

Intracellular motoneuronal recordings showed that a cutaneous stimulation evoked maximal EPSPs during the active phase of motoneurons during fictive locomotion while IPSPs were maximal during the reciprocal phase (Andersson et al 1978, Schomburg & Behrends 1978a). These results suggest a mechanism of central origin rather than an effect of afferent feedback. However, all cutaneous responses do not follow this simple scheme as illustrated by the stimulation of the superficial radial nerve, which evokes an excitatory response in the forelimb extensor triceps during the swing phase of locomotion even though it is generally active during stance (Drew & Rossignol 1987, see also Wand et al 1980). Thus, a cutaneous stimulation of the foot during different phases of the step-cycle evoke adapted phase-dependent responses to adequately compensate for the perturbation applied, even after a complete spinal lesion. This modulation is primarily attributed the convergence of primary afferents on common spinal interneurons. Indeed, cutaneous afferents have disynaptic excitatory connections with Ib interneurons and facilitate the interneuronal transmission in inhibitory and excitatory pathways from Ib afferents to motoneurons (Lundberg et al 1977).

Interneurons intercaled in cutaneous pathways. As mentioned in the preceding paragraph, cutaneous pathways from the hindpaw are trisynaptic in general although some exceptional disynaptic cutaneous pathways does exist (Moschovakis et al 1991, Degtyarenko et al 1996, 1998, Burke 1999, Burke et al 2001). Interneuron identification is rather difficult, particularly when several interneurons are intercalated between the afferent and the motoneuron. Thus state-dependent modulation of spinal reflexes was used as a tool to investigate the organization of spinal interneurons activated by the stimulation of cutaneous afferents of the hindpaw (Burke 1999). However, reflexes evoked by skin afferents recorded in forelimb motoneurons are known to be mediated by disynaptic pathways (some are also polysynaptic). Hence, a little more is known about those cutaneous interneurons and cervical (C_7) interneurons that mediate cutaneous reflexes in the forelimbs have been identified (Hongo et al 1989, Kitazawa et al 1993). These interneurons can be excitatory or inhibitory and receive excitatory inputs from corticospinal and rubrospinal tract fibres (Hongo et al 1989). Their activity during locomotion remains to be determined.

Cutaneous pathways and SCI Chronic spinal animals exhibit an increase in reflex activity to cutaneous input (Hultborn & Malmsten 1983). Changes in cutaneous pathways have been attributed to specific alterations in premotoneuronal mechanisms and not to the changes in passive membrane properties of motoneurons between acute and chronic spinal cats (Chandler et al 1984, Munson et al 1986, Baker & Chandler 1987b).

The participation of cutaneous input in locomotor recovery after SCI is poorly documented particularly because it has been difficult to separate the contribution of proprioceptive from cutaneous input. It has been shown that phasic cutaneous input improves motor recovery of the hindlimbs after a lateral spinal hemisection in chicks (Muir & Steeves 1995, 1997). It was shown that the selective phasic stimulation of cutaneous receptors from the plantar surface of the foot, without the activation of proprioceptors signaling limb loading, is sufficient to increase limb extension during swimming. An enhanced cutaneous feedback provided to the hindlimbs by buoyant tubes suspended from the bottom of the pool during swim-training has also been shown to improve motor recovery as compared to *standard* swim-training in contused rats (Smith et al 2006). In addition, cutaneous inputs have been shown to be essential to recover stepping in spinal cats after a cutaneous denervation of the hindlimb (Bouyer & Rossignol 2003b). Indeed, if a single source of cutaneous input to the hindlimb was left, foot placement was adequate. However, plantar foot placement was never recovered if this source was removed. A cutaneo-muscular stimulation of the medial plantar of the foot throughout the late swing and early stance phase of the step-cycle can also restore a normal reflex modulation in spastic SCI individuals by inhibiting the SOL H-reflex (Fung & Barbeau 1994). Together, these results suggest a crucial role for cutaneous feedback in the recovery of stepping.

3.3.3 Pathways from flexion reflex afferents (FRA)

The term *flexor reflex afferent (FRA)* was assigned to the ensemble of afferents that terminate on a common group of interneurons to generate the flexion reflex. These afferents include large and small cutaneous afferents, joints afferents and the high-threshold group II-III-IV muscle afferents. The flexion reflex is a polysynaptic and polysegmental spinal reflex that induces a complex flexion synergy of the ipsilateral limb with a reciprocal extension of the contralateral limb (cross extension reflex).

The Swedish group of Lundberg extensively studied these reflexes in the cat (Eccles & Lundberg 1959, Holmqvist & Lundberg 1961, Jankowska et al 1967ab, Engberg & Lundberg 1969, Lundberg 1979). The stimulation of FRA in acute spinal cats evokes a polysynaptic excitation in ipsilateral flexors and contralateral extensors. Following L-DOPA injection, FRA pathways are reorganized and produce suppression of the short latency flexor reflexes accompanied by the release of long latency and long lasting discharges in flexor and reciprocally organized extensor nerves. Lundberg and colleagues realized that the organization of FRA pathways in the L-DOPA preparation resembled Brown's (1911) *half center* organization for locomotion (section 2.2.1). After a further administration of nialamide (MAO inhibitor), FRA stimulation evokes alternating bursts of activity in flexors and extensors. On the basis of these observations, FRA pathways were proposed to be forerunners of locomotion and assumed to be part of the central core of rhythm generation.

FRAs terminate on spinal interneurons (FRA interneurons) which are subdivided in many subpopulation involved in various features of the reflex and have been recorded in the Rexed's lamina VII in the ventral horn of the spinal cord (Jankowska et al 1967b, Gossard et al 1994). The majority of FRA interneurons are contacted by a number of inhibitory and excitatory interneurons whereas some of them are first-order interneurons, monosynaptically contacted by converging FRA (Jankowska 1992). The same afferents can thus excite or inhibit FRA interneurons depending on the number and features of the intercalated interneurons. Yet, the excitatory effect of FRA is usually more powerful on ipsilateral flexor muscles while the predominant effect on ipsilateral extensors is inhibitory. Transmission in this pathway is modulated by descending input to adapt the response to motor activity. For example, during walking, the same stimulus evokes a flexion during stance and beginning of swing and an extension during the rest of the swing phase (Schomburg & Behrends 1978a).

Clearly, a simple alternation between the two *half-centers* or FRA interneurons is not an appropriate model to explain the variety of muscle activity patterns (section 2.2.1). Nevertheless, one could ask if FRA interneurons are part of the CPG? This issue is still controversial. In nialamide and L-DOPA acute spinal cats, it was shown that ipsilateral FRA could reset the rhythm by initiating or prolonging the flexion phase during locomotion and FRAs are considered to be part of the CPG. In contrast, the stimulation of contralateral FRA evokes activity in the extensors (Jankowska et al 1967ab, Lundberg 1979, Conway et al 1987, Gossard et al 1994). As originally described by Pearson in the locust and cat, tension in extensor muscles recruiting Ib afferents was shown to have a

profound effect on the extensor activity (Pearson 1995, see section 3.2.1.2). It was shown that coFRAs and group Ia-Ib inputs from extensors converge on common interneurons (Gossard et al 1994). It was suggested that the depolarization in extensor motoneurons following Ib stimulation was transmitted through the interneuronal network (CoFRA interneurons) responsible for extensor burst generation during locomotion (Conway et al 1987, Gossard et al 1994). However, the long-latency discharges evoked by the stimulation of FRAs in acute spinal cats injected with L-DOPA cannot be evoked in chronic spinal cats even if they have recovered locomotor movements. This suggests that FRA networks are not necessary to generate a locomotor pattern and those pathways transmitting inputs from FRAs are reorganized after SCI (Barbeau et al 1987). Notably, FRAs still evokes long latency and long duration discharges similar to those observed in acute spinal cats in individuals with a complete SCI (Roby-Brami & Bussel 1992).

3.3.4 Specific functional features of sensory feedback

Proprioceptive inputs provide a powerful source of modulation of the CPG and their role is well described in the generation and adaptation of locomotion (Pearson 2000, Dietz 2003). Two specific types of sensory input appear to have a direct access to the CPG and to be important for rhythm entrainment and phase transition: afferents signaling load and afferents originating from muscles around the hip. The importance of load signals and afferents involved in phase transition during locomotion will be described in the following sections. An additional section will succinctly illustrates how the afferent feedback can be modulated before reaching its spinal target and influencing the motor output.

3.3.4.1 Importance of load signals

Among the various types of sensory feedback, it has been shown that sensory feedback originating from load proprioceptors in the legs has a critical and powerful effect on CPG activity and gait regulation (reviewed in Duysens et al 2000, Dietz & Duysens 2000). Proprioceptive feedback from muscle and tendon afferents is especially increased during stance as the limb is loaded. This feedback is used as extensor reinforcing feedback as shown in many species including humans (reviewed in Duysens et al 2000). Load-related feedback during the stance phase contributes significantly to the generation of activity in

extensors (30 to 50%; Ghori & Luckwill 1985, Dietz et al 1992, Hiebert & Pearson 1999, Stephens & Yang 1999, Sinkjaer et al 2000). Consequently, unloading of the extensors such as stepping into a hole causes a marked reduction in the magnitude of extensor activity (Gorassini et al 1994, Hiebert & Pearson 1999). In humans, this type of response was ascribed to group Ib and II muscles afferents because an ischemic block (no transmission in Ia afferents) did not prevent a decrease in extensor activity after unloading (Sinkjaer et al 2000). Also, unloading of the leg is essential for the onset of swing in cats (Duysens & Pearson 1980, Pearson 1995, Whelan et al 1995, Pearson et al 1998) and humans, especially when supraspinal input is lacking (Harkema et al 1997, Yang et al 1998).

Load signals may be transmitted to the spinal cord not only by proprioceptors in extensor muscles but also by mechanoreceptors situated in the skin under the foot. It has been shown that cutaneous input from the sole of the foot and Ia-Ib inputs from the hip and ankle muscles are especially important drives for walking (Capaday 2002, Dietz 2003). The effects of load afferents on locomotion have also been implicated when supraspinal input are lacking in infants and SCI individuals. A body load applied during the stance phase of stepping prolongs the stance phase and delays the swing phase of gait in infants (Yang et al 1998, Pang & Yang 2000). The beneficial effects of locomotor training on functional recovery can be attributed, at least in part, to load application on the affected limbs: progressively increasing weight-bearing facilitates the generation of an adequate pattern of activity and stimulates plasticity (Barbeau & Rossignol 1987, Barbeau et al 1987, de Leon et al 1998b, 1999, Edgerton et al 1992, Harkema et al 1997). Several studies have shown that locomotor-like movements alone (without loading) generated by the application of a driven gait orthosis or by manually assistance are not sufficient to generate leg muscle activation in subjects with complete SCI (Harkema et al 1997, Dietz et al 2002, Ferris et al 2004). However, leg movements combined with loading lead to appropriate leg muscle activation. If limb loading is increased during the stance phase of stepping in the course of a step-training regimen, recovery occurs over a shorter period of time as compared to animals that have not experienced a larger limb loading (Edgerton et al 1992). Moreover, leg muscle activity has been shown to vary with the level of body weight support even following a complete SCI (Harkema et al 1997, Dietz & Duysens 2000, Dietz et al 2002). For example, the modulation of EMG amplitude in SOL and MG is related to peak load rather than muscle-tendon stretch (Harkema et al 1997). Further experiment illustrated that varying weight support provided to rats affect both the quantity and quality of stepping (Timoszyk et al 2005). Together, these studies suggest that load-

related feedback contributes to the generation of locomotion and recovery of function in both humans and animals.

3.3.4.2 Regulation of phase transition during stepping

The transition from stance to swing phase during locomotion is under powerful sensory influence. Certain mechanisms are of prime importance to prevent the initiation of the swing phase during the ongoing stance phase. The swing phase is only initiated when specific criteria are met (reviewed in Prochazka 1996, Duysens et al 2000).

Hip extension and unloading the ipsilateral limb are important sensory signals that promote the initiation of the swing phase in both invertebrates and vertebrates (Grillner & Rossignol 1978, Duysens & Pearson 1980, Whelan et al 1995, Hiebert et al 1996, Pang & Yang 2000). Indeed, many studies revealed the importance of afferents signals from the hip in controlling the initiation of the swing phase of locomotion. The hip needs to reach a certain amplitude of extension, normally attained at the end of the stance phase, and the controlateral limb to be in a position to accept body load for the swing phase to be initiated (Grillner & Rossignol 1978, Pang & Yang 2000). Conversely, a flexion of the hip abolishes stepping on the ispilateral side. During fictive locomotion, the static position of the hip markedly influenced the pattern recorded in the hindlimb muscle nerves and gradually extending the limb increases the vigor of the rhythm (Pearson & Rossignol 1991). Moreover, unloading the extensor muscles was shown to be critical to initiate the swing phase; the ankle extensor proprioceptors would signal weight support by the ankle and inhibit the flexor burst generator (Duysens & Pearson 1980). Little is known about the sensory receptors involved in mediating the transition, but they include Golgi tendon organs signaling load in ankle extensors (Duysens & Pearson 1980, Whelan et al 1995, Pearson et al 1998) and muscles spindles signaling length (group I and II afferents) from the hip flexors (Hiebert et al 1996).

The transition from swing to stance is less affected by sensory feedback, the activity in ankle and knee extensors precedes ground contact suggesting that sensory feedback carrying information about foot landing would unlikely trigger the transition (Engberg & Lundberg 1969, Halbertsma 1983). Thus, neuronal mechanisms responsible for initiating burst activity in the knee and ankle extensor muscles near the end of the swing phase may depend on mechanisms of central origin. This hypothesis is supported by other

investigations showing that when a trap opens under the feet just before foot contact, the initiation of extensor activity in ankle extensor muscles is still present whether the foot touches the ground or falls in the hole (Gorassini et al 1994).

Proprioceptive afferents play a significant role in setting the frequency of locomotion in animals and humans and can modify the timing of the step-cycle. This may explain how spinal cats and infants can adapt their walking speed to the treadmill (Forssberg et al 1980a, Barbeau & Rossignol 1987, Yang et al 1998). Experimentally, group I stimulation of knee, ankle and toe extensors are effective in resetting the fictive locomotor rhythm either by prolonging the extension phase and delaying flexion during stance or terminating the flexion phase during swing (Conway et al 1987, Gossard et al 1994, Guertin et al 1995). Both Ia and Ib afferents appear to contribute, but it seems that the dominant effect comes from Ib afferents (Conway et al 1987). A stimulation occurring during the swing phase terminates ipsilateral flexor activity and initiates extension whereas if it occurs during stance, the extensor activity will be prolonged and the onset of flexion delayed suggesting that these afferents have a facilitatory effect onto the extensor half center (Gossard et al 1994). Although not a general rule, Ia afferents can affect the rhythm. Indeed, stretch-evoked Ia input from plantaris during the stance phase have also been shown to prolong extension and delay flexion as Ib afferents but not to reset the step-cycle during swing (Guertin et al 1995).

It is assumed that Ib afferents from extensors directly access the rhythm generator, we thus believed that activity in these afferents following a step-training regimen after a complete SCI should induce plasticity in the Ib reflex pathway.

The effect of flexor on rhythm generation is less homogenous. During fictive locomotion, the stimulation of group I afferents from flexors had little effect on rhythmicity in spinal cats (Conway et al 1987), but more intense stimulation recruiting group II afferents can reset the rhythm in the same way as group I afferents from extensors in MLR-evoked locomotion (Perreault et al 1995). However, Pearson group (Hiebert et al 1996, Lam & Pearson 2001, 2002) reported that in decerebrated walking cats, Ia afferents from Srt, Ip and EDL and group II afferents from TA can interrupt the extension phase and reset the rhythm to flexion (Fig.3, right pathway 5). Only input from group I afferents of Srt and from group II afferents of EDL could prolong the flexion phase (Fig.3, right pathway 3). Thus it seems

that group II afferents from flexors can either promote extension or flexion depending on the preparation.

3.3.4.3 Presynaptic inhibition

Sensory input can be modulated through primary afferent inhibition or *presynaptic inhibition* (reviewed in Rudomin & Schmidt 1999). This may occur at various sites (Fig.3, yellow) to regulate the efficacy of the transmission by stopping or decreasing the sensory feedback before it reaches the spinal target. Presynaptic inhibition of sensory afferents is manifested by a depolarization of their terminals, which can be seen by a negative potential recorded in dorsal roots near the spinal cord (dorsal root potential, DRP) or intra-axonal depolarization (primary afferents depolarization, PAD). This depolarization is due to axo-axonic GABAergic synapses: $GABA_A$ receptors activate a chloride conductance leading to a movement of chloride to the extracellular compartment. Presynaptic inhibition can be highly specific and may reduce terminal release in a phase-dependent manner, in a chosen pathway or in selected collaterals of the same afferents (Rudomin & Schmidt 1999). Muscle group I-II and cutaneous afferents are under the control of presynaptic inhibition. The interneurons involved in presynaptic inhibition are under the excitatory and inhibitory influence of a wide group of afferents including most of the supraspinal pathways, FRAs, group Ia-Ib afferents and cutaneous afferents. Peripheral and supraspinal input can modulate presynaptic inhibition on Ia and Ib fibers in a different manner. This can be explained by the fact that different sets of interneurons would respectively exert their effect on Ia or Ib terminals to allow a selective control of information concerning the length or the tension of a given muscle (Rudomin & Schmidt 1999). It is believed that the modulation of the presynpatic inhibition may play more critical role in the control of transmission to Ib interneurons than to Ia inhibitory interneurons since Ib interneurons depend on peripheral input from a wider variety of afferents (Jankowska 1992).

Presynaptic inhibition is another source of modulation for sensory feedback and is thought to be involved in the gating of reflexes during locomotion. It is thus a potential site for plasticity to occur. However, very few data on the subject are available. PAD patterns in Ia and Ib afferents were shown to be modulated following peripheral nerve injury whereas PADs in cutaneous afferents almost dissappear (Rudomin & Schmidt 1999). Moreover, indirect evidence suggests that factors contributing to change in the gain of reflex

pathways in SCI individuals may include decreased presynaptic inhibition resulting from the disruption of supraspinal pathways (Calancie et al 1993, Faist et al 1994). The effect of SCI on presynaptic inhibition needs further investigations. However, presynaptic inhibition is likely to be modified after SCI and step-training.

3.4 Motoneurons

3.4.1 Localization

All afferent inputs involved in the control of locomotion, either from supraspinal or peripheral origin, ultimately converge on motoneurons, the final common pathway for motor output. Motoneurons are connected to muscles fibers, their effectors. Striated muscles fibers, responsible for muscle contraction, are innervated by α-motoneurons whereas γ-motoneurons innervate specialized striated muscle fibers of muscle spindles. The excitability of primary and secondary terminals of muscle spindles is modulated by γ-motoneurons, which are coactivated with α-motoneurons. α-motoneurons have the larger diameter (30-70μm) and receive up to 20000 to 50000 synaptic contacts allowing an important integration of the information. Motoneurons are contacted monosynaptically by Ia sensory afferent innervating muscle spindles, descending pathways, axons of spinal interneurons and propriospinal interneurons. All these synaptic contacts contribute to modulate the excitability of the motoneuron.

In this project, α-motoneurons from various motor pools including ankle and toe extensors (plantaris, lateral gastrocnemius-soleus, flexor hallucis longus, medial gastrocnemius), hip extensors (quadriceps, semimembranosus-anterior biceps), ankle and toe flexors (tibialis anterior, extensor digitorum longus, flexor digitorum longus) and a bifunctional muscle (posterior biceps-semitendinosus) were recorded during fictive locomotion. In the spinal cord, motoneurons are segregated according to their target muscle in the ventral horn gray matter (laminae IX and X) of a given lumbosacral segment of the ventral horn gray matter. A precise anatomical description of their localization was preformed in the cat by Vanderhost and Holstege (1997).

In this project, the motoneurons innervating the extensor and flexor muscles of the hindlimb were targeted. These pools are mainly localized in segments L5 to L7.

Motoneurons are depolarized by the summation of repeated excitatory input and respond with action potentials when the depolarization is sufficient to reach firing threshold. Motoneurons should not be considered as passive players simply involved in the spatial and temporal summation of afferent synaptic inputs originating from premotoneuronal networks. Several mechanisms affect motoneuronal excitability and may change the final motor output. Among them, motoneurons display intrinsic membrane properties able to shape the motor output and movement dynamics (reviewed in Kiehn et al 2000). Some of these properties are modulated following a chronic SCI (Cope et al 1986, Munson et al 1986, Hochman & McCrea 1994b) or altered during locomotion (Krawitz et al 2001). Hence, motoneurons participate actively in the patterning of motor commands during locomotion, with several intrinsic properties being turned "on" resulting in a non-linear input-output relationship (Kiehn et al 2000, Krawitz et al 2001, Powers & Binder 2001, Hultborn et al 2004).

3.4.2 Passive properties of motoneurons

The post-spike afterhyperpolarization (AHP) has a key function in transducing the processed synaptic input into a variable spike frequency depending on its duration that modulates the refractory period and return to excitability of the motoneuron. In anesthetized and acute decerebrate cats, a short duration AHP is typically associated to a fast conduction velocity, a high rheobase and low input resistance (R_{in}) within a motoneuron whereas a long duration AHP is associated to a slow conduction velocity, a low rheobase and a high R_{in} (Gustafsson & Pinter 1984b). This relation is maintained after an acute or chronic SCI (Mayer et al 1984, Cope et al 1986, Munson et al 1986, Baker & Chandler 1987a).

Membrane electrical properties of motoneurons such as R_{in}, rheobase, membrane time constant (τ_m) and the length constant (L) are important because they influence the shape and the size of PSPs (Lev-Tov et al 1983, Gustafsson & Pinter 1984a). Changes in motoneuronal properties following a complete SCI remain controversial. A significant increase in axonal conduction velocity, rheobase and threshold voltage (V_{th}) together with a decrease in AHP duration and R_{in} were reported in SOL motoneurons (Czéh et al 1978, Gallego et al 1978, Cope et al 1986) whereas others did not observe any significant changes in MG motoneurons (Czéh et al 1978, Gallego et al 1978, Mayer et al 1984, Munson et al 1986). It is possible that there is a differential effect of SCI on in SOL vs MG

and that SOL motoneurons are more likely to have their properties modified than MG because SOL is solely composed of slow muscle fibers (Czéh et al 1978, Gallego et al 1978). However, others reported that there is no difference in motoneuronal properties after chronic SCI in ankle extensors (grouped together). However, when an analysis was conducted according to the motor pool, V_{th} was significantly increased whereas the AHP was decreased only in MG motoneurons (Hochman & McCrea 1994b). Moreover, motoneurons properties are either reported to change (Czéh et al 1978) or not to be modified (Baker & Chandler 1987a) in comparative studies between acute and chronic SCI, revealing an uncertain effect of time post-injury. Although further experiments need to be conducted to elucidate the precise effect of SCI on motoneuronal properties; it is thought that in chronic spinal cats, these properties may not solely account for the variability in PSPs (Hochman & McCrea 1994b).

Motoneuron properties depend also partly upon factors associated with activity of the innervated muscles (reviewed in Gardiner et al 2006). Mimicking activity by stimulating the sciatic nerve periodically or performing passive cycling may prevent changes in motoneuronal properties after complete SCI (Czéh et al 1978, Beaumont et al 2004).

3.4.3 Dynamic properties of motoneurons

Motoneurons of many vertebrates, such as rodents, turtles, cats and humans, exhibit plateau potentials and have been described extensively (Kiehn 1991, Gorassini et al 1998, Kiehn & Eken 1998, Powers & Binders 2001, Hultborn 2002, Hultborn et al 2004). The plateau potential is used to described the fact that a short duration synaptic excitation (or brief depolarizing pulse) lead to a sustain shift in the membrane potential largely outlasting the initial depolarization. The plateau potential occurs predominantly in dendrites and is expressed in motoneurons as a shift in frequency discharge resulting from voltage-dependent non-inactivating Ca^{2+} currents due to a facilitation of L-type Ca^{2+} channels also known as persistent inward currents (PICs). The activation of the plateau potential evokes discharges that can be maintained for several minutes when the cell is sufficiently depolarized. These PICs contribute to shape motor output and amplify dramatically the effect of the synaptic input from descending and sensory afferents (Kiehn 1991, Kiehn & Eken 1998, Powers & Binder 2001, Hultborn 2002). Noteworthy, some authors also report the presence of plateau potentials in spinal interneurons (Hounsgaard & Kjaerulff 1992).

The presence of plateau potential in motoneurons seems to depend on the tonic activity of serotoninergic and/or noradrenergic descending pathways in the cat and turtle (Conway et al 1988, Hounsgaard et al 1988). Thus, PICs may be activated by the action of these neuromodulators to amplify synaptic input during locomotion. Plateau potentials are largely eliminated following an acute SCI but may be revealed with the application of drugs (Conway et al 1988, Hounsgaard et al 1988, Bennett et al 2001). It seems likely that similar PICs may be evoked in spinal motoneurons by other neuromodulators in chronic SCI animals in the absence of descending pathways because motoneurons may recover the ability to generate plateau potential in chronic spinal rats and cats (Eken et al 1989, Bennett et al 2001). This suggests a latent endogenous capacity to generate plateau potential.

During locomotion, motoneurons also exhibit a wave of depolarization of the membrane potential during its active phase that alternates with a wave of hyperpolarization during the antagonist phase: the locomotor drive potential (LDP) (Edgerton et al 1976, Schomburg & Behrends 1978ab, Perret & Cabelguen 1980, Chandler et al 1984, Shefchyk et al 1984, Pratt & Jordan 1987). The excitability of motoneurons is thus phasically modulated in part by the LDP excursion. The amplitude of the LDP is crucial to determine if PSPs reach the firing threshold and the depolarization facilitates the response occurring during the active period of the motoneuron.

Together, PICs and LDP contribute to a locomotor-dependent increase in motoneuronal excitability.

4. Nervous system plasticity

The amount of neurotransmitter released at a synapse or synaptic strength may change according to previous *experience*. Two main types of change in synaptic strength have now been associated with learning in a variety of systems and animal models. Short-term changes, without permanent structural modifications in the neurons themselves, last from seconds to hours. These involve strengthening existing synapses through modification of pre-existing proteins (ion channels, protein kinases, receptors, etc). Long-term changes (eg long-term potentiation or LTP) largely rely on the activation of gene expression and synthesis of new proteins in the nucleus. Continuous or repetitive synaptic activation can result in the activation of an intracellular cascade that initiates protein synthesis and results in alterations in the neuron itself. Changes in the efficiency, size and number of synapses imply that the adult nervous system is dynamic and that behavioral experience may ultimately sculpt synapse activation, addition or removal (Hawkins et al 1993). This concept is commonly referred as *synaptic plasticity*. The following sections will illustrate that the CNS displays a high level of plasticity, provide insights concerning possible molecular candidates mediating synaptic plasticity, with a focus on the spinal cord.

4.1 Spinal plasticity

It was long assumed that spinal reflex pathways were hardwired (Forssberg & Svartengren 1983), simply responding quickly and in a stereotyped fashion to descending and afferent inputs. There is now experimental and clinical evidence suggesting a certain level of spinal plasticity in response to central or peripheral lesions or operant conditioning (Mendell 1984, Durkovic 1996, Wolpaw 1997, Wolpaw & Tennissen 2001). The concept of spinal plasticity is critical to locomotor rehabilitation: it is critical to understand the precise mechanisms evoked both in response to a SCI or following a step-training regimen in order to enhance the residual potential of the spinal cord and build targeted and adapted pharmacological strategies that promote recovery of function. The following section will demonstrate briefly the plastic potential of the spinal cord in various conditions. It will be followed by 2 sections respectively describing observations made either after a SCI or after step-training.

4.1.1 Reflex conditioning and denervation

Peripheral and supraspinal inputs can evoke short- and long-term changes modeling the motor output generated in the spinal cord (Wolpaw & Tennissen 2001). The acquisition of simple extension and flexion responses in the hindlimb is caused by associative and non-associative mechanisms in spinal animals. Suggested several years ago by Shurrager and Culler (1940) and later confirmed by others, it was shown that a modulation in the amplitude of the withdrawal reflex can be acquired and maintained several hours after conditioning in spinal animals (Beggs et al 1983, Durkovic 1985, Durkovic & Damianopoulos 1986). The results obtained by Durkovic and colleagues exhibited the features of classical conditioning, including the typical dependence on the delay between the conditioned and the unconditioned stimulus. Their experiments suggested that the underlying spinal plasticity might involve interneurons conveying sensory input, rather than the sensory afferents or the motoneurons themselves. This spinal *learning* was maintained and somehow imprinted in the spinal cord: a subsequent acquisition was facilitated by the initial conditioning of the reflex. Wolpaw and colleagues also demonstrated the plastic potential of the spinal cord. In operant conditioning experiments, they trained rats and monkeys to either up- or down-regulate the H-reflex (reviewed in Wolpaw 1997, Wolpaw & Tennissen 2001). This modulation was further shown to produce a complex pattern of plasticity in the spinal cord itself that includes changes in motoneuron physiological properties as well as in synaptic terminals (Feng-Chen & Wolpaw 1996, Wolpaw 1997). Locomotor recovery following muscle (Bouyer et al 2001) or cutaneous (Bouyer & Rossignol 2003b) neurectomy is another significant example of adaptive spinal plasticity in chronic spinal cats.

Together, these studies suggest that the lumbar spinal cord, by itself, is able to memorize selected motor responses and that plastic modifications in spinal networks may be initiated by a specific pattern of afferent activity and acquired by sensory experience. The spinal cord has the capacity to adapt to major changes.

4.1.2 Spinal cord injury and plasticity

As summarized in Figure 5, a physiological, biochemical and functional reorganization occurs in the spinal cord caudal to the lesion following a SCI (Edgerton et al 1997a, 2004,

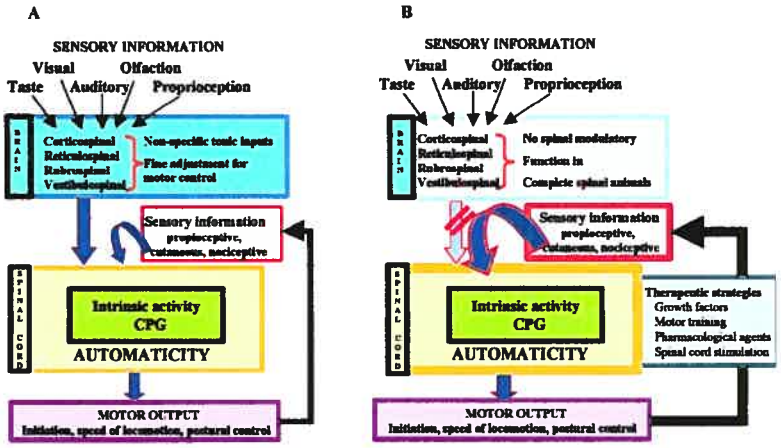

Figure 5: Plasticity of the spinal cord after SCI

A) Intact individual: Although the *basics* of the locomotor pattern and sensory processing are automatic to a certain extent (yellow), several inputs originating from supraspinal (blue) and sensory afferents (red) model the motor output (pink). They are integrated to adapt the locomotor pattern to the moment - to - moment environmental constraints.

B) SCI individual: spinal automaticity becomes evident following SCI. After supraspinal afferents are disconnected (gray arrow), the spinal networks adapt to an altered combination of inputs in order to facilitate motor output. For example, the locomotor pattern after step-training illustrates not only a high level of spinal automaticity but also the ability of the spinal cord to *learn* and perform motor tasks. As compared to intact individuals (A), the relative importance of the spinal networks and of the sensory inputs terminating upon them is greater (thick lines). Several strategies are employed to enhance locomotor recovery after SCI (gray box). From Edgerton et al 2004.

(Edgerton et al 1997a, 2004, de Leon et al 1999b, Tillakaratne et al 2000, 2002, Rossignol et al 2001). This reorganization involves, to different extent and with different time course, both excitatory and inhibitory neurotransmitter systems: monoaminergic, GABAergic, glycinergic, glutamatergic (Giroux et al 1999, Tillakaratne et al 2000, Chau et al 2002, Edgerton et al 2001, Giroux et al 2003) and probably others. However, species- and type-dependent discrepancies (complete vs partial SCI, contusion vs transection) have been observed drawing a quite complex portrait of lesion-induced plasticity.

Nacimiento and colleagues (1995) illustrated an example of synaptic reorganization in spinal networks caudal to the lesion after injury. Synaptophysin immunoreactivity on the ispilateral lumbar spinal cord of hemisected rats was significantly decreased for several weeks after the injury but returned to the intact side values within 90 days. Yet, the afferents forming these synapses are not identified (cutaneous, articular, tendinous, muscular or interneuronal). The supraspinal contribution of the intact side remains also largely undetermined although it is thought to be involved in this synaptic reorganization.

Edgerton and colleagues (2001) hypothesized that in the complete spinal cat, the number, size and distribution of synapses on motoneuron and interneuron membranes may change (Fig.6). These modifications together with the complete loss of supraspinal input could

contribute to modulate neuronal input efficiency and increase the relative effect of any segmental or peripheral synaptic input. Consequently, the sources of control during movement differ substantially from that which existed prior to the injury: the spinal cord is altered (Edgerton et al 1997b, 2001).

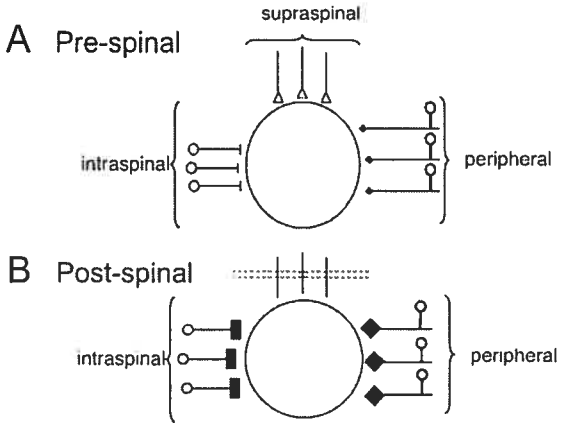

Figure 6: Schematic diagram of afferents (supraspinal, segmental and peripheral) to the spinal cord networks before and after SCI.

Before SCI (A), supraspinal and peripheral afferents enter the spinal cord and, together with segmental inputs (ie intraspinal), influence the motor output. After a complete SCI (B), the synapses of supraspinal origin are disrupted and the relative efficiency of the remaining synapses of segmental or peripheral origin is increased. From Edgerton et al 2001.

After a SCI, the interaction between the CPG network and peripheral inputs of various origin is critical because major sources of control, the brain and brainstem nuclei, have been eliminated. It is doubtful that the residual motor pathways ever executed locomotion independently and it seems plausible that these networks have to develop new strategies to perform stepping movements. The interpretation of a given sensory input may significantly differ from the intact animal (Fig.6, see also Edgerton et al 2001).

Similar to the developing nervous system which is structurally and functionally dynamic, the injured spinal cord undergoes a substantial process of reorganization that may make it especially responsive to be primed by external cues such as activity-dependent feedback.

4.1.3 Spinal plasticity and locomotion

4.1.3.1 Activity- and task-dependent

Early evidence for the beneficial effect of treadmill walking in spinalized kittens came as early as the 50s (Shurrager & Dykman 1951). This phenomenon was later confirmed in the adult and detailed by others (Lovely et al 1986, Barbeau & Rossignol 1987). Although anecdotic cases of spontaneous recovery have been reported (Pratt et al 1994, de Leon et al 1998b), step-training is usually essential to recover and enhance locomotor movements.

The recovery specifically depends on the task performed during training (Edgerton et al 1997a, de Leon et al 1998a) and is not simply due to an increased level of activity in neural networks or to an activity-related effect on musculature provided by training (Roy & Acosta 1986, Roy et al 1999). For example, spinal cats can either be trained to step or to stand (Pratt et al 1994). However, stand-training does not improve stepping recovery and conversely, the duration of standing episodes is not enhanced after a step-training regimen (de Leon et al 1999b). Another example of the specificity of a training regimen is illustrated by experiments in which spinal rabbits preferentially express a stepping pattern with an alternate or in-phase coupling of the hindlimbs (Viala et al 1986). The benefits of locomotor training can be retained for ~6 months to 6 years after the training is discontinued (de Leon et al 1999a, Wernig et al 1998) and re-learning is faster following the initial training (de Leon et al 1999a).

Taken together, these results suggest that practicing a given motor task using specific sensory pathways largely defines the subsequent ability to perform this task in spinal animals.

To date, mechanisms known to be involved in the *learning* of spinal reflex are not sufficient to elucidate the recovery of complex motor tasks such as locomotion in spinal, denervated or deafferented animals. Locomotor recovery requires a longer learning period and presumably the induction of long-term changes in spinal pathways controlling hindlimb movements.

4.1.3.2 Neurotransmitters and neuromodulators

The CPG is under the control of the reticulospinal pathways activated by various areas of the brainstem and also by monoaminergic pathways (5-HT, NA). Moreover, glutamate and glycine are essential neurotransmitters for CPGs in virtually all vertebrates whereas NA and 5-HT are modulators of the basic locomotor pattern (Rossignol 2000, Grillner 2003). Several pharmacological agents have been tested to assess the role of different modulatory neurotransmitter systems in initiating early and late spinal locomotion and subsequently maintaining the expression of the stepping pattern in various animals including rodents, cats and humans (reviewed in Rossignol et al 2001). The effect of a given neurotransmitter is not necessarily similar across species or preparations. It was

shown that only the activation of α_2-noradrenergic receptors could trigger locomotor movements in spinal cats (clonidine, tizanidine, oxymetazoline, NA, DOPA). Other systems (glutamatergic, serotoninergic, dopaminergic) have failed to trigger sustained locomotion in this preparation although they can in other species such as rats and mice (reviewed in Rossignol 2000). Indeed, locomotor movements are much more improved by 5-HT agonists or by transplantation of serotoninergic neurons in spinal rats (Ribotta et al 2000, 2002). They can also be triggered by excitatory amino acids (EAA), 5-HT in neonatal rats (Cazalets et al 1992) and by EAA in decerebrate cats (Douglas et al 1993). Thus, the focus in the next paragraphs is oriented toward results obtained in the spinal cat.

Inhibitory neurotransmitters: GABA and glycine. It is a generally accepted concept that movement-related afferent inputs result in synaptic activity-dependent alteration in synaptic strength, patterns of synaptic connectivity and structural modeling of spinal cord circuitry after SCI. For example, plasticity in the lumbar spinal inhibitory systems has an important impact on walking ability in complete SCIs. A global reduction of GABAergic (with bicuculline) or glycinergic inhibition (with strychnine) improves locomotor performance in spinal cats and dogs (Hart 1971, Robinson & Goldberger 1986). The specificity of these changes in inhibitory transmission is further supported by experiments in which strychnine improved the stepping ability of untrained and stand-trained cats but not of step-trained cats (de Leon et al 1999b). It was later shown that gephyrin (the protein responsible for the postsynaptic clustering of the glycinergic receptor), GAD_{67} (a GABA-synthesizing enzyme) and $GABA_A$ receptors are up-regulated in the lumbar spinal cord of SCI animals whereas they are near normal values in step-trained animals (Tillakaratne et al 2000, Edgerton et al 2001, Bravo et al 2003). Again, this suggests that spinal plasticity is not only activity-dependent, but also specifically task-related. Moreover, the GAD_{67} level around motoneurons is inversely correlated with stepping performance of step-trained spinal cats (Tillakaratne et al 2002) suggesting that step-training could reduce the overall inhibitory potential and result in a net excitatory effect on spinal networks controlling hindlimb stepping (de Leon et al 1999b). Notably, recent investigations showed that sensory stimulation mimicking sensory feedback provided by step-training increased reciprocal inhibition in humans (Perez et al 2003). In addition, step-trained cats were shown to have a better capacity to reciprocally inhibit antagonistic motor nuclei during walking as compared to stand-trained spinal or intact cats (Edgerton et al 1997a, de Leon et al 1999b). Some inhibitory pathways are thus enhanced whereas others are depressed.

This shows that spinal plasticity presents a high level of specificity and may be modulated according to a given reflex pathway or motor pool as a function of the specific needs of a particular task.

Monoaminergic pathways. The effect of the noradrenergic (NA) control of the spinal cord has been studied in animals with an acute spinal cord transection. The NA precursor, L-DOPA, increases the synthesis and liberation of NA from these terminals and mimics the effect of descending fibers. The effect of L-DOPA can be enhanced by nialamide, a MAO inhibitor, that blocks the enzyme responsible for NA breakdown. This technique has been used for years to favor the emergence of stepping in acute spinal cats (Grillner & Zangger 1979). Clonidine, an α_2-noradrenergic agonist, was also shown to facilitate the emergence of the fictive stepping pattern in spinal cats (Forssberg & Grillner 1973, Pearson & Rossignol 1991).

In chronic spinal cats, noradrenergic drugs such as clonidine were shown to trigger or improve the initiation and modulation of the locomotor pattern and accelerate stepping recovery (Barbeau et al 1987, Barbeau & Rossignol 1991, Chau et al 1998ab). Indeed, during early step-training, clonidine favored the emergence of coordinated stepping on the treadmill, reduced the need for sensory stimulation and improved the stepping rate. This effect is initiated only a few minutes after the injection and is maintained for 4-6 hours (Chau et al 1998a). A daily injection of clonidine also enables earlier recovery of locomotion (6-11 days vs 3-4 weeks) with plantar foot contact and weight support. In step-trained spinal cats that have already recovered locomotor movements, clonidine exerts a neuromodulatory effect and increases the duration of the step-cycle (Barbeau et al 1987, Barbeau & Rossignol 1991, Chau et al 1998b). Although doubts persist concerning the positive effect of clonidine for stepping recovery in humans SCIs, at least some patients showed an improved gait and decreased spasticity with clonidine and treadmill step-training (Fung et al 1990, Rémy-Néris et al 1999).

In cats, locomotion can be expressed in the total absence of NA descending fibers and yohimbine, a NA blocker, does not impair locomotion once the stepping pattern is recovered with step-training (Giroux et al 2001). In contrast, application of a serotoninergic (5-HT) agonist failed to initiate locomotion but enhanced the duration and amplitude of locomotor bursts in spinal cats that already recovered stepping (Barbeau & Rossignol

1991). Further autoradiographic receptor-binding studies showed elevated levels of α_1- and α_2-noradrenergic and 5-HT_{1A} receptors in selected laminae of the lumbar spinal cord following SCI (Giroux et al 1999). The respective role of SCI and step-training in this modulation remains to be determined. Together, these studies suggest that the basic locomotor rhythmicity in spinal cats is not dependent, but only modulated by neurotransmitters such as 5-HT and NA and that those pathways are candidates to be modulated by SCI and/or step-training.

In our experiments, clonidine was solely used during acute experiments to facilitate the emergence of fictive stepping but was not used during step-training to facilitate locomotor recovery.

Glutamatergic pathways. The role of glutamate-related amino acids in the control of locomotion is well documented. Glutamate release and subsequent activation of ionotropic glutamatergic receptors induces locomotion in a variety of species (Douglas et al 1993, Walwyn et al 1999). Application of NMDA together with an EAA uptake blocker (dihydrokainic acid) produces a well-coordinated locomotor pattern. Conversely, the administration of NMDA or non-NMDA antagonists (APV and CNQX) blocks fictive locomotion in the decerebrate cat (Douglas et al 1993). Although NMDA does not trigger locomotion in early spinal cats in the same way as NA agonists, the glutamatergic system is important in mediating locomotion in chronic spinal cats (Chau et al 2002). When locomotor movements are recovered, the NMDA blocker AP-5 completely abolishes locomotion and a further injection of NMDA reinitiates the motor pattern. These results suggest that the basic locomotor rhythmicity in spinal cats is NMDA-dependent (Giroux et al 2003). Even though the general decrease in total glutamate is observed after SCI is probably due to the disruption of glutamatergic supraspinal pathways, an increase in extracellular glutamate has been observed and significantly correlated to stepping performance in spinal rats. This suggests that extracellular glutamate in the dorsal horn is modulated at least in part by primary afferent depolarization during hindlimb stepping (Walwyn et al 1999). Further studies need to be conducted to understand how the organization of the spinal cord following transection changes the biochemical environment of the neural networks that generate stepping. It can be hypothesized that many if not all neurotransmitter systems are involved and modulated.

Other systems involved in synaptic plasticity. Although the mechanisms by which step-training improves gait and triggers and maintains spinal plasticity are not well understood, this most likely involves synaptic activity-dependent processes that can influence the ability of the spinal cord to *learn* and perform locomotion (Wolpaw & Tennissen 2001, Dobkin & Havton 2004, Edgerton et al 2004). Hence, investigations have recently been oriented toward molecules and processes involved in hippocampal learning to address the question of whether similar mechanisms are implicated in *spinal learning*. Recently, BDNF, initially known for its role in survival, growth and differentiation of neurons during development (Barde 1994) has emerged as a critical modulator of synaptic plasticity in the brain (Lo 1995, 1998, Patterson et al 2001). Promising experiments also showed that BDNF could promote recovery after SCI *in vivo*: it positively affects neuroprotection (Yan et al 1992, 1994), regeneration (Tuszynski et al 1994, Kishino et al 1997) and enhances locomotor recovery (Jakeman et al 1998). The next section succinctly describes neurotrophins, particularly BDNF and its downstream effectors, and other signaling pathways known to be involved in synaptic plasticity.

4.2 Neurotrophins

Neurotrophins (NTs), which in mammals include *nerve growth factor* (NGF), *brain-derived neurotrophic factor* (BDNF), NT-3 and NT-4/5, are by far the best-characterized family of neurotrophic factors (Lewin & Barde 1996). These small proteins share a common basic structure, along with variable domains, that determine the specificity of their biological actions resulting from the activation of their receptors. NTs are widely expressed in many cell types in the spinal cord including interneurons, α-motoneurons and glia (Dreyfus et al 1999, Scarisbrick et al 1999, Buck et al 2000). Indeed, motoneurons can accumulate NTs (Conner et al 1997, Yan et al 1997) and synthesize them (Dreyfus et al 1999, Scarisbrick et al 1999, Buck et al 2000, Copray & Kernell 2000). NTs effects are mediated by two types of receptors: $p75^{NTR}$ and the Trk family, a family of specific transmembrane tyrosine kinase receptors with conserved intracellular domains mediating relatively well described signal transduction pathways (reviewed in Kaplan & Miller 2000, Huang & Reichardt 2001). All NTs bind to $p75^{NTR}$; however, they show a high degree of specificity for the Trks. Individuals NTs activate different Trk receptors (NGF acting at TrkA, BDNF and NT-4/5 at TrkB and NT-3 acting primarily although not exclusively at TrkC (Barbacid 1994). An example of an intracellular cascade is simplified in Figure 7. BDNF, which binds to its specific receptor TrkB, induces its dimerization and further autophosphorylation. The

activation of TrkB creates a docking site for adapter proteins containing a Src-homology-2 domain (SH-2, Birge & Hanafusa 1993). Grb2-SOS, PI3K and PLCγ all contain SH-2 motifs, associate with Trk receptors as adapters (Pleiman et al 1993) and respectively propagate signals through the Ras/ERK (extracellular signal-regulated kinase) protein kinase pathway, the phosphatidylinositol-3-kinase (PI3K)/Akt kinase pathway and phospholipase Cγ (PLCγ) (Kaplan & Miller 2000, Huang & Reichardt 2001). Following the kinase cascade, transcription factors may subsequently be activated to regulate the transcription of selected genes.

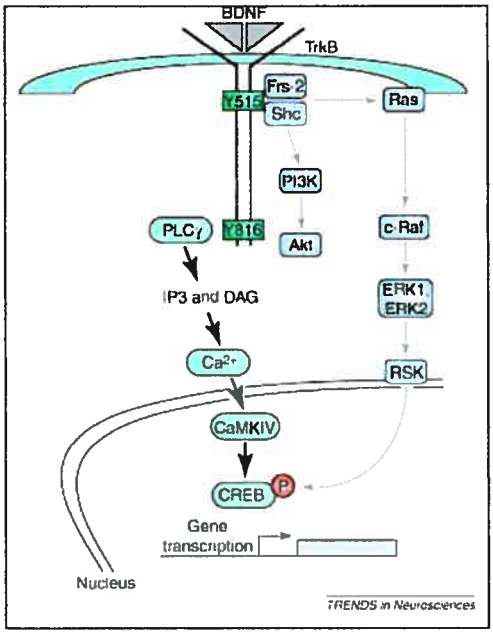

Figure 7 : Schematic diagram of BDNF-activated pathway via Trk receptor.

BDNF binds to its specific receptor, TrkB. Grb2-SOS, PI3K and PLCγ are known to associate with Trk receptors as adapters and propagate signals through the Ras/ERK protein kinase pathway, the PI3K/Akt kinase pathway and PLCγ pathway. Following the kinase cascade, transcription factors such as CREB may subsequently be activated to regulate the transcription of selected genes.

CaMK, calcium/calmodulin-dependent kinase; CREB, cAMP response element binding; DAG, diacylglycerol ; IP3, inositol (1,4,5)-triphosphate; PI3K, phosphatidyl-inositol-3-kinase; PLCγ, phospholipase Cγ; ERK, extra-cellular signal-regulated kinase. From Ernfors & Bramham 2003

Through the activation of these pathways, NTs may mediate cellular events to promote cell survival, differentiation and maintenance, neurite outgrowth and the activation of neurotransmitter synthesis and release. Recent studies strongly suggest a role for NTs in regulating synaptic function in the hippocampus, cortex and cerebellum of both developing and adult animals (reviewed in McAllister et al 1999, Thoenen 1995, 2000). They were also shown to be important in modulating activity-dependent neuronal plasticity and essential for the functional and structural refinement of neuronal circuits in the visual and somatosensory cortex, and in the hippocampus for learning and memory (McAllister 2001, Zhang & Poo 2001). Recent work from several laboratories demonstrated that the contribution of NTs to synaptic plasticity depends on the type of stimuli and involves pre and post-synaptic actions, as well as immediate and delayed effects (Patterson et al 2001).

We directed our investigation toward potential roles for BDNF for the following reasons: it is regulated in an activity-dependent manner and released in response to extracellular cues. Indeed, the secretion of NTs can either be regulated or constitutive. In constitutive secretion, NTs are spontaneously released shortly after being synthesized, thereby enabling a NT to be continuously available to a cell that requires it. In contrast, in the regulated pathway, once synthesized, NTs are stored in secretory granules and released in response to extracellular cues. BDNF is sorted in the regulated pathway as other NTs are mainly sorted in the constitutive pathway (Mowla et al 1999, Farhadi et al 2000). Moreover, BDNF seems to be especially susceptible to regulation by activity for both its expression and release (Neeper et al 1995, Lu & Chow 1999, Schinder & Poo 2000). Finally, only BDNF has previously been shown to exert complex actions at multiple synaptic levels, causing both translational and posttranslational changes in presynaptic proteins associated with exocytosis and in multiple postsynaptic receptors (Suen et al 1997).

BDNF-dependent signaling cascades emerge as potential candidates to mediate the effect of activity-dependent plasticity.

4.2.1 From the brain to the spinal cord

Although BDNF function in the developing animal has been extensively investigated and well documented in the last 20 years (Huang & Reichardt 2001), few reports account for its role in the adult. Increasing evidence suggests that BDNF is involved in synaptic plasticity in the adult CNS, particularly in the formation of LTP in the hippocampus (Lo 1998, Patterson et al 2001). Indeed, the hippocampal LTP is an extensively studied model for learning and memory and has been used as an effective paradigm to understand how the nervous system undergoes plasticity. Recent experiments point to the presence of similar mechanisms in the hippocampus and spinal cord: LTP like phenomena are also exhibited in spinal dorsal horn neurons in response to nociceptive stimuli (Ji et al 2003, Rygh et al 2005, Crown et al 2006) and in the gray matter of the ventral horn (Pockett & Figurov 1993). It is also known that several simple forms of learning such as sensitization and habituation can be induced in the spinal cord (Mendell 1984). More than 100 molecules have been implicated as mediators or modulators of hippocampal LTP and many are also involved in spinal sensitization. Like the consolidation of early-LTP into late-LTP in the

hippocampus, activity-dependent gene expression or transcription, which can increase the expression of pain-related receptors and signal proteins in the spinal cord, plays an important role in conversion from acute nociceptive injury to chronic pain states (Ji et al 2003, Ji 2004). The similarities between these 2 forms of synaptic plasticity are striking, particularly the post-translational regulation of AMPA and NMDA receptors and the activation of the ERK-CREB pathway (Ji et al 2003). In the hippocampus, BDNF and TrkB are required to strengthen LTP and may play a role in consolidating short-term memories into long-term memories (Xu et al 2000). Moreover, BDNF gene deletion or inhibition (Schinder & Poo 2000, Patterson et al 2001, Minichiello et al 2002) impairs hippocampal LTP. Not only does BDNF impact LTP in the hippocampus, but it may also facilitate synaptic efficacy via NMDA receptors to contribute to central sensitization in the spinal cord. Moreover, both the maintenance and performance of the flexion reflex is known to be NMDA-mediated in the spinal cord (Joynes et al 2004). Together, these observations suggest that learning phenomena can also occur in the spinal cord and be modulated by similar molecules. Whether *complex* spinal motor learning occurs via similar mechanisms is unknown.

The activation of NMDA receptors and their subsequent associated intracellular signal transduction cascades are involved in the induction, development and maintenance of synaptic plasticity in the hippocampus may also be effective in the spinal cord.

4.2.2 Intracellular cascade

The ras/ERK MAP kinase and PI3K/Akt pathways are the best-characterized signaling pathways activated by Trk receptors upon NT binding and will be described in the next section (see also Fig.7, Kaplan & Miller 2000, Huang & Reichardt 2001).

4.2.2.1 Ras/ERK MAPK pathway

Extracellular signal-regulated kinases (ERKs), members of the mitogen-activated protein kinase (MAPK) family, transduce a broad range of extracellular stimuli into diverse intracellular responses producing changes in the level of gene expression or transcription. Two isoforms of ERKs exist: 44kDa and 42kDa. In the spinal cord, ERK phosphorylation is regulated by the synaptic actions of both NTs and glutamate on neurons. ERK pathways

are major downstream signaling cascades for TrkB receptor stimulation through NT binding (Segal & Greenberg 1996). Briefly, Grb2-SOS, the adapter protein containing the SH-2 domain, was shown to link Ras-GTP and to lead to ERK. The activation of ERK1 and ERK2 requires sequential phosphorylation by Raf of MEK1 and MEK2, which in turn activate ERK1/2 through the phosphorylation of threonine and tyrosine residues (Kolch 2000, Ji & Woolf 2001). The activated ERK then translocates from the cytosol into the nucleus.

The phosphorylation of ERK proteins has been extensively used as a criterion of the degree of activation of the Ras/ERK kinase pathway. In this project, specific antibodies that recognize phosphorylated ERK1/2 (pERK1/2) were used.

BDNF is reported to activate ERK1/2 in various brain areas including cortical, hippocampal and cerebellar neuronal cells (Marsh & Palfrey 1996, Bonni et al 1999, Hetman et al 1999) and also in the spinal cord (Becker et al 1998, Pezet et al 2002). ERK signaling pathways are primarily mediators of axonal growth and neuronal survival (Kaplan & Miller 2000). Furthermore, ERK signaling promotes plasticity changes both in the hippocampus (Sweatt 2004) and in the spinal cord (Ji et al 1999, Ji 2004). The ERK1/2 cascade is involved in both the regulation of post-translational phosphorylation of key membranes receptors and transcriptional expression of critical genes, leading to short and long-term functional changes in spinal sensory neurons (Kolch 2000, Ji & Woolf 2001, Ji 2004). ERK is not only activated by BDNF (Jovanovic et al 1996, Ying et al 2002), but also exhibits a well-established interaction with NMDA receptors (Platenik et al 2000) and can be activated by glutamate by increasing intracellular calcium levels or by activating Ras (Lever et al 2003, Kawasaki et al 2004). To date, various kinds of molecules have been demonstrated to be downstream targets of ERK1/2, and these could be roughly divided in 4 groups: protein kinases, transcription factors such as CREB, cell surface molecules and cytoskeleton-associated molecules (Lewis et al 1998). Among them, ERK is notable for regulating CREB (Finkbeiner et al 1997) and synapsin I (Jovanovic et al 1996) to induce long-term changes in synaptic plasticity. ERK has also been shown to be required for long-term facilitation of excitatory transmission between sensory neurons and motoneurons in culture (Martin et al 1997). The ERK cascade not only amplifies extracellular stimuli but also integrates many signaling pathways and functions as a vehicle that imports the information into the nucleus.

4.2.2.2 PI3K/Akt pathway

The activation of PI3K by Ras is the major pathway by which NTs convey their survival-promoting signals (Vaillant et al 1999) leading to the activation of the serine/threonine kinase Akt (PKB). The autophosphorylation of Trk receptor and phosphorylation of Shc allows the recruitment of several adaptor proteins, which upon tyrosine phosphorylation interact with and activate PI3K. Akt then translocates to the nucleus. The 3 Akt isoforms (Akt1, Akt2, Akt3) mediates many of the downstream events regulated by PI3K. The PI3K/Akt signaling pathway has been shown to play an important role in cell death/survival pathways by stimulating both neuronal survival and axonal growth. Akt is a major mediator of cell survival by directly inhibiting different pro-apoptotic signals such as Bad, a Bcl-2 family member that promotes apoptosis, to prevent cytochrome C release (Datta et al 1997). Notably, Bad is also a substrate for MAPK, which similarly inactivates its apoptosis-promoting function (Bonni et al 1999). Other identified targets of Akt are pro-caspase9 and the Forkhead family of transcription factors.

4.2.2.3 cAMP response element binding

The transcription factor cAMP response element binding protein (CREB), one of the best-characterized transcription factors in the brain, is under the regulatory control of BDNF. Indeed, CREB was shown to be involved in several intracellular events associated with the action of BDNF on neuronal plasticity (Barde 1994). CREB can be phosphorylated by multiple intracellular kinases in response to a vast range of physiological and pathological stimuli. Both ERK and Akt pathways lead to CREB activation (Xing et al 1998). Briefly, the translocation of either ERK or Akt to the nucleus, will lead to the phosphorylation of CREB at serine residue 133 followed by its binding to the cAMP response element (CRE) of the target gene to regulate gene expression. More than 100 genes have been reported to be up-regulated following the activation of CREB (Lonze & Ginty 2002). These genes are implicated in different neuronal process such as survival, synaptic plasticity, memory, and learning (Silva et al 1998, Kandel 2001, Lonze & Ginty 2002). Many studies found that ERK-mediated CREB phosphorylation is required for synaptic plasticity associated with the induction of stable, late-phase LTP and long-term memory (Kelleher et al 2004, Thomas & Huganir 2004). CREB also appears to play a role in neuronal resistance to insult in conjunction with BDNF (Walton et al 1999). Noteworthy, not only can CREB be modulated by BDNF but CREB influences expression of BDNF itself via a calcium-dependent

mechanism (Finkbeiner 2000). In the spinal cord, CREB is a common target for multiple other intracellular kinases pathways including PKA, PKCγ and CaMKII.

4.2.2.4 Selected molecules involved in synaptic transmission

Synapsin I and synaptophysin are 2 molecules involved in synaptic transmission. Synapsin I is a member of a family of terminal-specific phosphoproteins involved in synaptic vesicle clustering and release, which mediate synaptic transmission (Jovanovic et al 1996). Synapsin I is a downstream effector for the action of BDNF on synaptic plasticity. BDNF phosphorylates synapsin I primarily through the TrkB receptor to activate the ERK1/2 signaling pathway, leading to modulation of neurotransmitter release (Jovanovic et al 2000). On the other hand, synaptophysin is a major integral protein of the membrane of presynaptic vesicles and is thought to be important for the biogenesis of synaptic vesicles, vesicle budding and endocytosis. Synaptophysin has been associated with synaptogenesis (Bergmann et al 1997). An increase in synaptophysin likely indicates that synaptic vesicles are formed either due to an increase in synapse formation or an increase in the number of vesicles in existing synapses (Sarnat et Born 1999). In the hippocampus, BDNF has been shown to act on presynaptic neurons and enhance vesicle release (Lu & Chow 1999). Synapsin and synaptophysin synthesis and phosphorylation are affected by BDNF via TrkB receptor resulting in an elevated transmitter release.

4.2.3 Lesion-induced plasticity

In the nervous system, cell death may involve aspects of both apoptosis and necrosis (Beattie et al 2000, 2002). Apoptotic cell death can be detected hours to several weeks after SCI and occurs in numerous cell types including neurons, glia and inflammatory cells (Crowe et al 1997, Liu et al 1997, Yong et al 1998, Beattie et al 2000, 2002). BDNF has been described as a key factor that regulates the survival and differentiation of selected neurons during CNS development (Huang & Reichardt 2001) and rescues a significant proportion of motoneurons that would otherwise die during the embryonic late period of massive cell death *in vivo* (Oppenheim et al 1992, 1993, Yan et al 1993). Some studies suggest a preservation of this role in adults. The action of BDNF on motoneurons has been extensively studied particularly in injury models. BDNF enhances survival and growth of motoneurons affected by several types of insults (Koliatsos et al 1993, Friedman et al

1995) and promotes functional recovery (Jakeman et al 1998, Bregman et al 2002). However, further investigations are needed to address if a physiological concentration of BDNF newly synthesized by motoneurons or transported from DRG, for example, would be sufficient to achieve such a functional change.

Apoptosis plays an important role in neuronal loss after SCI. It was shown that oligodendrocytes, neurons and glia undergo apoptosis (Crowe et al 1997, Liu et al 1997, Yong et al 1998) and that caspase cascades are involved in apoptosis after SCI (Springer et al 2001). To stimulate survival, TrkB activation by BDNF may lead to Akt-induced suppression of pro-apoptotic mechanisms and to ERK activation of anti-apoptotic proteins. Indeed, a transient increase in pAKT level is observed following a SCI returning to intact control values within 7 days (Yu et al 2005). Indeed, apoptosis is typically a rapid process (Bursch et al 1990). On the other hand, in CNS-derived cells cultured *in vitro*, the activation of ERK is implicated in both neuroprotective responses and in promoting cell death. The ERK1/2 pathway has been reported to be involved in a neuroprotective mechanism against the apoptosis of cortical neurons (Hetman et al 1999) and cerebellar granule neurons (Bonni et al 1999). Recent studies lend support to the hypothesis that excitotoxicity, neural apoptosis, inflammation, brain ischemia and nerve injury also induces activation of ERK1/2 cascades (Ji et al 1999, Ji & Woolf 2001, Ferrer et al 2001, Ji 2004). Moreover, BDNF is reported to protect neurons from cell death *in vivo* via the ERK pathways (Han & Holtzman 2000) including cholinergic maintenance of motoneurons (Kishino & Nakayama 2003). However, recent studies using *in vivo* models of cerebral ischemia (Namura et al 2001, Wang et al 2003) or traumatic brain injury (Mori et al 2002ab) have shown that inhibitors of MEK1/2 reduce neuronal loss. This suggests that the activation of ERK in response to acute CNS injury may also be detrimental. Evidence was also provided for an essential contribution of the PI3K/Akt pathway to motoneurons survival induced by BDNF contrary to the ERK pathway (Dolcet et al 1999). A role for the Ras/ERK kinase pathway in cell survival thus still remains controversial.

As briefly mentioned in section 4.1.3.2, it has been well documented that SCI also results in glutamate release (Nesic et al 2002) and up-regulation in gene expression of NMDA receptors (Grossman et al 2000). NMDA receptors are also transitory up-regulated following a complete spinal lesion in the cat and the basic locomotor rhythmicity in spinal cats is suggested to be NMDA-dependent (Giroux et al 2003). In the spinal cord, the phosphorylation of the NMDA receptor via the stimulation of ERK and PKC was shown to facilitate synaptic efficacy (Garraway et al 2003, Slack et al 2004). Moreover, an excessive

release of glutamate is also implicated in neuronal death associated with SCI. Importantly, EAA antagonists protect against deficits associated with SCI and loss of gray and white matter (Gomez-Pinilla et al 1989, Wrathall et al 1997, Beattie et al 2002). Glutamate is the major excitatory neurotransmitter of projection neurons and dorsal root afferents entering the spinal cord. It is thought that because BDNF is synthesized, stored and released from glutamatergic neurons (Lessmann et al 2003), BDNF may enhance the activation of NMDA receptors due to the increased release of glutamate.

Downstream effectors of BDNF induced synaptic plasticity were also found to be modulated after various kind insults to the CNS. In the isolated lumbar spinal cord, with a complete elimination of supraspinal and peripheral input, BDNF and synapsin I, are down-regulated (Gomez-Pinilla et al 2004). A similar down-regulation in BDNF, synapsin I and CREB expression was also observed in the lumbar spinal cord ipsilateral to the lesion in rodents with an hemisected spinal cord as compared to intact animals (Ying et al 2005). However, BDNF, TrkB, p75, MEK and ERK1 expression were shown to be up-regulated in motoneurons after a facial nerve injury (Kitahara et al 1994) contrary to PLCγ and PI3K expression which was not modified (Saika et al 1994). BDNF mRNA was also shown to be dramatically up-regulated in glial cells and neurons of the spinal cord after kainic acid delivery (Scarisbrick et al 1999). TrkB expression was also shown to be up-regulated in the astrocytes and motoneurons near the glial scar formed after an incomplete SCI in the adult rat and cat (Frisen et al 1992). Finally, an up-regulation of TrkB, BDNF, pERK1/2 and pCREB levels was also observed in DRG or dorsal horn of the spinal cord in a majority of segments after a complete SCI and the injury-induced CREB activation was suggested to be partly mediated by ERK pathway (Qiao & Vizzard 2005, Cruz et al 2006).

4.2.4 Exercise-induced plasticity

Both clinical and animal studies have repeatedly demonstrated that exercise enhances neuronal function in intact individuals. But what are the molecular mechanisms and signaling pathways through which activity promotes synaptic plasticity and functional recovery? In the CNS, the specific mechanisms remain largely unexplored, although recent data points to the involvement of NTs as a possible factor given their powerful role in modifying neuronal excitability and synaptic transmission (Kafitz et al 1999, Mendell et al 1999, Poo 2001). In an elegant work, the laboratory of Dr Gomez-Pinilla investigated changes in the relative expression of more than 1000 genes in the hippocampus of

running-wheel exercised rats (Molteni et al 2002). Their results suggest that exercise modulates molecular systems involved in maintaining neuronal function and plasticity in the brain (see Fig.8). BDNF is the only trophic factor gene modulated by exercise. Remarkably, most of the other genes affected by exercise have a recognized association with BDNF and are either members of synaptic trafficking machinery or part of signal transduction pathways. As summarized in Figure 8, exercise would impact the NT signaling pathways in the hippocampus. Blocking the action of BDNF during exercise was found to be sufficient to abolish the exercise-induced enhancement of both learning and memory (Vaynman et al 2004). Results from several experiments suggest that the same molecules may be involved in synaptic plasticity in other parts of the CNS. Various paradigms of locomotor training up-regulate BDNF and TrkB mRNA and protein expression in the hippocampus, cerebral cortex, cerebellum, spinal cord and muscles in otherwise intact rats (Neeper et al 1995, Gomez-Pinilla et al 2001, 2002, Molteni et al 2002, Hutchinson et al 2004, Klintsova et al 2004). In the spinal cord, further immunohistochemical experiments precisely demonstrated that BDNF (protein or mRNA)

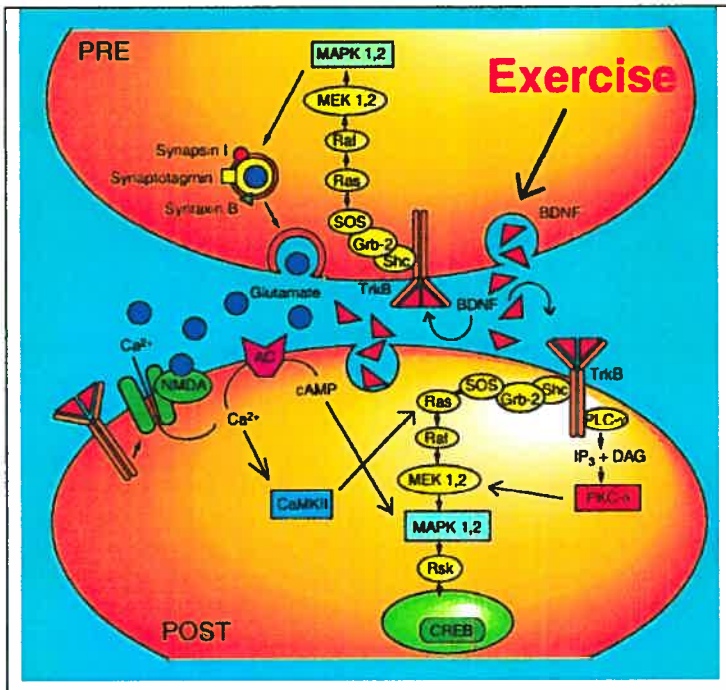

Figure 8: Hypothetic mechanisms by which exercise may impact neuronal plasticity.

Exercise increases the expression of BDNF and TrkB receptor. Ligand-receptor binding both at the presynaptic and postsynaptic terminals result in an up-regulation of several downstream effectors such as MAPK1/2, PKCγ and CaMKII. Furthermore, synapsin, synaptogamin and syntaxin at the presynaptic terminals modulate neurotransmitter release. NMDA receptor is also up-regulated at the postsynaptic level, increasing calcium influx and leading to the activation of the MAPK cascade via CaMKII. MAPK activation causes the up-regulation of transcription factors such as CREB. From Molteni et al 2002.

staining intensity was especially increased in motoneurons and their axonal processes in the ventral horn of the spinal cord (Gomez-Pinilla et al 2001, 2002). A parallel increase in TrkB and BDNF (protein or mRNA) expression in some large neurons of the lamina IX, presumably α-motoneurons, was also observed (Skup et al 2002).

Given these data, it appears that the BDNF system plays a central role in the molecular mechanisms by which exercise activates neuronal plasticity and translates into functional changes in the neuromuscular system. But how does an activity-related increase in BDNF modulate critical aspects of plasticity? It was shown that the modulation of TrkB mRNA was closely associated with changes in the levels of synapsin I in the spinal cord, which suggests that exercise could impact the synapse via the BDNF system by activating specific pathways to modify the way that information is transmitted across the synapse (Gomez-Pinilla et al 2001, 2002). Hence, synapsin I was shown to be primarily phosphorylated by BDNF through TrkB to activate the ERK pathway and modulate neurotransmitter release (see section 4.2.2.4, Jovanovic et al 2000). The time course of BDNF up-regulation is different in the spinal cord and in SOL muscle: after an initial up-regulation, a down-regulation is observed in the muscle with a concomitant up-regulation in the spinal cord suggesting that BDNF is retrogradely transported from the muscle to the spinal cord (Gomez-Pinilla et al 2002). The exercise-dependence of these changes is further supported by experiments where BDNF and synapsin I mRNAs were shown to be down-regulated in the spinal cord after the pharmacological inactivation of the SOL muscle. Moreover, in another set of experiments performed by the same group in the rat hippocampus, it was shown that exercise increased both synapsin I and synaptophysin levels and that blocking BDNF action was sufficient to prevent this change (Vaynman et al 2006). Both synapsin I and synaptophysin are thus under the regulation of BDNF and it was suggested that this may contribute to the ability of BDNF to regulate both the number of synapses and the complexity of the axonal arborization in the hippocampus. Moreover, there is a positive correlation between synapsin I and synpatophysin in exercised rats and also between synapsin I and the amount of exercise they received (Gomez-Pinilla et al 2002, Vaynman et al 2006). Synapsin I and synaptophysin may thus be involved in events characterizing synaptic function during exercise.

In the brain, exercise impacts downstream effectors of BDNF action on gene expression through CREB activation. The ability of exercise to activate transcription factors is fundamental to the proficiency of activity to induce long-lasting or permanent changes in function of the nervous system and the activation of CREB may be a critical step. CREB seems to be an important link in the BDNF-mediated cascade responsible for the effects of exercise on learning and memory. Indeed, the modulation of BDNF and CREB mRNA levels were positively correlated and associated with memory recall performance following exercise. CREB activation may serve as a molecular switch to transform short lasting into long-lasting synaptic plasticity in the hippocampus. Moreover, blocking the NMDA receptor

prevented the exercise-induced mRNA up-regulation of BDNF, TrkB, CREB and Synapsin I (Vaynman et al 2003) suggesting that the action of BDNF may depend on an interaction with the NMDA receptor. CREB mRNA expression was also shown to be up-regulated in the spinal cord after exercise (Gomez-Pinilla et al 2002) suggesting again that a similar cascade may be induced by exercise both in the hippocampus and the spinal cord. Notably, similar mechanisms have been demonstrated in the spinal cord for the development and maintenance of chronic pain (Ji et al 2003). Indeed, in the dorsal horn, CREB has been suggested to contribute to central sensitization associated with persistent pain states. It has been proposed that NMDA activation-induced Ca^{2+} influx can trigger an early phase of CREB phosphorylation and a persistent phase of CREB phosphorylation is mediated by a delayed ERK cascade (Crown et al 2006).

Furthermore, recent experiments showed that the expression of several neurotrophic factor genes was affected by voluntary exercise with differential time-profile (Molteni et al 2002). CaMK pathways, closely regulated by the NMDA receptor system, were shown to be markedly up-regulated with short-term exercise in rats trained in running wheels. In acute *in vitro* exposure of cortical neurons to BDNF, a rapid enhancement of NMDA receptor activity by increasing channel open probability is observed (Levine & Kolb 2000). Similarly, the increase in NMDA receptor subunits in the spinal cord could represents the downstream effect of exercise during the acute phase (Molteni et al 2002). On the other hand, ERK pathways seem to become more important with time, when exercise extends for longer periods (Molteni et al 2002).

The objective of this thesis was to investigate the effect of long-term step-training, ERK pathway will be especially targeted.

4.2.5 Step-training induced plasticity after SCI

Are the molecular mechanisms involved in exercise-dependent plasticity the same in the intact state and following SCI? Exogenous administration of BDNF has been shown to stimulate locomotor activity in rats after incomplete SCI suggesting the potential to modulate the excitability of spinal networks (Jakeman et al 1998). Whether a physiological concentration of BDNF is sufficient to facilitate stepping is not known. However, after a spinal cord hemisection in rodents, a down-regulation of BDNF, synapsin I and CREB is

observed in the lumbar spinal cord ipsilateral to the lesion as compared to intact animals (Ying et al 2005). After being exposed to voluntary wheel running (up to 28 days), BDNF mRNA and protein expression was shown to be up-regulated in motoneurons and their axonal process in the lesion side of the spinal cord as compared to the unlesioned side. Thus, physical activity may restore the expression of these proteins near normal levels after incomplete SCI. Contrary to exercise enhanced expression of these molecules in intact animals; exercise did not increase expression in lesioned animals as compared to control. It was suggested that the lesion might limit the effectiveness of exercise (Ying et al 2005).

Moreover, motoneurons from rats with complete SCI displayed a marked atrophy with loss of dendritic membrane and elimination of branching within a few days after the injury, which is not observed in step-trained animals (Gazula et al 2004). This suggests that the functional benefits of exercise may involve stabilizing or remodeling processes in the dendritic tree of motoneurons below the injury site following the loss of excitatory drive from descending input on segmental interneurons.

5. Model and hypothesis

The introduction underlined the complexity of locomotor control together with the capacity of the spinal cord to reorganize in response to insult or muscle activity. Our general interest in both locomotion and plasticity of the spinal cord led us to investigate the changes that occur in the spinal cord that might provide insight into methods that will promote stepping recovery after SCI.

5.1 The model: Step-training in chronic spinal cats

The isolated spinal cord, deprived of descending influence from the brain, has been a popular and productive experimental model for more than a century. Our model is based on earlier studies that revealed the effectiveness of a step-training program in the adult cat to promote the capability to walk following a SCI (Lovely et al 1986, Barbeau & Rossignol 1987, Bélanger et al 1996). During the recuperation process, the spinal networks are continuously stimulated by sensory feedback and the individual progressively recovers rhythmic and alternate locomotor movements.

5.2 Project I: Plasticity of spinal reflexes

The decerebrate cat paralyzed with curare to prevent movement-related sensory feedback is a widespread model to investigate the control of locomotion. In this preparation, the locomotor activity is recorded by electrodes directly positioned on the muscle nerves (electroneurogram, ENG). The curare-evoked paralysis prevents the occurrence of any movement and rhythmic patterns of nerve activities are reported as *fictive locomotion*. The pattern of ENG bursts during fictive locomotion is roughly similar to overground stepping or locomotion on the treadmill (Grillner & Zangger 1979, Fleshman et al 1984).

Two to 4 weeks after a complete SCI and onset of step-training, adult cats are able to perform proper plantigrade contact of the paw with the treadmill belt and execute weight-bearing on the hindlimbs during stepping (Lovely et al 1986, Barbeau & Rossignol 1987, Bélanger et al 1996). In this project, the terminal experiment took place one month after the spinal transection (spinal group) and step-training (trained group). Fictive locomotion is

a privileged tool to study details of synaptic transmission mechanisms and presents 2 main advantages: the absence of movement-related rhythmic sensory feedback and stable intracellular recordings. Fictive locomotion was monitored with ENGs and intracellular recordings of lumbar motoneurons were obtained at rest and during fictive locomotion evoked by i.v. injection of clonidine (Pearson & Rossignol 1991) in step-trained and untrained spinal cats.

It was previously suggested that locomotor recovery depended, at least in part, on slow modifications of the CPG in chronic spinal cats (Pearson & Rossignol 1991). Indeed, the complexity of the fictive locomotor pattern increased as a function of time post-spinalisation suggesting that a progressive increase in the transmission of afferent pathways may enhance the excitability of the spinal networks inducing slow modifications of the CPG. Although it is generally assumed that plasticity occurs within the CPG, we **hypothesize** that changes in sensory afferent pathways could also occur. In the complete absence of supraspinal commands, it is known that the repeated sensory stimulation provided by step-training induces long-term plastic changes in the spinal cord but the role of the different sensory inputs and neurophysiological mechanisms leading to locomotor recovery are still poorly understood.

The **first objective** of this thesis is thus to examine the effect of step-training on transmission in specific sensory pathways originating either from muscle group I afferents or cutaneous afferents. We tested our hypothesis by comparing motoneuronal responses to nerve stimulation in trained and untrained cats spinalized 3-5 weeks prior to the acute experiment.

Proprioceptive input can act directly on the CPG, particularly those transmitted by group I afferents from extensors (Conway et al 1987, Gossard et al 1994, McCrea 1998, Pearson 1998). We speculated that transmission in these pathways, which are involved in body weight support during stance, is especially modified by step-training. We further **hypothesize** that transmission in muscle group I afferents of extensors to extensor motoneurons would be increased in excitatory pathways and decreased in inhibitory pathways to facilitate weight-bearing.

Contrary to proprioceptive feedback, cutaneous inputs usually do not have such a powerful action on rhythm generation. We therefore expected less plasticity in these pathways. However, because cutaneous inputs have been shown to be involved in locomotor

recovery after a SCI (Muir & Steeves 1995, 1997, Bouyer & Rossignol 2003b), we **hypothesize** that at least some cutaneous pathways would be modified by step-training, particularly those that may be involved in proper foot placement.

5.3 Project II: Modulation of intracellular signaling pathways associated with activity-dependent plasticity

These studies are based on the assumption that the activation of specific neural networks by physical activity leads to the expression of molecules related to synaptic plasticity in the spinal cord of intact animals. ERK was chosen as the main target of investigation as its 2 main activators, BDNF and glutamate, have been shown to be involved in exercise-induced plasticity in intact animals. ERK may therefore be required for the functional reorganization of spinal networks following SCI and step-training.

This second project takes advantage of a completely spinalized preparation in which no supraspinal influence is possible and allows for the investigation of the induction of intracellular cascades, using the neurotransmitters that are intrinsic to the neuromuscular system, in order to recover an adequate locomotor pattern. The **second objective** of this thesis is to investigate signal transduction pathways through which long-term step-training may affect spinal cord plasticity after a complete SCI. However, little is known about the involvement of these molecules following a SCI. The various injury models (contusion, hemisection, peripheral nerve transection, kainic acid delivery) have generated highly variable results probably as a result of the complex interaction of sensory and spinal interneuronal pathways together with the remaining supraspinal fibers that may influence such plasticity. Moreover, many lumbar segments were merged together in most studies and this prevents the capacity to detect any significant segmental difference in modulation, such as we hypothesized to occur. Thus in addition to the effect of step-training, we investigated the effect of a chronic and complete SCI.

As decribed above, 2 to 4 weeks of step-training after a complete SCI allows adult cats to perform proper plantigrade placement of the paw and execute weight-bearing stepping (Barbeau & Rossignol 1987, Lovely et al 1986, Bélanger et al 1996). However, if step-training is prolonged beyond that time window, the improvement in stepping ability further progresses with an increase in the maximum walking speed and in the number of plantar steps performed. Stepping performance typically reaches a plateau at approximately 3

months after the onset of step-training (Lovely et al 1986, Barbeau & Rossignol 1987). For these reasons, 1 and 3 months were chosen as time points for investigation. Protein expression was compared between intact, spinal (1 or 3 months) and spinal and step-trained cats using western blot analysis of homogenates of spinal cord segments. The study focussed on assessing relative levels of ERK and pERK protein. We **hypothesize** that ERK activation may participate in the synaptic events associated with locomotion after SCI and that the most important changes would take place in spinal segments known to be important for locomotion. Additional experiments assessed possible changes in expression of the transcription factor CREB, known to be activated by ERK and changes in expression of Akt, a protein kinase known to be activated by BDNF.

PUBLICATION #1: SPINAL CATS ON THE TREADMILL: CHANGES IN LOAD PATHWAYS

Côté MP, Ménard A and Gossard JP (2003). Spinal cats on the treadmill: changes in load pathways. J. Neurosci. 23:2789-2796.

Abstract

Treadmill training and clonidine, an α-2 noradrenergic agonist, have been shown to improve locomotion after spinal cord injury. We speculate that transmission in load pathways, which are involved in body support during stance, is specifically modified by training. This was evaluated by comparing two groups of spinal cats; one group (n=11) was trained to walk until full-weight-bearing (3-4 weeks), and the other (shams; n=7) was not. During an acute experiment, changes in group I pathways, monosynaptic excitation, disynaptic inhibition, and polysynaptic excitation were investigated by measuring the response amplitude in extensor motoneurons before and after clonidine injection. Monosynaptic excitation was not modified by clonidine but was decreased significantly by training. Disynaptic inhibition was significantly decreased by clonidine in both groups, but more significantly in trained cats, and significantly reduced by training after clonidine. Also, clonidine could reverse group Ib inhibition into polysynaptic excitation in both groups but more frequently in trained cats. We also investigated whether fictive stepping revealed additional changes. In trained cats, the phase-dependent modulation of all three responses was similar to patterns reported previously, but in shams, modulation of monosynaptic and polysynaptic responses was not. Overall, training appears to decrease monosynaptic excitation and enhance the effects of clonidine in the reduction of disynaptic inhibition and reversal to polysynaptic excitation. Because it is believed that polysynaptic excitatory group I pathways transmit locomotor drive to extensor motoneurons, we suggest that the latter changes would facilitate the recruitment of extensor muscles for recovering weight-bearing during stepping.

Introduction

Treadmill training has been shown to successfully enhance and maximize residual locomotor capacities of spinal cord injured (SCI) patients (Fung et al 1990, Wernig et al 1995, Harkema et al 1997, Harkema 2001). Previous studies first demonstrated this

beneficial effect in adult spinal cats that have a remarkable capacity to recover locomotion (Lovely et al 1986, Barbeau & Rossignol 1987, Bélanger et al 1996, de Leon et al 1998b). Moreover, clonidine, an α-2 noradrenergic agonist, improves and accelerates the recovery of stepping early after spinalization in cats (Forssberg & Grillner 1973, Barbeau & Rossignol 1991, Chau et al 1998a) and, when combined with treadmill training, improves walking patterns in SCI humans (Fung et al 1990, Rémy-Néris et al 1999). The repeated sensory stimulation provided during treadmill training is the only source of input that the transected spinal cord can use to trigger recovery and underlying plastic changes (de Leon et al 1999a). But which sensory input is most important for recovery? It has been shown in many species, including humans (Prochazka 1996, Duysens et al 2000), that sensory feedback from load receptors in the legs has a particularly powerful effect on the activity of the central pattern generator (CPG) for locomotion. Of particular interest is the reflex reversal occurring when Ib inhibition (negative feedback) in extensors is replaced by excitation (positive feedback), reinforcing weight support during the stance phase of stepping (Gossard et al 1994, Prochazka 1996). This reversal is state dependent [i.e., it occurs only when the spinal cord is generating locomotion (Gossard & Hultborn 1991, Stephens & Yang 1996) or after injection of L-DOPA (Gossard et al 1994) or clonidine (McCrea et al 1995)]. Here, we hypothesize that transmission of group I (Ia plus Ib) pathways is specifically modified by training to assist extensors during stance. We tested this by comparing two groups of cats transacted at T13; one group was trained on a treadmill until "full-weight-bearing" (3-4 weeks), and the other was spinalized but not trained. Synaptic transmission was evaluated during an acute experiment using intracellular recordings of motoneurons before and after clonidine injection. We found that treadmill training did induce plastic changes in the transmission of group I pathways from extensors that could be helpful for recovering weight-bearing during stance.

Materials and Methods

All procedures were conducted according to the Guide for Care and Use of Experimental Animals of Canada using protocols approved by the Ethics Committee of Université de Montréal.

Spinalization and locomotor training. Eighteen adult female cats (2.5-4.1kg) were used for this study. After administration of preoperative medication, the cats were anesthetized (isoflurane, 2%; Abbott Labs, Montréal, Canada) and spinalized at T13 under aseptic

conditions. Protocols for spinalization procedures and subsequent postoperative care were analogous to those described previously (Chau et al 1998a). A patch of fentanyl (Duragesic, 25µg; Janssen-Ortho, Markham, Canada) was sutured on the back of the cat for continuous and stable delivery of analgesic over a 2d period. The first group of cats (sham) was only spinalized, whereas the second group (trained) was also trained to walk until they could support the weight of their hindquarters (referred to as full-weight-bearing, as in previous reports), which took ~1 month (mean, 28d). Training on the treadmill (0.2-0.4m/sec) started 2d after surgery and consisted of one to four daily training sessions for periods of 10min. In early training, hindquarters were sustained by the experimenter to provide weight support, and perineal stimulation was used to induce and maintain locomotion. The animal gradually became able to support its hindquarters, and perineal stimulation was no longer needed. No drugs were used to assist the locomotor training. The training was stopped when the cat was able to walk continuously on the treadmill for >5 min while the experimenter assisted only for lateral stability by holding the tail.

Acute experiment. Cats were first anesthetized by inhalation of an oxygenated mixture (50%) of nitrous oxide (50%) and halothane (2-3%; MTC Pharmaceuticals, Cambridge, Canada). Cannulas were inserted in the right common carotid artery to monitor blood pressure and in the jugular and cephalic veins for administration of pharmacological agents or fluids. Cats were then decerebrated and curarized (Pavulon, 0.2mg/kg, 45min; Sabex, Boucherville, Canada) and artificially ventilated as detailed previously (Ménard et al 1999, Leblond et al 2000). The following muscle nerves from the left hindlimb were dissected free, cut, and mounted on bipolar silver chloride electrodes for recording [electroneurogram (ENG)] and stimulation: posterior biceps-semitendinosus (PBSt), semimembranosus-anterior biceps (SmAB), lateral gastrocnemius-soleus (LGS), medial gastrocnemius (MG), plantaris (Pl), flexor hallucis longus (FHL) and flexor digitorum longus together, tibialis anterior, extensor digitorum longus, and the sciatic nerve (uncut). Quadriceps nerves (Quad) were not cut and were inserted in a polymer-cuff electrode. SmAB and PBSt nerves from the right hindlimb were also mounted for recording and stimulation.

Stimulation, recordings, and analysis. The cord dorsum potential (CDP) was recorded with a silver chloride-ball electrode located near the dorsal root entrance at the L6-L7 border. Stimulation intensity required to just evoke a deflection in the CDP determined the threshold for the most excitable fibers for each nerve (1T). Stimulus intensity will be expressed as a multiple of the threshold. Intracellular potentials evoked by the stimulation

of group I afferents of extensors [PI, LGS, MG, sometimes together (gastrocnemii-soleus, GS), Quad; six pulses (p), 1.4–1.8T, 200-300Hz] were recorded in identified motoneurons (Leblond et al 2000) with glass micropipettes filled with K^+-acetate (2M) and *N*-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium bromide (100mM; Alamone Laboratories, Jerusalem, Israel) to prevent sodium spikes. The duration of the afterhyperpolarization (AHP) was measured in every cell, from the spike onset to the point at which the AHP crosses the baseline (Gustafsson & Pinter 1984b). Stimulation trains of peripheral nerves were given every 0.3, 0.4, or 0.5sec. The amplitude of EPSPs and IPSPs in motoneurons evoked by monosynaptic, disynaptic, and/or polysynaptic pathways was measured (Fig.1). A "trial" is the averaged response in one motoneurons evoked by the stimulation of a given pathway (an afferent-motoneuron pair). Several trials could be studied from the responses of a given motoneuron.

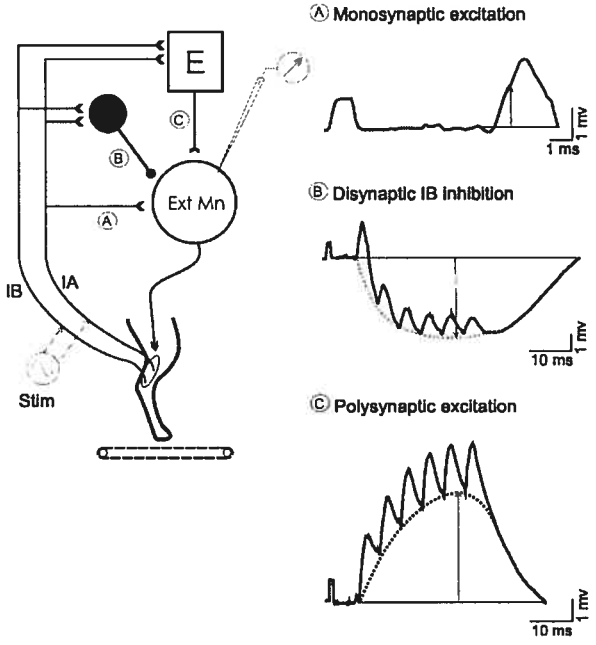

Figure 1. Spinal proprioceptive pathways under study. A schematic representation of three sensory pathways transmitting inputs from muscle group I afferents to extensor motoneurons (*ExtMn*) is shown to the *left*: the monosynaptic (stretch reflex) pathway (from group Ia afferents originating in muscle spindles of extensors), the disynaptic inhibitory pathway (from group Ib afferents of extensors originating in Golgi-tendon organs plus some group Ia fibers), and the polysynaptic excitatory pathway (from group Ib and Ia afferents of extensors). In the acute spinal cat, this latter pathway shares interneurons with the network generating the excitatory locomotor drive in extensors (*box E*). Sample records of motoneuronal postsynaptic potentials used for measurements are on the *right*. ***a***, The amplitude of monosynaptic EPSPs was measured at a latency of 1.4 msec (rising phase in this example; i.e., just before the onset of possible disynaptic components). ***b***, The disynaptic Ib inhibition was evoked by a short train of stimuli (6p, 1.4-2.0T, 200-300Hz), and the IPSP amplitude was measured at the maximal negative deflection in the intracellular trace. Note that there were often monosynaptic EPSPs (*six positive humps*) overriding the inhibitory trough (*dotted line*). ***c***, Polysynaptic excitation was evoked by a similar short train of stimuli, and the amplitude was measured at the maximal positive deflection (*dotted line*) underlying monosynaptic EPSPs.

The amplitude of monosynaptic EPSPs was measured at a latency of 1.4msec (i.e., just before the onset of possible disynaptic components) (McCrea et al 1995, Gosgnach et al 2000). A train of stimuli evoked either disynaptic inhibition or polysynaptic excitation

depending on the conditions (Gossard et al 1994). The amplitude of IPSP attributable to Ib inhibition was measured at the maximal negative deflection in the intracellular trace in response to the stimulation train, and the amplitude of EPSP resulting from polysynaptic excitation was measured at the maximal positive deflection, as illustrated by *dotted lines* in Figure 1. McCrea et al (1995) have shown that polysynaptic excitation is not just masking the Ib inhibition, but that the latter completely disappears when there is a reversal. Thus, in our calculation, the finding of an excitation (reversal) was considered a 100% reduction of inhibitory transmission. Conversely, a cell showing Ib inhibition was considered to have zero transmission in excitatory pathways. We also studied the long-lasting motor responses to the stimulation of flexor reflex afferents (FRA) from each leg. For this, the PBSt and SmAB nerves of either leg were stimulated together with a train of 50 pulses at 50T. All responses were also studied during a period of 2hr after 500µg/kg intravenous clonidine injection (Sigma, St. Louis, MO) and during fictive locomotion induced by perineal stimulation. Up to two doses of clonidine were injected in an experiment, and data were recorded for the next 2hr. Once clonidine was injected, there was no return to control conditions, and all subsequent recordings were considered postclonidine data. Bursts of ENG activities were used to divide the step cycle into flexion (corresponding to swing) and extension (corresponding to stance) phases. The locomotor cycle, defined as the period between the onsets of two successive bursts of ENG activity in extensors, was normalized to the duration of the averaged cycle. Postsynaptic potentials evoked during flexion and extension were separated and averaged to study phase-dependent modulation.

Statistical analysis. Results in figures are expressed as means ±SEM. Statistical analysis was performed to disclose differences between the sham and trained groups, between the averaged responses in all motoneurons obtained before and after clonidine injection, between rest and fictive locomotion (state-dependent changes), and between flexion and extension phases (phase-dependent changes). The Kolmogorov-Smirnov-Liliefors (KSL) test was used to compare the shape and location of the distribution of responses with a normal distribution. If KSL confirmed that the sample variables did fit a normal distribution, a one-way ANOVA was performed; if not, the Kruskal-Wallis one-way ANOVA on ranks was used. The χ^2 test with the Yates correction factor was used to compare the occurrence of polysynaptic excitation between groups. Significant differences are indicated by asterisks (*p<0.05, **p<0.01, ***p<0.001).

Results

Changes in the transmission of group I pathways from extensors were monitored by measuring the peak amplitude of EPSPs and IPSPs at specific latencies in several extensor motoneurons of 11 trained (22 LGSs, 18 MGs, 12 PIs, 14 FHLs, 19 SmAB) and seven nontrained (12 LGSs, 20 MGs, 10 PIs, 9 FHLs, 13 SmAB, 3 Quad) cats. Overall, we measured the responses evoked by 314 afferent-motoneuron pairs (134 in shams, 180 in trained cats) with a mean of 2,29 pairs (range, 1-5) per motoneuron. Although responses varied between motoneurons, similar trends were observed among shams and trained cats. Data pooled according to motor nuclei or stimulated nerves did not show significant trends. For this reason, and because there is extensive convergence and divergence in the three pathways under study (Jankowska 1992), we grouped all extensor motoneurons in the different conditions for additional analysis. In the first part, we compared the effects of clonidine in trained and nontrained cats. In the second part, responses were studied during fictive locomotion, which occurs in a curarized cat (i.e., without movement-related sensory feedback or reafference).

Training and clonidine

The monosynaptic stretch reflex is thought to make a major contribution to the level of EMG activities during stepping (Stein et al 2000), although this role in humans was questioned previously (Sinkjaer et al 2000). Clonidine did not affect the amplitude of monosynaptic EPSP significantly (Fig.2). When preclonidine and postclonidine values were grouped together, it was found that the amplitude of monosynaptic excitation was significantly decreased (by 36%) by training (Fig.2). Motoneurons, divided in two groups

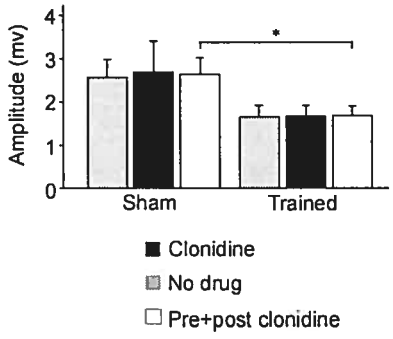

Figure 2. Clonidine did not modify monosynaptic excitation. The mean amplitude of monosynaptic EPSPs (109 trials in 73 cells) evoked by the stimulation of knee and ankle extensor group I afferents (Quad, PI, LGS, MG) was not changed significantly by clonidine injection. If we grouped all values together (preclonidine and post-clonidine), there is a significant decrease in the amplitude monosynaptic EPSPs (*p<0.05) caused by training. *Filled bars*, Clonidine; *gray bars*, no drug; *open bars*, preclonidine and postclonidine.

according to their AHP duration, corresponding approximately to slow (>50 msec) and fast (20-50msec) motor units, were also compared before and after clonidine and between shams and trained cats, but no significant changes were observed.

To evaluate transmission in the Ib inhibitory pathways, we measured and compared disynaptic IPSPs in response to a short train of stimuli (appropriate to recruit interneurons) in group I (Ia plus Ib) (Jankowska & McCrea 1983, Jankowska 1992) afferents from extensors in sham and trained cats. In Figure 3, the trough of Ib inhibition was reduced in the extensor motoneurons after clonidine in both groups of cats (Fig.3*a*,*b*). Overall (Fig.3*c*), Ib inhibition was decreased by clonidine injection in sham cats (by 30.5%; $p<0.05$) and, even more so, in trained cats (by 61.0%; $p<0.001$). Training was able to enhance the reduction of Ib inhibition for responses evoked after clonidine ($p<0.01$).

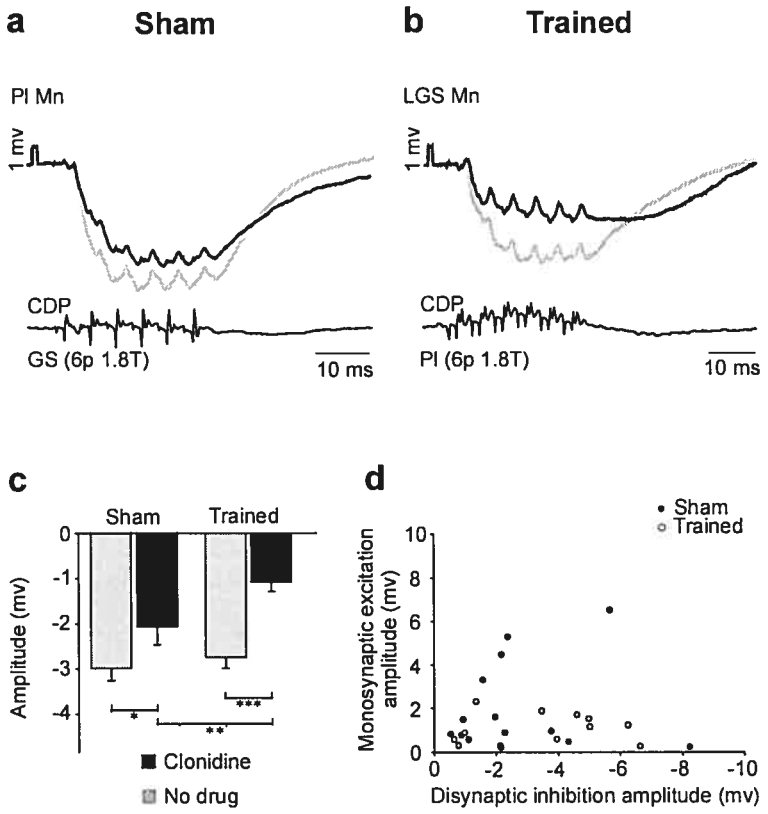

Figure 3. Training plus clonidine injection decreased disynaptic Ib inhibition. *a*,*b*, IPSPs evoked by stimulation of GS group I afferents [6p 1.8T] in a PI motoneuron in a sham (***a***) and PI group I afferents (6p 1.8T) in an LGS motoneuron (similar AHP as the PI cell) in a trained cat (***b***) before (*gray trace*) and after (*black trace*) clonidine. Clonidine decreased Ib inhibition in both groups of cats. *Mn*, Motoneuron. ***c***, Afferent volley was monitored by the CDP. Overall, disynaptic IPSPs (314 trials in 143 cells) evoked by stimulation of knee and ankle extensor group I afferents (Quad, PI, LGS, MG, GS) were significantly decreased by clonidine in shams (30.5%; $*p<0.05$) and even more significantly in trained cats (61.0%; $***p<0.001$). Training enhanced significantly the reduction of Ib inhibition after clonidine ($**p<0.01$). ***d***, Plot of EPSP amplitude versus IPSP amplitude measured from the same cell in shams (*filled circles*) and trained cats (*open circles*).

Decreases in both monosynaptic excitation and disynaptic inhibition could result from a similar modification in motoneuronal properties (e.g., a decrease in membrane resistance). In Figure 3*d*, we plotted the amplitude of monosynaptic EPSP against the amplitude of disynaptic IPSP measured from the same cell in shams (*filled circles*) and trained cats

(*open circles*). If both responses were to change together, because of the same motoneuronal modification, one would expect the values from shams to be grouped in the top right corner (i.e., large EPSP and large IPSP together) and the values from trained cats, which are both significantly reduced, to be grouped in the bottom left corner. The considerable scattering of points in this graph suggests on the contrary that these two pathways were modified independently.

The reversal of Ib inhibition into excitation was first described in acute spinal cats (Gossard & Hultborn 1991, Gossard et al 1994, McCrea et al 1995). In this system, group I afferents from knee and ankle extensors converge on pathways to produce the excitatory drive to extensor muscles during stance. Here, we investigated the occurrence and amplitude of polysynaptic excitation of extensors in chronic spinal cats after clonidine and training. Surprisingly, there were instances of reversals without drugs or locomotion in both sham (8 of 94 trials) and trained (9 of 103 trials) cats. This indicates that after 3-4 weeks of spinalization, interneurons in the polysynaptic excitatory pathways recovered some level of excitability. As expected, clonidine injection succeeded in reversing IB inhibition into excitation in motoneurons from both groups of cats, as shown in Figure 4. In shams, the occurrence of reversals was more frequent (21.5%; $p<0.01$) and its amplitude was greatly increased (from 0.09 to 0.60 mV; 535.6%; $p<0.001$) with clonidine. In trained cats, there was a significant increase in amplitude of polysynaptic excitation (from 0.17 to 0.68 mV;

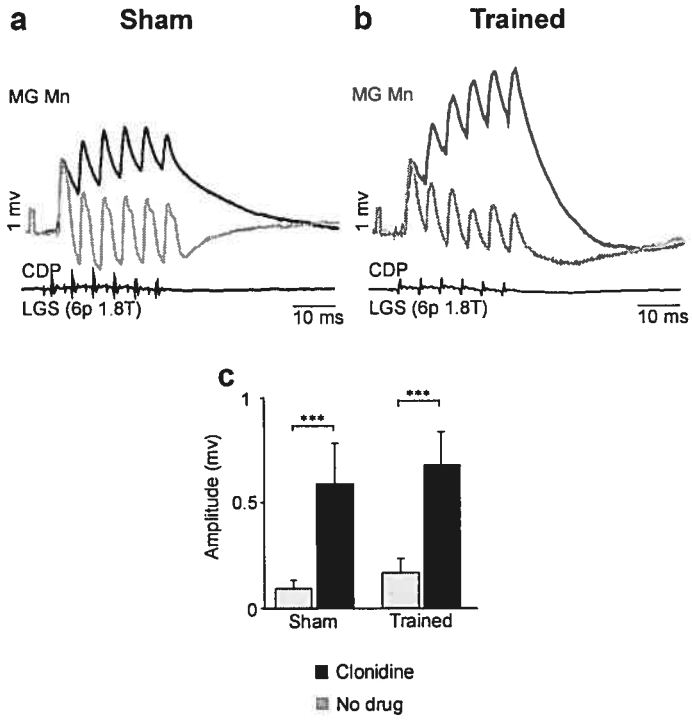

Figure 4. Clonidine increased polysynaptic group I excitation in both groups of cats.

a, b, EPSPs evoked by stimulation of LGS afferents [6p 1.8T] recorded in MG motoneurons (with similar AHPs) in a sham cat (*a*) and a trained cat (*b*) before and after clonidine. Here, clonidine reversed Ib inhibition (*gray trace*) to polysynaptic excitation (*black trace*) both in sham and trained cats. *Mn*, Motoneuron. ***c,*** Overall, the amplitude of polysynaptic EPSPs (313 trials in 143 cells) evoked by stimulation of knee and ankle extensor group I afferents (Quad, PI, LGS, MG, GS) was increased by clonidine in sham (535.6%; ***$p<0.001$) and in trained (307.8%; ***$p<0.001$) cats.

307.8%; $p<0.001$) and a highly significant increase in occurrence (30.2%; $p<0.001$) attributable to clonidine.

We succeeded in keeping intracellular recordings of four motoneurons while injecting clonidine and had the opportunity to observe changes in responses. In a sham, a PI cell showed a decrease in Ib inhibition (from -7.2 to -4.1 mV), and in another sham, an FHL cell showed a reversal from inhibition to excitation (from -2.2 to 1.4 mV). In a trained cat, an LGS cell showed a decrease in Ib inhibition (from -1.6 to -0.8 mV) and, in another trained cat, an MG cell showed a reversal (from -2.7 to 1.6 mV). Similar results were found in the overall population, as reported above. Table 1 gives the mean amplitude of monosynaptic excitation, disynaptic inhibition, and polysynaptic excitation in shams and trained cats before and after clonidine injection.

Table 1. The effects of clonidine and training on the mean amplitude of responses in the specified pathway and on occurrence of reversals

	Sham			Trained		
	No drug	Clonidine	Effect	No drug	Clonidine	Effect
Effect of clonidine						
Monosynaptic excitation	2.55	2.69		1.65	1.66	
Disynaptic inhibition	-3.01	-2.09	↓30.5%	-2.75	-1.07	↓61.0%
Polysynaptic excitation	0.09	0.60	↑535.6%	0.17	0.68	↑307.8%
Occurrence of reversals	8 of 94	12 of 40	↑21.5%	9 of 103	30 of 77	↑30.2%
	Without drug			With clonidine		
	Sham	Trained	Effect	Sham	Trained	Effect
Effect of training						
Monosynaptic excitation	2.55	1.65		2.69	1.66	
Disynaptic inhibition	-3.01	-2.75		-2.09	-1.07	↓48.7%
Polysynaptic excitation	0.09	0.17		0.60	0.68	
Occurrence of reversals	8 of 94	9 of 103		12 of 40	30 of 77	

Only significant changes are indicated

The long-lasting reflexes evoked by stimulating FRA after administration of L-DOPA in spinal cats are believed to be part of the locomotor circuitry (Jankowska et al 1967ab, Schomburg et al 1998). Moreover, it was shown that group I afferents from extensors and contralateral FRA (coFRA) converge on common interneurons to excite extensors after L-DOPA injection (Gossard et al 1994). The involvement of the FRA pathways in chronic spinal cats has been questioned (Grillner 1973, Barbeau et al 1987). In this study, we found that primarily flexors (16 of 18 cats) and not extensors were excited by coFRA stimulation, with or without clonidine. Similar patterns were observed both in sham cats (18

of 24 trials) and trained cats (30 of 38 trials). This strongly suggests that pathways mediating flexion reflexes are deeply reorganized after chronic spinalization.

Training and fictive locomotion

Before clonidine injection, rhythmic bursts of ENG activity were scarcely evoked by perineal stimulation (Barbeau & Rossignol 1987, Bélanger et al 1996) in shams (two of seven cats) (Fig.5*a*). Surprisingly, training did not increase significantly the occurrence of fictive locomotor activities (7 of 11 cats) (Fig.5*b*). After clonidine, perineal stimulation induced robust and well organized episodes of fictive locomotion in both groups of cats (Fig.5*c*,*d*). As exemplified by reflex reversals, it is now well established that transmission in several sensory pathways is deeply modified during locomotion (Rossignol 1996). We thus investigated whether fictive stepping disclosed additional effects of training on the transmission of group I pathways (state-dependent changes). We also studied whether training modified the CPG-dependent modulation in reflex transmission (phase-dependent changes). The amplitude of monosynaptic EPSPs in motoneurons was reported to be decreased during fictive locomotion (by 34%) induced by mesencephalic stimulation in decerebrate cats because of a tonic level of presynaptic inhibition (Gosgnach et al 2000). In this study with chronic spinal cats, fictive stepping did not induce a significant decrease in monosynaptic EPSP amplitude compared with rest in both sham (by 31.2%) and trained (by 29.2%) cats. During walking, transmission in the monosynaptic reflex pathway is phasically modulated in the cat (Forssberg & Grillner 1973, Akazawa et al 1982, Gossard 1996,

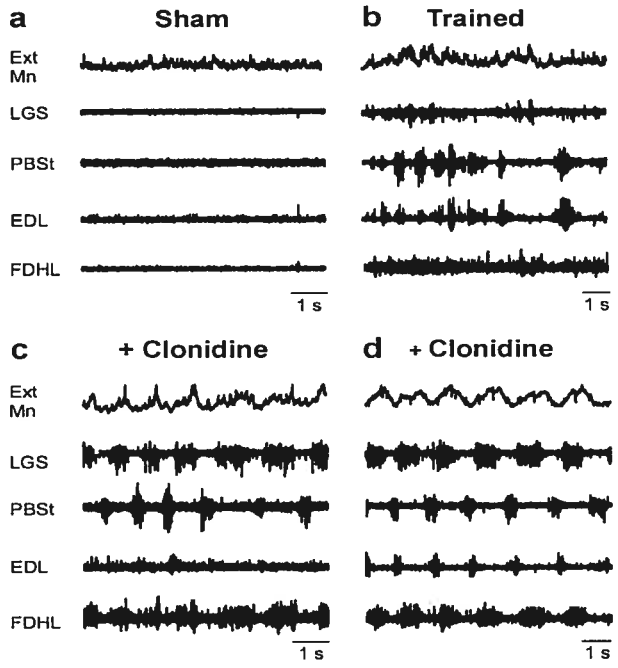

Figure 5. Fictive locomotion can be induced in shams and trained cats. ***a***, ***b***, Motoneuronal intracellular potential and ENG activity in flexor and extensor muscle nerves in a sham (***a***) and a trained (***b***) cat. Rhythmic bursts of activity evoked by perineal stimulation before clonidine injection were observed in trained cats (7 of 11) and in shams (2 of 7). ***c***, ***d***, After clonidine, perineal stimulation induced robust locomotor episodes in both groups of cats. *ExtMn*, Extensor motoneuron.

Ménard et al 1999) and in humans (Capaday & Stein 1986, Simonsen & Dyhre-Poulsen 1999), being maximal during stance in extensors when motoneuronal pools are depolarized. Figure 6 illustrates that the phases for maximal amplitude of monosynaptic EPSPs are opposite in a sham and a trained cat. Phase-dependent modulation was found to be significant only in a few trials (5 of 29 in 4 of 22 cells) (Gosgnach et al 2000). Among those, it was found that training significantly modified the pattern of modulation ($p<0.01$), the maximum amplitude occurring during the depolarized active phase (Fig.6*c*). This very limited sample suggests that training may modify the monosynaptic Ia-transmission pathway to extensor motoneurons so that it is maximally transmitting during the extensor (stance) phase.

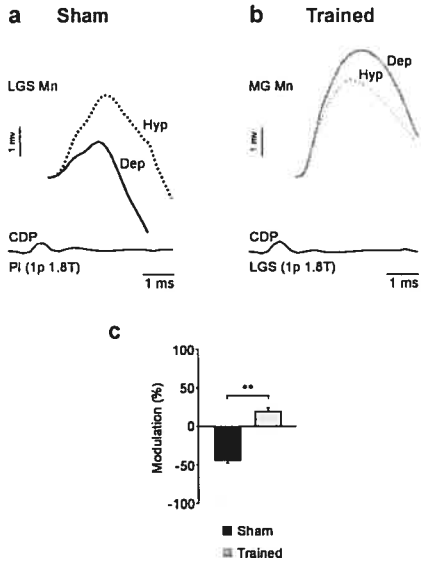

Figure 6. Training could change the pattern of CPG-related modulation of monosynaptic excitation. ***a***, The amplitude of monosynaptic EPSPs evoked by PI stimulation [1p, 1.8T] was larger during the hyperpolarized (*Hyp*) phase in an LGS motoneuron from a sham cat. ***b***, The amplitude of monosynaptic EPSPs evoked by LGS stimulation (1p, 1.8T) was larger during the depolarized (*Dep*) phase in an MG motoneuron from a trained cat. *Mn*, Motoneuron. ***c***, Training modified significantly the pattern of phase-dependent modulation of monosynaptic EPSPs (5 trials in 4 cells; $^{**}p<0.01$) evoked by group I afferents of ankle extensors (PI, MG, LGS), with the maximum amplitude occurring during the hyperpolarized phase in sham cats and during the depolarized phase in trained cats.

We also investigated whether fictive stepping disclosed additional effects of training on the transmission of Ib inhibitory pathways. It was found that fictive locomotion did not change significantly the amplitude of disynaptic IPSPs compared with rest in both sham (48 trials in 27 cells) and trained (35 trials in 26 cells) cats. We also assessed the phase-dependent modulation in IPSP amplitude. Figure 7 illustrates that the amplitude of IPSPs was larger during the depolarized phase in motoneurons in both sham (Fig.7*a*) and trained (Fig.7*b*) cats. From motoneurons presenting a significant phasic modulation (40 of 59 trials in 33 of 41 cells) between depolarized (active) and hyperpolarized phases, it was found that the average depth of modulation was not significantly changed by training (sham, 28.8%; trained, 29.3%). Analysis also showed that the IPSP reduction was not related to the amplitude of locomotor depolarization in motoneurons. This suggests that the CPG-related

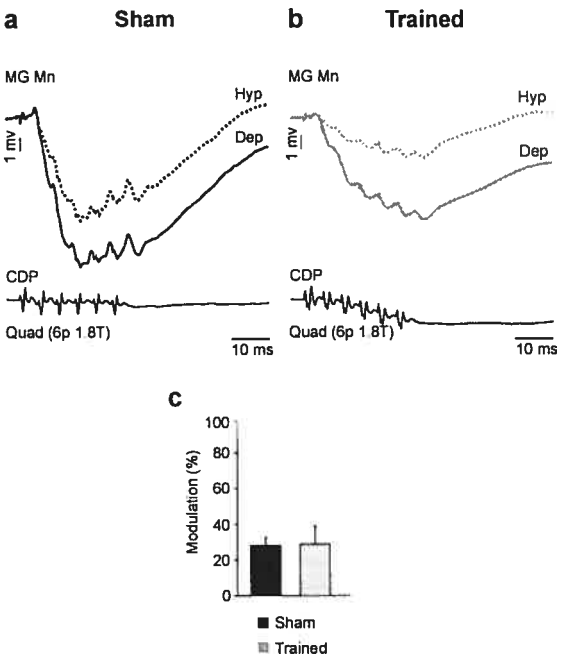

Figure 7. Training did not change the pattern of CPG-related modulation of Ib inhibition. ***a, b,*** IPSPs evoked by Quad (6p, 1.8T) in MG motoneurons during fictive locomotion in a sham (***a***) and a trained (***b***) cat. The amplitude of IPSPs (trough) was increased during the depolarized (*dep*) phase in the sham (*black trace*) and the trained (*gray trace*) cat. *Mn*, Motoneuron. ***c,*** The depth of modulation in IPSPs (40 trials in 33 cells) was not significantly changed by training. *Hyp*, hyperpolarized.

modulation that was similar in both groups probably occurred in Ib interneurons. Compared with rest, the occurrence of reversals from Ib inhibition to excitation was more frequent during fictive locomotion in shams (25.1%; $p<0.001$) but not in trained cats (11.7%; not significant). Also, the amplitude of responses was much increased during fictive stepping in shams (48 trials, by 225.5%; $p<0.05$), whereas it was unchanged in trained cats (36 trials). We also assessed its phase-dependent modulation. For example, in Figure 8, the amplitude of polysynaptic EPSPs was increased during the depolarized phase in the motoneuron from a sham (Fig. 8*a*), whereas it was decreased during that same phase in a motoneurons from a trained cat (Fig. 8*b*). From motoneurons presenting a significant phasic modulation (19 of 29 trials in 15 of 21 cells), it was found that the average depth of modulation was not significantly changed by training. However, the polysynaptic excitation was larger (by 8.1%) during the depolarized phase in sham cats, whereas it was decreased (by 19.3%) during that same phase in trained cats (Fig.8*c*). The fact that, in trained cats, the amplitude of polysynaptic excitation is smaller during fictive stepping compared with rest and smaller during the depolarized phase may be attributable to the occlusion of this pathway caused by its recruitment by the CPG to produce extensor activities (Gossard & Hultborn 1991, Gossard et al 1994). This was evaluated by comparing in both groups of cats the linear regressions relating the amplitude of polysynaptic excitation and the amplitude of locomotor bursts of activity of the parent extensor nerve. In shams, the amplitude of polysynaptic excitation was growing with

increasing ENG-burst amplitude (upward slope), whereas in trained cats, it decreased with increasing ENG bursts (downward slope), and this difference was significant (p<0.03).

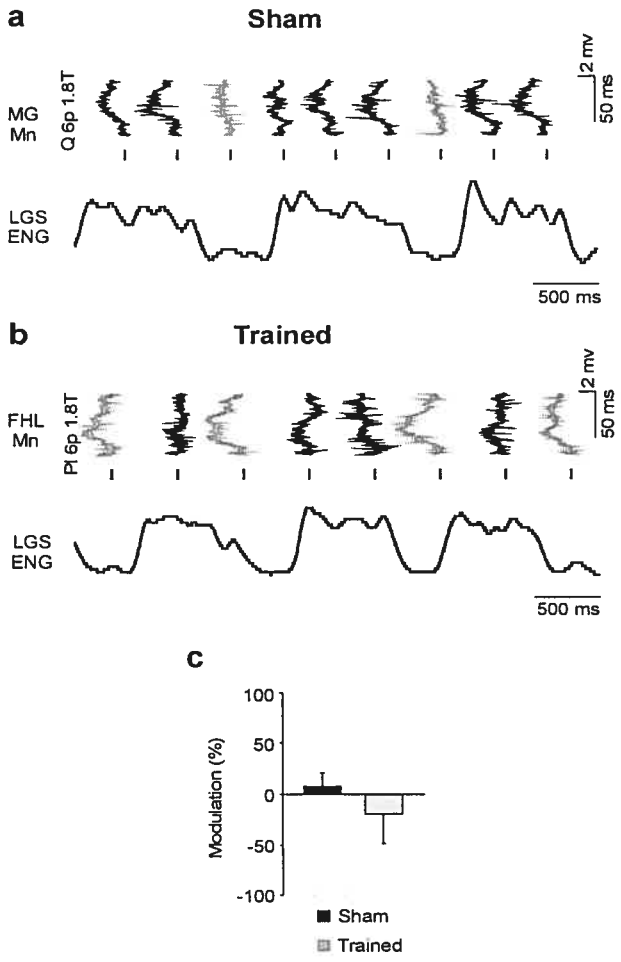

Figure 8. Different patterns of CPG-related modulation of polysynaptic excitation. ***a***, EPSPs recorded in an MG motoneuron (tilted 90°) in a sham were evoked by Quad stimuli [6p 1.8T] at different moments in the step cycle illustrated by the rectified and filtered ENG activity of the LGS nerve. The amplitude of polysynaptic EPSPs was maximal (*gray trace*) when occurring during the active period of LGS (i.e., during the extension phase). ***b***, The amplitude of polysynaptic EPSPs evoked by PI stimulation and recorded in an FHL motoneuron (tilted 90°) from a trained cat (6p, 1.8T) was minimal (*black trace*) during the extension phase when LGS was maximally active. *Mn*, Motoneuron. ***c***, Overall, the pattern of phase-dependent modulation of polysynaptic EPSPs (19 trials in 15 cells) tended to be opposite in shams and trained cats, but this difference was not statistically significant.

Discussion

Acute experimentation in curarized animals is advantageous to investigate transmission of sensory pathways because it allows stable intracellular recordings, and responses can be solely attributed to the operation of central networks. The effects of training or clonidine observed in this study can then be attributed to changes occurring in spinal pathways and not to an alteration in peripheral sensory events or muscle fibers. There is now growing evidence that reflex pathways are not "hard-wired" (Forssberg & Svartengren 1983), and that they can display a certain level of plasticity in response to central or peripheral lesions or operant conditioning (Mendell 1984, Durkovic 1996, Wolpaw 1997, Wolpaw & Tennissen 2001). The recovery of stepping with treadmill training has been attributed solely to plasticity of the CPG (Lovely et al 1986, Rossignol 1996, Harkema 2001). This

study is the first to report that recovery of locomotion may also involve changes in several reflex pathways. Plastic changes in a reflex arc can occur in motoneurons, interneurons, or primary afferents. Our results showed that stimulation of the same group I afferents could elicit opposite response patterns in two different pathways (monosynaptic and polysynaptic), one being increased and the other decreased in amplitude, in the same motoneuron. Also, decreases in monosynaptic and disynaptic responses did not appear to covary in the same motoneuron. Additionally, clonidine injection significantly modified transmission in disynaptic pathways without affecting monosynaptic transmission. Moreover, AHP duration, which varies systematically with input resistance and membrane time constant (Gustafsson & Pinter 1984b), was found not to be modified by 1 month of training (data not shown). Therefore, premotoneuronal mechanisms can most easily explain our response patterns. Finally, there is an unknown contribution and plastic modification of recurrent inhibition in our recordings. However, Ib inhibition and its reduction caused by training were observed between motoneurons (e.g., Quad) and group I fibers from muscle nerves (e.g., Pl) known to lack recurrent inhibitory connections (Baldissera et al 1981). We thus believe that plasticity induced by training in load pathways was occurring primarily in interneurons of the group I pathways to extensors and interneurons of presynaptic inhibition. The first finding of this work is that training decreases monosynaptic excitation by 36%. This was apparent when we pooled all amplitude values because of the lack of significant effect of clonidine on this transmission. In the few cells in which it was possible to test, the phase-dependent modulation showed a maximum monosynaptic transmission occurring during the extensor phase in trained cats in which it could help the excitation of motoneurons. Intrathecal injection of clonidine also failed to change the H-reflex in incomplete paraplegic subjects (Rémy-Néris et al 1999). Also, treadmill training decreased and improved the gating of IA reflexes in spinal-cord-injured humans compared with normal subjects (Trimble et al 1998). Transmission in this pathway can be changed by presynaptic inhibition and/or motoneuronal properties. As explained above, postsynaptic changes alone cannot easily explain all of the response patterns observed in this work. We thus believe that training may have increased the level of presynaptic inhibition in Ia terminals ending in the ventral horn. Such an increase was inferred to explain a general (non-muscle-specific) tonic decrease in Ia-EPSPs (by 34%) in a majority of hindlimb motoneurons during fictive locomotion evoked by mesencephalic stimulation in the cat (Gosgnach et al 2000). We thus suggest that training could help reduce spasticity by decreasing IA transmission and improve phase-dependent modulation of the stretch reflexes during stepping. Another main finding from this work is that the decrease in Ib inhibition after clonidine is enhanced by training. A normalization of

inhibitory systems in the spinal cord may be of prime importance in recovering stepping (Robinson & Goldberger 1986, de Leon et al 1999a). For example, it was recently described that the number of cells stained for GAD67 mRNA was specifically decreased by step training in laminas V and VI (in which Ib interneurons are located) in spinal cats (Tillakaratne et al 2002). Note that during reflex reversal, the disappearance of disynaptic inhibition precedes the appearance of polysynaptic excitation (Gossard et al 1994, McCrea et al 1995). We thus interpret the observed reduction of IB inhibition as a first step toward reversals. The results also showed that clonidine increased more significantly the occurrence of polysynaptic excitation in trained cats than in shams. However, it was surprising not to see more effects of training on the amplitude of polysynaptic excitation. Perhaps smaller doses of clonidine would have revealed more differences. Indeed, the dose used (500 µg/kg) was determined from previous reports on acute spinal cats and is possibly more than sufficient to evoke reflex reversals in all spinal cats. Fictive locomotion did not reveal additional training-related changes in Ib inhibition amplitude or phase-dependent modulation patterns. However, it showed that the minimal amplitude in polysynaptic excitation occurred during the extensor phase when the locomotor excitation is maximal in trained cats. We interpret this pattern as being attributable to the occlusion of the pathways by the action of the CPG during the extensor phase as it was proposed in the acute spinal cat (Gossard et al 1994). We interpret this as being another step toward the establishment of locomotor-related polysynaptic excitatory pathways to extensors caused by training. The same reasoning may help explain why the occurrence and amplitude of polysynaptic excitation in shams were increased during fictive stepping. If locomotor circuitry is not as well established in shams as in trained cats, there is less occlusion in these pathways and the segmental responses become more apparent. In the decerebrate cat walking on a treadmill, it was estimated that up to 50% of the force generated during the stance phase was caused by muscle reflexes (Hiebert & Pearson 1999, Stein et al 2000). We may presume that the isolated spinal cord would depend even more on sensory feedback to generate force during stepping. Although modest, the reported plastic changes indicate that after spinal cord injury, load pathways would have a larger contribution in the control of stance if trained regularly and together with pharmacological intervention. Our results support previous reports that load receptors may contribute to the activation of leg extensors during walking in humans (Ghori & Luckwill 1985, Dietz et al 1992, Stephens & Yang 1999, Sinkjaer et al 2000, Stein et al 2000). For example, it was proposed that afferent inputs from receptors signaling contact forces during the stance phase are essential for the activation of spinal locomotor centers in SCI subjects (Harkema et al 1997). Moreover, the improvement in treadmill and overground

locomotor patterns was attributed to the repetitive alternating-limb loading using body-weight support (Wernig et al 1998). Whether treadmill training or repetitive loading revived the previous (prespinalization) CPG or whether it set up a new locomotor circuitry is still debatable. Our results indicate clearly that some pathways involved in locomotion in the acute spinal cat, namely the FRA networks (Jankowska et al 1967ab), are reorganized after chronic spinalization (Barbeau et al 1987). Moreover, fictive stepping sometimes occurred without concomitant appearance of group I polysynaptic excitation in some extensor motoneurons, which was not seen in the acute cat injected with L-DOPA (Gossard et al 1994). Thus, as proposed previously (Hodgson et al 1994, de Leon et al 1999a), our results support the idea that the isolated spinal cord "learned" how to walk by establishing new locomotor pathways.

Acknowledgments

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PUBLICATION #2: STEP TRAINING-DEPENDENT PLASTICITY IN SPINAL CUTANEOUS PATHWAYS

Côté MP and Gossard JP (2004). Step training-dependent plasticity in spinal cutaneous pathways. J. Neurosci. 24:11317-11327.

Abstract

Plasticity after spinal cord injury can be initiated by specific patterns of sensory feedback, leading to a reorganization of spinal networks. For example, proprioceptive feedback from limb loading during the stance phase is crucial for the recovery of stepping in spinal-injured animals and humans. Our recent results showed that step training modified transmission from group I afferents of extensors in spinal cats. However, cutaneous afferents are also activated during locomotion and are necessary for proper foot placement in spinal cats. We therefore hypothesized that step training would also modify transmission in cutaneous pathways to facilitate recovery of stepping. We tested transmission in cutaneous pathways by comparing intracellular responses in lumbar motoneurons (n=136) in trained (n=11) and untrained (n=7) cats spinalized 3-5 weeks before the acute electrophysiological experiment. Three cutaneous nerves were stimulated, and each evoked up to three motoneuronal responses mediated by at least three different pathways. Overall, of 71 cutaneous pathways tested, 10 were modified by step training: transmission was reduced in 7 and facilitated in 3. Remarkably, 6 of 10 involved the medial plantar nerve innervating the plantar surface of the foot, including two of the facilitated pathways. Because the cutaneous reflexes are exaggerated after spinalization, we interpret the decrease in most pathways as a normalization of cutaneous transmission necessary to recover locomotor movements. Overall, the results showed a high degree of specificity in plasticity among cutaneous pathways and indicate that transmission of skin inputs signaling ground contact, in particular, is modified by step training.

Introduction

Growing experimental and clinical findings provide evidence for activity-dependent plasticity of spinal networks (Wolpaw & Tennissen 2001). These studies indicate that physiological, biochemical, and functional reorganization of lumbar spinal cord occur over time (Nacimiento et al 1995, Edgerton et al 1997ab, Giroux et al 1999). Consistent with

this, strategies to recover motor functions after spinal cord injury (SCI) include the management of sublesional spinal cord based on the reorganization of the remaining undamaged neural pathways.

Sensory feedback plays a crucial role in the recovery of function after SCI in humans and animals. This is well illustrated by the ability to regain rhythmic locomotor patterns after repetitive sensory stimulation provided by step training (Lovely et al 1986, Barbeau & Rossignol 1987, Fung et al 1990, de Leon et al 1998b, Harkema 2001, Leblond et al 2003). Improvement depends on specific activity-dependent sensory feedback (de Leon et al 1998a) and not on the effect of training on musculature (Roy & Acosta 1986, Roy et al 1999). For instance, step-trained spinal cats improve their gait pattern but are not better at standing, and conversely, stand-trained animals are not better at stepping (de Leon et al 1998a, 1999b). Also, functional stepping recovery and precise limb placement in spinal hemisected cats is correlated with sprouting of primary afferents (Helgren & Goldberger 1993). Thus, both anatomical and behavioral evidence suggests that the sensory feedback can be used to compensate for the loss of supraspinal inputs to spinal circuits. However, little is known about the mechanisms and pathways underlying the beneficial action of sensory feedback.

Proprioceptive feedback during limb loading contributes to the recovery of stepping. In both humans and cats recovering from SCI, progressively increasing weight-bearing improves stepping ability (Barbeau & Rossignol 1987, Barbeau et al 1987, Edgerton et al 1992, Harkema et al 1997). Moreover, we recently showed that step training modified transmission from group I afferents of extensors in spinal cats (Côté et al 2003a). However, cutaneous afferents are also activated by locomotor movements and may participate in recovery. Cutaneomuscular stimulation can partially restore normal reflex modulation in spastic SCI patients (Fung & Barbeau 1994). Previous experiments showed that the selective phasic stimulation of cutaneous receptors from the plantar surface of the foot (without activation of proprioceptors signaling limb loading), was sufficient to permanently increase limb extension during swimming in spinal hemisected chicks (Muir & Steeves 1997). Also, progressive cutaneous denervation of the hindlimb in spinal cats indicates that proper foot placement during stepping requires a minimum cutaneous input (Bouyer & Rossignol 2003b). Because of its influence on locomotor networks, we hypothesized that step training would also modify transmission in cutaneous pathways. We tested this by comparing motoneuronal responses to cutaneous nerve stimulation in trained and untrained cats spinalized 3-5 weeks before an acute experiment. Our findings

indicate that plasticity occurs only in specific cutaneous pathways, with decreased transmission detected in most. The results also reveal selective modification of skin inputs signaling ground contact, suggesting that plasticity of these connections may be of particular importance during step training.

Materials and Methods

All procedures were conducted according to the *Guide for Care and Use of Experimental Animals* (Canada), using protocols approved by the Ethics Committee of the Université de Montréal.

Spinalization and locomotor training. Eighteen adult female cats (2.5-4.1kg) were used for this study. After administration of preoperative medication, the cats were anesthetized (2% isoflurane; Abbott Laboratories, Montréal, Québec, Canada) and spinalized at T13 under aseptic conditions. Protocols for spinalization procedures and subsequent postoperative care were analogous to those described previously (Chau et al 1998a, Côté et al 2003a). A patch of fentanyl (Duragesic; 25 µg; Janssen-Ortho, Markham, Ontario, Canada) was sutured on the back of the cat for continuous and stable delivery of analgesic over a 2d period. The first group of cats was only spinalized [sham operated (sham)], whereas the second group was locomotor trained until they could support the weight of their hindquarters (mean, 28d). Training on the treadmill (0.2-0.4m/sec) started 2d after surgery and consisted of two to four daily training sessions for periods of 10 min. In early training, hindquarters were supported by the experimenter to provide weight support, and perineal stimulation was used to increase central excitability and to maintain locomotion. The animal became gradually able to support its hindquarters and to walk, and perineal stimulation was no longer needed in most cases. No drugs were used to assist the locomotor training. The training was stopped when the cat was able to walk continuously on the treadmill for >5 min while the experimenter assisted only for lateral stability by holding the tail.

Acute experiment. Cats were first anesthetized by inhalation of an oxygenated mixture (50%) of nitrous oxide (50%) and halothane (2-3%; MTC Pharmaceuticals, Cambridge, Ontario, Canada). Cannulas were inserted in the right common carotid artery to monitor blood pressure and in the jugular and cephalic veins for administration of pharmacological agents or fluids. Cats were then decerebrated and curarized (Pavulon; 0.2 mg/kg/45 min;

Sabex, Boucherville, Ontario, Canada) and artificially ventilated as detailed previously (Ménard et al 1999, Leblond et al 2000).

To monitor locomotor episodes and antidromically identify motoneurons, the following muscle nerves from the left hindlimb were dissected free, cut, and mounted on bipolar silver chloride electrodes for recording [electroneurogram (ENG)] and stimulation: posterior biceps-semitendinosus (PBSt), semimembranosus-anterior biceps (SmAB), lateral gastrocnemius-soleus (LGS), medial gastrocnemius (MG), plantaris (Pl), flexor hallucis longus (FHL) and flexor digitorum longus (FDL) together, tibialis anterior (TA), extensor digitorum longus (EDL), and sciatic nerve (uncut). Three cutaneous nerves were also dissected free for subsequent stimulation: caudal cutaneous sural (CCS), medial plantar (MPL), and superficial peroneal (SP).

Stimulation, recordings, and analysis. The cord dorsum potential (CDP) was recorded with a silver chloride ball electrode located near the dorsal root entrance at the L6-L7 border. Stimulation intensity required to just evoke a deflection in the CDP determined the threshold for the most excitable fibers for each nerve (1T). Stimulus intensity was expressed as a multiple of the threshold. Intracellular potentials evoked by the stimulation of low-threshold cutaneous afferents (CCS, MPL, SP; one pulse; 2T) were recorded in identified extensor and flexor/bifunctional motoneurons (Leblond et al 2000) with glass micropipettes filled with K^+-acetate (2M) and QX314 [*N*-(2,6-dimethylphenylcarbamoyl-methyl) triethylamonium bromide; 100mM; Alamone Laboratories, Jerusalem, Israel] to prevent sodium spikes. FDL and FHL motoneurons could be distinguished by their responses to SP stimulation and the phase of peak depolarization during fictive stepping (Burke 1999). The duration of the afterhyperpolarization (AHP) was measured in every cell, from the spike onset to where the AHP crosses the baseline (Gustafsson & Pinter 1984b). Stimulation of peripheral nerves was given every 0.3, 0.4, or 0.5sec.

All responses were further studied during a period of 2hr after intravenous clonidine injection (α_2-noradrenergic agonist; 500µg/kg; Sigma, St. Louis, MO) and during episodes of fictive locomotion induced by perineal stimulation. Up to two doses of clonidine were injected in an experiment, and data were recorded for the following 2hr. Once clonidine was injected, there was no return to control conditions, and all subsequent recordings were considered post-clonidine data.

A "trial" is the averaged response in one motoneuron evoked by the stimulation ($n \geq 40$) of a given pathway (a cutaneous afferent-motoneuron pair). Several pathways could be studied in a given motoneurons corresponding to different cutaneous stimulation. The transmission of cutaneous pathways was monitored by measuring the peak amplitude of IPSP and EPSP in motoneurons. The amplitude of IPSPs (R2) was measured as the maximal negative deflection from the baseline in the intracellular trace in response to the stimulation, and the amplitude of EPSPs (R1-R3) was measured as the maximal positive deflection from the baseline, as illustrated in Figure 2 (upward and downward arrows) and described in Results. Changes attributable to training, clonidine, or locomotion were determined by comparing the average amplitude obtained in each of these three conditions.

Data collected during rest (silent ENGs) and during fictive stepping were compared to study state-dependent changes in cutaneous transmission. During locomotor episodes, bursts of ENG activities were used to divide the step cycle into flexion (corresponding to swing) and extension (corresponding to stance) phases. The locomotor cycle, defined as the period between the onsets of two successive bursts of ENG activity in extensors, was normalized to the duration of the averaged cycle. PSPs evoked during flexion and extension were separated and averaged to study phase-dependent modulation.

Statistical analysis. Histograms in the figures are expressed as means ± SEM. Statistical analysis was performed to disclose differences between responses obtained (1) in sham-operated and trained groups (training-dependent plasticity), (2) at rest and during fictive locomotion (state-dependent changes), (3) during flexion and extension phases of locomotion (phase-dependent changes), and (4) before and after clonidine injection. The Kolmogorov-Smirnov-Liliefors test was used to compare the shape and location of the distribution of responses to a normal distribution, and the Levene median test was used for equal variance. If these two tests confirmed that the sample variables did fit a normal distribution and were equally variant, a one-way ANOVA was performed; if not, the Kruskal-Wallis one-way ANOVA on ranks was used. The χ^2 with the Yates correction factor or Fisher's exact test evaluating frequency distributions was used to further identify differences in the occurrence of type of responses between groups. For all statistical tests, the significance level was set to $p<0.05$. In histograms, significant difference is indicated as follows: $*p<0.05$, $**p<0.01$, or $***p<0.001$. When no significant changes were found in data measured before and after clonidine injection, the data were merged together.

Results

In the first section, we present the effect of training on response amplitude recorded at rest and during fictive locomotion episodes. In the next section, we compare response amplitude between locomotion versus rest in shams and also in trained cats. In the last section, the effect of clonidine is compared with the effect of training. In each section, changes in the transmission of cutaneous pathways were monitored by measuring the peak amplitude of IPSPs and EPSPs in several motoneurons. AHP duration, which varies systematically with input resistance and membrane time constant (Gustafsson & Pinter 1984b), was compared between sham and trained cats as an indication of the size and membrane resistance of motoneurons. Figure 1*a* shows the similarity of AHP duration distribution for both groups of cats. Overall, there was no significant difference between mean AHP duration (±SE) measured in the two groups of cats (sham, 76±4.3 msec; trained, 68±3.7 msec), even if grouped according to the motor pool (data not shown). Also, a change in PSP amplitude could be partly attributable to differences in membrane potential (Powers & Binder 1985, Coombs et al 1955). We therefore compared the levels of membrane potential at rest of all motoneurons of shams (average, 62.1 ± 1.1 mV) and trained cats (average, 60.5±0.9 mV) and found no significant difference (p=0.24). Figure 1*b* shows the similarity of membrane potential distribution for both groups of cats.

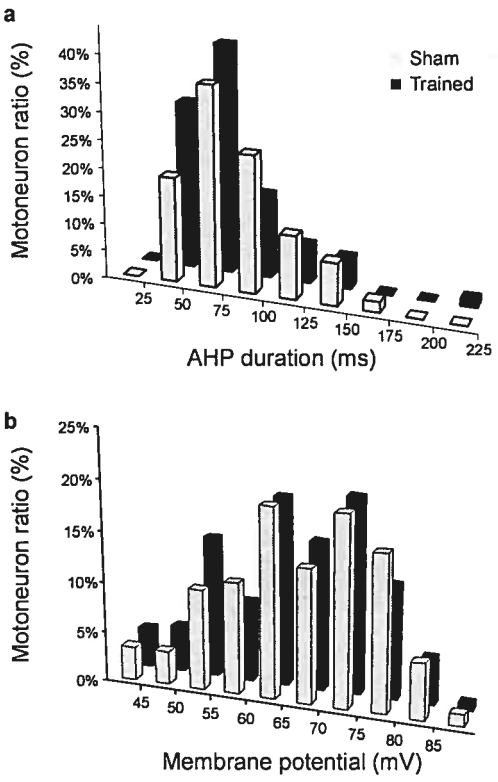


Figure 1. Training did not modify AHP duration and membrane potential. The histograms of AHP duration (***a***) and membrane potential (***b***) of motoneurons in shams (gray) and trained cats (black) show a similar distribution.

Cutaneous PSP patterns

We evaluated the effect of training on patterns of PSPs evoked by cutaneous afferents recorded at rest in five extensor (22 FHL, 17 MG, 16 SmAB, 15 LGS, 12 PI) and three flexor/bifunctional (21 PBSt, 20 EDL, 13 FDL) motoneuronal pools of 7 shams and 11 trained cats. Figure 2 illustrates six different patterns of PSPs elicited by a single shock in cutaneous afferents in various motoneurons. These multiphasic records were typical of the effects observed intracellularly. Responses were composed of one to three components: early excitation (R1), inhibition (R2), and late excitation (R3) (Baker & Chandler 1987b). More precisely, type A response was composed of R1-R2-R3 (Fig. 2*a*), type B response was composed of R2-R3 (Fig. 2*b*), type C response was composed of R2 (Fig. 2*c*), type D response was composed of R1-R3 (Fig. 2*d*), type E response was composed of R1-R2 (Fig. 2*e*), and type F response was composed of R1 (Fig. 2*f*). The relative frequency of type A–F responses is reported in Table 1. As noted in previous reports (Baker & Chandler 1987b), the most represented type of response was composed of all three components (i.e., the pattern referred to as type A here). In shams, the type A

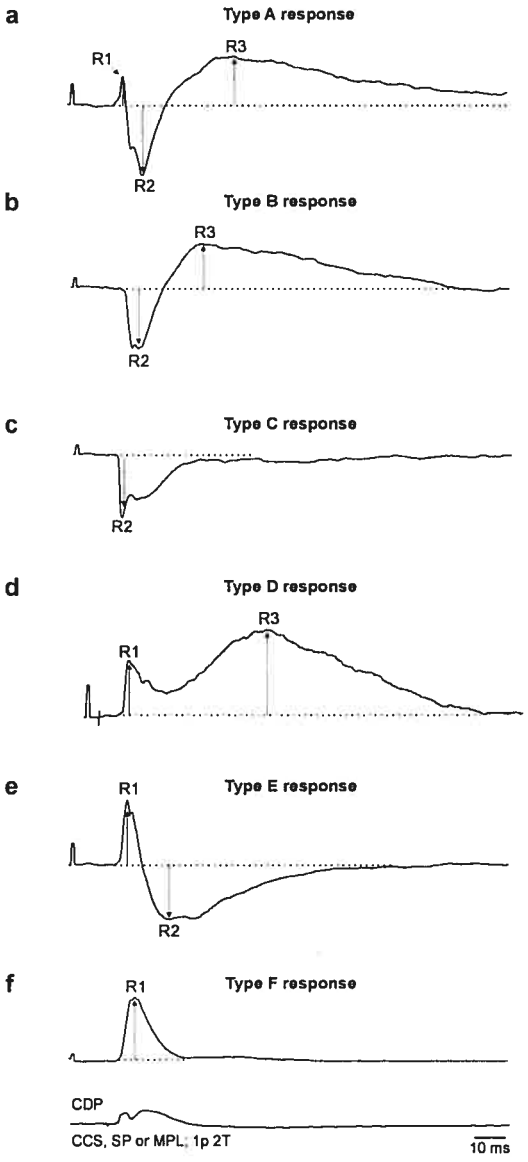


Figure 2. Type of responses to cutaneous stimulation recorded in motoneurons. Representative averaged PSP patterns (n=62) evoked by cutaneous afferents (CCS, MPL, or SP) recorded in extensor or flexor/bifunctional motoneurons are shown. The initial depolarization is referred to as R1, the subsequent hyperpolarization referred to as R2, and the following depolarization referred to as R3. ***a***, Type A response: R1-R2-R3; ***b***, type B response: R2-R3; ***c***, type C response: R2; ***d***, type D response: R1-R3; ***e***, type E response: R1-R2; *f*, type F response: R1. Baseline is represented by a dotted line from which amplitude is measured for each component (upward and downward arrows). Calibration pulse, 1 mV.

response was most prevalent in seven of eight motoneuronal pools, except for PBSt (Table 1, gray highlight), whereas in trained cats, the type A response was most prevalent in six of eight motoneuronal pools (Table 1, black highlight), except for MG and PBSt. In those two cases, type D response (R1-R3) was the most prevalent. We then performed an analysis of frequency of type A-F responses to assess the effect of training.

Table 1. Effect of training on the type of responses to cutaneous stimulation according to motoneuronal pool

	FHL		LGS		MG		SmAB		PI		EDL		FDL		PBSt		TOTAL	
	Sham	Trained	Sham	Trained	Sham	Trained	Sham	Trained	Sham	Trained	Sham	Trained	Sham	Trained	Sham	Trained	Sham	Trained
	n=23	n=31	n=15	n=25	n=21	n=28	n=16	n=27	n=22	n=11	n=12	n=38	n=12	n=26	n=24	n=28	n=145	n=214
Type A (R1-R2-R3)	78.3%	67.7%	100.0%	72.0%	61.9%	42.9%	75.0%	70.4%	63.6%	63.6%	50.0%	57.9%	100.0%	61.5%	25.0%	17.9%	66.2%	56.1%
Type B (R2-R3)	8.7%	9.7%		8.0%	19.0%	3.6%				27.3%	8.3%	2.6%					4.8%	4.7%
Type C (R2)	4.3%	3.2%							9.1%								2.1%	0.5%
Type D (R1-R3)	8.7%	19.4%		16.0%	19.1%	50.0%	25.0%	18.5%	18.2%	9.1%	25.0%	36.9%		30.8%	41.7%	71.4%	18.6%	33.6%
Type E (R1-R2)				4.0%				7.4%	9.1%		8.3%	2.6%		7.7%	8.3%		3.5%	2.8%
Type F (R1)						3.5%									25.0%	10.7%	4.1%	1.8%
No response								3.7%			8.3%						0.7%	0.5%

The proportion of the type of responses evoked by cutaneous stimulation (CCS, MPL, and SP) is reported according to the motor pool (extensor: FHL, LGS, MG, SmAB, and PI; flexor/bifunctional: EDL, FDL, and PBSt) in shams and trained cats. The number of observations or trials (*n*) for a given type is expressed as the percentage of the total trials recorded in that motor pool in shams or in trained cats. The most represented type is highlighted in gray for shams and in black for trained cats. Note that the training did not modify the distribution of types of cutaneous responses evoked in most motor pools. Training did change the distribution of responses evoked in FDL motoneurons ($p<0.05$) with the appearance of type D and type E responses (dotted square).

Overall, training did not modify the distribution of the different types of responses (Table 1), the type A response being the most common finding in both groups (66.2% sham, 56.1% trained). Also, the type A response was the most represented type in both extensor (74.2% sham, 63.1% trained) and flexor/bifunctional (50.0% sham, 46.7% trained) motoneurons. When grouped according to motoneuronal pools, training tended to increase the occurrence of type D responses in six of eight motor pools. However, only the distribution of responses recorded in FDL motoneurons was significantly modified by training ($p<0.05$); the type A response was recorded in 100% of trials in shams, whereas in addition to type A, both types D and E were recorded in trained cats (Table 1). Type D has no inhibitory response (R2), and this absence is not simply attributable to a difference in membrane potential levels in FDL motoneurons between shams (average, 71.0±3.3 mV) and trained cats (average, 61.4±3.9 mV; $p=0.16$).

Because the type A response was the most common finding both in shams and in trained cats, as reported for chronic and acute spinal cats (Baker & Chandler 1987b), the amplitude of all three components were collected for additional analysis. Here the three components were considered as the outcome of three different pathways involving a different number of interneurons inserted between cutaneous afferents and motoneurons (Pinter et al 1982). Data were pooled according to motor nuclei and stimulated cutaneous nerve (see below). The mean amplitude for each of the three components was compared between sham and trained cats. The absence of one of the components (Fig.2*b-f*) was considered as 0mV of amplitude for that component and 100% reduction in synaptic transmission in that pathway.

Effect of training recorded at rest

Significant changes in transmission in cutaneous pathways attributed to training were calculated by comparing data measured in the sham and trained groups. Table 2 depicts the effect of training on cutaneous responses evoked by CCS, MPL, or SP in various extensor and flexor/bifunctional motoneuronal pools. Among all the possible afferent-motoneuron pairs, we succeeded in testing 71 of 72. Of the 71 pathways tested at rest, 10 were significantly modified by training. In a majority of these cases (7 of 10), training decreased the mean amplitude of responses. Each cutaneous source tested was significantly modified in at least one pathway, and MPL was the most potent (6 of 10 pathways). Training barely affected the amplitude of R2 (2 of 10). Also, when significantly modified by training, R3 amplitude was reduced in extensor motor pools (CCS-MG-R3, MPL-MG-R3) and increased in flexor/bifunctional motor pools (CCS-EDL-R3, MPL-PBSt-R3). Data from several cats were used to reveal the modifications attributable to training on pathways to MG (4-5 cats), PI (3-5 cats), EDL (8-12 cats), and PBSt (7-11 cats) motor pools.

The effect of training was predominantly observed in MG motoneurons. Of the 10 pathways significantly modified, six had MG as a target (Table 2). Actually, training significantly modified the mean amplitude of the responses in six of nine pathways recorded in MG motoneurons as reported in Table 2. All significant changes were a decrease in amplitude (Fig.3*a–c*) (CCS-MG-R1, MPL-MG-R2, SP-MG-R2, CCS-MG-R3, MPL-MG-R3), except for MPL-MG-R1, which increased in amplitude (Fig.3*b*). Moreover, one should note that training reduced R2 amplitude only in MG motoneurons (MPL-MG-

R2, SP-MG-R2). This training-induced reduction in the amplitude of R2 in MG motoneuron was not attributable to a reduced occurrence of inhibition. As noted above, a change in IPSP amplitude could also be attributable to a difference in membrane potentials in the two groups. We therefore analyzed the relationship between IPSP amplitude and membrane potential levels in all MG cells and found no significant linear relationship [as did Powers & Binder (1985)] in shams (r=0.20; p=0.45) or trained cats (r=0.50; p=0.08). Restricting comparisons to cells with membrane potential between 50-65 and 65-80 mV did not eliminate differences in IPSP amplitude either.

It was reported before that cutaneous stimulation produced a differential distribution of early EPSPs (referred to as R1 here) within the three extensor motor nuclei comprising the triceps surae in decerebrate cats (LaBella et al 1989), semichronic spinal cats (LaBella et al 1992), and humans (Duysens et al 1996). CCS afferent stimulation preferentially evoked excitation in MG motoneurons, whereas SP afferents preferentially evoked excitation in LG

Table 2. Effect of training on the mean amplitude of responses in specific cutaneous pathways

		Nb of trials	CCS	MPL	SP
Extensor Mn	FHL	13-19			
	LGS	12-15			
	MG	9-17	R1↓47% R3↓61%	R1↑89% R2↓54% R3↓46%	R2↓67%
	PI	10-12		R1↓80%	
	SmAB	14-15			
Flexor Mn	EDL	15-19	R3↑274%	R1↓43%	
	FDL	12-13			
	PBST	15-20		R3↑582%	

The effect of training is reported according to the afferents (CCS, MPL, and SP) and motoneurons (FHL, LGS, MG, PI, SmAB, EDL, FDL, and PBSt). Pathways (R1, R2, or R3) significantly modified (10 of 71 tested pathways) by training are represented as circled numbers. Upward arrows indicate an increase and downward arrows indicate a reduction in mean amplitude between shams and trained cats. These are followed by the mean percentage of change in amplitude as calculated by comparing the mean amplitude (on the basis of all trials) obtained in trained cats to the one obtained in shams. The number of trials for each pathway for the corresponding motor pool is shown in the second column.

(LaBella et al 1989, 1992). We therefore tested whether the differential distribution of PSPs in MG and LGS motoneurons was maintained both in chronic spinal shams and trained cats. In agreement with these previous reports, the amplitude of R1 evoked by CCS was greater in MG than in LGS both in shams ($p<0.05$) and in trained cats ($p<0.001$). In addition, we further looked at R2 and R3 amplitude. R2 amplitude was smaller in MG than in LGS in trained cats ($p<0.01$) but not in shams, whereas R3 amplitude was greater in MG than in LGS in shams ($p<0.01$) but not in trained cats. Also, CCS afferent stimulation more frequently produced responses without R2 (type D) in MG than in LGS in shams ($p<0.05$) but not in trained cats. The results suggest that CCS afferents are more likely to transmit excitation (early and late) to the MG than LGS motor pool and that no dramatic difference was observed because of training. However, training did change the differential excitation from SP afferents to MG and LGS motoneurons. As in decerebrate cats (LaBella et al 1992), R1 amplitude was more frequently absent in MG than in LGS motoneurons in shams ($p<0.05$) (LaBella et al 1992). Conversely, in trained cats, R2 amplitude was more often absent in MG than in LGS motoneurons ($p<0.05$).

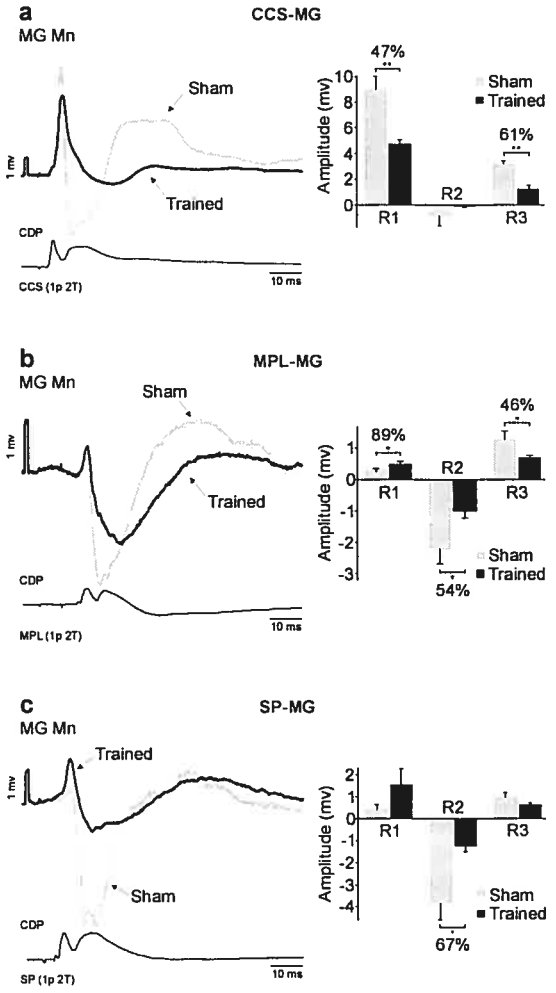


Figure 3. Training specifically modified transmission from cutaneous afferents to the MG motor pool. Left, PSPs ($n \geq 40$) evoked by stimulation of CCS (***a***), MPL (***b***), and SP (***c***) afferents recorded in MG motoneurons with similar AHPs (range, 70-91 msec) in a sham (gray) and a trained cat (black). Right, Histograms of the mean amplitude of responses evoked by CCS (***a***), MPL (***b***), and SP (***c***) afferents recorded in all MG motoneurons in shams (gray) and trained cats (black). Six of the nine pathways tested in MG motoneurons were modified by training. Significant differences are indicated as follows: $^{*}p<0.05$, $^{**}p<0.01$. Overall, training decreased both CCS-MG-R1 (by 47%) and CCS-MG-R3 (by 61%) (***a***) amplitude, increased MPL-MG-R1 amplitude (by 89%), decreased MPL-MG-R2 (by 54%) and MPL-MG-R3 (by 46%) (***b***) amplitude, and decreased SP-MG-R2 amplitude (by 67%) (***c***). Mn, Motoneuron.

Locomotion versus rest in sham and trained cats

As shown in other pathways (Conway et al 1987, Gossard et al 1994, McCrea et al 1995), modification in cutaneous transmission could emerge only during the operation of locomotor networks. We therefore evaluated state-dependent changes in the transmission of cutaneous pathways by comparing responses recorded at rest and during fictive locomotion both in shams and in trained cats. Intracellular recordings during locomotor episodes (either spontaneous or after perineal stimulation) and rest were performed in five extensor (13 MG, 10 FHL, 10 SmAB, 6 LGS) and one flexor (10 EDL) motor pools of six shams and seven trained cats. Among all possible afferent-motoneuron pairs, we succeeded in testing 38 of 45 pathways both in shams and in trained cats.

Table 3*a* depicts the effect of locomotion obtained without drugs on cutaneous responses evoked either by CCS, MPL, or SP in various motor pools in shams and trained cats. More pathways were modified during locomotion in shams (13 of 38) compared with trained cats (4 of 38; $p<0.05$). The amplitude of most of these 17 responses decreased during locomotor episodes (10 of 13 shams, 3 of 4 trained), except for MPL-MG-R1, SP-MG-R1, and CCS-EDL-R3 in shams and CCS-MG-R2 in trained cats. Again, change in transmission from cutaneous afferents to MG motoneurons was the major modification caused by locomotion (6 of 13 shams, 2 of 4 trained). Note that R2 amplitude was rarely modified by locomotor networks in shams (2 of 13) compared with trained cats (3 of 4). Altered transmission in pathways originating from CCS afferents was the major modification in shams (6 of 13), whereas those originating from CCS (2 of 4) and SP (2 of 4) afferents exhibited the largest changes in trained cats.

Effect of training during fictive locomotor episodes

We also assessed the effect of training by comparing responses obtained during locomotor episodes in two to five shams and two to nine trained cats depending on pathways. We first compared the overall amplitude evoked by a given pathway during entire locomotor episodes in shams or trained cats. Of the 31 pathways tested during locomotor episodes, training reduced transmission in only two: CCS-MG-R1 and SP-MG-R1 (data not shown).

Table 3. State-dependent (locomotion vs rest) changes in mean amplitude of responses in specific cutaneous pathways

a no drug

		Nb of trials	CCS	MPL	SP
Sham	FHL	10			
	LGS	7-8	R1 ▼63%		
	MG	8-16	R1 ▼58% R3 ▼85%	R1 ▲612% R2 ▼82%	R1 ▲580% R3 ▼90%
	SmAB	8-13	R1 ▼48% R3 ▼88%	R3 ▼57%	
	EDL	6-8	R3 ▲310%		R2 ▼74% R3 ▼76%
Trained	FHL	9-17			R2 ▼67%
	LGS	10-12			
	MG	10-15	R1 ▼64% R2 ▲127%		
	SmAB	11			
	EDL	17-20			R2 ▼81%

b with clonidine

		Nb of trials	CCS	MPL	SP
Sham	FHL	4			
	MG	5-6			
	EDL	4			
Trained	FHL	7-8			R2 ▲181%
	LGS	4			
	MG	5-10	R2 ▲100%		R1 ▼67%
	SmAB	6			
	EDL	8-9			R3 ▼87%

Same display as in Table 2. The effect of locomotion is reported according to the afferents (CCS, MPL, and SP) and motoneurons (FHL, LGS, MG, SmAB, and EDL) without drug (***a***) and after clonidine injection (***b***). ***a***, Pathways (R1, R2, or R3) significantly modified (17 of 38 tested pathways) with no drug are represented as filled circles in shams and as empty circles in trained cats. ***b***, Pathways significantly modified (4 of 26) after clonidine are only in trained cats and are represented as empty circles. The percentage is calculated by comparing the mean amplitude recorded during locomotion to the one recorded at rest. The number of trials for each pathway in the corresponding motor pool is shown in the second column.

We then analyzed whether training modified the phase-dependent modulation pattern of cutaneous transmission. Transmission through cutaneous reflexes is modulated in a phase-dependent manner during the locomotor step cycle in intact cats (Forssberg et al 1975, Drew & Rossignol 1987) as well as during fictive stepping in immobilized, decerebrate semichronic (LaBella et al 1992) and chronic (Forssberg et al 1975, 1977, Andersson et al 1978) spinal cats. Overall, we found that, when significantly modulated during the fictive step cycle, SP-EPSPs and CCS-EPSPs (R1) were of maximal amplitude

during flexion in a majority of cases both in flexors/bifunctional (5 of 6) and in extensors (20 of 23), whereas MPL-EPSPs (R1) were of maximal amplitude in extension both in flexors/bifunctional (9 of 9) and in extensors (2 of 4). Moreover, contrary to previous reports (Andersson et al 1978, Schomburg & Behrends 1978a), the maximum amplitude of SP-IPSPs, MPL-IPSPs, and CCS-IPSPs (R2) occurred during the extension phase in extensors (39 of 44) and during the flexion phase in flexors/bifunctional motoneurons (6 of 6). For example, in Figure 4 we superimposed the averaged responses to CCS stimulation in a MG motoneurons evoked during the depolarized (gray traces) and the hyperpolarized (black traces) phases of the fictive step cycle in a sham (Fig.4*a*) and in a trained cat (Fig.4*b*). The amplitude of the IPSP in both cases was increased during the depolarized phase. As mentioned above, the IPSP amplitude (R2) could depend on variations in membrane potentials. Figure 4*c* shows the R2 amplitude plotted against the locomotor-drive potential (LDP) amplitude for all motoneurons, and there was no linear relationship between these two values neither in shams (r=0.41) nor in trained cats (r=0.48). We also found that training

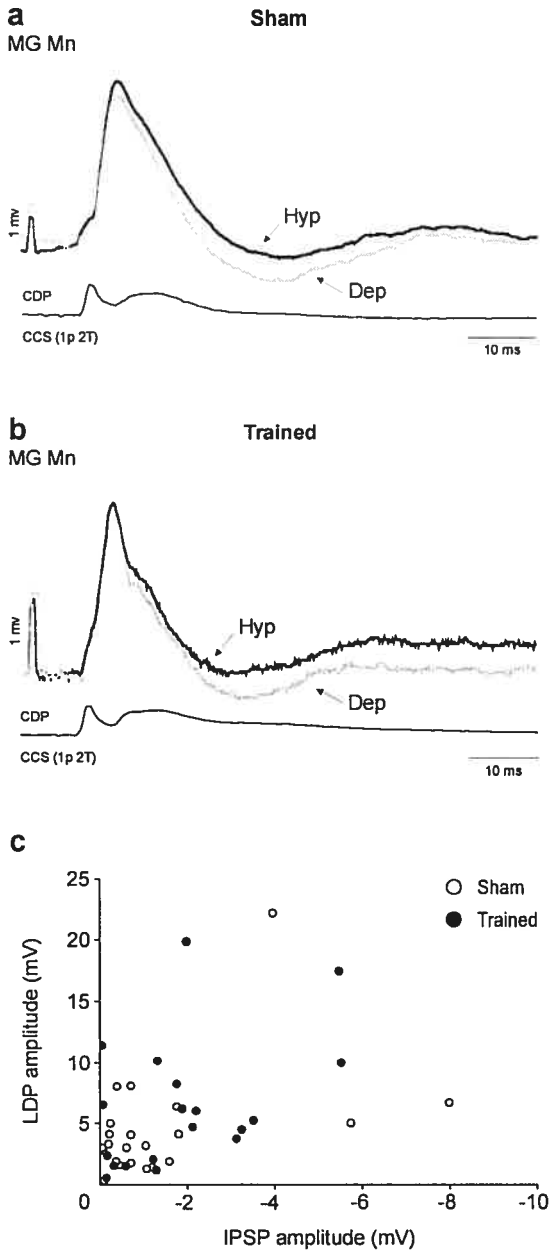


Figure 4. Training did not modify the phase dependency of cutaneous responses. *a,b,* PSPs recorded in MG motoneurons evoked by CCS stimuli during the depolarized (gray traces) and hyperpolarized (black traces) phases of the fictive step cycle in the sham (***a***) and the trained cat (***b***). The amplitude of the IPSP was decreased in the sham (by 50%) and in the trained cat (by 92%) during the hyperpolarized phase. ***c***, IPSP amplitude was plotted against the LDP for all tested motoneurons in shams (gray) and trained cats (black). No linear relationship was observed between the two values neither in shams (r=0.41) nor trained cats (r=0.48). Mn, Motoneuron.

did not significantly modify the peak-to-peak amplitude of LDPs (data not shown). Overall, there were no significant differences in the ratio of cells significantly modulated, in the phase-dependency patterns or in the depth of modulation between shams and trained cats.

Effect of clonidine

Clonidine is an α_2-noradrenergic agonist known to improve the initiation and modulation of locomotor patterns, to accelerate locomotor recovery in chronic spinal cats (Barbeau et al 1987, Barbeau & Rossignol 1991, Chau et al 1998a), and to facilitate the emergence of fictive walking patterns (Forssberg & Grillner 1973, Pearson & Rossignol 1991). Moreover, reduced spasticity and facilitation of locomotor recovery was seen in two SCI subjects after clonidine and cyproheptadine (serotoninergic antagonist) treatment (Fung et al 1990). Clonidine also reduced spasticity dramatically in eight SCI subjects and improved gait patterns in three subjects (Rémy-Néris et al 1999). Overall, two to five shams and two to eight trained cats were used to test the effect of clonidine on pathways ending on the different motor pools. In most cases, we compared responses from motoneurons recorded before drug injection with those from motoneurons recorded after injection. However, we succeeded in maintaining intracellular recordings of seven motoneurons while injecting clonidine and had the opportunity to follow changes in cutaneous responses. In Figure 5, we compared responses in five of these motoneurons (superimposed traces) obtained before (gray) and after (black) clonidine injection. The mean amplitude of the overall population of motoneurons (before and after injection) is reported as histograms. For example, SP-FHL-R2 amplitude was decreased by clonidine in an FHL motoneuron in a sham cat (Fig.5*a*, traces), and MPL-FHL-R2 amplitude was also decreased in an FHL motoneuron in a trained cat (Fig.5*b*, traces). The membrane potential in the same motoneuron did not change after clonidine injection. Note that the decrease in R2 amplitude was also significant in the overall FHL population of shams (Fig. 5*a*, histogram) and trained cats (Fig.5*b*, histogram). There was also an increase in MPL-FHL-R1 amplitude attributable to clonidine in FHL motoneurons of shams (Fig.5*a*, histograms). The effects of clonidine on SP-, MPL-, and CCS-evoked PSPs are also illustrated in one MG motoneuron of a trained cat (Fig.5*c-e*). Only SP-MG-R2 amplitude was significantly reduced by clonidine in this cell, as well as in the overall population (Fig.5*c*).

Table 4 depicts the effect of clonidine on cutaneous responses. Among all the afferent-motoneuron pairs, we succeeded to test 65 of 72. Of the 65 pathways tested at rest, five

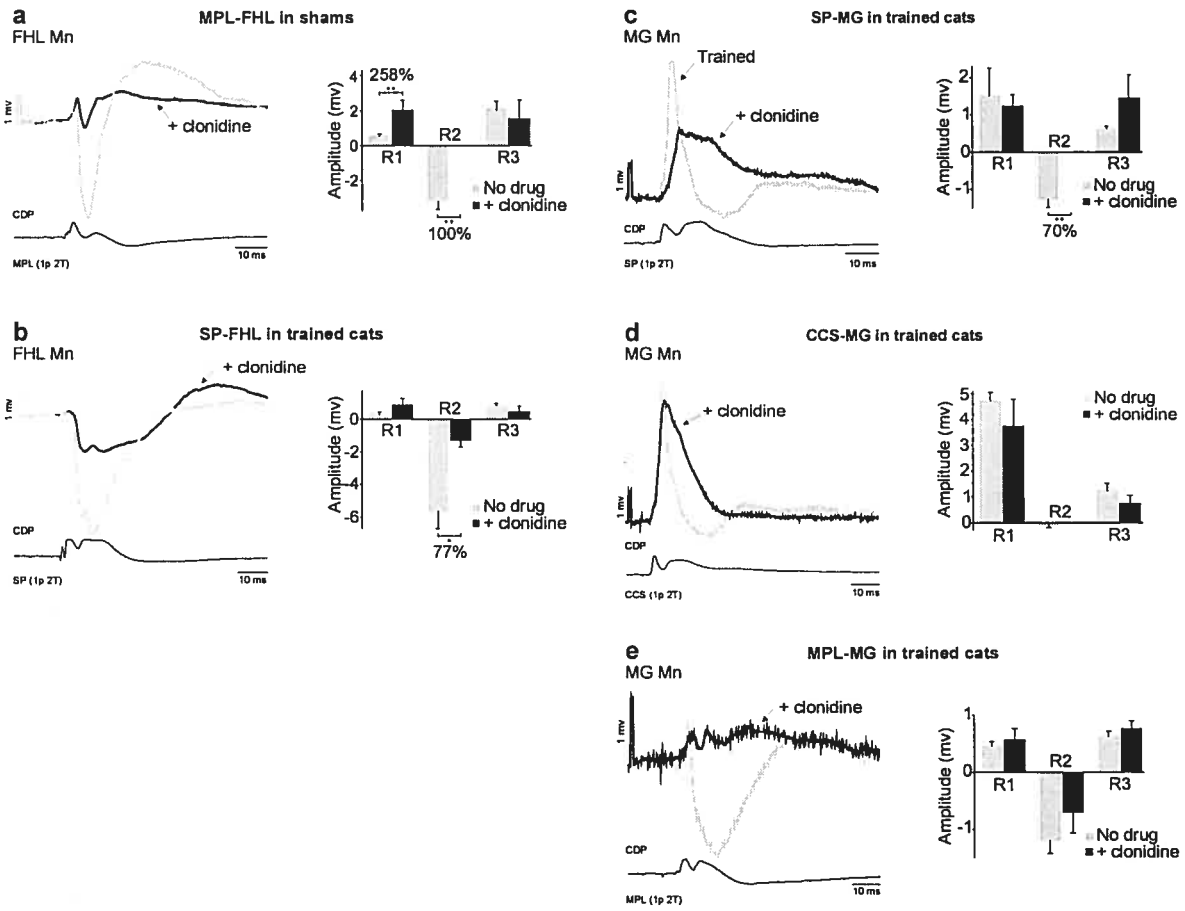
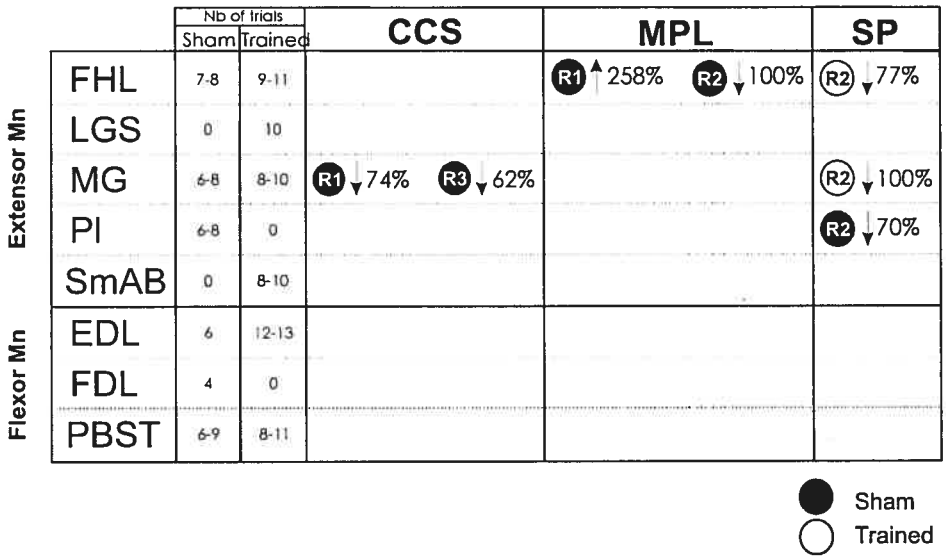


Figure 5. Clonidine specifically modified transmission from cutaneous afferents to extensor motor pools. Left, PSPs evoked in the same motoneuron before (gray) and after (black) clonidine injection by the stimulation of SP (***b,c***), MPL (***a,e***), and CCS (***d***) in FHL (***a,b***) and MG (***c-e***) motoneurons of shams (***a***) and trained cats (***b-e***). Right, Histograms of the mean amplitude of responses recorded in all motoneurons in shams (gray) and trained cats (black). Significant differences are indicated as follows: *p<0.05, **p<0.01. Overall, clonidine increased MPL-FHL-R1 (by 258%) and decreased MPL-FHL-R2 (by 100%) amplitude in shams (***a***), decreased SP-FHL-R2 amplitude in trained cats (by 77%) (***b***), decreased SP-MG-R2 amplitude in trained cats (by 100%) (***c***), did not modify amplitude in CCS-MG pathways in trained cats (***d***), and did not modify amplitude in MPL-MG pathways in trained cats (***e***). Mn, Motoneuron.

were significantly modified by clonidine in shams and two were significantly modified by clonidine in trained cats. The number of modified pathways in shams (two of seven) was not significantly different from the number of pathways in trained cats (five of seven). Overall, in shams, MPL-FHL-R1 amplitude was increased (Fig.5*a*), and the amplitude of the following pathways was decreased: MPL-FHL-R2 (Fig.5*a*), CCS-MG-R1, CCS-MG-R3, and SP-PI-R2. Overall, in trained cats, the amplitude of SP-FHL-R2 and SP-MG-R2 was decreased (Fig.5*a,c*). Interestingly, only pathways activated by SP afferents were significantly modified by clonidine in trained cats.

Table 4. Effect of clonidine on the mean amplitude of responses in specific cutaneous pathways



Same display as in Table 2. The effect of clonidine is reported according to the afferents (CCS, MPL, and SP) and motoneurons (FHL, LGS, MG, PI, SmAB, EDL, FDL, and PBSt). Pathways (R1, R2, or R3) significantly modified by clonidine (7 of 65 tested pathways) are represented as filled circles in shams and as empty circles in trained cats. The percentage is calculated by comparing the mean amplitude obtained after clonidine to the one obtained before clonidine. The number of trials for each pathway in the corresponding motor pool is reported in the second (sham) and third (trained) columns. A zero indicates that there was not enough data to perform statistical tests in that group.

In contrast to training, clonidine more powerfully modified transmission in R2 pathways (four of seven). Clonidine was more likely to act on R2 not only to diminish its amplitude but also to reduce it to zero. Moreover, with clonidine there was significantly less occurrence of inhibition in two of four pathways: MPL-FHL in shams ($p<0.05$) and SP-MG in trained cats ($p<0.01$). No significant modification was observed in flexor/bifunctional motor pools of either sham or trained cats. Although clonidine and training both had a major influence on pathways to MG motoneurons (three of seven modified pathways), clonidine produced a supplementary effect on transmission in pathways to FHL motoneurons (three of seven).

We also assessed the additional effects of clonidine on state-dependent changes in the transmission of cutaneous pathways by comparing responses obtained at rest and during fictive locomotion both in shams and in trained cats (Table 3b). Among all the possible afferent-motoneuron pairs, we succeeded in testing 14 in shams and 26 in trained cats. Additional locomotor-dependent changes attributable to clonidine were observed solely in

4 of 26 pathways in trained cats (Table 3b) and mostly for pathways from SP (2 of 3). Again, changes in transmission to MG motoneurons were the most frequent (two of three). Thus, once locomotor networks are activated (Table 3a) (17 modified pathways), the effect of clonidine per se is minimal (4 modified pathways). We finally assessed the effect of clonidine on state-dependent changes in shams and trained cats that displayed locomotor episodes before and after clonidine injection. Of the 23 pathways tested, clonidine significantly decreased inhibitory transmission in only one pathway in shams: CCS-MG-R2.

Discussion

Plasticity in spinal pathways

It is now recognized that reflex pathways exhibit plasticity in response to central or peripheral lesions or operant conditioning (Mendell 1984, Durkovic 1996, Wolpaw & Tennissen 2001). Recovery of function may occur after spinal lesion; however, the role of plastic reflex pathways underlying the recovery remains to be defined.

The preparation in this study (i.e., acute experiments in curarized animals) allows stable intracellular recordings of responses in motoneurons that are not influenced by rhythmic sensory feedback. The effect of training can therefore be attributed to changes occurring in spinal pathways and not to alterations in peripheral sensory events or muscle properties (Roy & Acosta 1986, Roy et al 1999). Changes in cutaneous reflexes have been previously attributed to specific alterations in premotoneuronal mechanisms and not to changes in passive membrane properties of motoneurons between acute and chronic spinal cats (Chandler et al 1984, Munson et al 1986, Baker & Chandler 1987a). Moreover, modified properties of motoneurons could not explain increased monosynaptic reflexes in chronic spinal animals (Hochman & McCrea 1994b). In this study, training did not modify AHP duration, which varies with input resistance and membrane time constant (Gustafsson & Pinter 1984b, Côté et al 2003a). Moreover, a general change in membrane responsiveness is unlikely to explain the simultaneous increase in R1 amplitude and decrease in R3 amplitude observed in the same motoneuron (Table 2). We therefore consider that most of the plasticity after step training resulted from interneuronal mechanisms. However, a complete investigation of motoneuronal properties after chronic spinalization and step training is clearly needed to understand their contribution to the

plasticity of sensory transmission. Latencies of cutaneous responses in hindlimb motoneurons are minimally trisynaptic (Lundberg et al 1977, Baker & Chandler 1987b, Fleshman et al 1988, LaBella et al 1989, LaBella & McCrea 1990), and such linkage is appropriate for R1 responses in this study. There is an exceptional disynaptic linkage between SP and FDL motoneurons during the depolarized phase of fictive stepping in decerebrate cats (cf. Burke 1999). Later responses (R2 and R3) most probably involve longer chains of spinal interneurons, the exact number of which is precarious to estimate.

Previous work suggested that removal of cutaneous inputs does not exert an important effect on rhythm generation in intact quadrupeds because they may use alternative inputs to compensate (Sherrington 1910, Forssberg et al 1977, Duysens & Stein 1978). However, in spinal cats, at least one source of cutaneous information is necessary to recover proper foot placement during stepping (Bouyer & Rossignol 2003ab). In this study, we present experimental evidence that step training induced robust plasticity in the transmission of particular cutaneous pathways that was apparent at rest, without neuromodulators or locomotor network configuration. Moreover, the results show that the addition of clonidine or the activity of locomotor networks induced additional changes in cutaneous transmission, most of them in shams (see above). This study therefore supports the idea that cutaneous feedback from the hindlimbs is important for the full expression of locomotion after spinal transection.

Cutaneous transmission was modified by training only in a few specific pathways (10 of 71), transmission being decreased in the majority (7 of 10). The changes observed may represent training-related plasticity superimposed on spinalization-induced plasticity or a removal or prevention of spinalization-induced plasticity. For example, there is a persistent hyperexcitability of several reflexes following SCI because of the removal of inhibitory descending inputs from the brainstem (Hultborn & Malmsten 1983, Holmqvist & Lundberg 1961, Lundberg 1964). A recent study showed that some flexor reflex components mediated by thick sensory afferents increased permanently their excitability after SCI in rats (Malmsten 1983, Valero-Cabré et al 2004). A similar hyperexcitability seen in withdrawal reflexes would contribute to spasms and spasticity (Bennett et al 1999, Rémy-Néris et al 1999, Ashby & McCrea 1987). Thus, because chronic spinalization induces an enhanced cutaneous reflex responsiveness, step training may compensate for this by normalizing the level of cutaneous transmission. In such a case, the comparison between trained and untrained spinal cats would show up as a decrease in cutaneous transmission.

Plasticity in cutaneous pathways is highly specific

Training alone evoked modifications in 10 of 71 pathways involving three of three tested cutaneous nerves and four of eight motor other pathways between the two groups was far from reaching statistical significant difference (54 of 61; $p>0.1$). We therefore believe that these changes represent a true task-specific plasticity in cutaneous transmission (de Leon et al 1998a, 1999b). It is not known whether longer periods of step training or a larger sample would have revealed changes in additional pathways. Nerve-specific reflex responses to cutaneous stimulation were previously observed during locomotion in cats (Abraham et al 1985, Moschovakis et al 1991, Pratt et al 1991, LaBella et al 1992, Degtyarenko et al 1996) and humans (van Wezel et al 1997) to provide location-specific information from the skin of the foot. However, a common synergy of flexor responses in the swing phase and of extensor responses in the stance phase was also observed, independent of the location of the stimulus in cats walking on a treadmill (Duysens & Loeb 1980, Abraham et al 1985, Duysens & Stein 1978). Thus, during locomotion, both common and nerve-specific controls of cutaneous reflex responses were observed. Our results did show specificity but no simple swing- or stance-related patterns during fictive stepping.

Clonidine markedly reduces cutaneous excitability while accelerating the recovery of stepping in spinal cats (Barbeau et al 1987, Chau et al 1998ab). Here clonidine was not used during training but only during the acute experiment to facilitate the emergence of fictive stepping (Pearson & Rossignol 1991). As expected, when significantly modified by clonidine, transmission in cutaneous pathways was decreased in all cases but one (Table 4). It is remarkable that clonidine modified pathways mostly in shams (five of seven). This is surprising because we expected training to favor the responsiveness of spinal networks to clonidine (Côté et al 2003a). An alternative interpretation for these findings is that both training and clonidine target the same pathways so that clonidine only added a small effect because training already decreased cutaneous transmission. We may further speculate that some cutaneous reflexes are detrimental to recovery of stepping and that clonidine or step training is a different mean to normalize the cutaneous feedback onto spinal locomotor networks.

Recent studies (Edgerton et al 2001, Tillakaratne et al 2002) reported that a complete spinal section in cats increased levels of GABA and glycine in spinal tissues and that step training (but not stand training) decreased these levels toward normal values. Our results

showed that only 2 of 10 (Table 2, R2) inhibitory cutaneous pathways were modified by step training, so global changes in inhibitory systems may be affecting other pathways.

Possible role of modified cutaneous transmission

A major finding is that training predominantly modified transmission in pathways to MG motoneurons, particularly when activated by MPL afferents. MG is an ankle extensor muscle involved in weight support during stance. The plantar surface of the foot (MPL receptive field) presumably provides phasic information about the ground surface. Previous studies show that extensor reflexes evoked by the plantar surface of the foot can promote extension during stance and stop the swing phase of locomotion (Duysens & Pearson 1976, Duysens 1977, Guertin et al 1995). Our results indicate that both training (Table 2) and fictive locomotion in shams (Table 3a) result in a net excitatory action from MPL to MG motor pool. Thus, we suggest that at least some of the modified pathways would result in a better recruitment of the MG pool during ground contact that may help to recover weight-bearing.

Our study also showed there was no remarkable change in the distribution of types of motoneuronal responses after training. Step training is therefore upregulating or downregulating transmission in existing pathways. However, a significant change in transmission to FDL motoneurons was observed (all cutaneous nerves pooled together): going from type A to type D response (i.e., without inhibition, R2). FDL is a toe plantar flexor active just at the onset of swing (Fleshman et al 1984, Schmidt et al 1988, Moschovakis et al 1991, Degtyarenko et al 1996) to clear the toes from the ground (as St burst) (Rossignol 1996). We suggest that inhibitory transmission to FDL is decreased in general to facilitate the excitatory effects of cutaneous signals related to paw drag during early training sessions. Together with the increase in transmission in the MPL-PBSt-R3 excitatory pathway, this would result in a better clearing of the toes at the onset of swing.

However, it is difficult to interpret the exact role of modified cutaneous transmission in the control or recovery of locomotion, because there is little understanding of its role in normal locomotion. For example, some receptors from the plantar surface of the foot were found to fire during swing and to be silent during stance (Loeb et al 1977). Thus, the influence of cutaneous inputs on spinal pathways in a particular phase of stepping cannot be predicted solely based on its anatomical localization.

Load or skin?

Proprioceptive inputs can act directly on the CPG, particularly those evoked by group I afferents from extensors (Conway et al 1987, Gossard et al 1994, McCrea 1998, Pearson et al 1998). Our recent experiments demonstrated that step training decreased group IB inhibition and increased polysynaptic group I excitation of extensors after clonidine injection in spinal cats, suggesting a better recruitment of antigravity muscles to assist the recovery of weight-bearing (Côté et al 2003a). Cutaneous inputs do not have such a powerful action on rhythm generation, and we therefore expected less plasticity in these pathways than in proprioceptive pathways. However, significant changes in cutaneous transmission could be seen even without clonidine or fictive locomotion. The plasticity in cutaneous pathways was therefore more robust than in group I pathways studied in the same animals (Côté et al 2003a). The relative contribution of different sensory modalities for the recovery of stepping after SCI is still unclear. This knowledge is necessary to focus and improve training strategies in SCIs (Dietz et al 2002, Ferris et al 2004).

Acknowledgments

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PUBLICATION #3: LESION-INDUCED ERK ACTIVATION IS DECREASED FOLLOWING STEP-TRAINING IN CHRONIC SPINAL CATS

Côté MP, Gossard JP and Kennedy TE (2006) Lesion-induced ERK activation is decreased following step-training in chronic spinal cats. To be submitted.

Abstract

Step-training has been shown to enhance locomotor pattern recovery after spinal cord injury (SCI). Plasticity in spinal networks that impact on walking ability involves several neurotransmitters including monoamines, GABA, glycine, and glutamate. However, the precise mechanisms and transduction pathways through which step-training promotes synaptic plasticity and translates into functional changes are not well understood. It has become evident that spinal networks can be affected by activity-dependent processes that influence the ability to recover, perform and maintain an adequate locomotor pattern. Recent investigations address the possibility that molecules involved in forms synaptic plasticity, such as long-term potentiation in the hippocampus, might also be implicated in spinal motor *learning*.

Brain-derived neurotrophic factor (BDNF) has recently emerged as a critical modulator of synaptic plasticity. BDNF-induced ERK activation may facilitate the expression of genes essential for short and long-term functional changes. Moreover, ERK activation is also regulated by glutamate through NMDA receptors to facilitate synaptic efficacy. Importantly, the glutamatergic system is important in mediating locomotion in chronic spinal cats. Given the predominant effect of ERK on synaptic plasticity and function, we hypothesized that ERK might mediate the beneficial effects of step-training and participate in the synaptic events associated with locomotor recovery after SCI.

To evaluate potential modulation of ERK activation, Western blots assessing the expression of ERK1/2 and pERK1/2 in spinal segments (L3-L7) were run and results compared between intact, spinal (1 or 3 months after a complete SCI) and trained (1 or 3 months after SCI and step-training onset). We report that ERK activation is up-regulated following chronic SCI and down-regulated by step-training in SCI cats.

Introduction

Gait is frequently reported to be impaired in individuals with a spinal cord injury (SCI). The beneficial effect of step-training over a treadmill belt on locomotor pattern recovery is now well established in both animals and humans (Lovely et al 1986, Barbeau & Rossignol 1987, Wernig et al 1995, Harkema et al 1997, de Leon et al 1998b, Harkema, 2001). Plasticity in the lumbar spinal networks that impact on walking ability involves both excitatory and inhibitory neurotransmitter systems: monoaminergic (reviewed in Rossignol et al 2001), GABAergic (Robinson & Goldberger 1986, Tillakaratne et al 2000, Bravo et al 2003), glycinergic (Hart 1971, de Leon et al 1999, Edgerton et al 2001), glutamatergic (Giroux et al 2003) and probably others. However, the precise mechanisms and signal transduction pathways through which step-training promotes synaptic plasticity and translates into functional changes are not well understood. Nevertheless, it has become evident that spinal circuits can be affected by activity-dependent processes that influence the ability to recover, perform and maintain an adequate locomotor pattern (Wolpaw & Tennissen 2001, Dobkin & Havton 2004, Edgerton et al 2004). Recent investigations have begun to address the possibility that molecules involved in forms of CNS synaptic plasticity, such as long-term potentiation (LTP) in the hippocampus, might also be implicated in spinal motor *learning*.

Brain-derived neurotrophic factor (BDNF), initially described for its important role during development, regulating the survival, growth and differentiation of immature neurons (Barde 1994), has emerged as a critical modulator of synaptic plasticity in the brain (Lo 1995, 1998, Patterson et al 2001). Importantly, it has also been shown to promote recovery after SCI *in vivo* by enhancing neuroprotection (Yan et al 1992, 1994), regeneration (Tuszynski et al 1994, Kishino et al 1997) and locomotor recovery (Jakeman et al 1998). The key role of BDNF and TrkB in activity-dependent synaptic plasticity is strongly supported by experiments in which their expression is up-regulated in the hippocampus, cerebral cortex, cerebellum, spinal cord and muscles of intact animals by various locomotor training paradigms (Neeper et al 1995, Gomez-Pinilla et al 2001, 2002, Molteni et al 2002, Hutchinson et al 2004, Klintsova et al 2004). BDNF was also shown to be down-regulated following incomplete and complete SCI. Notably, step-training was shown to either prevent this decrease, or in fact, increase BDNF expression (Gomez-Pinilla et al 2004, Ying et al 2005). BDNF activates ERK in neurons in brain and spinal cord (Marsh & Palfrey 1996, Becker et al 1998, Bonni et al 1999, Hetman et al 1999, Pezet

et al 2002) and this activation promote synaptic plasticity (Sweatt 2004, Ji 2004). Indeed, ERK activation leads to the phosphorylation of key membrane receptors and the expression of gene products essential for the short and long-term functional changes in spinal sensory neurons (Ji & Woolf 2001, Kolch 2000). ERK has also been shown to be required for long-term facilitation of excitatory transmission between sensory neurons and motoneurons *in vitro* (Martin et al 1997). In the spinal cord, ERK activation is regulated both by BDNF (Jovanovic et al 1996, Ying et al 2002) and also by glutamate via a well established interaction with NMDA receptors (Platenik et al 2000) ultimately contributing to facilitation of synaptic efficacy (Garraway et al 2003, Slack et al 2004). Notably, the glutamatergic system is important in mediating locomotion in chronic spinal cats (Chau et al 2002, Giroux et al 2003). Given the predominant effect of ERK on synaptic plasticity and function and its role in integrating signals from the cell surface to transcription factors, we hypothesized that ERK might mediate the beneficial effects of step-training and participate in the synaptic events associated with locomotor recovery after SCI.

The spinal cord undergoes major biochemical and functional reorganization following SCI (de Leon et al 1999, Edgerton et al 1997b, Rossignol et al 2001, Tillakaratne et al 2000, 2002) involving at least two possible activators of ERK, BDNF and glutamate (Gomez-Pinilla et al 2001, 2004, Ying et al 2005). For example, SCI has been documented to result in glutamate release (Nesic et al 2002) and up-regulation of NMDA receptor gene expression (Grossman et al 2000, Rossignol et al 2004). Moreover, an excessive release of glutamate has been implicated in neuronal death associated with SCI and excitatory amino acid antagonists protect against deficits associated with SCI and loss of gray and white matter (Wrathall et al 1997, Beattie et al 2002, Gomez-Pinilla et al 1989).

Together, these results suggest that ERK activation may not only be involved in plasticity following a spinal lesion but also following step-training. However, the effect of a complete SCI on ERK activation has not been previously reported in the adult cat. We thus considered that investigating the occurrence of lesion-dependent plasticity in the lumbar spinal cord to be a critical step in accurately assessing the effect of step-training on ERK activation in a spinal cord that has been altered by the loss of supraspinal afferents.

To evaluate potential modulation of ERK activation, Western blots assessing the expression of total ERK1/2 and pERK1/2 (activated phosphorylated form) in spinal segments L3 to L7 were run and results compared between different groups of cats: intact, spinal (1 or 3 months after a complete spinal lesion) and trained (1 or 3 months after the

lesion and step-training onset). We report that ERK activation is up-regulated following chronic injury and down-regulated by long-term step-training in completely spinalized cats.

Material and Methods

All procedures were conducted according to the *Guide for Care and Use of Experimental Animals* (Canada), using protocols approved by the Ethics Committee of Université de Montréal.

Experimental groups. Twenty three adult female cats (2.5-5.2kg) were used for this study divided in 5 groups: a) a first group was spinalized and step-trained over a treadmill belt until weight-bearing by the hindlimbs without assistance (1-month-trained, n=4, μ=29d); b) a second group was spinalized, and step-training was maintained for a longer period in order to strengthen weight-bearing and locomotor pattern (3-months-trained, n=6, μ=84d); c) a third group was spinalized and the terminal experiment took place at an equivalent period of time after spinalization as group A and served as control to assess the effect of 1 month of step-training (1-month-spinal, n=3, μ=30d); d) a fourth group was spinalized and the terminal experiment took place at an equivalent period after spinalization as group b and will serve as control to assess the effect of 3 months of step-training (3-months-spinal, n=6, μ=86d); e) the fifth group is composed of intact cats which served as control to assess the effect of spinal transection (intact, n=4). But for the intact group, all cats were previously used in acute electrophysiological experiments (see Côté et al 2003a, Côté & Gossard 2004).

Spinalization. Following administration of preoperative medication, the cats were anaesthetized (isoflurane, 2%; Abbott Laboratories; Montreal, Canada), a small laminectomy was performed at T_{13} under aseptic conditions. The dura was incised, fold apart and lidocaine hydrochloride (xylocaine 2%; Astra Zeneca, Mississauga, Canada) was applied topically on the area of spinal cord to be transected. The spinal cord was completely transected with a pair of surgical scissors so that the spinal canal could be clearly visualized. The space between the rostral and caudal ends of the spinal cord was filled with absorbable hemostat (Surgicel; Ethicon, Somerville, NJ) helping local hemostasis, blood coagulation and preventing any regrowth of the spinal cord. Back muscles and skin were then sutured in layers. Subsequent postoperative care was analogous to previously described (Chau et al 1998a). A patch of fentanyl (Duragesic 25

μg; Janssen-Ortho; Markham, Canada) was sutured on the back of the cat for continuous and stable delivery of analgesic over a two-day period.

Step-training. Training on the treadmill (0.2-0.4m/s) started 2 days after surgery and consisted of 2-4 daily training sessions for periods of 10 minutes. Early in the recovery, the hindquarters were supported by the experimenter to provide weight support and perineal stimulation was used to induce and maintain locomotor movements. Over days, the animal became gradually able to walk and support its hindquarters and perineal stimulation was no longer needed. The training was stopped when the cat was able to walk continuously on the treadmill for more than five minutes while the experimenter assisted only for lateral stability by holding the tail for the 1-month-trained group whereas the training regimen was continued for two more months after these criteria were reached for the 3 month trained group. This allowed 2 additional months of step-related sensory feedback and weight-bearing to stimulate the spinal cord networks. No drugs were used to assist locomotor training during sessions, but clonidine was used during the acute electrophysiological experiments prior to tissue collection (Côté et al 2003a, Côté & Gossard 2004).

Tissue processing. Cats were killed by an overdose of sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Canada) and the spinal cord was removed, dissected and transversely sectioned according to lumbar segments (≈10mm, L3 to L7) for subsequent regional analysis. Spinal cord tissues were rapidly frozen by immersion in 2-methylbutane and stored at −80°C until further processing. Every spinal cord segment was embedded in optimal cutting temperature (O.C.T.) compound (Tissue Tek O.C.T.; Sakura Finetek, Torrance, CA) and 20 μm cryostat sections of the spinal cord were performed, homogenized in lysis buffer (RIPA; 150 mM NaCl, 1% NP-40, 0.5% deoxycholate sodium, 0.1% SDS, 10 mM Tris–HCl pH 8.0) and manually ground with a Teflon-in-glass homogenizer. The total protein concentration of homogenates was quantified using BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard. Spinal cord homogenates were then prepared in 4x Laemmli sample buffer (0.24 M Tris–HCl pH 6.8, 8% SDS, 3% β-mercaptoethanol, 20% glycerol, 30.8mM dithiothreitol (DTT), bromophenol blue), denatured at 95 °C for 5 minutes and clarified by centrifugation at 13,000g for 2 minutes.

Western blotting. The following antibodies were used for Western blot analysis: monoclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) (1:2000; Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-p44/42 MAP kinase (1:1000; Cell

Signaling Technology), rabbit polyclonal anti-Akt (1:1000; Cell Signaling Technology), rabbit polyclonal anti-CREB (1:1000; Cell Signaling Technology) and monoclonal anti-synaptophysin (1:4000,Sigma-Aldrich, Mississauga, Ontario, Canada). Briefly, the equivalent of 10µg of total protein was separated on a 8-12% acrylamide resolving gel (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). After a brief wash in 0.1% Tween-20 in Tris-buffered saline (TBST), membranes were blocked for 1–3h at RT in TBST with 5% non-fat dry milk and incubated with primary antibodies overnight at 4°C. The blots were then extensively washed with TBST and incubated for 1h at room temperature with an HRP-conjugated donkey anti-rabbit (1:5000) or donkey anti-mouse secondary antibody for 1h at RT (1:7500; Jackson Immunoresearch, West Grove, PA). Reactive bands were visualized using Chemiluminescence Reagent Plus protein kit (Perkin Elmer, Boston, MA) and optical density quantified on scanned images of immunoblots using Adobe Photoshop software (Adobe Systems, San Jose, CA). Molecular weights were estimated using broad range prestained molecular weight markers (New England BioLabs).

Statistical analysis. Results are presented as the mean ± SEM. Statistical analysis was carried out using one-way ANOVA to disclose differences between mean optical density obtained for different groups: 1) spinal vs. intact group (lesion dependent-plasticity); 2) spinal and step-trained group (training dependent-plasticity) and 3) intact and step-trained group (combined effect of spinal lesion and training). The Kolmogorov-Smirnov-Liliefors test was used to compare the shape and location of the distribution of responses to a normal distribution and the Levene median test was used to examine for equal variance. If these two tests confirmed that the sample variables did fit a normal distribution and were equally variant, a one way analysis of variance (ANOVA) was performed. If not, the nonparametric Kruskal-Wallis one-way analysis of variance was performed with Dunn's *post-hoc* multiple comparison test. In all tests, the α level for statistical significance was set at $p<0.05$. In histograms, significant differences are illustrated by * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

Results

Lesion-induced plasticity of ERK activation

Antibodies that specifically recognize the phosphorylated active form of ERK1/2 (pERK) on Western blots provide a straight forward method to assess the relative levels of ERK activation in tissue homogenates and have been extensively used as a criterion to judge the degree of activation of the ERK pathway. In the present study, we used a complete spinal cat model to determine if ERK signaling was modulated 1 and 3 months after a complete thoracic (T13) spinal lesion. Western blot analyses of protein homogenates derived from spinal lumbar segments L3 to L7 were carried out and results for intact and completely spinalized animals compared. Figure 1 illustrates 44kDa and 42kDa immunoreactive bands detected on a Western blot incubated with an antibody recognizing endogenous p44 and p42 MAPK dually phosphorylated at threonine 202 and tyrosine 204 (pERK1 and pERK2). Protein homogenates on this blot were derived from an intact, a spinal and a step-trained cat, 1 month (Fig.1a) or 3 months (Fig.1b) after spinalization and/or onset of step-training. Increased optical density of p44 and p42 bands was detected in the 1-month-spinal cat (Fig.1a, lane 2) that was even more pronounced in the 3-months-spinal (Fig.1b, lane 2) as compared to the intact (lane 1). In the overall population, no modulation in pERK1/2 expression was observed in any lumbar segment in 1-month-spinal as compared to intact cats (Fig.2ab, gray). However, the expression of pERK1 was up-

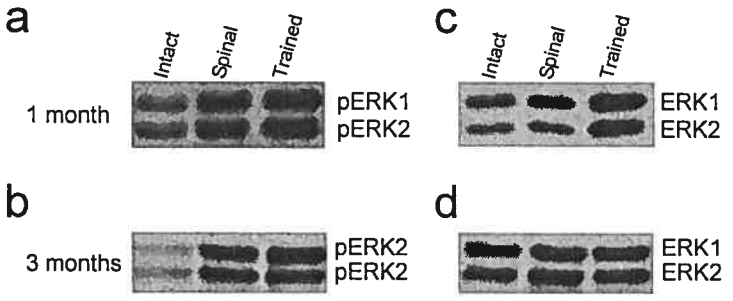

Figure 1. Western immunoblots analysis showing pERK1/2 and total ERK1/2 expression in the lumbar spinal cord of cats. ***a-b***, Western blots incubated with anti-pERK1/2 (1:2000) showing the expression of pERK1/2 in spinal homogenates of L3 segments of ***a***, an intact (lane 1), a 1-month-spinal (lane 2) and a 1-month-trained cat (lane 3) and of ***b***, an intact (lane 1), a 3-months-spinal (lane 2) and a 3-months- trained cat (lane 3); ***c-d***, Western blots incubated with anti-ERK1/2 (1:1000) showing the expression of total ERK1/2 in spinal homogenates of L3 segments of ***c***, an intact (lane 1), a 1-month-spinal (lane 2) and a 1-month-trained cat (lane 3) and of ***d***, an intact (lane 1), a 3-months-spinal (lane 2) and a 3-months-trained cat (lane 3).

regulated in all segments (L3, 249.1%; L4, 153.7%; L5, 491.1%; L6, 148.0%; L7, 125.2%) and pERK2 in all segments but L7 (L3, 183.2%; L4, 99.0%; L5, 231.7%; L6, 134.0%) in 3-months-spinal when compared to intact cats (Fig.2ab, black).

To determine if the increase in pERK1/2 detected after the spinal lesion was due to increased activation of ERK1/2 or simply an increase in expression, increasing the total pool of ERK1/2 available, we used, in another set of experiments, an antibody recognizing total ERK1/2. This antibody detects p44 and p42 MAPK (ERK1 and ERK2), irrespective of phosphorylation state. Increased ERK1 and ERK2 immunoreactivity (Fig.1cd) was detected in homogenates of 3-months-spinal cat (Fig.1d, lane 2) as compared to an intact (lane 1) in the L3 spinal segment. Notably, a corresponding difference was not detected in 1-month-spinal cats (Fig.1c, lane 2). Overall, total ERK1 expression was up-regulated specifically in L6 segments (16.6%) in 1-month-spinal (Fig.2c, gray) while no significant change in ERK2 expression was observed as compared to intact cats (fig. 2d, gray). In 3-months-spinal, ERK1 expression was up-regulated (Fig.2c, black) in all segments but L7 (L3, 39.1%; L4, 68.5%; L5, 28.1%; L6, 38.3%). An increase in ERK2 expression was detected (Fig.2d, black) in L3 segments only (21.6%) as compared to intact cats. Finally,

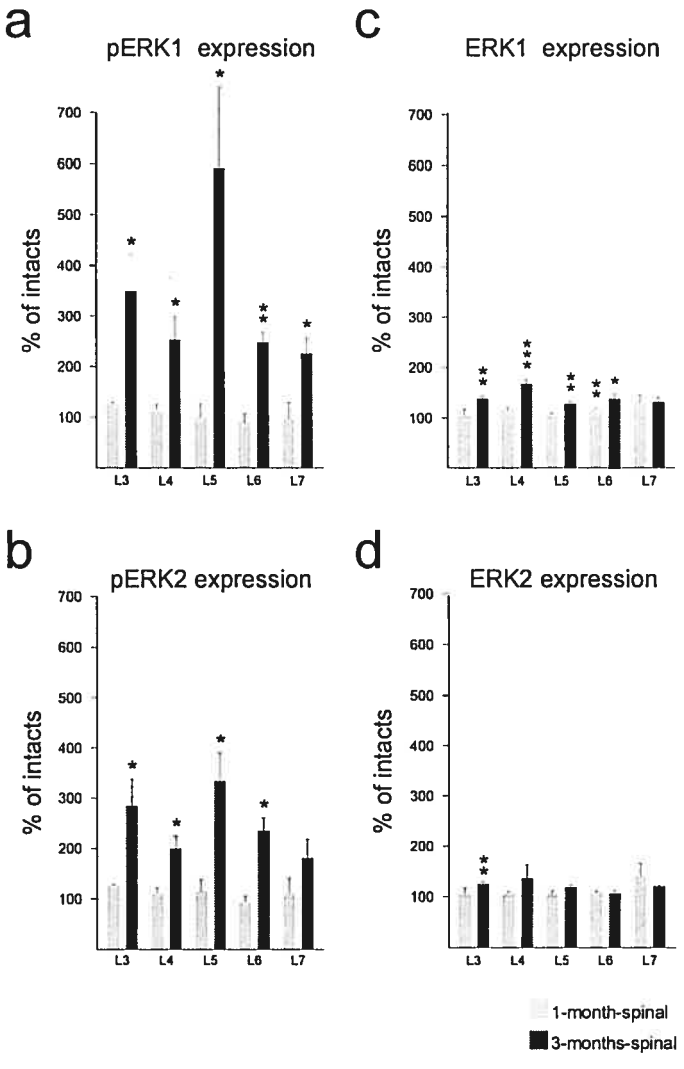

Figure 2. The expression of pERK1/2 and total ERK1/2 is increased following a complete spinal lesion.
a-d, Averaged pERK1/2 (***a,b***) and total ERK1/2 (***c,d***) protein level in the spinal lumbar segments (L3 to L7) of 1-month-spinal (gray) and 3-months-spinal cats (black). Data in histograms are represented as percent of intact cats +/- SEM. ***a***, pERK1 was up-regulated in L3 (249.1%), L4 (153.7%), L5 (491.1%), L6 (148.0%) and L7 (125.2%) 3 months after SCI (black) but was not modulated 1 month after SCI (gray); ***b***, pERK2 was up-regulated in L3 (183.2%), L4 (99.0%), L5 (231.7%) and L6 segments (134.0%) 3 months after SCI (black) but was not modulated 1 month after SCI (gray); ***c***, total ERK1 was up-regulated only in L6 spinal segment (16.6%) 1 month after SCI (gray) and in L3 (39.1%), L4 (68.5%), L5 (28.1%) and L6 (38.3%) spinal segments 3 months after SCI (black); ***d***, ERK2 expression was up-regulated only in L3 segments (21.6%) 3 months after SCI (black) but was not modulated 1 month after SCI (gray). Significant differences are indicated by *($p<0.05$), **($p<0.01$) or *** ($p<0.001$).

assessment of all lumbar segments as a single group indicate that, total ERK1 and ERK2 expression increased of 44% and 22% respectively, while pERK1 and pERK2 increased 233% and 162% in 3-months-spinal as compared to intact cats (data not shown).

Step-training induced plasticity of ERK activation

Assessment of relative levels of pERK1/2 in homogenates of L3 segments of an intact cat (lane 1), a 1-month-trained (lane 3, Fig.1a), and a 3-months-trained (lane 3, Fig.1b) revealed an increase in pERK1/2 immunoreactivity as compared to the intact cat (lane 1) but not as compared to the 1-month- or 3-months-spinal (lane 2). Overall, no modulation in pERK1/2 expression was observed in any lumbar segment following one month of step-training as compared to spinal cats (Fig.3ab, gray). After 3 months, step-trainingselectively down-regulated pERK1 (69.3%) and pERK2 (74.5%) expression in L5 segments (Fig.3ab, black). Further Western blots analysis of total ERK1/2 expression demonstrated an increased optical density in L3 spinal segments of a 1-month-trained (Fig. 1c, lane 3) as compared to 1-month-spinal cat (lane 2). Overall, step-training

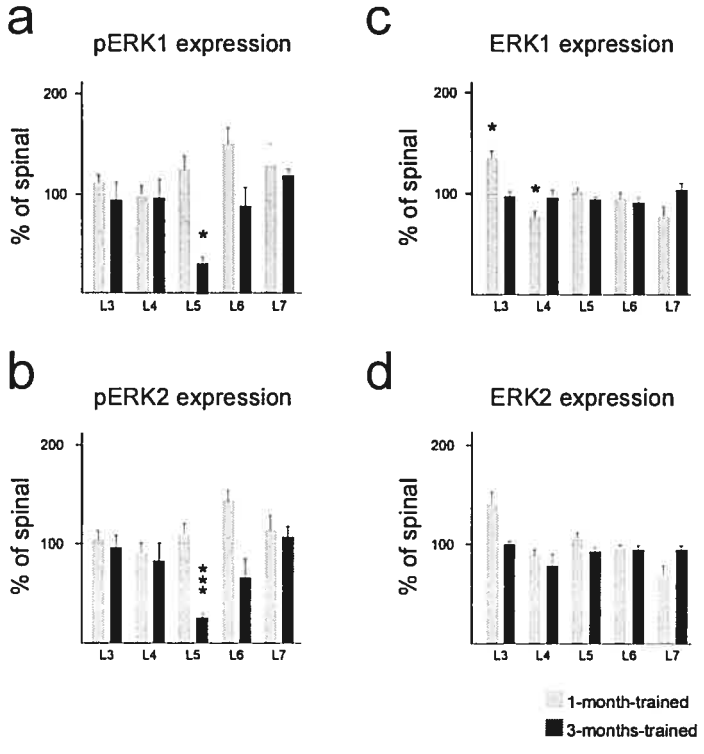

Figure 3. Step-training following a complete spinal transection modulates pERK1/2 and total ERK1/2 expression in the lumbar spinal cord of cats.
a-d, Averaged pERK1/2 (***a,b***) and total ERK1/2 (***c,d***) protein level in the spinal lumbar segments (L3 to L7) of 1-month-trained (gray) and 3-months-trained cats (black). Data in histograms are represented as percent of 1-month-spinal for 1-month-trained cats and of 3-months-spinal for 3-months-trained cats +/- SEM. ***a***, pERK1 was down-regulated in L5 (69.3%) after 3 months of step-training (black) but was not modulated after 1 month (gray); ***b***, pERK2 was down-regulated in L5 (74.5%) after 3 months of step-training (black) but was not modulated after 1 month (gray); ***c***, total ERK1 was up-regulated in L3 (34.8%) and down-regulated in L4 (23.3%) after one month of step-training (gray) whereas no modulation was observed after 3 months (black); ***d***, No modulation in ERK2 expression was observed after 1 month (gray) or 3 months of step-training (black). Significant differences are indicated by *($p<0.05$) or ***($p<0.001$).

selectively modulated the expression of ERK1 being up-regulated in L3 (34.8%) and down-regulated in L4 segments (23.3%) in 1-month-trained as compared to 1-month-spinal cats (Fig.3c, gray). This change was transient and was not detected in 3-months-trained cats (Fig.3c, black).

CREB up-regulation after a complete spinal cord injury independent of step-training

CREB is a transcription factor known to have roles promoting neuronal survival, regulating neural plasticity, and an involvement in learning and memory (Silva et al 1998, Kandel 2001, Lonze & Ginty 2002). ERK-mediated CREB phosphorylation is required for synaptic plasticity associated with the induction of stable, late-phase LTP and long-term memory (Kelleher et al 2004, Thomas & Huganir 2004). Here, we observed modulation of pERK expression was mainly at 3 months after a complete spinal lesion with or without step-training. We then investigated the possibility that CREB expression might be similarly regulated at the 3 months post-injury time point. Examination of endogenous CREB protein in homogenates of spinal cord segments revealed an appropriate single ~43kDa immunoreactive band. Figure 4a illustrates increased CREB immunoreactivity detected in

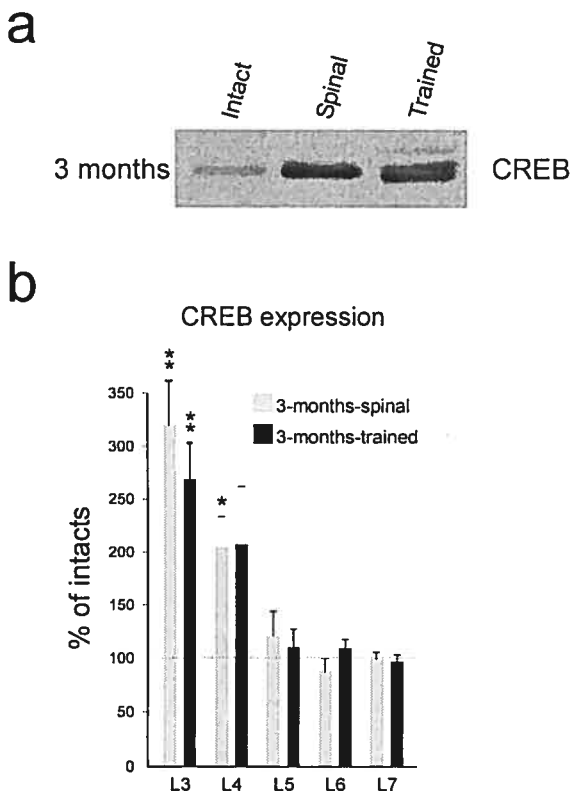

Figure 4. CREB expression is up-regulated following a complete spinal lesion in the lumbar spinal cord of cats. ***a***, Western blot incubated with anti-CREB (1:1000) showing the expression of CREB protein in homogenates of L4 spinal segments of an intact (lane 1), a 3-months-spinal (lane 2) and a 3-months-trained cat (lane 3); ***b***, Averaged CREB protein level in the spinal lumbar segments (L3 to L7) of 3-months-spinal (gray) and 3-months-trained cats (black). Data in histograms are represented as a percent of intact cats +/- SEM. CREB protein level was specifically up-regulated in L3 (219.0%) and L4 (103.8%) in 3-months-spinal as compared to intact cats (gray). A similar up-regulation was observed in L3 segments (138.8%) in 3-months-trained as compared to intact cats. No further effect of step-training was observed in any lumbar segment when 3-months-trained were compared to 3-months spinal cats. Significant differences are indicated by *($p<0.05$) or ** ($p<0.01$).

3-months-spinal and 3-months-trained animals as compared to intact cat. Overall, CREB protein levels were specifically up-regulated in L3 (219.0%) and L4 (103.8%) in 3-months-spinal, and also up-regulated in L3 spinal levels (138.8%) in 3-months-trained as compared to intact cats (Fig.4b). No additional effect of step-training was observed when step-trained were compared to spinal cats. Attempts to assess levels of phospho-CREB expression were unsuccessful. No phospho-CREB immunoreactivity was detected in any condition, possibility due to incompatibility between available phospho-specific CREB antibodies and feline CREB protein (data not shown).

PI3K/Akt signaling not detectably altered by long-term spinal injury or step-training

In addition, we investigated the expression of Akt, a protein kinase activated by neurotrophins and well characterized to function upstream of CREB activation. Figure 5 illustrates a Western blot of protein homogenates from L4 spinal segments of an intact, a 1-month-spinal and a 1-month-trained cat with an antibody recognizing Akt1, Akt2 and Akt3 proteins. A single ~60kDa immunoreactive band was detected for all groups of cats, 1 month (Fig.5a) or 3 months (Fig.5b) with or without step-training. These findings reveal no modulation of Akt expression following a complete spinal lesion with (black) or without

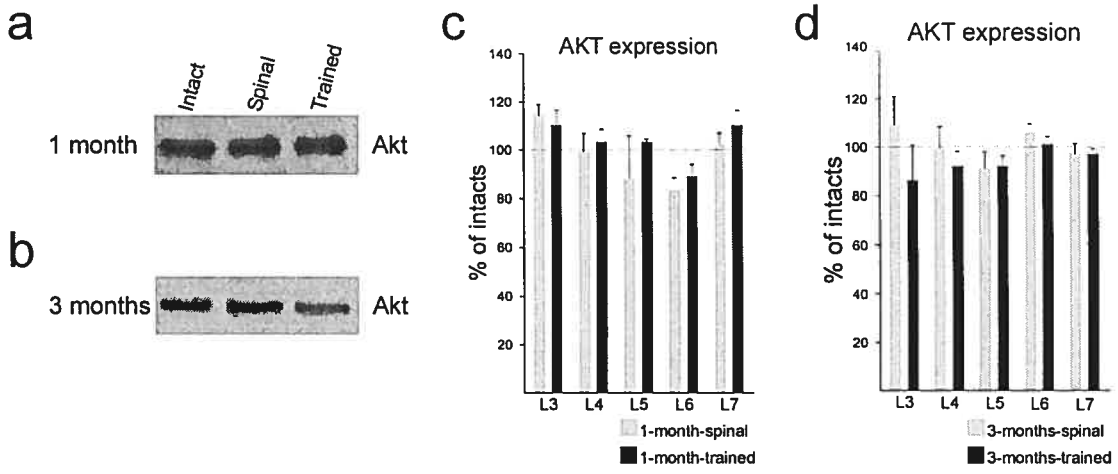

Figure 5. Akt expression is not modified because of a complete spinal lesion or step-training in the lumbar spinal cord of spinal cats. *a-b*, Western blots incubated with anti-Akt (1:1000) showing the expression of Akt protein in homogenates of L3 spinal segments of ***a***, an intact (lane 1), a 1-month-spinal (lane 2) and a 1-month-trained cat (lane 3) and of ***b***, an intact (lane 1), a 3-months-spinal (lane 2) and a 3-months-trained cat (lane 3); ***c,d***, Averaged Akt protein level in the spinal lumbar segments (L3 to L7) of ***c***, 1-month-spinal (gray) and 1-month-trained cats (black) and of ***d***, 3-months-spinal (gray) and 3-months-trained cats (black). Data in histograms are represented as a percent of intact cats +/- SEM. No difference in the expression of Akt protein in any spinal segment was observed because of the spinal lesion or step-training after 1 or 3 months.

step-training (gray) in any spinal level either after one (Fig. 5c) or three months (Fig. 5d). This result provides evidence that plasticity induced by lesion and step training specifically impacts the ERK pathway, and does not reflect a general increase in the expression of kinase pathways related to BDNF and TrkB activated signaling.

Synpatophysin is down-regulated after spinal cord injury with or without training

In the hippocampus, BDNF acts on presynaptic neurons and enhances vesicle release via TrkB activation (Lu & Chow 1999). Increased leveld of synaptophysin protein is a molecular correlate of increased numbers of synaptic vesicles, either due to increases in synapse formation or an increase in the number of vesicles in existing synapses (Sarnat & Born 1999). In the spinal cord, exercise leads to the modulation of TrkB mRNA expression, which is closely associated with changes in the levels of protein components of the synaptic machinery, potentially providing a mechanistic link between exercise and the modification of information transmission across the synapse (Gomez-Pinilla et al 2001,

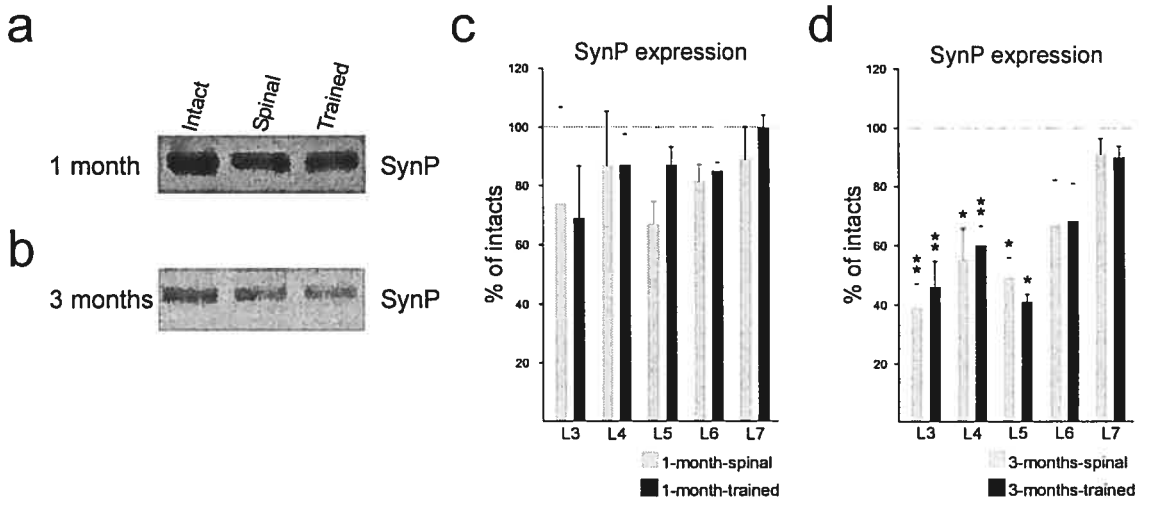

Figure 6. Synaptophysin (SynP) expression is down-regulated following a spinal lesion in the lumbar spinal cord of cats. ***a-b***, Western blot incubated with anti-SynP (1:4000) showing the expression of SynP protein in homogenates of L4 spinal segments of ***a***, an intact cat (lane 1), a 1-month-spinal (lane 2) and a 1-month-trained cat (lane 3) and of ***b***, an intact cat (lane 1), a 3-months-spinal (lane 2) and a 3-months-trained cat (lane 3); ***c,d***, Averaged SynP protein level in the spinal lumbar segments (L3 to L7) of ***c***, 1-month-spinal (gray) and 1-month-trained cats (black) and of ***d***, 3-months-spinal (gray) and 3-months-trained cats (black). Data in histograms are represented as a percent of intact cats +/- SEM. One month (**c**) was not sufficient to reveal a modulation in the expression of SynP whether the spinal cats were step-trained (black) or not (gray). However, SynP protein level was specifically down-regulated in L3 (60.6%), L4 (44.7%) and L5 (50.8%) in 3-months-spinal and in L3 (54.1%), L4 (40.2%) and L5 spinal segments (59.0%) in 3-months-trained as compared to intact cats (*d*, gray). No further effect of step-training was observed in any lumbar segment when step-trained cats were compared to spinal cats. Significant differences are indicated by * ($p<0.05$) or ** ($p<0.01$).

2002). As illustrated in Figure 6, an antibody against synaptophysin recognizes a single ~38kDa immunoreactive band that is significantly reduced in spinal and step-trained as compared to intact cats 1 month (Fig.6a) and 3 months (Fig.6b) after the complete spinal lesion (gray) and onset of step-training (black). Overall, the level of synaptophysin protein was significantly down-regulated in L3 (60.6%), L4 (44.7%) and L5 (50.8%) in 3-months-spinal and in L3 (54.1%), L4 (40.2%) and L5 spinal segments (59.0%) in 3-months-trained as compared to intact cats (Fig.6d). Although it was not sufficient to reach statistical significance, a similar trend was observed one month after injury (Fig.6c). No further effect of step-training was observed in any lumbar segment when step-trained cats were compared to spinal cats.

Discussion

Spinal networks were long assumed to be hardwired, simply responding quickly and in a stereotyped fashion to afferents or descending inputs (Forssberg & Svartengren 1983). Substantial experimental and clinical evidence now supports the conclusion that the spinal cord exhibits a remarkable capacity for plasticity in response to central or peripheral lesions, operant conditioning or step-training (Mendell 1984, Durkovic 1996, Wolpaw & Tennissen 2001, Côté et al 2003a, Côte & Gossard 2004). The preparation used in this study, a complete spinal cord transaction model, allowed us to attribute lesion- and training-induced plasticity in ERK pathways to the intrinsic competence of the spinal cord to reorganize without any contribution of regeneration or supraspinal influence to induce or model this rearrangement.

In an elegant series of studies, Dr Gomez-Pinilla and colleagues investigated the molecular mechanisms and signaling pathways by which activity promotes synaptic plasticity and functional recovery in the hippocampus, spinal cord and muscles in intact and SCI animals (Neeper et al 1995, Gomez-Pinilla et al 2001, 2002, Molteni et al 2002, Hutchinson et al 2004). In an exercised but otherwise intact animal, their results suggest that exercise modulates molecular systems involved in maintaining neuronal function and plasticity in the brain: BDNF was the single trophic factor whose expression was determined to be modulated by exercise. Additionally, the majority of other genes found to be affected by motor activity had a recognized association with BDNF (Molteni et al 2002). Further experiments indicated that the same molecular mechanisms were involved in

short-term exercise-dependent plasticity in incomplete SCI animals, BDNF, CREB and synapsin I being down-regulated in the lumbar spinal cord as compared to intact animals and up-regulated after exercise suggesting that motor training may restore the expression of these proteins to near normal levels (Ying et al 2005). Here, we aimed to investigate the involvement of pathways activated by BDNF, in particular ERK pathways, in long-term step-training and chronic spinal cord transection in the cat.

During locomotor recovery, spinal networks are continuously stimulated by sensory feedback and the injured animal progressively recovers rhythmic and alternate locomotor movements. The ability to relearn motor tasks such as stepping in the absence of any descending influence has been attributed to the reorganization of local connections within the spinal cord (Edgerton et al 2004). Two to 4 weeks after a complete spinal lesion and onset of step-training, adult cats are able to perform proper plantigrade contact of the paw with the treadmill belt and execute weight-bearing on the hindlimbs during stepping (Lovely et al 1986, Barbeau & Rossignol 1987, Bélanger et al 1996). If step-training is prolonged beyond that time window, stepping ability further progresses: the maximum walking speed and the number of steps performed on the plantar surface of the foot increase. Stepping performance typically reaches a plateau approximately 3 months after step-training onset (Lovely et al 1986, Barbeau & Rossignol 1987). We therefore chose to assess the training-induced plasticity in ERK pathway at these 2 time points. It is important to consider that any potential plasticity induced by step-training will be superimposed on an initial massive plastic change induced by the complete spinal injury itself, data which are not available in the literature. It was thus of critical importance to first characterize changes associated with the injury-induced plasticity alone in order to accurately identify plasticity solely attributable to step-training.

In this study, modulation of the expression of proteins in signaling pathways in the spinal cord were solely observed after 3 months, one month being insufficient to reveal detectable biochemical correlates of plasticity change. Recent findings have revealed that the expression of several genes can be affected by voluntary exercise, with a number of different temporal-profiles, in otherwise intact animals (Molteni et al 2002). CaMK signaling, closely regulated by the NMDAR system, was shown to be highly up-regulated by short-term wheel running and was suggested to represent the downstream effect of exercise during the acute phase (Molteni et al 2002). On the other hand, ERK pathways were not modulated in the first days following the onset of exercise and a significant up-regulation was solely observed when exercise extend for longer periods of time (Molteni et

al 2002). Our results support the hypothesis that modulation of ERK pathway signaling is involved in the chronic effect of exercise, being predominantly modulated 3 months after step-training onset.

Modulation of ERK signaling following injury and step training-induced plasticity

Here we provide the first evidence for increases in ERK1/2 and ERK activation, inferred from levels of pERK1/2, in the feline lumbar spinal cord, from L3 to L7, as a result of step-training, in the injured spinal cord. Western blot analyses revealed that pERK1/2 and ERK1 protein levels were up-regulated in a majority of lumbar spinal segments 3 months after a complete spinal transection. Furthermore, our results suggest that the increased level of pERK was not only due to synthesis of new proteins but also to a higher rate of phosphorylation. Indeed, it was shown that following a facial nerve lesion, ERK1 protein expression was up-regulated until at least 8 weeks after the injury although ERK1 mRNA levels returned to levels similar to control within the 4th week (Kitahara et al 1994). In vitro, ERK1/2 signaling is implicated in neuroprotective mechanisms that inhibit apoptosis of cortical neurons (Hetman et al 1999) and cerebellar granule neurons (Bonni et al 1999). Moreover, recent studies lend support to the hypothesis that excitotoxicity, neural apoptosis, inflammation, brain ischemia and nerve injury also induce activation of ERK1/2 cascade (Ji et al 1999, Ferrer et al 2001, Ji & Woolf 2001, Ji 2004). Additionally, the increase in ERK phosphorylation following SCI might be as a result of a loss of supraspinal modulation leading to a loss of inhibitory control to spinal neurons. The observed down-regulation in synaptophysin expression supports such a possibility. The decrease detected was specific to L3-L5 spinal segments suggesting a substantial loss of synaptic transmission in this area. Synaptophysin expression is associated with synaptogenesis (Bergmann et al 1997) and an increase in synaptophysin likely indicates that synaptic vesicles are formed either due to an increase in synapse formation or an increase in the number of vesicles in existing synapses (Sarnat & Born 1999). Our results suggest that step-training does not induce an increase in "net" synaptogenesis, but may simply reflect the loss of the multiple supraspinal contacts projecting onto these lumbar segments.

We also showed a step-training induced down-regulation in pERK expression selective to L5 spinal segment. In this specific case, it is doubtful that this reduction would subserve a decreased neuroprotective effect evoked by step-training. Several recent studies using *in vivo* models of cerebral ischemia (Namura et al 2001, Wang et al 2003) or traumatic brain

injury (Mori et al 2002ab) have shown that inhibitors of MEK1/2, the upstream activator of ERK, reduce neuronal loss suggesting that the activation of ERK in response to acute CNS injury may also be detrimental. The down-regulation in pERK expression in step-trained spinal cats supports this hypothesis and suggests an interfering effect of ERK activation with locomotor recovery. To date, various kind of molecules have been demonstrated to be downstream targets of ERK1/2 ranging from cytoskeleton-associated molecules to other kinases, phosphatases, enzymes, transcription factors and cell surface molecules (Lewis et al 1998). ERK cascade not only amplifies extracellular stimuli but also integrates many converging signaling pathways and functions as a vehicle that imports the information into the nucleus. This may explain the variety of functions transmitted by ERK pathway. Together, these results suggest that the decrease observe in pERK in L5 expression is triggered by sensory afferents activated by repetitive walking on the treadmill surface. How a decrease in ERK activation contributes to locomotor recovery in chronic spinal cats remain to be determined.

CREB expression is altered by injury but not step-training

Our findings reveal detectable levels of CREB protein in the feline lumbar spinal cord, from L3 to L7, whether intact or injured. A significant up-regulation in total CREB expression 3 months after a complete SCI was detected in the lumbar cord. In conjunction with BDNF, CREB plays a key role in neuronal resistance to insult (Walton et al 1999). Injury-elicited CREB activation in the spinal dorsal horn or in DRG, is at least partly mediated by ERK signaling following a spinal cord injury (Qiao & Vizzard 2005, Cruz et al 2006). In addition, several studies have found that ERK-mediated CREB phosphorylation is required for synaptic plasticity associated with the induction of stable, late-phase LTP and long-term memory in the hippocampus (Kelleher et al 2004, Thomas & Huganir 2004) and plasticity associated with neuropathic pain in the spinal cord (Ji et al 2003, Rygh et al 2005, Crown et al 2006). Indeed, CREB was shown to be involved in several intracellular events associated with the action of BDNF on neuronal plasticity (Barde 1994). Our results suggest that lesion-induced plasticity impacts on CREB expression in cell populations specifically located in L3 and L4 spinal segments. However, the up-regulation of pERK expression in all lumbar segments examined (L3-L7) may not, in itself, account for the specific increase in CREB expression. CREB can be phosphorylated by multiple intracellular kinases in response to a vast range of physiological and pathological stimuli. Among them, both ERK and PI3-K/Akt kinase pathways lead to CREB activation (Xing et

al 1998) to regulate gene expression. Together, these results suggest that other intracellular kinases pathways known to activate CREB in the spinal cord such as PKA, PKCγ and CaMKII may also be involved in chronic lesion-induced plasticity.

Moreover, CREB was shown to be up-regulated after short-term step-training in intact and incomplete SCI animals (Gomez-Pinilla et al 2002, Ying et al 2005). No long-term step-training-induced modulation in CREB expression was observed in this study as previously observed (Molteni et al 2002). Whether CREB is solely involved in plasticity occurring in the first weeks following step-training or if a complete spinal lesion prevents a modulation in CREB expression is not known.

Specificity of plasticity induced by long-term injury and step-training to ERK pathways

Based on evidence that exercise preferentially modulates genes associated with BDNF (Molteni et al 2002) and also impacts on CREB expression in the hippocampus and spinal cord (Gomez-Pinilla et al 2002, Vaynman et al 2003), we choose to investigate PI3K/Akt, a pathway known to be activated by BDNF, and lead to regulation of CREB. Our results suggest that this pathway is not involved in long term lesion-induced or step-training induced plasticity and that the cascades downstream of BDNF/TrkB may be differentially modulated following injury or step-training. The PI3K/Akt signaling pathway has been shown to be activated by different tyrosine kinase receptors and plays an important role in cell/death survival pathways by stimulating neuronal survival of many populations of neurons and axonal growth. It was also shown to play an important role in motoneuronal survival induced by BDNF (Dolcet et al 1999). Akt phosphorylation was shown to be transiently up-regulated in the spinal cord following SCI for 7 days (Yu et al 2005). Apoptosis being typically a rapid process (Bursch et al 1990), our results suggest that PI3K/Akt pathway might not be involved in long term plasticity.

Segmental distribution of spinal plasticity

Our results suggest that lesion-induced modulation of ERK signaling is spread throughout the lumbar spinal cord while step-training plasticity exhibits a more restricted local modulation. One month following the onset of the step-training regimen, the expression of

ERK1 was transiently increased in L3 and decreased in L4 spinal segments. However, concomitant changes were not observed in pERK levels and were not present in 3-months-trained cats. The main observation regarding modulation in this pathway following step-training was a decrease in pERK expression selectively observed in L5 spinal segment. The L5 spinal segment contains motoneuronal pools that innervate proximal muscles of the hip (Vanderhorst & Holstege 1997) which is particularly important in initiating the swing phase of locomotion and entraining the locomotor rhythm (Grillner & Rossignol 1978, Andersson & Grillner 1983, Lam & Pearson 2002). Moreover, the L5 spinal level contains a population of interneurons activated by the mesencephalic locomotor region (MLR, Noga et al 1995), an area known to induce locomotion (Jordan 1998). Together, these results suggest that L5 level exhibits particularly high levels of plasticity following step-training.

Chronic lesion-induced spinal plasticity particularly impacts on CREB expression in cell populations localized in L3 and L4 spinal segments. In cats, midlumbar segments provide essential inputs to organize the locomotor pattern. The integrity of the L3-L4 spinal segments is necessary to sustain locomotor activity suggesting they may contain interneurons strongly involved in stepping generation (Marcoux & Rossignol 2000, Langlet et al 2005). Moreover, L3 and L4 segments contain group II afferents-activated interneurons suggested to target a great variety of other interneurons and ascending tract cells (Bannatyne et al 2006). These interneurons are thought to be involved in the adequate activation of motoneurons in a variety of centrally initiated movements mediated by reticulospinal neurons and commissural interneurons (Edgley et al 2004). These results suggest that the complete disruption of supraspinal afferents to the lumbar spinal cord in chronic spinal cats may particularly impact gene transcription in areas containing these 2 types of interneurons.

Notably, plasticity occurring in these areas of the lumbar spinal cord may be due to regulation of expression in different cell types. Although plasticity in ERK pathways has been previously demonstrated in motoneurons (Kitahara et al 1994, Kishino & Nakayama 2003), several other cell types express these proteins, including interneurons, glial cells and endothelial cells. Western blot analysis is constrained to identifying changes in overall expression. Thus, small changes in expression within a subpopulation of cells may not be detected and will require additional immunohistochemical analysis.

Spinal cord plasticity is highly specific

Spinal plasticity may depend on the duration of injury (acute vs. chronic), extent (complete or partial) and type (contusion, hemisection, transection) and has also been shown to be task-dependent (de Leon et al 1998a, Edgerton et al 1997a). The amount of activity performed, for example the amount of wheel running by rodents, has also been found to correlate with spinal plasticity (Bravo et al 2003, Vaynman et al 2006). Previous studies have reported that the majority of proteins involved in activity-dependent plasticity rapidly returned toward normal values within few days (Molteni et al 2002). In the past, lesion-induced and activity-related plasticity in the pathways studied here have been largely investigated only in the few minutes or hours following lesion, a single bout on a treadmill or few days of locomotor activity (Gomez-Pinilla et al 2001, 2002, Ying et al 2003, 2005, Bravo et al 2003). Here we demonstrated that regulation of ERK and CREB protein levels may also be involved in long-term plastic changes in the CNS following a chronic and complete spinal lesion and also following a long-duration step-training regimen in the case of pERK. These changes does not exclude an acute modulation in these pathways but suggest that they may also be involved in chronic spinal plasticity occurring several weeks after the injury and step-training onset since it is observed after 3 months, but not after 1 month. From previous experiments, we expected a decrease in ERK and CREB following injury and an increase after step-training. Although pERK returned to normal values in L5 homogenates after 3 months, an increase in ERK, pERK and CREB was observed following injury. This suggests the involvement of spared supraspinal fibers in triggering or modulating spinal plasticity following a partial (hemisection or contusion injury) spinal lesion and also in motor recovery. Notably, such a down-regulation following exercise has been observed following locomotor training and traumatic brain injury, but only if training begins immediately after the injury onset without a recovery period and is associated with cognitive impairment (Griesbach et al 2004). Finally, our detailed segmental analysis confirms that it is fruitful to carefully interpret changes in protein expression following injury, as general increases or decreases in protein level in the whole spinal cord observed when several segments are merged together, may not reveal more dramatic changes that are specifically localized to a subset of spinal segments. The findings obtained here identify changes in protein expression and activation limited to specific regions along the longitudinal axis of the spinal cord and provide further evidence for the remarkable plasticity of the spinal cord following injury.

Acknowledgments

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The introduction described the plasticity of spinal reflex pathways after SCI, particularly related to improvements in motor function with activity-dependent rehabilitation. Such paradigms aim at maximizing the residual functions of the spinal cord caudal to the lesion. Indeed, several mechanisms which modulate spinal reflex activity are deficient in individuals with SCI. Consequently, the motor output is disrupted and the gait abnormal. It is generally assumed that plastic changes related to step-training are located within the CPG. Without excluding this possibility, we illustrate that spinal plasticity may also occur in both muscle (1st manuscript) and cutaneous reflex pathways (2nd manuscript). In addition, we demonstrate that it might involve plastic changes in intracellular cascades located in lumbar segments not necessarily associated to the localization of the CPG in the cat (3rd manuscript). It is believed that plastic changes in these pathways can impact and model the excitability of the CPG to generate a more functional locomotor output. It is worth noting that the modulation observed in reflex pathways may represent a training-related plasticity superimposed on spinalization-induced plasticity, or a prevention of spinalization-induced plasticity. Additional experiments are needed to address this complex question.

Functional stepping recovery after SCI suggests that properties of networks and cells involved in the control of locomotion have been modified to optimize the motor output through a reduced circuitry. Our studies highlight the complexity of spinal plasticity: it can occur at multiple sites, involve several systems throughout the spinal cord, may target specific pathways or molecules, and engage dynamic processes that have specific and different time course. It is believed that all these changes are interrelated and integrated to ultimately contribute to improve the motor output after SCI.

Incomplete SCI may stimulate the reorganization of synaptic connections such as increasing collateral branching or shifting the representation of the hindlimbs in the motor cortex. Moreover, indirect evidence suggests that at least part of the reorganization of the spinal networks caudal to the spinal lesion depend on spared supraspinal afferents (Helgren & Goldberger 1993, Nacimiento et al 1995, Bregman et al 2002, Schucht et al 2002). Here, a complete SCI model was chosen to assess the plastic potential of the spinal cord excluding any plasticity driven by supraspinal pathways. Although partial spinal injury such as contusion are reminiscent of what is seen in the clinic, we consider that it is important to reveal the extent of the plasticity that can occur independently, within the

spinal cord, and without the influence of the brain. This knowledge is crucial to design the basics of a successful rehabilitation programs after SCI. It could serve as building blocks to identify the important physiological features of the recovery that may be targeted by the spared supraspinal pathways, if any, and further enhance the motor recovery.

Step training-related plasticity does occur in both load and skin reflex pathways

Load-related inputs can act directly on the CPG, particularly those evoked by group I afferents from extensors (Conway et al 1987, Gossard et al 1994, McCrea 1998, Pearson et al 1998). In addition, it has been shown that phasic load input related to stepping are required because stand-training, which provides static load, is not sufficient to improve locomotor movements (de Leon et al 1999b). We thus hypothesized that this pathway is a potential candidate for step training-related plasticity. In addition, we believed that widespread and evident changes in transmission would take place to reinforce weight-bearing. The preparation used in this study for electrophysiological recordings allows the locomotor *program* to be active. The actual movements are prevented by blocking the neuromuscular transmission to perform stable intracellular recordings. Moreover, motoneuronal responses are not submitted to the dynamic influence of sensory feedback related to stepping movements. The effect of step-training can thus be attributable to changes within the spinal pathways and not to alteration in peripheral sensory events or muscle properties (Roy & Accosta 1986, Roy et al 1999).

In these experiments, we showed that transmission in the monosynaptic pathway is decreased following step-training. Most studies reported the effect of SCI alone on monosynaptic EPSP amplitude and revealed plastic changes that seem to be highly dependent on the context, preparation, level of spinal lesion and motor pool recorded (*cf monosynaptic reflex and SCI*, p21). This issue was not investigated in our study. To our knowledge, this is the first study to report the effect of step-training on the amplitude of the monosynaptic EPSP after a complete SCI. However, locomotor training has been shown to decrease and improve the gating of the H-reflex (Trimble et al 1998). Whether the decrease in monosynaptic EPSP amplitude in our experiment would result in a decrease in the amplitude of the monosynaptic reflex is probable but this requires further investigation.

We also illustrated that step-training decreased Ib non-reciprocal inhibition and improved polysynaptic excitation evoked by Ia/Ib afferents of extensors in synergistic motoneurons. However, plasticity in this pathway seems less robust than expected because it needed clonidine to be revealed. We expected that stimulation of these pathways would be so strong that plasticity would be measurable at rest. More data collected during locomotor episodes may have revealed more plastic changes. Most of the intracellular recordings were recorded at rest because it is difficult to obtain fictive locomotion in this preparation. In addition, one month of step-training may not be sufficient for plasticity to fully develop in these pathways. Preliminary data suggest that plasticity in group I reflex pathways is more robust at rest in extensor motoneurons of animals that were trained for 3 months (Côté et al 2003b).

Plasticity in each pathway measured separately appears to be modest considering the remarkable effect of step-training. Notably, during locomotion multiple pathways would be active simultaneously and their input converge on common interneurons (Baldissera et al 1981, Jankowska 1992). Spatial facilitation is known to occur at the interneuronal level and amplifies the motor response. So, taken altogether, the modified transmission in sensory pathways would certainly contribute to the recovery of stepping.

Cutaneous inputs are also activated by locomotor movements and several studies reported that cutaneous feedback might take part in the recovery of motor function after SCI (Fung & Barbeau 1994, Muir & Steeves 1995, 1997, Bouyer & Rossignol 2003b, Smith et al 2006). However, cutaneous inputs do not have a powerful effect on rhythm generation such as group I muscle afferent inputs. We thus expected less step training-dependent plasticity in skin reflex pathways. Results confirm that, in addition to plasticity in group I muscle pathways, step-training induces significant plastic changes in cutaneous transmission from at least three different skin territories (CCS, SP, MPL) to motoneurons of flexor and extensor muscles acting at the hip, knee, ankle and toe joints. Surprisingly, plasticity in these pathways turned out to be more vigorous than we expected: it was apparent at rest, without the activity of the CPG (fictive locomotion) or the addition of clonidine.

Proteins involved in learning phenomena are present in the cat spinal cord and up-regulated after SCI

Importantly, locomotor recovery may either be seen as a recovery of existing function or as a novel *learning* that could potentially involve mechanisms implicated in different forms of learning processes in the hippocampus (sensitization, habituation, LTP, etc). It is worth noting that this second project did not aim to correlate changes in the biomolecular content of the spinal cord with the plasticity in spinal reflex pathways but rather to investigate the same issue from another point of view.

We chose the ERK pathway as a potential candidate involved in spinal plasticity for several reasons. It has been associated with learning mechanisms in the hippocampus (Sweatt 2004) and is activated by BDNF, the expression of which is regulated in an activity-dependent manner both in the brain and in the spinal cord (Neeper et al 1995, Gomez-Pinilla et al 2001, 2002, Molteni et al 2002, Hutchinson et al 2004, Klintsova et al 2004). ERK activation is also regulated by the action of glutamate/NMDA receptors to facilitate synaptic efficacy (Garraway et al 2003, Slack et al 2004). It is worth noting that glutamate is an important neurotransmitter for stepping generation in chronic spinal cats (Chau et al 2002, Giroux et al 2003) and that NMDA receptors were shown to be up-regulated following SCI and step-training (Rossignol et al 2004). Also, sensory afferents activated by step-training release glutamate on their postsynaptic targets. Antibodies that specifically recognize the phosphorylated active form of ERK1/2 (pERK) on Western blots provide a straightforward method to assess the relative levels of ERK activation in tissue homogenates and have been extensively used as a criterion to judge the degree of activation of the ERK pathway. We first investigated the plasticity after SCI given the initial massive plastic changes induced by the complete SCI itself, data that is not available in the literature. It was thus of critical importance to first characterize changes associated with the injury-induced plasticity alone in order to accurately identify plasticity solely attributable to step-training. We chose to investigate changes in this pathway at two different critical time post-injury, 1 month and 3 months because weight-bearing is known to be recovered approximately one month after the onset of step-training and improvements in the locomotor pattern were shown to reach a plateau after 3 months.

We demonstrate for the first time that ERK1/2, pERK1/2, CREB and Akt are present in the spinal cord both in the intact and completely spinalized adult cat. The activation of ERK was shown to be increased after a complete SCI. We also provide evidence that plasticity induced by SCI and step-training specifically impacts the ERK pathway, and does not reflect a general increase in the expression of kinase pathways related to BDNF and TrkB activated signaling. The increased ERK activation observed 3 months after SCI is consistent with previous investigations that illustrate a persistent up-regulation of NMDA, AMPA and kainate receptors, contrary to NA and 5-HT receptors which eventually returns to control values (Rossignol et al 2004).

We also show an increase in CREB expression 3 months after a complete SCI. This increase is restricted to L3 and L4 spinal segments contrary to the widespread increase in ERK activation (L3 to L7). In the spinal cord, CREB is also a common target for multiple intracellular kinases including PKA, PKCγ and CaMKII. Because CREB appears to play a role in neuronal resistance to insult (Walton et al 1999) it could imply that a population of cells in these areas is undergoing neuroprotective processes. On the other hand, these segments were shown to provide essential inputs to organize the locomotor pattern. The integrity of L3 and L4 spinal segments was shown to be necessary to sustain locomotor activity suggesting they may contain interneurons strongly involved in stepping generation in cats (Marcoux & Rossignol 2000, Langlet et al 2005). Moreover, L3 and L4 segments contain group II afferents-activated interneurons suggested to target a great variety of other interneurons and ascending tract cells (Bannatyne et al 2006). These interneurons are thought to be involved in the adequate activation of motoneurons in a variety of centrally initiated movements mediated by reticulospinal neurons and commissural interneurons (Edgley et al 2004). These results suggest that the complete disruption of supraspinal afferents to the lumbar spinal cord in chronic spinal cats may especially target gene transcription in these areas.

The specific character of step training-induced spinal plasticity

Our studies greatly illustrated that step training-dependent plasticity is specific and target particular pathways and areas of the spinal cord.

Inhibitory systems: an example The inhibitory potential is increased in the neurons located in the ventral and dorsal horn of the lumbosacral spinal cord below the SCI and is related to the loss of supraspinal inputs which are mainly excitatory to the spinal cord (Edgerton et al 2001, Tillakaratne et al 2000, 2002). Step-training would either prevent this increase in inhibition to happen or return those levels of inhibitory molecules towards intact values (Tillakaratne et al 2002). Precise measurements illustrate that the labeling of $GABA_A$ receptors ($\alpha_{1\text{-}2\text{-}3}$-, β_2, γ_2) of sensory and motor neurons associated with SOL and TA muscles is inversely correlated to stepping performance (Bravo et al 2003). These experiments carried by the group of Edgerton suggest that by reducing inhibition of spinal networks, sensory inputs can be integrated to generate locomotor activity. One could thus think that the activity in reflex pathways would result in less inhibition and more excitation of motoneurons after step-training. Our study shows that plasticity in reflex pathways is by far more complex. On one hand, there is a decreased transmission in the Ib inhibitory pathway together with an improved polysynaptic excitation in step-trained cats that follows this general rule. On the other hand, inhibitory transmission is facilitated in some cutaneous pathways and the monosynaptic reflex decreased after step-training. In those cases, the net result is more inhibitory after step-training. Moreover, indirect evidence also suggests a better reciprocal inhibition after step-training in human SCIs (Maegele et al 2002). Among other spinal synaptic actions involving inhibitory neurotransmitter is presynaptic inhibition of primary afferents and interneurons. An increased in presynaptic inhibition is believed to be one of the mechanisms involved in step-training plasticity (further discussed in *Potential mechanisms involved in step-training plasticity in reflex transmission* section, p133). Together, these results illustrate that spinal plasticity is highly specific.

Cutaneous transmission We also illustrate that plasticity in cutaneous pathways is highly specific depending on the stimulated nerve and target motoneuron. Among the pathways tested (n=71), transmission was modified in only 10 and the other pathways were far from reaching statistical significance. We thus believe this is a true activity-dependent plasticity in very specific cutaneous pathways. As reported in the introduction, nerve-specific responses are observed in response to cutaneous stimulation during stepping (Abraham et al 1985, Moschovakis et al 1991, Pratt et al 1991, LaBella et al 1992, Degtyarenko et al 1996, Van Wezel et al 1997) to provide location specific information from the skin of hindlimb. On the other hand, a common synergy enhancing flexion during swing and facilitation extension during stance can also occur and this, independently on the location

of the stimulus (Duysens & Stein 1978, Duysens & Loeb 1980, Abraham et al 1985). Our results show specificity in the responses and no simple stance- or swing-related patterns. For example, step-training induce an increase in early excitation amplitude (R1) in MG motoneurons and a decrease in a synergist motor pool such as PI. Moreover, most of the plastic changes occur in the MG motor pool in response to a stimulation of MPL. This could suggest a preferential role of the plantar skin during weight-bearing. However, the role of different skin territories in the recovery of locomotion is difficult to interpret because patterns of activity of skin receptors during the different phases of the step cycle remain mostly unknown. There is little understanding of its role in normal locomotion. For example, some receptors from the plantar surface of the foot were found to be silent during stance and fire during swing (Loeb et al 1977). Thus the influence of cutaneous inputs on spinal pathways during a given time of locomotion cannot rely solely on its anatomical localization.

Another example of specificity of step-training related plasticity is illustrated again in changes in cutaneous transmission. The only motor pool in which the occurrence of responses without an inhibitory component (type D) increases following step-training is FDL (0% in untrained, 30.8% in step-trained). FDL is a toe plantar flexor active at the onset of swing (Fleshman et al 1984, Schmidt et al 1988, Moschovakis et al 1991, Degtyarenko et al 1996) to help clear the toes from the ground (Rossignol et al 1996). It acts as an extensor of the toes and is active in early flexion. We suggest that the inhibitory transmission to FDL is decreased to facilitate extension of the toes and reduce paw drag in early swing. There is indeed a paw drag at the beginning of the locomotor recovery in chronic spinal cats (Lovely et al 1986, Barbeau & Rossignol 1987, Bélanger et al 1996) and babies with immature descending tracts (Yang et al 2004). It seems to be associated with the inappropriate timing of flexion movements (hip, knee and ankle) at the beginning of the swing phase. This is thought to result from the disruption of the corticospinal and rubrospinal tracts (Jiang & Drew 1996). Notably, paw drag is especially pronounced in spinal cats when cutaneous inputs from the paw are removed (Bouyer & Rossignol 2003b).

ERK activation The assumption of our study was that, to exert their beneficial effect on the spinal cord networks, step-training would affect ERK pathway in the spinal cord. Furthermore, we expected that the clearest effect of training would be detected in the spinal interneuronal networks involved in the control of locomotion. In fact, step-training do

affect the expression and activation of ERK in the spinal cord of cats, but the most striking change occur in the L5 segment and not in the rostral lumbar segments potentially associated to the CPG (Kiehn 2006). In the cat, the L5 spinal segment contains a population of interneurons presumably activated by the MLR. Cord dorsum potential evoked by MLR stimulation and recorded at the dorsal root entry zone at the medial level of the spinal cord is maximal near the L5 level (Noga et al 1995). Accordingly, c-*fos* labeled cells following MLR-induced locomotion are most numerous in the spinal segments with the largest MLR-evoked field potentials (Dai et al 2005). Consistent results have been obtained after locomotion on the treadmill in corresponding spinal segments of the rats (Anh et al 2006). In these experiments, the number of *fos*+ neurons was directly related to the duration of treadmill stepping. In addition, preliminary results from the group of Dr Edgerton illustrate that the *fos*+ neurons activated by locomotion in the lumbar spinal cord of intact and spinal rats overlap with those neurons that express molecules such as CREB, NMDA receptor subunits and CaMKII (see Anh et al 2006). ERK-mediated CREB activation has been shown to be required for synaptic plasticity associated with the induction of stable, late-phase LTP and long-term memory in the hippocampus (Kelleher et al 2004, Thomas & Huganir 2004) and to short- and long-term synaptic changes in spinal sensory neurons (Kolch 2000, Ji & Wolf 2001, Ji 2004). However, we did not observe any change in CREB expression in step-trained animals.

It is notable that the rhythmogenic capacity is highest in the rostral spinal cord where hip motoneurons are located. This includes the L5 spinal segment in the cat. It has been suggested that the rhythmogenic network controlling hip movements act as a leading oscillator, entraining the more caudal and less excitable oscillators (Stein et al 2005). Further experiments may investigate if changes in ERK activation specifically target interneuronal and motoneuronal networks associated with hip muscles.

The general assumption is that the easier access of spinal neurons to neurotrophins following step-training might lead to the potentiation of the excitatory drive to motoneurons through an interaction with glutamatergic receptors (Gomez-Pinilla et al 2001, 2002, Skup et al 2002, Molteni et al 2002, Vaynman et al 2003). Surprisingly, we obtain a decrease in ERK activation. It has been reported that SCI might limit the effectiveness of exercise to enhance the expression of BDNF and CREB proteins (Ying et al 2005). Importantly, it is rather difficult to contrast data from previous and current experiments. There is unfortunately a great variety in the results from one preparation to the other, depending on the extent of the spinal lesion, on the time post-injury, and also on the exercise regimen

(chronic and repetitive or acute treadmill bout). Also, most of previous studies merged all lumbar spinal segments together. Our study shows that significant changes are specifically localized to a subset of spinal segments. In addition, our results suggest that in chronic spinal cats, ERK may be detrimental for stepping generation.

Potential mechanisms responsible for step training-induced plasticity in reflex transmission

A variety of mechanisms may explain the occurrence of modulation in the transmission of input in reflex pathways after SCI and step-training including morpho-functional changes in motoneurons and/or changes at the premotoneuronal level arising from dishinibition. The first type could include changes in intrinsic properties of the motoneurons, changes in motoneurons morphology and or synaptic growth. The second type might be attributable to a modulation of presynaptic inhibition and or postsynaptic transmission from afferent fibers. Plastic changes in a reflex pathway can occur in motoneurons, interneurons, or primary afferents.

Motoneuronal properties In previous reports, changes in spinal reflex pathways have been previously attributed to specific alterations in premotoneuronal mechanisms and not to changes in membrane properties of motoneurons between acute and chronic spinal cats (Chandler et al 1984, Munson et al 1984, Baker & Chandler 1987a). The same conclusion was reached as to explain the increase in monosynaptic reflexes in chronic spinal cats as compared to intacts (Hochman & McCrea 1994b).

In our experiments, a concomitant decrease in monosynaptic excitation, disynaptic inhibition and early and late cutaneous excitatory transmission could result from a general change in membrane responsiveness. However, we show that monosynaptic excitation and disynaptic inhibition do not covary in the same motoneuron. Additionally, the AHP duration, which varies systematically with R_{in} and membrane time constant (Gustafsson & Pinter 1984b), is found not to be modified by step-training even when motoneurons are grouped according to motor pools. Changes in resting membrane potential could also be responsible for a change in PSP amplitude (Powers & Binder 1985, Coombs et al 1955) but are shown not to be modified. Moreover, our results indicate that the stimulation of the same group I afferents could elicit opposite response patterns in two different pathways: a

decrease in monosynaptic excitation together with an increase in polysynaptic excitation in the same motoneuron. Also, a general change in membrane responsiveness is unlikely to explain the simultaneous increase in cutaneous early excitation amplitude (R1) and decrease in late excitation amplitude (R3) observed in a single motoneuron. In addition, changes in PSP amplitude are shown not to be related to changes in membrane potential levels during fictive locomotion (LDP) and step-training do not modify LDP peak-to-peak amplitude. In agreement with others, (Baker & Chandler 1987ab, Hochman & McCrea 1994a-c, Gosgnach et al 2000, Shefchyk & Jordan 1985), we consider that most of the plasticity after step-training result from premotoneuronal mechanisms. The exact contribution of motoneuronal properties to reflex transmission following SCI and step-training remains to be elucidated.

Presynaptic inhibition The previous results suggest that premotoneuronal mechanisms are mostly responsible for the changes in PSP amplitude in step-trained cats. These include plasticity induced by training in interneurons of group I pathways and cutaneous pathways and/or interneurons of presynaptic inhibition. For example, the decrease in monosynaptic excitation, a pathway in which there is no intercaled interneuron, support that there could be a role for presynaptic inhibition in step-training related plasticity. Indeed, presynaptic inhibition is associated with a decrease in transmission in this pathway during MLR-evoked locomotion as compared to rest (Gosgnach et al 2000) but has not been reported in spontaneous fictive locomotion in the same preparation (Ménard & Gossard, personal communication). Cyclic variations of afferent depolarization that could represent phasic presynaptic inhibition are also observed in many muscle and cutaneous afferents during locomotion in cats and other species (Nusbaum et al 1997). However, the extent of plasticity in presynaptic inhibitory networks is still very much unknown. There is one report of modified presynaptic inhibitory patterns following a nerve crush (Enriquez et al 1996). Because presynaptic inhibition may be regulated in a highly selective fashion (Rudomin & Schmidt 1999), we believe it has a potential role in the plasticity observed after step-training. Further experiments should be designed to specifically address this question.

Functional considerations

Following SCI, supraspinal centers will no longer excite interneurons in inhibitory pathways from Ia-Ib afferents and Renshaw cells. Enhancement of the actions of excitatory

interneurons could on the other hand be secondary to the loss of inhibitory control by descending tract neurons. Indeed, when supraspinal control of spinal reflexes is impaired, the inhibition of the monosynaptic reflex is missing in addition to a reduced facilitation of polysynaptic reflexes. In the next sections, we suggest that step-training may help to counter the lesion-induced modifications in reflex pathways to re-express stepping.

Step training-related plasticity may decrease reflex hyperexcitability associated with clonicity and spasticity. There is a persistent hyperexcitability of several spinal reflexes following SCI because of the removal of inhibitory descending inputs from the brainstem (Holmqvist & Lundberg 1961, Lundberg 1964, Hultborn & Malmsten 1983, Malmsten 1983). For example, some components of the flexor reflex mediated by low threshold sensory afferents increased permanently their excitability after SCI in rats (Malmsten 1983, Valero-Cabré et al 2004). The withdrawal reflex was also shown to be hyperexcitable after SCI and suggested to contribute to spasms and spasticity (Ashby & McCrea 1987, Bennett et al 1999, Rémy-Néris et al 1999). Similarly, an enhanced monosynaptic reflex has been associated to spasticity and reported to interfere with the generation of locomotion after SCI (Calancie et al 1993, Faist et al 1994, Trimble et al 2001). In our experiment, step-training decrease the monosynaptic EPSP. Also, cutaneous pathways that show a significant modulation in transmission are mostly depressed by step-training. Because chronic spinalization induces a facilitation of reflex responsiveness, step-training may compensate by normalizing the level of transmission in these pathways. We suggest that a concomitant decrease in the amplitude of cutaneous EPSPs and of the monosynaptic EPSPs following step-training would serve to counter the hyperexcitability of reflexes after SCI and lead to a more functional locomotor output.

Step-training may facilitate the recruitment of extensors to help recover weight-bearing. A role for group I afferents in the recovery of motor functions after a lesion to the nervous system has previously been demonstrated. Indeed, reflexes regulating the timing of phase transitions during stepping and also those reinforcing the generation of extensor activity are enhanced by partial denervation of ankle extensor muscles (Pearson & Misiasek 2000) and this would depend on group I afferent feedback (Pearson et al 2003).

Inputs from group I afferents from extensors, Deiter's nucleus, some reticulospinal fibers in the medial longitudinal fasciculus (MLF) and pyramidal tract are transmitted through

common polysynaptic pathways that can enhance and promote extensor activity following L-DOPA injection (Leblond et al 2000, 2001). In spinal animals, the facilitatory effect of descending tracts on extensors during the stance phase is lost and weight-bearing is greatly impaired. Thus, it is likely that after SCI, Ib interneurons are mainly excited by group I afferents and also from cutaneous afferents. Our results showed that step-training appears to enhance the effects of clonidine in the reduction of disynaptic group I inhibition and reversal to polysynaptic excitation. Because it is believed that polysynaptic excitatory group I pathways transmit locomotor drive to extensor motoneurons (Gossard et al 1994) and that load-related feedback during the stance phase contributes significantly to the generation of activity in extensors (Ghori & Luckwill 1985, Dietz et al 1992, Hiebert & Pearson 1999, Stephens & Yang 1999, Sinkjaer et al 2000), we suggest that the latter changes would facilitate the recruitment of antigravity muscles to assist the recovery of weight-bearing during stepping. In addition, longer periods of locomotor training (3 months) further decreased the group Ib disynaptic inhibition and increase polysynaptic excitation in extensors, and this even without clonidine (Côté et al 2003b). This further suggests that plasticity in group I pathways after step-training is not transient, but persist over months, and it develops and progresses over a period of time. This would further facilitate extensor activity during the stance phase of stepping.

Normally, the monosynaptic reflex in ankle extensors reaches its maximum soon after the onset of the EMG, at the time the foot normally touches the ground. Conversely, the reflex is minimal late in the extension phase, just before the foot leaves the ground so that a perturbation will not interfere with the forthcoming swing phase (Akazawa et al 1982, Capaday & Stein 1986, Crenna & Frigo et al 1987, Simonsen & Dyhre-Poulsen 1999). Intracellular recordings show that the maximal amplitude of the monosynaptic EPSP occurs during the depolarized phase of the motoneuron when the muscle is active (Schomburg & Behrends 1978b, Perret & Cabelguen 1980, Shefchyk et al 1984, Gossard 1996, Ménard et al 1999, 2003). We report that the maximal amplitude of monosynaptic EPSPs occurs in the opposite phase in the chronic spinal cat. It was shown that the monosynaptic stretch reflex might contribute to the level of EMG activity during stepping (Yang et al 1991b, Sinkjaer et al 1996, 2000, Stein et al 2000). A disrupted modulation of monosynaptic transmission could lead to a decrease in ankle extensor muscle activity during stepping and this may interfere with functional walking in untrained animals. We also report that step-trained animals recovered a normal modulation pattern of the monosynaptic reflex indicating a maximal Ia-motoneuron transmission during the stance phase to enhance weight-bearing.

In addition, a major finding in our studies is that cutaneous transmission is predominantly modified in pathways to MG motoneurons, particularly when activated by MPL afferents. MG is an extensor muscle involved in weight support during the stance phase of stepping. The plantar surface of the foot, MPL receptive field, presumably provides phasic inputs during ground contact and also convey information concerning ground surface. Reflexes evoked by the plantar surface of the foot can promote extension during stance and interrupt the swing phase during locomotion (Duysens & Pearson 1976, Duysens 1977, Guertin et al 1995). Our results indicate that both step-training or CPG activity in untrained spinal cats (fictive locomotion) result in a net excitatory action from MPL to MG motor pool. Thus, we suggest that plasticity in at least some cutaneous pathways would result in a better recruitment of the MG motor pool during ground contact. This may further help to recover weight bearing.

Do plastic changes in reflex pathways contribute to locomotor recovery? During locomotion, reflexes function as to preserve balance and ensure a stable walking pattern throughout the cycle. Muscle and cutaneous reflexes act together in an integrated manner to adapt the motor output but also contribute significantly to the generation of force. After SCI, reflex may no longer function as efficiently during stepping. Modifications are observed in both muscle and cutaneous pathways following step-training, but one may ask if these changes are effectively involved in the recovery of locomotion and if the electrophysiological assessment of spinal cord reflexes is a useful tool to investigate spinal cord functional reorganization following SCI and step-training. It was recently shown that the restoration of stepping, when facilitated by epidural stimulation of the spinal cord, coincide with the restoration of late polysynaptic responses (Lavrov et al 2006). Moreover, a contribution of enhanced reflex function in improving locomotor performance is indicated by recent experiments in which adult spinal rats were treated with olfactory ensheathing cells (Ramon-Cueto et al 2000). The locomotor performance on an incline grid was strongly correlated to the appearance of cutaneous and proprioceptive reflexes. This may suggest a contribution of enhanced reflex function in improving the locomotor performance.

However, some cases were reported in which alterations in descending and segmental reflexes did not correlate with functional recovery (Norrie et al 2005). The recovery of a *skilled* locomotor task in the trained group occurred before the recovery in ground support and proprioceptive reflexes. The authors thus suggest that reflexes had little impact on the

improvement in accurate stepping. Accurate placing movements may indeed recruit direct cortical pathways. However, it is important to note that in this latter experiment, reflexes were studied in a stationary position and might have needed the locomotor program to reveal further changes in reflex pathways. Another important factor is the time course of plastic processes. It is quite possible that plasticity involving upper motor areas precedes plasticity in lower levels. For example, the increase strength of reflexes regulating the timing of stance to swing transition and reinforcing the generation of extensor activity in denervated animals was shown to take days to weeks to fully develop and are established while animals are behaving normally (Whelan & Pearson 1997, Pearson & Misiaszek 2000, Gritsenko et al 2001). Plasticity is a dynamic process and involve mechanisms that evolutes over time. An additional example is the peak facilitation of the H-reflex that was observed 45 days post-lesion in SCI rat and then decreased over time (Valero-Cabré et al 2004).

In our study, reflex transmission was evaluated one month after the complete spinal cord lesion and/or onset of step-training. Further studies are needed to directly address the question whether the changes in reflex pathways parallel the improvement of the locomotor pattern over time. Also, the extent of plasticity in so many sensory pathways (group II, articular, nociceptive) and circuits (propriospinal, recurrent and reciprocal inhibitory) is still unknown. It is most likely that several mechanisms and networks (including CPG plasticity) are involved to ultimately lead to stepping recovery and none of them taken independently may fully correlate with the improvement in the stepping pattern.

Future directions

Among the many unknowns, issues and experiments that our study raised, some of the most important include the unknown gamma bias and the role of converging Ib and cutaneous inputs.

The parallel excitatory control of α- and γ-motoneurons projecting to one muscle and the corresponding Ia inhibitory interneuron activation allows a coordinated activation and relaxation of antagonist muscle pairs. Moreover, another way to alter proprioceptive feedback is to change the γ drive to muscle spindles. Different explanations have been proposed for the alterations observed in the spinal cord after SCI such as γ-motoneuron depression (Weaver et al 1963) because of the loss of excitatory drive by supraspinal

afferents. From studies on H-reflexes and monosynaptic EPSPs during fictive locomotion we better understand the central contribution to sensory modulation. However, during real locomotion, the Ia transmission can possibly be determined by the gamma drive. What happens to gamma activation after SCI and /or step-training is still very much unclear. This is also an important issue regarding training and stimulating SCI subjects on the treadmill.

Afferent inputs from load receptors are involved in the modulation of cutaneous reflex responses which were decreased in amplitude with increasing load. The authors suggest that a higher body load would improve stability and less reflex activity in cutaneous pathways is needed (Bastiaanse et al 2000). Conversely, Ib reflex pathway is strongly depressed in motoneurons innervating muscles of the knee following a cutaneous stimulation (Pierrot-Deseilligny et al 1981a). There is a control of Ib interneuronal transmission evoked by the plantar surface of the foot. Are those patterns maintained after SCI? It was shown that plantar cutaneous afferent inputs modulate the SOL H-reflex differently in intact and SCI subjects (Knikou 2007) suggesting that it may not be the case. Further research is needed to evaluate the sensorimotor integration between Ib and cutaneous pathways and their relative contribution to the motor output after step-training.

Activity-dependent plasticity after SCI involves essentially all the elements of the nervous system from neuromuscular junction to the brain. It most likely extend beyond neurons and synapses to involve glia and vasculature. Our results suggest that stimulation of particular sensory pathways could contribute to the plasticity underlying locomotor recovery. This basic knowledge may help refine therapeutic strategies by means of electrical stimulation or neurorehabilitation to decrease spasticity, reduce neuropathic pain, or optimize the walking pattern in SCI patients. Experimental protocols can be conceived to emphasize some sensory modalities.

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