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Modifications of excitability of spinal networks in healthy subjects and patients with central nervous system lesions

Wanalee Klomjai

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Université Pierre et Marie Curie

Ecole doctorale : Cerveau Cognition et Comportement

*Laboratoire d'Imagerie Biomédicale / Equipe Systèmes dynamiques anatomo-fonctionnels chez
l'Homme : altération et récupération fonctionnelle*

Modifications d'excitabilité des réseaux neuronaux de la moelle épinière chez des sujets sains et des patients porteurs de lésions du système nerveux central.

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กำลังใจและสนับสนุนลูกสาวคนนี้อมาตลอด รักแม่ค่ะ*

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ตลอดระยะเวลาที่เรียนที่นี่ทุกคนมีความสำคัญกับพี่มากและขอบคุณอาร์ค
สำหรับทุกอย่างค่ะ

ขอบคุณทุกคนมากจริงๆ

Je vous aime!!!

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Introduction

Motor function (i.e. muscle tone, posture and movement) depends on the firing of the motoneurons (MNs) "the final common pathway" (Sherrington, 1906). Features of MN firing (number of MNs activated, frequency of firing and pattern of activation) reflect an integration of the upstream activity in different structures of the central nervous system (CNS) (motor cortex, cerebellum, basal ganglia, vestibular system, and spinal cord circuitry).

Most available data concerning the knowledge of neural pathways involved in motor control come from experiments performed in animal preparations (anaesthetised, decerebrated or spinalised) using invasive methods that cannot be used in humans. Due to phylogenetic evolution, data obtained in animals cannot be always transposed in man without caution for several reasons, for example:

1. Both the descending control of spinal pathways (e.g. the appearance of the monosynaptic cortico-motoneuronal connections in primates) and the distribution of afferent inputs to MN pools have changed with the appearance of bipedal stance while the upper limb is free from postural tasks.
2. The consequences of a corticospinal lesion are much more dramatic in humans (hemiplegia following stroke) than in cats which recover quasi-normal mobility in a few weeks.
3. The knowledge of normal neural organisation human is a pre-requisite to harness the residual motor capacities of the spinal cord after disconnected from upper motor neurons.

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My research projects fit into these frameworks and focus on two aspects:

1. The study of changes in spinal inhibitory network induced by furosemide in healthy subjects and patients with spinal cord injury (SCI).

Recent studies in animals have shown that down-regulation of potassium chloride co-transporter KCC2 after spinal cord transection reverses the effect of gamma aminobutyric acid (GABA) and glycine back to their immature (excitatory) state (Vinay & Jean-Xavier, 2008) and thus results in a reduction of inhibitory synaptic efficiency (Bouleguez et al. 2010). If this phenomenon also exists in humans following SCI, it may contribute to the reflex hyper-excitability exhibited in SCI patients. To address this question, we demonstrated effect of a diuretic (furosemide), a cation-chloride co-transporter antagonist, on spinal network excitability in healthy subjects and SCI patients.

2. Ipsi- and contralateral corticospinal control on reciprocal inhibition between forearm muscles in healthy subjects and hemiplegic stroke patients as demonstrated by transcranial direct current stimulation.

Transcranial direct current stimulation (tDCS) has emerged as a method for exploring cortex excitability (Priori et al., 1998; Nitsche & Paulus, 2000), and has also begun to be used as a tool to enhance recovery from hemiplegia. Recent studies in healthy subjects have shown that anodal tDCS over the contralateral hand motor cortex can induce changes in spinal network excitability (i.e. reciprocal inhibition) (Roche et al., 2009, 2010, 2012). It is unknown whether motor activity from the unaffected cerebral hemisphere could be employed after semi-brain damage in patients with hemiplegia. Moreover, little is known about the non-affected limb if it always functions like 'normal' after unilateral stroke. To address this question, the ipsi- and contralateral corticospinal controls on reciprocal inhibition between forearm muscles were explored using anodal tDCS applied over the motor cortex of non-lesioned hemisphere of stroke patients and then compared to healthy subjects.

The common objective of these projects is to disclose pathophysiology of motor impairment following CNS lesions so as to develop new therapeutics treatments (i.e. new rehabilitation programs relying on neurophysiological bases, pharmacological treatments). This thesis is organized into four chapters. The first chapter is dedicated to basic knowledge of physiology and functioning of spinal networks studied in this thesis. The second chapter firstly presents the basic electrophysiological method used in common by the two studies together with its advantages and limitations, followed by two sections separating the methods used in each study. The third chapter is also divided into two sections: i) the first section reports results obtained in the study observing changes in spinal inhibitory network induced by a diuretic (furosemide) in

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healthy subjects and paraplegic patients ii) the second section reports results from study of ipsi- and contralateral corticospinal control on reciprocal inhibition in healthy subjects and hemiplegic stroke patients as demonstrated by transcranial direct current stimulation. The final chapter is divided into two sections as well, both involving discussion and conclusion of each study.

Part of the work presented in this thesis is included in two following articles.

[1] Klomjai, W., Lackmy-Vallée, A., Katz, R., Bussel, B., Bensmail, D., Lamy, J., Roche, N. Changes in spinal inhibitory networks induced by furosemide in humans. *Journal of Physiology*. Accepted 30 April 2014

[2] Lackmy-Vallée, A., Klomjai, W., Bussel, B., Katz, R., Roche, N. Anodal transcranial direct current stimulation of the motor cortex induces opposite modulations of reciprocal inhibition in wrist extensors and flexors. *Journal of Neurophysiology*, in revision.

Chapter 1

Background of physiology

1. Presynaptic inhibition of Ia fibres

It has been shown that the synaptic efficacy of the primary sensory afferent (Ia fiber) - α -motoneuron synapse can be regulated by presynaptic inhibition (PSI) at the spinal cord. The sensory information can be thereby modified before reaching the target neuron to optimise motor performance and sensory discrimination.

In 1957, Frank and Fuortes, have shown in the cat that stimulation of sensory afferent fibres leads to a reduction in excitatory postsynaptic potentials (EPSPs) generated in motoneurons in the spinal cord. This decrease occurs without any accompanying change in the postsynaptic membrane.

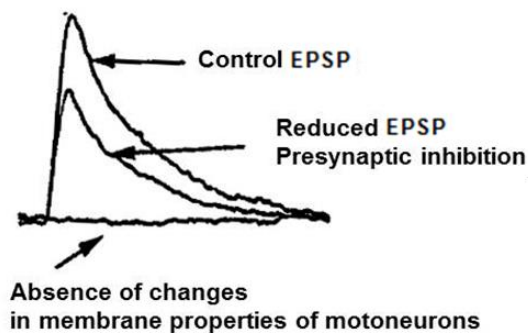


Figure 1 : Response in motoneurons in the spinal cord corresponds with presynaptic inhibition.

Reproduced from Frank & Fuortes, 1957.

The presynaptic inhibition is mediated by interneurons acting on primary afferent terminals through axo-axonic synapses (reviewed in Eccles, 1964). This results in a depolarization of primary afferents, termed primary afferent depolarization (s) which leads to a reduction in transmitter release at synaptic junction, thus inducing a decrease in excitatory postsynaptic potentials (EPSPs). Pharmacological studies have shown that presynaptic inhibition is reduced by the GABA_A antagonists (Eccles et al., 1963; Curtis et al., 1971). The existence of GABAergic axo-axonic synapses on the terminal of Ia afferent fibres is proposed as a mechanism underlying presynaptic inhibition (reviewed in Rudomin, 1999). Activation of GABA_A receptors increases the efflux of Cl⁻ ions and thus inducing depolarisation of the terminal of afferent fibres. This induces a reduction of the amplitude of propagated action potential that blocks or reduces Ca²⁺ influx, thereby inhibiting the release of neurotransmitter from Ia afferent axons (reviewed in Rudomin & Schmidt, 1999).

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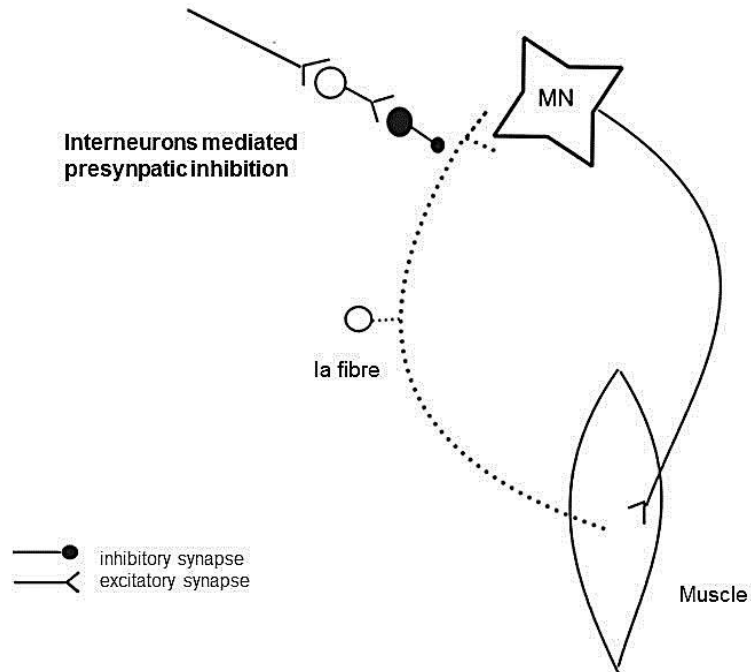


Figure 2 : Sketch of presynaptic inhibition of Ia fibre.

It has been shown in the cat that the latency of presynaptic inhibition was 1.5-1.8 ms and at least two interneurons (first-order PAD interneuron and last-order PAD interneuron) were basically required in this control (reviewed in Jankowska, 1992). The long-duration presynaptic inhibition has been suggested to be due to sustained activity of PAD interneurons (Eccles et al., 1962b) or the slow dynamic of GABA release and/or uptake (reviewed in Rudomin et al., 1998).

Electrophysiological studies in animals showed that PAD interneurons can be activated by repetitive electrical stimulation, vibration or stretching of the muscle tendon (Eccles, 1964; Devanandan et al., 1965, 1966; Gillies et al., 1969).

1.1 Inputs to PAD interneurons

1.1.1 Peripheral inputs to PAD interneuron

Interneurons mediating presynaptic inhibitions are facilitated by group I afferents. In humans, there is no direct evidence showing that Ib afferents also contribute to the activation of the PAD interneurons inhibition as demonstrated in animals. However, the fact that the threshold of inhibition "D1" of the soleus motoneurons induced by conditioning stimuli of the common peroneal nerve ($0.9-1 \times$ motor threshold) (Mizuno et al., 1971; Iles, 1996) is higher than that of disynaptic reciprocal Ia inhibition ($0.6-0.7 \times$ motor threshold) would be compatible with the involvement of Ib afferents. Studies in animals and humans showed that low-threshold cutaneous afferent volleys induce a decrease of presynaptic inhibition of Ia fibres (Iles & Robert, 1987; Nakashima et al., 1990; Meunier & Pierrot Desseilligny, 1998; Rudomin et al., 1983). Anaesthesia of the hand in human subjects increased the amount of presynaptic inhibition. This confirms that cutaneous input can modulate transmission in presynaptic inhibitory pathways in humans (Nakashima et al., 1990). At least in the cat, articular afferents also induce a decrease on presynaptic inhibition and both cutaneous and articular afferents depress transmission in PAD pathways at the level of the first-order PAD interneurons (Lund et al., 1965; Rudomin et al., 1983)

1.1.2 Descending inputs to PAD interneuron

Inhibitory descending control

The main descending control influencing PAD interneurons mediating presynaptic inhibition of Ia fibres is depressive. Corticospinal fibres and cutaneous afferents converge onto inhibitory interneurons and suppress the activity of first-order PAD interneurons (reviewed in Lundberg & Vycklicky, 1963; Rudomin et al., 1983). Last-order PAD interneurons are tonically depressed via different reticulospinal pathways (reviewed in Rudomin & Schmidt, 1999). The interruption of this strong tonic inhibitory control results in net increase in the excitability of PAD interneurons presynaptic inhibition after spinalization in decerebrate animals. The brainstem structures responsible for tonic depression of presynaptic inhibition of Ia fibers are controlled by inhibition effect from the higher centers. Therefore, presynaptic inhibition is diminished in decerebrate animals. In humans, presynaptic inhibition is consistently decreased in the lower limb after spinal cord lesions (Faist et al., 1994; Nielsen et al., 1995; Pierrot-Desilligny, 1990). Study in humans showed that motor cortex stimulation reduces D1 inhibition of the soleus H-reflex (Meunier & Pierrot-Desseilligny, 1998) confirming corticospinal depression of PAD interneuron of presynaptic inhibition of soleus Ia terminals.

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Facilitatory descending control

Facilitatory descending control on PAD interneuron of presynaptic inhibition probably exists, but it is generally less dominant than inhibitory effects (reviewed in Lundberg, 1992). The first-order PAD interneurons could be excited via vestibular nuclei (Carpenter et al., 1966).

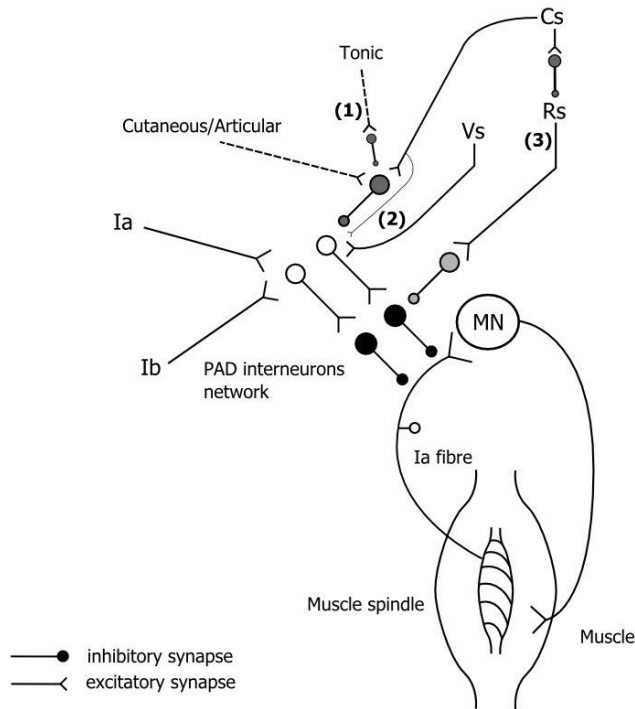


Figure 3 : Pathways of presynaptic inhibition with primary afferent depolarisation (PAD) of Ia terminals in the cat.

First-order PAD INs receive excitation from Ia and Ib afferents and the VS pathway (2). They receive inhibition through the same inhibitory INs from cutaneous afferents and the CS pathway (however, there is also facilitation effect from corticospinal pathway on first-order PAD INs, indicated by the thin line). Inhibitory INs inhibiting first-order PAD INs receive descending tonic inhibition (dotted line (1)). Last-order PAD INs receive inhibition from the RS pathways, themselves inhibited from higher centres (3). *Adapted from Pierrot-Deseilligny & Burke, 2012.*

VS = vestibulospinal pathway, CS = corticospinal pathway, RS = reticulospinal pathways

2. Postsynaptic inhibition induced by cutaneous stimulation

Postsynaptic inhibition induced by cutaneous stimulation plays a role in withdrawal reflex/flexor reflex. This is a spinal reflex, causing a contraction of limb flexors in response to nociceptive stimulus to protect the body from damage. Flexor reflex was firstly introduced by Sherrington in 1906. In decerebrate animals, noxious skin stimuli on the plantar of foot induce contraction of flexor muscles of hip and knee and dorsiflexor of the foot while antagonist extensor muscles are inhibited (Sherrington 1906, 1910). Sherrington also states that the flexion movement at this joint is according to the skin-point stimulated, introducing the concept of 'local sign'. The connection between sensory neuron (impulses from nociceptors) with motoneurons in the flexion reflex circuit is shown to be polysynaptic involving several interneurons (reviewed in Hunt & Perl, 1960). The flexion reflex was confirmed in humans by Hagbarth (1960). Noxious stimuli applied to the plantar of foot evoke reflexes, which closely resemble with the flexion reflex of Sherrington. It was suggested by the author that flexor reflex can be elicited in all lower limb muscles when the adequate receptive field is stimulated. The latencies of these reflexes were relatively short which indicate that they are probably mediated by spinal reflex arc (reviewed in Kugelberg, 1948; Pedersen, 1954).

Influence of cutaneous volley on the modulation of soleus monosynaptic reflex was demonstrated by Hugon (1973). After electrical stimulation of the sural nerve (cutaneous foot afferents), reflex response appear at a latency of about 80 ms in different muscles of both legs and also induces an inhibition of the monosynaptic soleus H-reflex. The effects of single cutaneous stimuli on soleus motoneuron excitability depend on the site of stimulation and the inhibition recorded from ankle tendon jerk is greater when cutaneous stimuli applied over the lateral dorsum or the plantar surface of the foot than over the mid-dorsum of the foot. Burke et al. (1991) and Nielsen et al. (1997) presented the evidence that the cutaneous effect induced in the lower limb with latency shorter than 70 ms, is spinal in origin. Their results are also supported by the fact that the cutaneous stimuli induced spinal motoneuron inhibition exists in spinal animals and complete spinal cord lesions in humans (Longchamp & Chanelet, 1968; Logigian et al, 1999).

The descending control seems to have a net facilitatory effect on cutaneous inhibition. A study in patients with spinal cord lesion demonstrated that soleus motoneurons could be inhibited by high-intensity cutaneous stimuli of sural nerve over the same ISI range as normal subject, but the degree of inhibition is less than in normal subjects (Logigian et al, 1999).

No direct evidence demonstrates neurotransmitter involved in postsynaptic inhibition induced in soleus motoneuron by cutaneous afferents. Few pharmacological studies have shown that strychnine, an antagonist of spinal postsynaptic inhibition, blocked the effects of glycine on spinal motoneurons and interneurons (Curtis, 1969) and inhibition of motoneuron involved in flexor reflex has been shown to be mediated by glycine (Dhawan, 1972).

3. Post-activation depression (synaptic transmission between Ia fibre and motoneuron)

It has been shown in the cat that the amplitude of monosynaptic reflex decreases during repetitive stimulation of afferent fibres (Eccles & Rall, 1951). A frequency related depression of this reflex has been described at stimulus intervals as long as 10-20 s (Lloyd & Wilson, 1957). Using intracellular recordings of motoneurons in the cat, Curtis & Eccles (1960) have demonstrated changes in the EPSP generated monosynaptically in gastrocnemius motoneuron following repetitive activation of Ia afferent of tibial nerve (Figure 4).

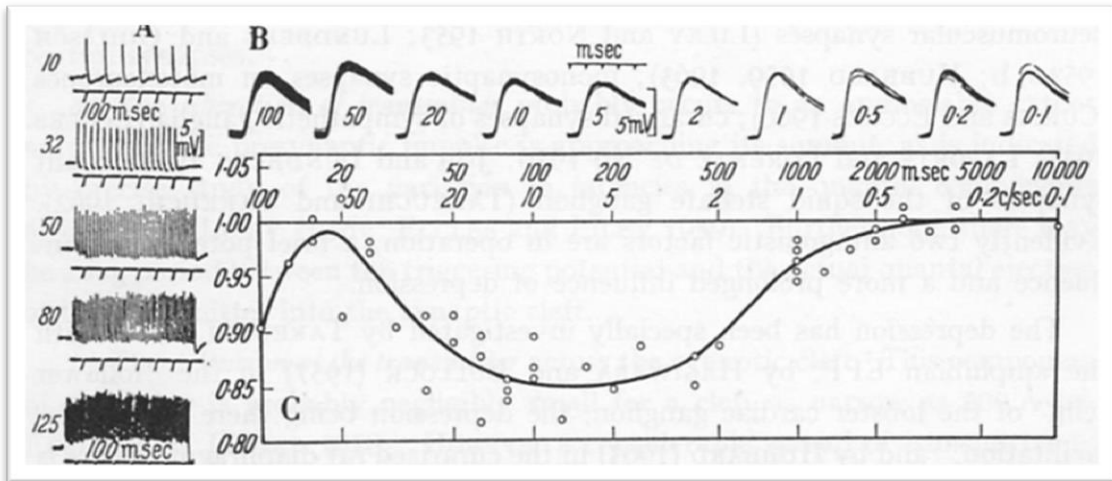


Figure 4 : Intracellular recording of a cat motoneuron the EPSP generated monosynaptically in gastrocnemius motoneuron following repetitive activation of Ia afferent of tibial nerve.

A) Repetitive monosynaptic EPSP's recorded from gastrocnemius motoneuron in a cat with amplifier and the frequency of the maximum group I volley from posterior tibial nerve of each record B) Repetitive monosynaptic EPSP's at the indicated frequencies (Hz) when the EPSP had attained a steady state. C) Repetitive monosynaptic EPSP's plotted against the stimulus frequencies. Above the frequency scale, the corresponding stimulus intervals are shown in msec. *Reproduced from Curtis & Eccles, 1960.*

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The recording reveals changes in amplitude of EPSP following changes in frequencies. There is no change in the EPSP's size until the frequency is in excess of 0.4/sec. There is a progressive depression of EPSP as the frequency is raised to 5-10/sec, whereas, at higher rates, EPSPs increase and reach a maximum at approximately 50/sec. It has been suggested that the depression results from reduced release of neurotransmitter from Ia afferent fibers to motoneurons (Kuno, 1964). Repetitive activation of the Ia afferents can be expected to induce a reduction of neurotransmitter stores in the Ia afferent terminal. If this reduction becomes pronounced, the neurotransmitter release would be insufficient for subsequent activation. The post-synaptic depression appears to depend on the intrinsic neuronal properties of the motoneuron. Facilitation effect is dominant for high-threshold fast motoneuron, while depression is dominant for low-threshold slow motoneuron (Honig et al., 1983).

It has been also shown in humans that the amplitude of H-reflex decreases during repetitive stimulation (Magladery & McDougal, 1951; Paillard, 1955; Rothwell et al., 1986; Hultborn & Nielsen, 1998; Hultborn et al., 1996; Aymard et al., 2000; Lamy et al., 2005). This depression can be attributed to the previous activation of Ia afferent because it is observed after a conditioning stimulus that is subthreshold for the H-reflex or tendon jerk (Katz et al., 1977; Hultborn & Nielsen, 1998). A long lasting inhibition of the soleus H-reflex can also be evoked by a preceding soleus H-reflex, by a brief of voluntary ankle flexor or extensor muscle contraction or by a tap applied to Achilles tendon. The time course of this long lasting inhibition was similar in all cases suggesting that the mechanism responsible for this depression is the same. At stimulus intervals between the preceding conditioning stimulus and the soleus H-reflex test longer than 12 s, the size of the H-reflex did not increase, whereas a slight decrease of the H-reflex is observed at a conditioning-test interval of 10 s and the depression increases rapidly when the frequency of stimulation below 10 s (Crone & Nielsen, 1989) (see Figure 5).

In addition, post-activation depression is reported to be decreased in spastic patients compared to healthy subjects (Nielsen & Hultborn, 1993, Nielsen et al., 1995, Hultborn & Nielsen, 1998). The amount of post-activation depression of the flexor carpi radialis (FCR) and soleus H-reflex is significantly reduced on the affected side of hemiplegic patients, whereas no changes were observed on the unaffected side and also in normal subjects (Aymard et al., 2000, Lamy et al., 2009).

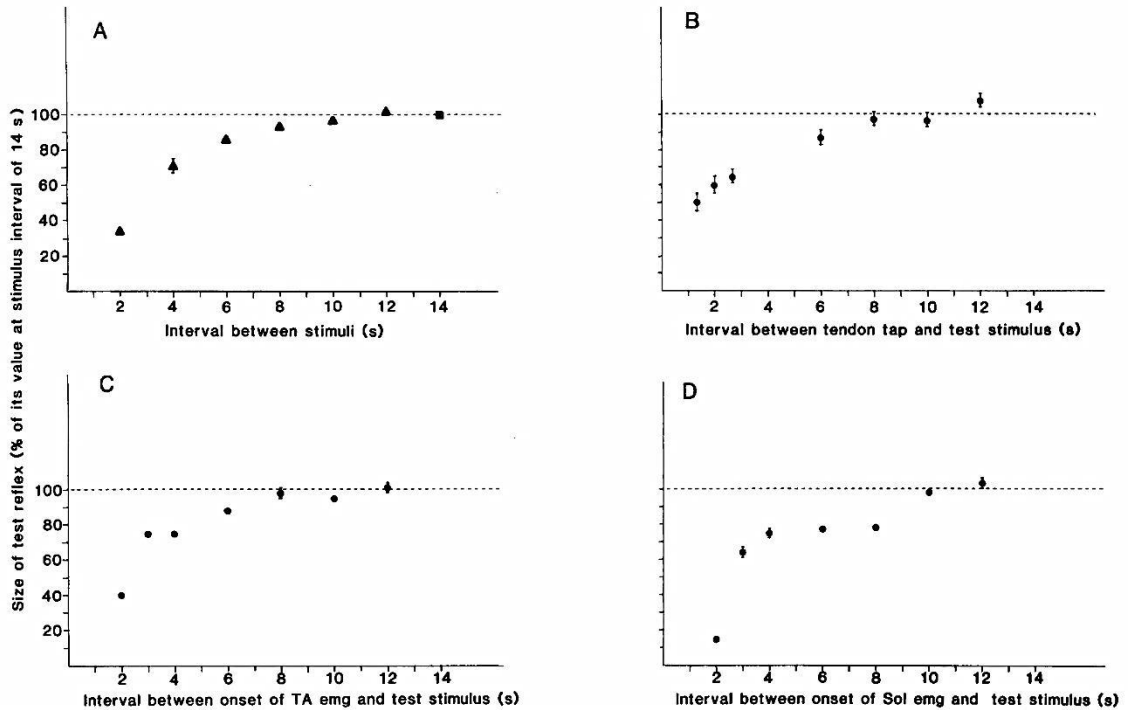


Figure 5 : Size of the soleus H-reflex preceded by difference conditioning stimuli activating soleus Ia fibres.

A) A subthreshold tendon tap applied to the Achilles tendon B) A brief voluntary contraction of tibialis anterior C) A brief voluntary contraction of soleus D) Sustained contraction of soleus. In A-D the test reflex was elicited every 14s and the conditioning stimulus/movement was elicited at varying intervals (1-12 s) before the test reflex. The size of conditioned H-reflex was expressed as a percentage of its control value measured at a stimulus frequency of 1 stimulus per 14 s. *Reproduced from Crone & Nielsen, 1989.*

4. Reciprocal inhibition

The concept of reciprocal inhibition was firstly introduced by Sherrington in 1897. Sherrington's law of reciprocal innervation states that: When a muscle contracts, its direct antagonist relaxes to an equal extent allowing smooth movement. Pathway of reciprocal Ia inhibition was firstly investigated in the cat lumbar motoneurons using monosynaptic test reflexes, suggesting that Ia afferents had 'direct effect' on antagonistic motoneuron, introducing 'direct inhibition' (Lloyd, 1946). However, the hypothesis of direct inhibition was failed since intracellular recording subsequently showed that an interneuron is interpolated in the Ia inhibitory pathway (Eccles et al., 1956) and activity in this pathway can inhibit the monosynaptic reflex (Araki et al., 1960). The interneuron has been called 'Ia interneuron' (Eccles et al., 1960). The Ia interneuron was introduced as a mechanism changing the excitatory action of Ia afferents into inhibitory action, determining as a simple 'integrative centre' (Eccles & Lundberg, 1958). Individual Ia inhibitory interneuron inhibits approximately up to 20% of motoneuron of a given nucleus. However, each interneuron induces only simply small inhibitory postsynaptic potentials (IPSPs) in motoneurons, and so at least 70 interneurons are required to inhibit individual motoneurons (Jankowska & Robert, 1972).

In general, Ia interneuron directly inhibits antagonist motoneuron. However, this rule cannot be applied in some cases. Intracellular recordings showed that reciprocal Ia inhibition is not found between adductors and abductors (Eccles & Lundberg, 1958) or expiratory and inspiratory intercostal muscles of the same segment (Sears, 1964). In contrast, there is an inhibition from knee extensor to motoneuron of the hip flexor (Eccles & Lundberg, 1958). It was shown in decerebrated cat that in the hindlimb that maximal Ia IPSPs are much larger in flexor than in extensor motoneuron (Eccles & Lundberg, 1958). Besides its connection to antagonist motoneuron, Ia inhibitory interneuron also projects to opposite Ia interneuron (Hultborn et al., 1976). For example, Ia interneuron activated from flexor Ia afferents inhibits Ia interneuron activated from extensor Ia afferents.

It was shown in human ankle (Mizuno et al., 1971), wrist (Day et al., 1984), and elbow (Katz et al., 1991), that group I afferent fibres are involved in reciprocal inhibition. As stressed in the cat by Hultborn et al. (1971), Ia inhibitory interneurons can be inhibited by Renshaw cell. Except from Renshaw cells themselves, Ia interneurons are the only spinal interneurons that receive recurrent inhibition. This specific characteristic allows to distinct between reciprocal Ia and non-reciprocal group I inhibition (see Pierrot-Deseilligny & Burke, 2012). In humans, reciprocal inhibition at ankle and elbow levels have been considered as 'true' reciprocal Ia inhibition since i) the

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muscles are 'real' antagonists ii) the inhibition can be evoked by a pure Ia volley, and iii) the inhibition is depressed by recurrent inhibition (Katz et al., 1991; Baret et al., 2003). Unlike ankle and elbow levels, the reciprocal inhibition between wrist flexors and extensors is not inhibited by Renshaw cells activation and there is no evidence that the inhibition can be evoked by pure Ia volley (Aymard et al., 1995). Indeed, flexor carpi radialis (FCR) and extensor carpi radialis (ECR) are not real antagonists since they operate as synergistic muscles in wrist abduction movement. The inhibition of ECR motoneurons by activation of Ia afferent fibres from the median nerve is not only inhibition between antagonistic muscles since the median nerve also innerves finger flexors muscles that are synergists of wrist extensor in clenching and grasping. Moreover, the fact that the interneurons mediating reciprocal inhibition between wrist flexors and extensors also receive excitation from group I afferent of the other nerve, including homonymous nerve, suggesting that this inhibition is likely non-reciprocal group I inhibition (reviewed in Pierrot-Deseilligny & Burke, 2012). Further investigation supported the evidence that Ib afferents probably contribute to the activation of interneuron mediating the reciprocal inhibition from ECR to FCR.

4.1 Inputs to non-reciprocal interneurons

4.1.1 Segmental inputs

a) Group I afferent fibres

Convergence from group I elbow muscle afferents and antagonistic group I afferents onto interneurons mediating reciprocal inhibition from ECR to FCR was revealed in post-stimulus time histogram (PSTH) of single voluntarily activated motor units (Aymard et al., 1995).

Selectively raising the electrical threshold of Ia fibres in the conditioning volley during the prolonged vibration has been shown to increase the electrical threshold of reciprocal Ia inhibition between elbow muscles (Katz et al., 1991). However, such prolonged vibration did not alter the electrical threshold of the inhibition at the wrist level suggesting that the dominant input to the relevant interneurons is Ia in origin at the elbow level but Ib in origin at the wrist level (Wargon et al., 2006). These results could be likely explained by the convergence of Ia and Ib afferents onto the relevant non-reciprocal 'Ib' interneurons, described in cats (Harrison & Jankowska, 1985).

b) *Mutual inhibition*

In animal studies, mutual inhibition between opposite-side interneurons has been identified as a general central nervous system mechanism. Baldissera et al. (1987)

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also demonstrated that mutual inhibition exists in humans at the wrist level. Stimulating of group I afferents in the radial nerve results in an inhibition of the FCR H-reflex. Similarly, stimulating of group I afferents in the median nerve results in an inhibition of the ECR H-reflex

c) Recurrent inhibition

Ia interneurons are only spinal interneurons that can be blocked by recurrent inhibition. Contrary to what was observed both in the cat hindlimb and in human elbow muscles, the interneuron mediating reciprocal inhibition between wrist flexors and extensors is not inhibited by Renshaw cells (Aymard et al., 1995). Activation of Renshaw cells by intravenous administration of levo-acetylcarnitine blocked Ia reciprocal inhibition from triceps to biceps muscles but not disynaptic inhibition from ECR to FCR (Rossi et al., 1995).

4.1.2 Descending inputs

Studies in animals showed that non-reciprocal group I inhibitory interneurons (Ib) receives excitatory supraspinal control from corticospinal and rubrospinal tracts and that they are inhibited by the dorsal reticulospinal and the noradrenergic reticulospinal system (Jankowska & Lundberg, 1981). Studies in humans show that TMS does not increase the reciprocal inhibition from ECR to FCR and sometimes even decreases. It was suggested that the corticospinal excitatory inputs to inhibitory interneurons mediating reciprocal in the human wrist are weak (Mercuri et al., 1997; Munier & Pierrot-Deseilligny, 1989; Kato et al., 2002).

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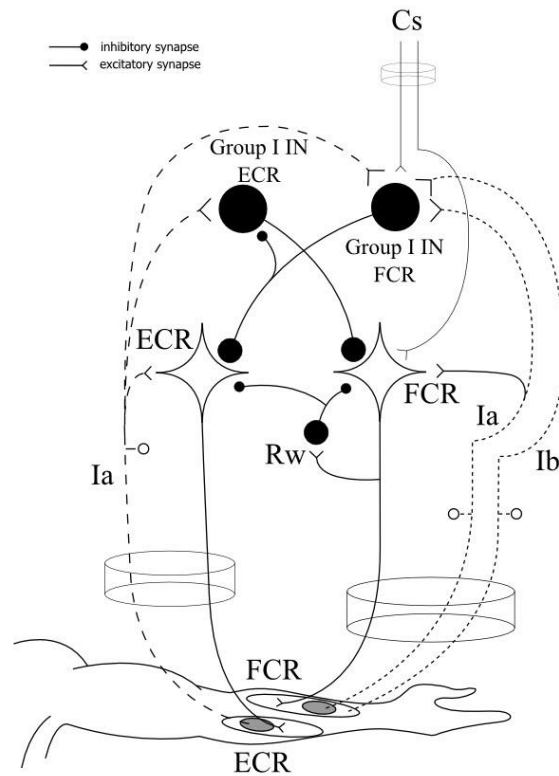


Figure 6 : Pathways of non-reciprocal group I inhibition at wrist level.

FCR-coupled group I inhibitory interneurons receive their excitatory group I input from FCR and ECR Ia and Ib afferents (projections from ECR Ib afferents have been omitted), are not inhibited from Renshaw cells, and project to ECR motoneurons and ECR-coupled group I inhibitory interneurons (inhibitory projections of ECR-coupled group I inhibitory interneurons to FCR-coupled group I interneurons have been omitted). Renshaw cells activated by recurrent collaterals from FCR motoneurons project to both FCR and ECR motoneurons. Reproduced from Wargon et al., 2006.

Ia = Ia fibre afferents, Ib = Ib fibre afferents, FCR = flexor carpi radialis, ECR= extensor carpi radialis, IN = interneuron, CS = Corticospinal control

5. Ionic theory

5.1 Conductance increase during an impulse

Important insights into the mechanism of action potential were obtained by Cole & Curtis (1939). Using an electrode inserted along the squid giant axon, these authors showed that the membrane resistance dramatically decreases when the potential action occurs. A resistance of about $1000 \Omega/\text{cm}^2$ at rest falls to a minimum value of $25 \Omega/\text{cm}^2$ during the action potential, implying an increase of conductance of nerve impulse. This experiment partly confirmed Bernstein's hypothesis (1902) that a nerve impulse represents a temporary "breakdown" of the membrane resistance.

5.2 Reversal in membrane polarity "Na hypothesis"

As a conductance is a measure of the ion permeable aspect of the membrane, it has been believed that the depolarisation of a nerve fibre during excitation involves an increase of permeability to ions. The importance of ionic movements in excitable tissue has been shown by a number of animal experiments. Overton's work in 1902 demonstrated the irritability of frog muscles in relation to the sodium ion (Na^+). The frog muscles quickly lost their power to respond to direct stimulation during they were immersed in a solution containing 10% less of sodium concentration than normally found in the external medium. Hodgkin & Katz (1949) have proposed that the net entry of Na^+ in one impulse is probably large enough to charge the membrane capacity to the full extent of the action potential, the entry of Na^+ is the direct cause of the change in membrane potential. It has also been proposed that the rate of rise and amplitude of the action potential are determined by the ratio of concentration of Na^+ inside and outside of the axon (Hodgkin & Katz, 1949; Huxley & Stämpfl, 1951). These experiments are consistent with the theory that nervous conduction depends on a specific increase in permeability which allows Na^+ to move from outside of nerve fibre where its region of concentration is high to its region of low concentration inside it. Hodgkin (1977) stated that "*In all of these theoretical action potentials the reversed potential difference at the crest of the spike depended on a selective increase in Na permeability and a low internal concentration of Na ions*". Moreover, it was also considered that a selective increase in the sodium conductance would induce not only for the depolarization but also for the reversal of the membrane potential.

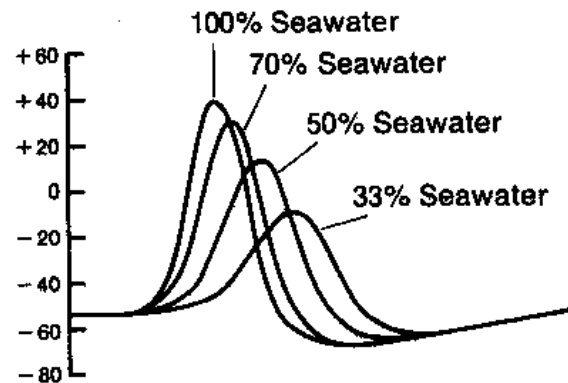


Figure 7 : The relationship of the height of the spike and the ratio of the concentration of Na inside and outside the axon. Reproduced from Hodgkin and Katz's work in 1949.

5.3 Resting Membrane Potential and Ionic Distribution during neuron activity

Bernstein (1902) suggested that resting membranes are highly permeable to potassium ions (K^+). Hodgkin & Katz (1949) has confirmed Bernstein's hypothesis demonstrating that there is much more K^+ inside than outside the cell, and the potassium-selective channels are open in resting neuron implying the resting membrane is more permeable to K^+ than to the other ions. The flow of ions through membrane channels is dependent on ion gradients across the membrane. For example, Na^+ is actively pumped out of cells while K^+ is pumped in (Na^+/K^+ -ATPase). The concentrations of chloride ions (Cl^-) and calcium ions (Ca^{2+}) are also maintained at greater levels outside the cell. The ionic composition inside the cell is substantially different from that of extracellular fluids, leaving negative charges within the cell.

Using measurement of electric potential across the membrane and radioactive tracers, Keynes & Lewis (1951) showed that sodium and potassium are important to have reliable values for the intracellular ionic concentrations since a net entry of sodium during nervous activity approximately balances the loss of potassium. The diffusion of Na^+ into the cell during depolarisation reduces the resting membrane potential creating an excitatory postsynaptic potential (EPSP). Until reaching the threshold, the reduced voltage now opens up a great amount of the sodium channels in the membrane resulting in an increase of Na^+ influx into the cell; the action potential is then generated.

An outflow of potassium ions has been proposed to be related with repolarisation during the falling phase and this phase may be accelerated by a process which increases the potassium permeability after the action potential has reached its crest (Hodgkin et al., 1949). Chloride conductance has also been suggested to be

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activated by hyperpolarisation in *Aplysia* neurons. Increasing the intracellular Cl^- concentration markedly increased the steady state conductance (Chesnoy-Marchais, 1983). Hyperpolarisation (inhibitory postsynaptic potential (IPSP)) is thus thought to be caused by an efflux of K^+ or influx of Cl^- .

6. Chloride channel

Cl⁻ has been reported to be both distributed passively and actively transported through membrane channel. In resting skeletal muscle and erythrocytes, Cl⁻ permeability is extremely high, Cl⁻ distributes passively due to the membrane potential, and thereby the intracellular Cl⁻ concentration is close to electrochemical equilibrium causing the active transports of Cl⁻ hardly work to counteract this high permeability (Hodgkin & Horowicz, 1959; Hutter & Nobel, 1960; Adrian, 1960, 1961). While, in squid axon, it was shown that the intracellular Cl⁻ concentration was 2-3 times higher than equilibrium (Keynes, 1963). Studies in neuron cell reveal GABA hyperpolarised neuron response by opening ligand-gated Cl⁻ channel showed that the chloride equilibrium potential (E_{Cl}) was not equal to resting membrane potential (Kuffler & Edwards, 1958; Boistel & Fatt, 1958), suggesting that active Cl⁻ transport would exist. Active Cl⁻ transport mechanisms have consequently been suggested. In skeletal muscle and red blood cell, Cl⁻ is in equilibrium since Cl⁻ permeability is high that masks active Cl⁻ transport. In immature neuron, Cl⁻ intruder such as the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC) establish a higher intracellular Cl⁻ concentration than electrochemical equilibrium, whereas Cl⁻ extruder such as the Na⁺-Cl⁻ cotransporter (KCC) induces a lower intracellular Cl⁻ concentration than electrochemical equilibrium in mature neuron (For reference see Alvarez-Leefmans & Delpire, 2009).

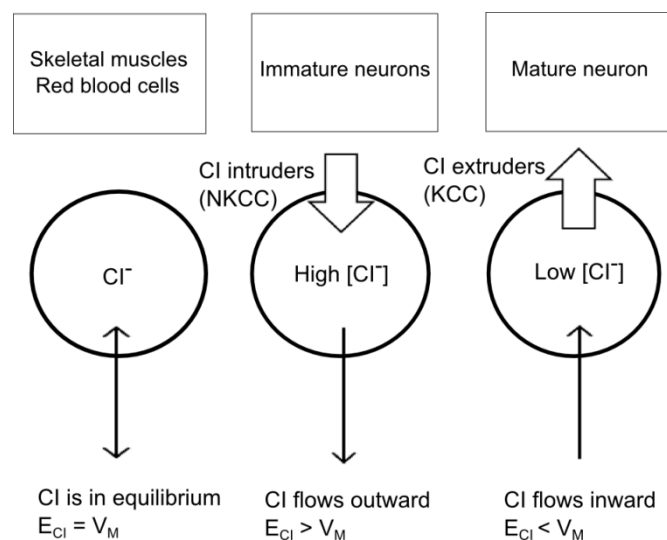


Figure 8 : Control of cytosolic Cl⁻ in different cell type.

Reproduced from Alvarez-Leefmans & Delpire, 2009.

7. Cation-chloride cotransporter

7.1 General characteristics

During the recent years, there has been an increase in the studies focusing on the role of cation chloride cotransporters (CCCs). The CCCs are secondary active transporters that mediate the couple movement of Na⁺ and/or K⁺ to that of Cl⁻ across cell membrane, known as Na⁺-K⁺-2Cl⁻ cotransporter (NKCCs) and Na⁺-Cl⁻ cotransporter (KCCs). They move Cl⁻ across membrane against their concentration/or electrochemical gradient using the potential energy stored in the electrochemical transmembrane gradient of Na⁺ (generated by the Na⁺ pump). Two isoforms of NKCC (NKCC1 and NKCC2) and four isoforms of KCC (KCC1-4) have been identified. Chloride exporters are the KCCs, while the NKCCs act as chloride importers.

7.2 Neuronal function

The role of CCCs in neurons is currently intensively investigated. It was shown in animal studies that CCCs are determining in the control of the intracellular Cl⁻ concentration (Payne et al., 2003). Chloride homeostasis is important for a range of neurophysiological process in mammals. It is known that gamma aminobutyric acid (GABA) and glycine, common inhibitory neurotransmitters in the adult CNS, activate chloride-permeable ionotropic glycine and GABA_A receptors (GlyRs and GABA_ARs) (Legendre, 2001; Fritschy & Brünig, 2003). During development of neurons, the expression of CCCs plays an important role in changes of GABA and glycine actions. In embryonic and early postnatal life of the rat, neurons in the brain show strong expression of NKCC1, whereas expression of KCC2 is low (Plotkin et al., 1997; Clayton et al., 1998; Wang et al., 2002), resulting in high intracellular Cl⁻ levels. This forces GABA_A receptors to open, inducing an efflux of Cl⁻ through their channel, thus depolarizing of GABA_A/Glycine responses. As development progresses, the expression of NKCC1 decreases while the expression of chloride exporters, like KCC2 increases. This results in a decrease of the intracellular chloride concentration, inducing an influx of Cl⁻, thereby hyperpolarizing of GABA_A/Glycine response (reviewed in Ben-Ari, 2002; Blaesse et al, 2009). A negative shift in the GABA reversal potential (E_{GABA}) is paralleled by a strong up-regulation of KCC2 near the end of the second postnatal week in the rat hippocampal and neocortical pyramidal neuron (Rivera et al., 1999). It has been demonstrated in humans that KCC2 expression in the neocortex begins to increase at 40 weeks after conception (Dzhala et al., 2005). From these evidences, the developmental shift in GABA and glycine actions from excitatory to inhibitory is likely due to strong expression of co-transporters KCC2 and down-regulation of co-

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transporters NKCC1. In addition, it was shown that after neonatal complete spinal cord transection in the rat, thereby removing the influence of the brain on the lumbar spinal cord, prevents or at least delays the switch from depolarizing to hyperpolarizing IPSPs that normally occurs during postnatal development (Jean-Xavier et al., 2006). This reversal appears related to down-regulation of KCC2, thus decreases in intracellular chloride concentration.

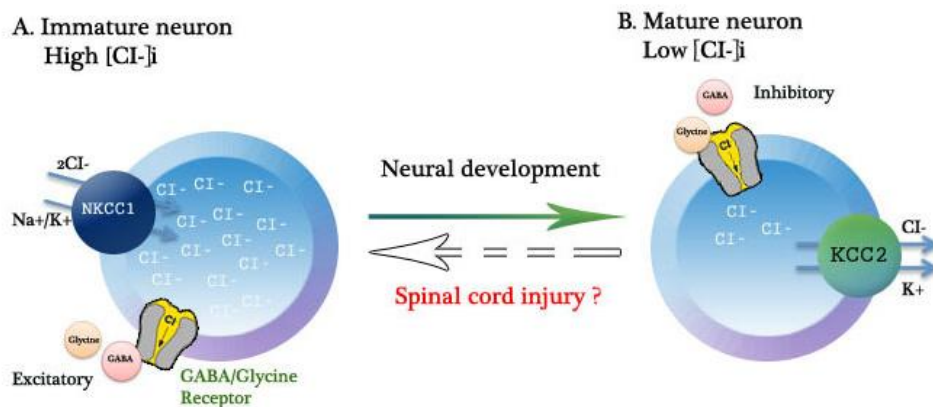


Figure 9 : The expression of NKCC1 and KCC2 in neurons.

(a) Early expression of NKCC1 and (b) Late expression of KCC2 determine the change in chloride intracellular concentration in response to GABA and glycine during development. Glycinergic and GABAergic synapses are first excitatory before become inhibitory during development while a transection of the spinal cord does not allow this transformation. Adapted from Ben-Ari, 2002; Jean-Xavier et al, 2006.

7.3 Physiopathology in the brain

7.3.1 Animal studies

Two members of the CCCs family, KCC2 and NKCC1 have been much interest in studies on GABAergic signaling under pathophysiological conditions, such as seizures, and stroke (reviewed in Kahle et al., 2008; Löscher et al., 2013). The contribution of altered NKCC1 and KCC2 activity to epileptogenesis has been extensively explored. As the NKCC1 expression is high during the neonatal period, elevated intracellular Cl^- level induces the excitatory action of GABA, this has been proposed to contribute to increased efficacy of neonatal seizure (Dzhala & Staley, 2003; Khazipov et al., 2004). Hypersensitivity of GABA response in the hippocampus is also observed after KCC2 knockdown, inducing generalized seizure in the adult mice (Hübner et al., 2001; Woo et al., 2002). A study in the rat revealed a sustained down-regulation of KCC2 after focal cerebral ischemia (Jaenisch et al., 2009). It was proposed that this down-regulation of KCC2 would lead to positive shift of the chloride equilibrium potential, which is expected to reduce functional GABAergic inhibition. As epileptic seizure occasionally occur after stroke, the post ischemic KCC2 reduction might be involved in the pathogenesis of epilepsy after brain lesion. However, it is not unclear whether this reduction of KCC2 is a result or the cause of epilepsy. Another aspect is also proposed, the down-regulations of KCC2 after stroke that looks similar with those in the development process, might initiate reorganization of neuronal networks (see *Di Filippo et al., 2008*).

7.3.2 Human studies

Cohen et al. (2002) have demonstrated *in vivo* in patients with temporal lobe epilepsy that perturbed neuronal chloride transport induces excitatory GABAergic signaling and that might result in epileptogenesis. The excitatory GABAergic signaling was later found to be related to a down-regulation of KCC2 (Huberfeld et al., 2007). A dysfunction in GABA system has been proposed in autism. Using bumetanide, a high specific NKCC1 antagonist, reduces intracellular chloride and thus reinforcing GABAergic inhibition. Bumetanide significantly reduced the score of Childhood Autism Rating Scale in sixty children with autism or Asperger syndrome (Lemonnier et al., 2012).

7.4 Physiopathology in the spinal cord

In the animal spinal cord, the lack of KCC2 expression induces a rise of intracellular Cl⁻ concentration, thereby depolarizing of GABA and glycine (Vinay & Jean-Xavier, 2008), and also results in reduction of inhibitory synaptic strength (Boulenguez et al. 2010). In KCC2 knockout animals, sciatic nerve recordings reveal abnormal spontaneous electrical activity and altered reflex responses to peripheral electrical stimuli (Hübner et al. 2001). KCC2 was also reported to be involved in pain perception through its effects on postsynaptic inhibition in spinal cord neurons. Several studies demonstrated that decreased KCC2 expression in the spinal cord is associated with increased nociception (Coull et al., 2003; Mantyh et al., 2004). Inhibition of KCC2 in the mouse spinal cords led to decreased heat-evoked withdrawal latency, whereas inhibition of NKCC1 resulted in increased response latencies to heat stimuli (Austin & Delpire, 2011).

7.5 Physiopathology in the peripheral nerve

After sciatic nerve crush in animals, reduction of KCC2 is observed at the injured side of the spinal cord (lumbar spinal motoneurons) compared to intact animals whose spinal cord shows well defined line surrounding motoneurons where is KCC2 located in non-injured condition (Modol et al., 2014).

Chapter 2

Materials and methods

1. The monosynaptic reflex

The stretch reflex, also known as myotatic reflex or tendon reflex is a muscle contraction in response to stretching the muscle. The stretch reflex was firstly defined by Liddell & Sherrington (1924) in the decerebrate cats. This spinal reflex provides automatic regulation of skeletal muscle length. When a muscle is stretched, sensory fibres (Ia fibres) from the muscle spindles excites monosynaptically the α -motoneurons that are located in the ventral horn of the spinal cord (Lloyd, 1943, 1946 a,b ; Granit, 1950; Hunt, 1955). The α -motoneuron sends the motor output back to the muscle fibres to contract the muscle. In animal studies, the method of the monosynaptic reflex was introduced in the 1940s as a tool for investigating the excitability changes in the motoneuron pools (Renshaw, 1940; Lloyd, 1943). Since then, the technique of the monosynaptic reflex has used to reveal various properties of the spinal circuitry that is necessary for the integration of peripheral afferents and descending pathways. The monosynaptic reflex from Ia afferents to motoneurons is probably the best known reflexes. The technique of the monosynaptic reflex constitutes one of the simplest tools available to investigate spinal pathways in both animals and humans.

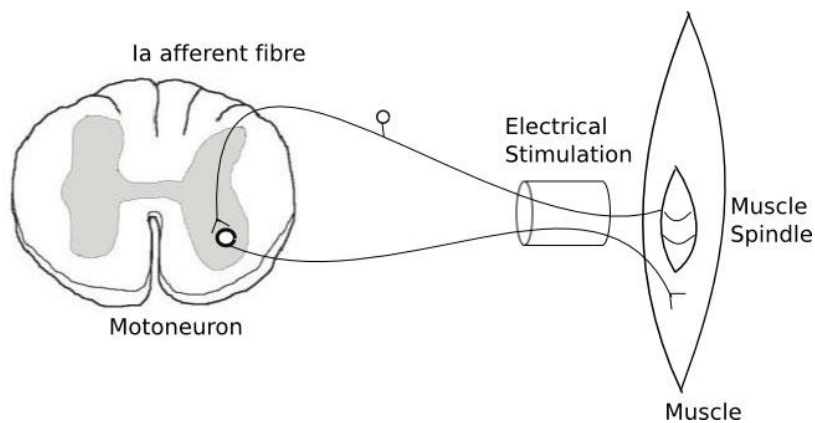


Figure 10 : Sketch of pathway of the monosynaptic reflex.

Ia sensory fibre from muscle spindle primary ending has monosynaptic projections to alpha motoneuron innervating the corresponding muscle.

2. The H-reflex

2.1 Principles of the method

The Hoffmann reflex or H-reflex is the electrical equivalent of the monosynaptic stretch reflex in animal studies and has become the main tool in many motor control investigations in humans (for a review, see Pierrot -Deseilligny & Burke, 2012). Sherrington (1910) showed in the cats that the monosynaptic reflex is predominant in the antigravity muscles like soleus, quadriceps and also flexor carpi radialis muscle (FCR) in the forelimb. Similar results were also found in humans. In most healthy subjects at rest, the H-reflex can usually be recorded from the soleus (Hoffman, 1918), quadriceps (Gassel, 1963), in the lower limb and from FCR in the upper limb (Deschuytere et al., 1976). The soleus H-reflex was firstly introduced as a monosynaptic response that is the most reliably elicited in the lower limb in humans by electrical stimulation of the posterior tibial nerve in the popliteal fossa (Hoffman, 1918). Electromyographic recording from the soleus muscle via an electrode percutaneously placed over the muscle shows a direct motor (M) response that is followed by the H-reflex. The latency of the M response is about 5-10 msec, while the soleus H-reflex appears later, with a latency of 25-35 msec (Figure 11). In the upper limb, the FCR reflex is the most reliably elicited. It appears with a latency of 16-22 msec (Figure 12). The latency of the H-reflex is different amongst individuals also depending upon on the height of subjects. The neural circuitry responsible for the H-reflex is characterized by the monosynaptic projection of the group Ia afferents onto the α motoneurons. Using intrathecal recordings Magladery et al. (1951) demonstrated in humans that activation of Ia afferent fibres induced by percutaneous electrical stimulation sends the signal to the spinal cord with a conduction time from the dorsal root to the ventral root of only 1.5 ms. The authors proposed that this delay is too short for interneuronal transmission, determining a monosynaptic connection between the Ia fibre and motoneuron.

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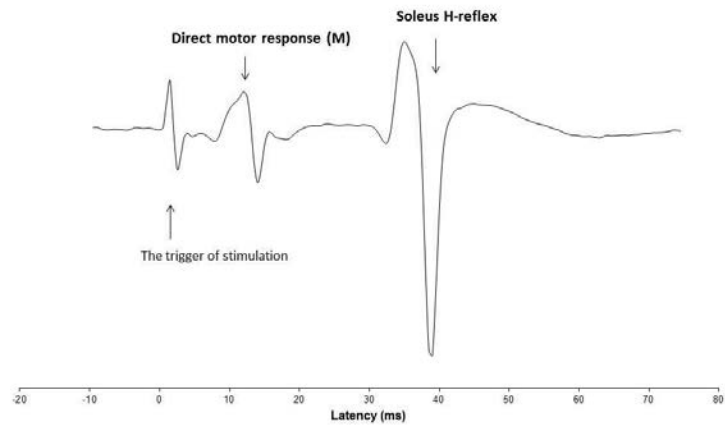


Figure 11 : Example of M response and H-reflex in the soleus muscle.

The soleus H-reflex is elicited by selectively stimulating the Ia fibres projecting onto the homonymous motoneuron, while the direct motor response (M) is due to direct activation of the axon motor. The latency of M response is about 5-10 msec, while the soleus H-reflex appears with a latency of 25-35 msec.

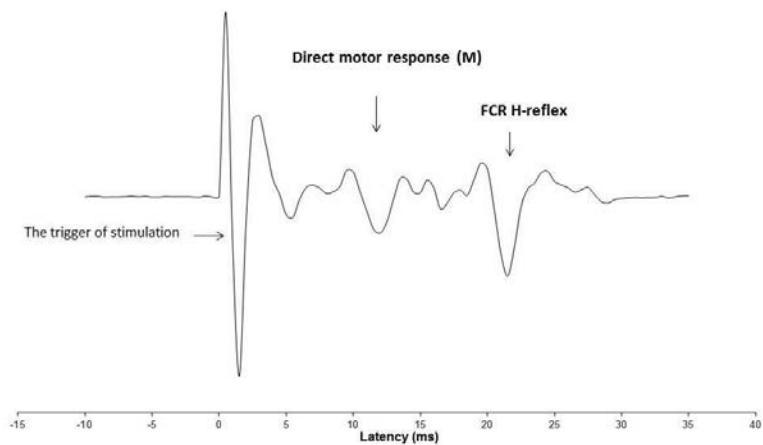


Figure 12 : Example of M response and H-reflex in FCR muscle.

The FCR H-reflex is elicited by selectively stimulating the Ia fibres projecting onto the homonymous motoneuron, while the direct motor response (M) is due to direct activation of axon motor. The FCR H-reflex appears with a latency of 15-22 msec.

2.2 H-reflex and M-wave recruitment curves

The recruitment curve

The relationship between the intensity of stimulation and the size of the H-reflex and M response has been studied in both animals and humans. It was shown that the modulations of these two responses depend on the intensity of stimulation. At low stimulus intensity, the H-reflex appears at a lower threshold than the motor response, since Ia afferents (larger diameter) corresponding to the H-reflex are more excitable than the motor axons. Progressive increase of the intensity of stimulation induces an increase of the H-reflex until reaching its maximum (Hmax) and then decreases. Meanwhile, the M response appears at a stimulus intensity corresponding to the motor threshold, which is above the threshold of the H-reflex since the axons of the α -motoneuron are smaller than those of the Ia afferents. Further increase in the intensity of stimulation leads to M response increasing until reaching its maximum response (Mmax); the H-reflex is completely suppressed. This results from the antidromic motor volley set up in motor neurons colliding with and eliminating the H-reflex (Hoffman, 1922).

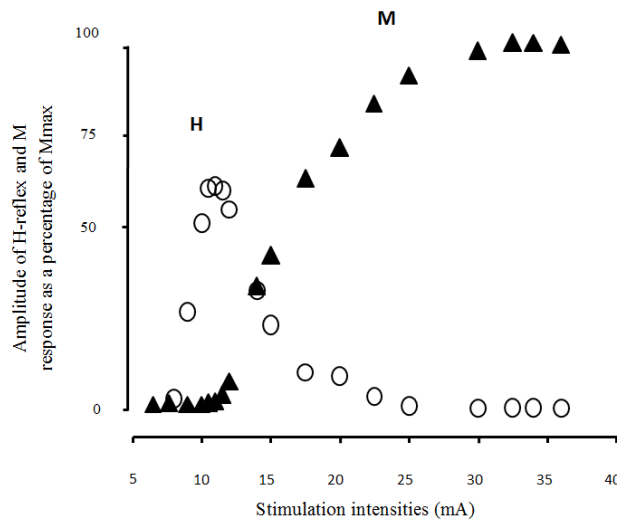


Figure 13 : H-reflex and M-wave recruitment curve. Maximal M wave (Mmax)

The amplitude of the motor response (Δ) and the H-reflex response expressed as a percentage of the M_{max} (O) is plotted against the intensity of the electrical stimulation of the posterior tibial nerve (expressed in multiples of motor threshold). *Reproduced from Pierrot-Deseilligny & Mazevet, 2000.*

The M response represents the direct activation of the axon motor by the electrical stimulation. The maximum M response indicates the recruitment of all motor

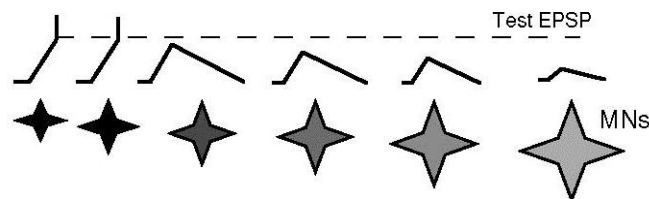
CHAPTER 2 MATERIALS AND METHODS

axons. It provides an estimate of the response given by the entire motoneuron pool tested by the monosynaptic reflex. Expressing the H-reflex as a percentage of M_{\max} allows us to compare the results between individual subjects.

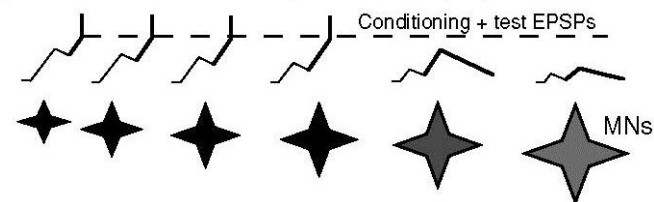
2.3 The orderly recruitment of α -motoneurons in the monosynaptic reflex

In general, motoneurons are recruited in an orderly sequence from the smallest to the largest size according to the size principle (Henneman et al., 1965; Henneman & Mendell, 1981). These authors demonstrated that the smallest motoneurons represented by the smallest impulse amplitude have lower thresholds and thus are recruited first, while larger motor neurons have higher thresholds and are recruited last. The facilitation input will begin to affect motoneurons that just failed to be discharged in the test reflex, while inhibition will affect those that had just been recruited in the test reflex.

A) Test reflex alone



B) Test reflex conditioned by an excitatory input



C) Test reflex conditioned by an inhibitory input

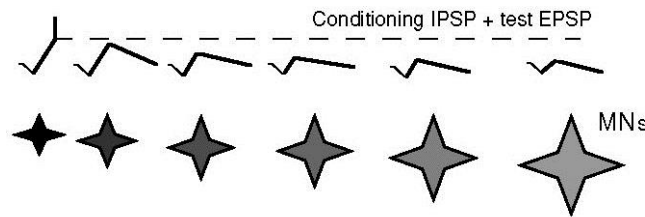


Figure 14 : Orderly recruitment of motoneuron (MNs).

A) Given Ia input, the size of monosynaptic Ia EPSP decreases while motoneuron size increases. The dotted line represents the threshold for discharge of the MNs. Only the two smallest motoneurons (black) are fired by the test Ia volley. B) When an excitatory input is applied, there is a summation of the conditioning EPSP (thin line) and test (thick line) EPSP. As a result, MNs which had failed to discharge in the control reflex are raised to firing threshold and the size of the reflex is increased. C) When an inhibitory input is applied, there is a summation of the conditioning IPSP (thin line) and test (thick line) EPSP. As a result, MNs which had been recruited in the control reflex cannot be discharged, thus the size of the reflex is reduced. *Reproduced from Pierrot -Deseilligny & Burke, 2012*

2.4 Use of the H-reflex

The H-reflex technique is used to test the changes in the excitability of motoneurons in response to a conditioning stimulus. Modulations of the H-reflex amplitude depend on the number of motoneurons involved in the test H-reflex under conditioning stimulation, reflecting changes in the excitability of the motoneuron population. If a conditioning stimulus induces an inhibitory effect, the Ia afferent test volley recruits fewer motoneurons than in the control situation, thus the amplitude of the H-reflex is decreased. In contrast, if motoneurons are facilitated by a conditioning stimulus, those that have not been recruited in the control H-reflex will be fired. This increases the size of the H-reflex as a result of temporal summation of the conditioning and test EPSP.

2.5 Advantages and disadvantages of the method

2.5.1 Advantages of the method

The monosynaptic reflex is a simple and non-invasive method, and can be used to estimate the excitability of spinal motoneurons at rest and during movement. This method is widely used to study spinal circuits in both animals and humans, and also in patients with CNS lesions. However, it presents some limitations.

2.5.2 Limitation of the method

a) Mechanisms controlling the Ia afferent volleys

H-reflex is mediated by Ia afferents so the mechanisms controlling the Ia afferent volleys such as alteration of the excitability of Ia afferents, presynaptic inhibition, post-activation depression, curtailment of the compounded EPSP by disynaptic IPSP have to be taken in consideration. Some of these limitations can be overcome: for example post-activation at the fibre Ia –motoneurons synapse rely on the H-reflex frequency (see physiological background). However, keeping the frequency identical for conditioned and unconditioned H-reflex allow discarding its effect.

Possible changes in the amount of presynaptic Ia inhibition, alteration of Ia excitability fibre are much more important during voluntary movement that at rest. However, our experiments were performed at rest. Furthermore, previous experiments have demonstrated that presynaptic Ia inhibition is not modified by tDCS (Roche et al., 2009)

b) Limitation due to the property of the motoneuron pool and in homogenous distribution of afferents within the motoneuron pool.

Sensitivity of different sizes of H-reflex

A conditioning stimulation causes facilitatory or inhibitory effects on the H-reflex. These effects vary with the intensity of the conditioning stimulus, and also depend on the size of the test H-reflex itself (Crone et al., 1990; Meinck 1980). These different sensitivities of the reflexes with respect to their different sizes reflects a non-linear input-output relationship within the motoneuron pool. It was shown that small and large sizes of monosynaptic reflex have a lower sensitivity to facilitatory and inhibitory input than reflexes of intermediate size (Crone et al., 1990).

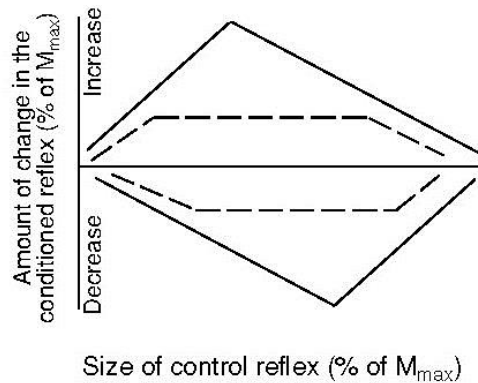


Figure 15 : Schematic of Sensitivity of the different size of the H-reflex.

The sensitivity of the monosynaptic reflex to facilitation (upper part) or inhibition (lower part). *Reproduced from Crone et al., 1990*

When the conditioning stimulus is strong (solid line), the number of additionally recruited (facilitation) or derecruited (inhibition) motoneurons will increase with the size of the test H-reflex, and then decrease. Meanwhile, when the conditioning stimulus is weak (dashed line), a plateau region appears between phases of increase and decrease. Given this, it is therefore necessary to adjust the intensity of the stimulation test to maintain and adjust the size of the H-reflex to where it is sensitive to response to facilitatory or inhibitory effects (20-30% of M_{max} , see figure above) and keep a similar size throughout the experiment.

4. Plateau potential

It has been shown in animals that α -motoneurons could develop the plateau potential due to persistent inwards currents (for reference see Hultborn et al., 2003; Heckman et al., 2009). They amplify the response of synaptic inputs leading to a sustained increase of motoneuron activity. This phenomenon may influence the relationship between excitatory synaptic inputs and discharge frequency of motoneurons (Hultborn et al., 2003). The existence of plateau potential might be considered to affect the results obtained with the technique of the H-reflex. Studies in humans using a stimulus applied directly to the calf muscle suggested that a stimulation frequency of at least 10 Hz is required to disclose the plateau potentials (Collins et al., 2001, 2002). However, there is no direct evidence proving in humans that the stimulation evoked H-reflex is sufficient to generate plateau potentials, and the monosynaptic reflexes studied in the present study was likely not contaminated with plateau potential since the maximum stimulation frequency used was only 1 Hz.

To sum up, the H-reflex technique is nevertheless the most valuable method to investigate changes in motoneurons excitability in healthy subjects and patients with

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CNS lesions but the following rules have to be taken into account: i) the amplitude of the unconditioned reflex must be within the ascending part of the recruitment curve (Figure 15) ii) the H-reflex amplitude has to be expressed of the percentage of M_{\max} iii) the unconditioned H-reflex amplitude has to be the same in all situations iv) conditioned and unconditioned H-reflex have to randomly alternated.

3. Methods used in the project 1

3.1 Electrophysiological recordings

3.1.1 Stimulation test: Soleus H-reflex

Spinal inhibitory circuits were studied using non-invasive electrophysiological techniques based on the H-reflex. The H-reflex was evoked in the soleus muscle by percutaneous stimulation of the posterior tibial nerve (PTN) at the popliteal fossa with a rectangular 1 ms duration stimulus, delivered at 0.33 Hz to the PTN. The H-reflex was recorded from the EMG signal of the soleus muscle using bipolar surface electrodes positioned on the skin parallel to the muscle belly.

The maximum motor response (M_{\max}) and maximum H-reflex response (H_{\max}) were recorded at the beginning of each experiment. The stimulus intensity was then adjusted to elicit an H-reflex amplitude of 20-35% of M_{\max} , which corresponded to ~ 50%, of H_{\max} . The unconditioned H-reflexes were set to have similar size across subjects and kept constant throughout the experiment, since the sensitivity of the H-reflex to facilitation or inhibition conditioning effects depends on its non-conditioned size (Crone et al. 1990).

3.1.2 Inhibitory conditioning stimulations.

a) Presynaptic inhibition

Several non-invasive methods have been developed to study presynaptic inhibition in humans. The first study on presynaptic inhibition has begun with a phenomenon called the "vibration paradox." Effects of prolonged tendon vibration result in two phenomena i) a discharge in the homonymous Ia afferent fibres acting on the motoneuron which causes the tonic vibration reflex (TVR) (reviewed in De Gail et al, 1966; Hagbarth & Eklund, 1966) and ii) an inhibition of the H-reflex of that muscle (De Gail et al., 1966; Delwaide, 1971, 1973). The coexistence of these two phenomena has led to attribute to presynaptic inhibition of Ia terminals with PAD (Delwaide 1973). However, application of vibration to the homonymous tendon activates other mechanisms such as homosynaptic depression (see Background of physiology). Another method was subsequently developed to eliminate the lack of specificity is the prolonged vibration of the homonymous tendon. Application of vibration to the tendon of a heteronymous muscle (tibialis anterior or bicep femoris) induces a clear inhibition of the soleus H-reflex (Morin et al., 1984, Hultborn et al., 1987; Nielsen & Petersen, 1994; Nakashima et al., 1989).

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Presynaptic inhibition of Ia fibres may also be evoked by a conditioning stimulus to the nerve innervating the antagonist muscle. This induces an inhibition of the H-reflex in the agonist muscle called D1/D2 inhibition. A conditioning stimulus applied to the common peroneal nerve at $1-1.4 \times$ motor threshold (MT) induces an inhibition of the H-reflex of the soleus. The inhibition is long lasting and composed of two phases, called 'D1' at 5-30 ms, and 'D2' at 70-200 ms (Mizuno et al., 1971). It has been shown that the same conditioning volley that inhibition the H-reflex does not modify the motor evoked potential (MEP) amplitude evoked by cortical stimulation in the soleus at an interstimulus interval (ISI) corresponding to D1 (21 ms) (Faist et al., 1996). This suggests that at this ISI, common peroneal nerve induced H-reflex inhibition is presynaptic in origin.

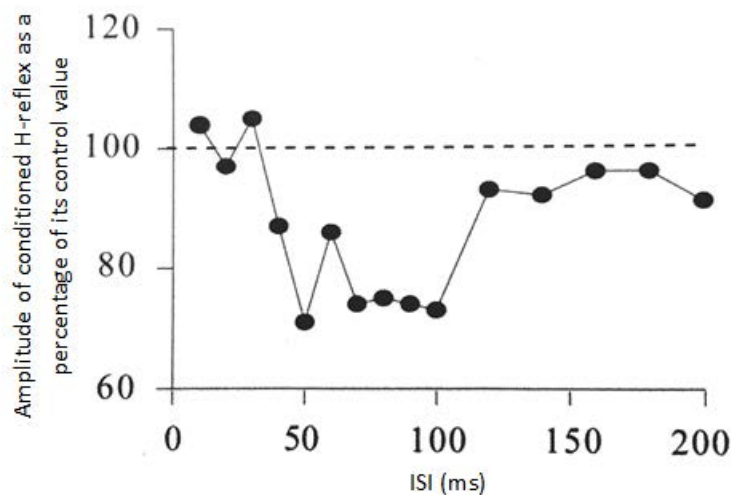


Figure 16 : Time course of presynaptic inhibition induced by conditioning stimulus of common peroneal nerve.

The amplitude of soleus H-reflex expressed as a percentage of its control value is plotted against the interstimulus interval. *Reproduced from Morita et al., 1998.*

Another method called heteronymous facilitation was proposed to measure the presynaptic inhibition of Ia fibres. A facilitation of the soleus H-reflex induced by conditioning stimulus to the nerve innervating the quadriceps muscle is purely monosynaptic within the first 0.6 ms (Hultborn et al., 1987). A constant conditioning stimulus elicits a constant EPSP in motoneurons, and thus produces a constant reflex facilitation, unless the amount of presynaptic inhibition of Ia fibres mediating the conditioning volley changes. Thus, modification of motoneurons excitability can result in changes in the reflexes H sizes, which reflects a change in the level of presynaptic inhibition. The more that the facilitation reflex is important, the more that presynaptic

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inhibition is low. However, this method presents some limitations: i) it requires a fairly high level of heteronymous facilitation to highlight changes in presynaptic inhibition, and ii) to avoid risk of contamination with non-monosynaptic effects, the ISI used to record the monosynaptic facilitation of the H-reflex ought to be chosen between 0.2-0.6 ms. This implies an accurate definition of the ISI that maybe time consuming.

To conclude, as D1 inhibition is the easiest method, it was chosen to investigate presynaptic inhibition of Ia fibres in healthy and patient subjects in the present study. The soleus H-reflex was conditioned with a preceding stimulation of the common peroneal nerve (CPN) through hemispherical bipolar electrodes placed 2 cm below the neck of the fibula. The stimulus consisted of a train of 3 rectangular pulses, each of 1 ms duration, an interpulse interval of 3 ms, with an intensity 1.2 times the tibialis anterior (TA) motor threshold (MT), delivered 21 ms before the test stimulation. The motor threshold was determined by tendon palpation and by the oscilloscope EMG display.

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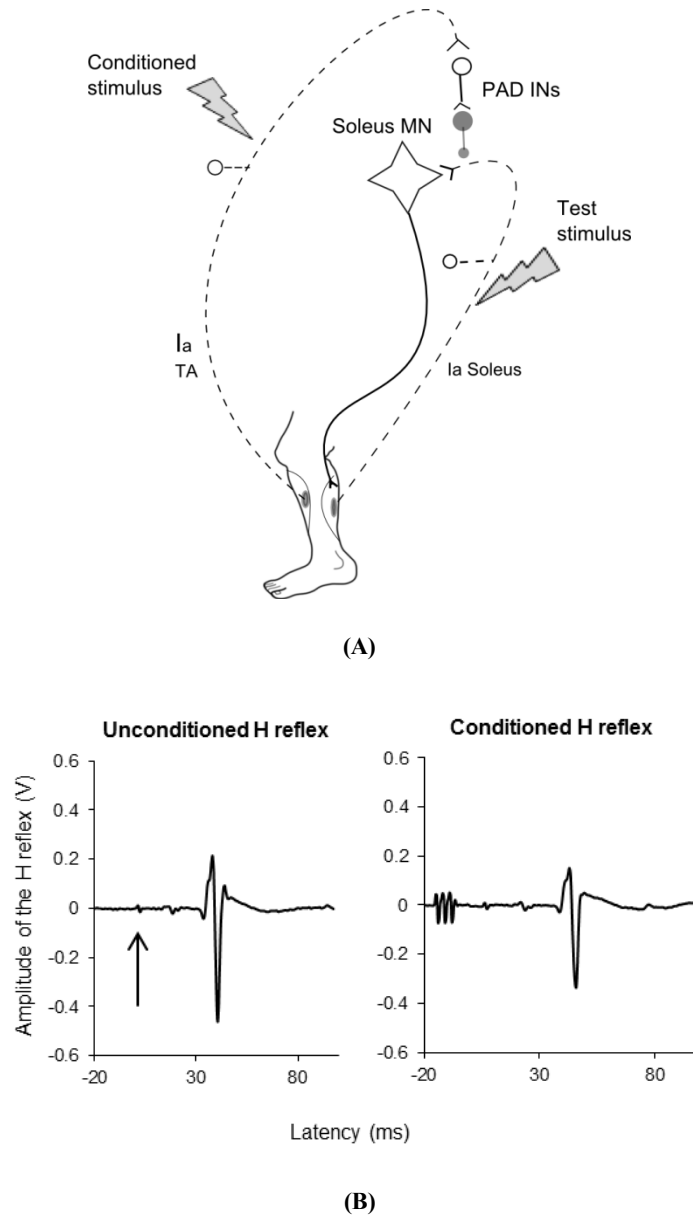


Figure 17 : Schematic diagram of the presynaptic inhibition of soleus Ia fibres and example of waveforms of the soleus unconditioned H-reflex and the soleus conditioned H-reflex induced by conditioning stimulus of common peroneal nerve.

(A) The conditioning stimulus induces the afferent volley in the tibialis anterior (TA) Ia afferent fibres and activates primary afferent depolarization (PAD) interneurons responsible for the presynaptic inhibition of Ia soleus fibres before the synapse with the soleus motoneuron. *Adapted from Pierrot-Deselligny & Burke, 2012.* (B) Examples of waveforms of the soleus unconditioned H-reflex (left), and the soleus conditioned H-reflex (right) when preceded by the TA conditioning stimulus. The arrow indicates the trigger of the test stimulation. The conditioning stimulus consists of a train of 3 rectangular pulses, each of 1 ms duration, with an intensity 1.2 times the TA motor threshold (MT), delivered 21 ms before the test stimulation (see the beginning of the trace).

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b) Postsynaptic inhibition

Postsynaptic inhibition elicited in soleus motoneurons by electrical stimulation of sural cutaneous afferents is one of the most robust reflexes in animals and humans. It has been shown that stimulation of the sural nerve (a purely cutaneous nerve) induces an inhibition of the soleus H-reflex (Hugon et al. 1965; Hugon & Bathien, 1967). The inhibition of the soleus H-reflex appears when cutaneous stimuli applied over the lateral dorsum or the plantar surface of the foot. The average distance from the point of stimulation on the distal of foot to the mid-popliteal region is 50 cm (reviewed in Gassel & Ott 1970).

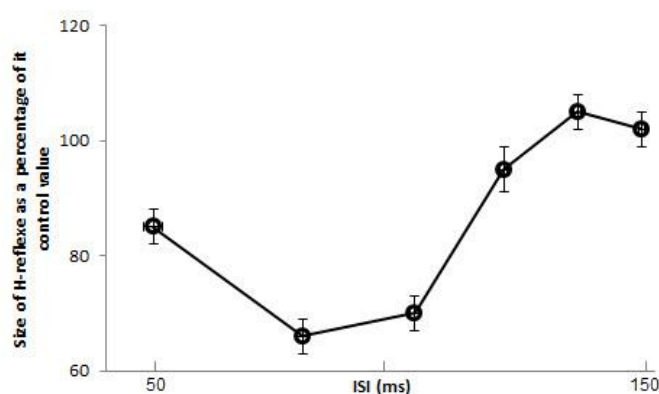


Figure 18 : Time course of postsynaptic inhibition induced by cutaneous stimulation applied at the lateral side the fifth toe.

The amplitude of soleus H-reflex expressed as a percentage of its control value is plotted against the interstimulus interval Adapted from Pierrot-Deseilligny et al., 1973.

In humans, the amount of this inhibition is marked (around 40 % of the unconditioned reflex) (Pierrot-Deseilligny et al., 1973). The soleus H-reflex was conditioned by electrical stimuli applied to cutaneous afferents of the sural nerve through bipolar adhesive electrodes placed on the lateral side of the fifth toe. The stimuli consisted of trains of 17 pulses, each of duration 1 ms, with an interpulse interval of 3 ms, delivered 50 ms before the test stimulus. In such conditions, the induced inhibition is likely caused by postsynaptic inhibition of soleus motoneurons (for reference see Pierrot-Deseilligny & Burke, 2012). The intensity of stimulation was based on the subject's perception threshold (PT) defined as the lowest stimulus intensity at which the subject reported the sensation of the stimulation using a single pulse. As it was shown in our laboratory that this postsynaptic inhibition is significant when the

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intensity of cutaneous stimulus is greater than 3 times the threshold of perception, the intensity of the stimulus train was slowly increased to 3-5 times the perceptual threshold in order to induce a clear-cut inhibition of the soleus H-reflex. To reproduce the same conditions of stimulation in SCI patients, we have demonstrated an intensity curve of postsynaptic inhibition induced by cutaneous stimulation in six healthy subjects to define the minimum intensity inducing a significant inhibition with the stimulator (D7SA Digitimer Ltd, Welwyn Garden City, UK) used for this protocol. The inhibition is significant in this group of subjects when the intensity of stimulation is greater than 6.5 mA, then reinforces for the currents between 6.5-8.5 mA and remains constant for the currents between 8.5-10 mA reflecting a saturation of inhibition. The intensity of the conditioning stimuli inducing postsynaptic inhibition was therefore set in all SCI patients at approximately 8.5 mA.

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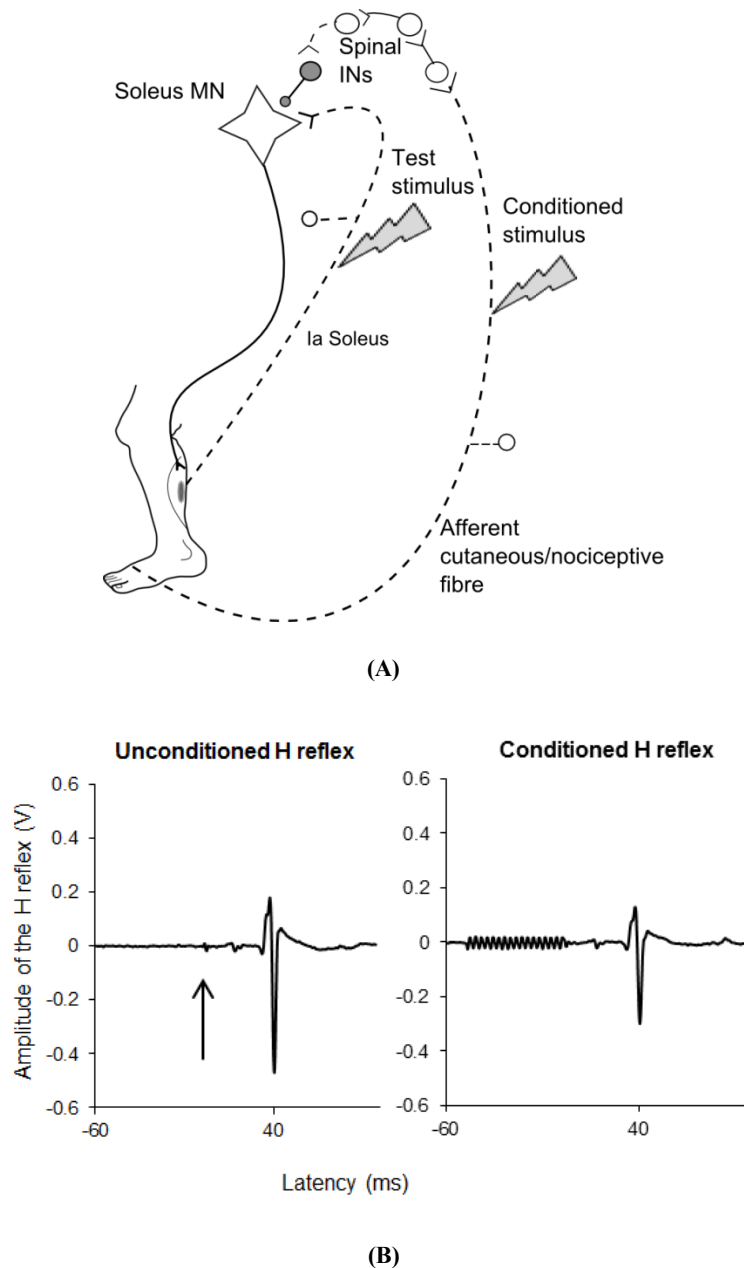


Figure 19 : Schematic diagram of the postsynaptic inhibition induced by cutaneous stimulation and example of waveforms of the soleus unconditioned H-reflex, and the soleus conditioned H-reflex induced by conditioning stimulus of cutaneous afferents of sural nerve.

(A) A conditioning stimulus induces the afferent volley in the cutaneous/nociceptive fibres (dotted line) in the sural nerve from the skin of the lateral side of the fifth toe activating spinal interneurons (INs) projecting onto the soleus motoneuron. *Adapted from Pierrot-Deseilligny & Burke, 2012.* (B) Examples of waveforms of the soleus unconditioned H-reflex, and the soleus conditioned H-reflex by electrical stimuli applied to cutaneous afferents of the sural nerve evoking postsynaptic inhibition. The conditioning stimulus consists of trains of 17 pulses, each of duration 1 ms, with an interpulse interval of 3 ms, delivered 50 ms before the test stimulus. The arrow indicates the triggering of the test stimulus.

3.1.3 Excitatory conditioning stimulation.

Post-activation depression

Post-activation depression was chosen in the present study to test the possible effect of furosemide on excitatory synapses since it reflects the efficiency of the monosynaptic excitatory transmission between Ia fibre and motoneuron and is a pure excitatory effect. Alternative possibilities to test pure excitatory effects should be either homonymous or heteronymous monosynaptic facilitation of the soleus H-reflex. However, homonymous monosynaptic facilitation cannot be used in humans to assess purely excitatory effects, due to several factors, including the mixed excitatory and inhibitory actions and duration of after-hyperpolarisation. Heteronymous monosynaptic Ia facilitation from the quadriceps to the soleus requires an accurate determination of the time interval between the conditioning and test stimulus to ensure its monosynaptic origin (Hultborn et al., 1987). For other excitatory pathways, for example group II disynaptic excitatory pathways, the indirect method we used cannot eliminate possible contamination by monosynaptic afferent projections or by inhibitory projections involved in the pathway. Moreover, testing group II excitatory pathways at rest relying on the use of the quadriceps H-reflex is not as easy as evoking the H-reflex in the soleus muscle.

It has been shown in both animals (Eccles, 1964) and humans (Magladery et al., 1951; Paillard, 1955; Crone & Nielsen, 1989; Hultborn & Nielsen, 1998) that post-activation depression at the Ia fibre-motoneuron synapse reflects the efficiency of the monosynaptic excitatory transmission between Ia fibre and motoneuron and is thus a simple and reliable tool to assess the functioning of excitatory synaptic transmission without contamination of other synaptic mechanisms (Meunier et al., 2007). Post-activation depression can be demonstrated by the depressive effect of increasing the stimulus rate on the size of H-reflexes. The H-reflex is dramatically depressed at short intervals (1 Hz) with gradual recovery as the interval increases to over 10 s (Crone & Nielsen, 1989). In the present study, the H-reflex evoked every 3 s (0.33 Hz) was initially adjusted at $H_{\max}/2$. The soleus H-reflex evoked at low frequency (every 6 s or 0.16 Hz) and high frequency (every 1 s or 1 Hz) was measured in each case (see Figure 20). Twenty soleus H-reflexes were evoked for each stimulus rate (1 Hz and 0.16 Hz). The amount of post-activation depression of the H-reflex was evaluated as the size of the H-reflex elicited at 1 Hz (high stimulus rate) expressed as a percentage of its value when elicited at 0.16 Hz (low stimulus rate). This is referred to as the 1/0.16 ratio. This ratio was used to determine the amount of post-activation depression of the H-reflex (Aymard et al., 2000).

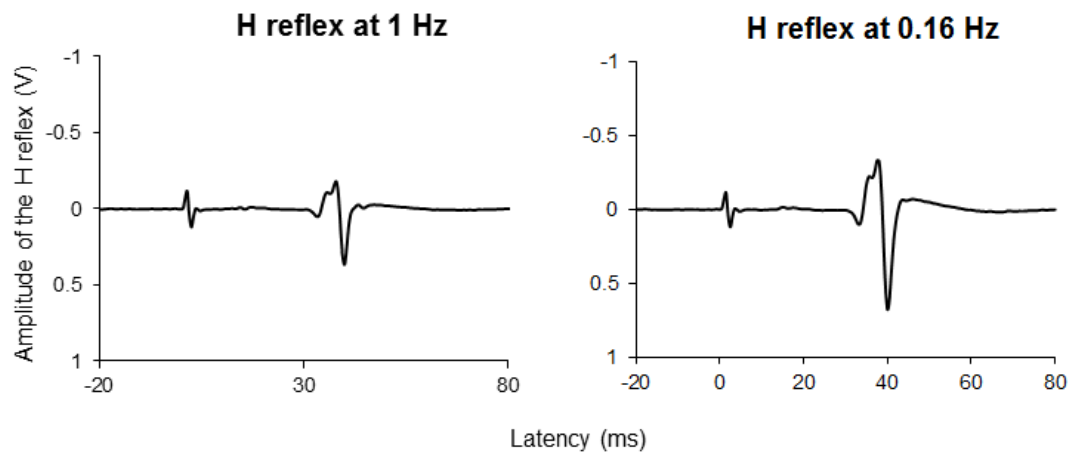


Figure 20 : Examples of waveforms of the soleus H-reflex preceded by another soleus H-reflex at 1 Hz and at 0.16 Hz

3.2 Furosemide

The loop diuretics, furosemide and bumetanide, are thought to modulate inhibitory synapses through their antagonist effect on cation-chloride co-transporters. It has been suggested that furosemide antagonises the KCC2 co-transporter but also the NKCC1 co-transporter. Nevertheless, its effect on the NKCC1 co-transporter is less potent than bumetanide (Gillen et al. 1996; Russell, 2000). To date, no substance has been identified that selectively inhibits KCC2 (Kahle et al. 2008; Löscher & Schmidt, 2011), and some of the KCC2 blockers used in animal studies require very high concentrations (Payne, 1997) that are likely to be toxic in humans.

Furosemide was reported to reduce GABA_A inhibition in adult neurons by reducing the neuronal transmembrane chloride gradient (Thompson et al., 1999). Nicoll (1978) demonstrated using intracellular recordings from the frog spinal cord that furosemide selectively and reversibly blocked inhibitory postsynaptic potentials (IPSPs) mediated by GABA and glycine without affecting excitatory postsynaptic potentials (EPSPs) mediated by glutamate. It has also been shown in both animals and humans that furosemide has clinically relevant antiepileptic actions. Studies on animal hippocampal slices revealed that furosemide potently blocks epileptiform activity without suppressing neuronal excitability (Hochman et al., 1995, 1999; Hochman & Schwartzkroin, 2000). It was proposed that the action of furosemide is mediated through nonsynaptic mechanisms modulating the size of extracellular space since modulation of electric field interactions via the extracellular space (ECS) might also contribute to neuronal hypersynchrony and epileptogenicity. In humans, a small clinical trial showed that oral furosemide (120 mg daily during 4 months) significantly decreased the frequency of seizures in patients with severe focal epilepsy (Ahmad et al.,

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1976). Recently, it was shown that a single intravenous injection of 20 mg furosemide crosses the blood-brain barrier and completely blocks epileptic activity in the brain (Huglund & Hochman, 2005). Together with its effects described above and given that furosemide is a widely used diuretic which has been employed with a good safety record for many years in humans, we have chosen furosemide to investigate the functioning of inhibitory synapses in the present study.

Furosemide used in the present study

The usual starting oral dose of furosemide in the clinic in adults is 20-80 mg per day. In the present study, the smallest dose, furosemide 20 mg was firstly tested on the spinal inhibitory networks in a small sample of subjects. Orally-administered furosemide 20 mg induced no significant changes in spinal inhibitory networks (see Results), so furosemide 40 mg was subsequently tested using the same experimental design. For the subjects convenience, due to its diuretic effect, the amount inhibition was monitored through repetitive measurements over a time period limited to 70 minutes after administration of the drug since furosemide pharmacokinetics and pharmacodynamics in healthy subjects show that furosemide (40-80 mg) appears in the serum within 10 minutes and peaks from 60 to 90 minutes after oral administration (Branch et al. 1976, 1977; Tilstone & Fine, 1978).

3.3 Recording chain

The test stimulus evoking the soleus H-reflex and the conditioning stimuli were delivered using constant-current stimulators (D7SA Digitimer Ltd., Welwyn Garden City, UK). The stimulations were computer-triggered using a customised script (Signal 4.20, Cambridge Electronic Design, Cambridge, UK). The H-reflex was recorded from the EMG signal of the soleus muscle using bipolar surface electrodes (Delsys Inc., Boston, USA, Ag electrodes DE-2.1) positioned on the skin parallel to the muscle belly. The EMG signals were amplified ($\times 1000$), band pass filtered at 20-450 Hz (Delsys, Inc., Boston, MA, US), digitised at 1 kHz (Power 1401 A/D board, Cambridge Electronic Design, Cambridge, UK) and then transmitted to an oscilloscope for visualization and to a computer for storage and offline analysis (Figure 21).

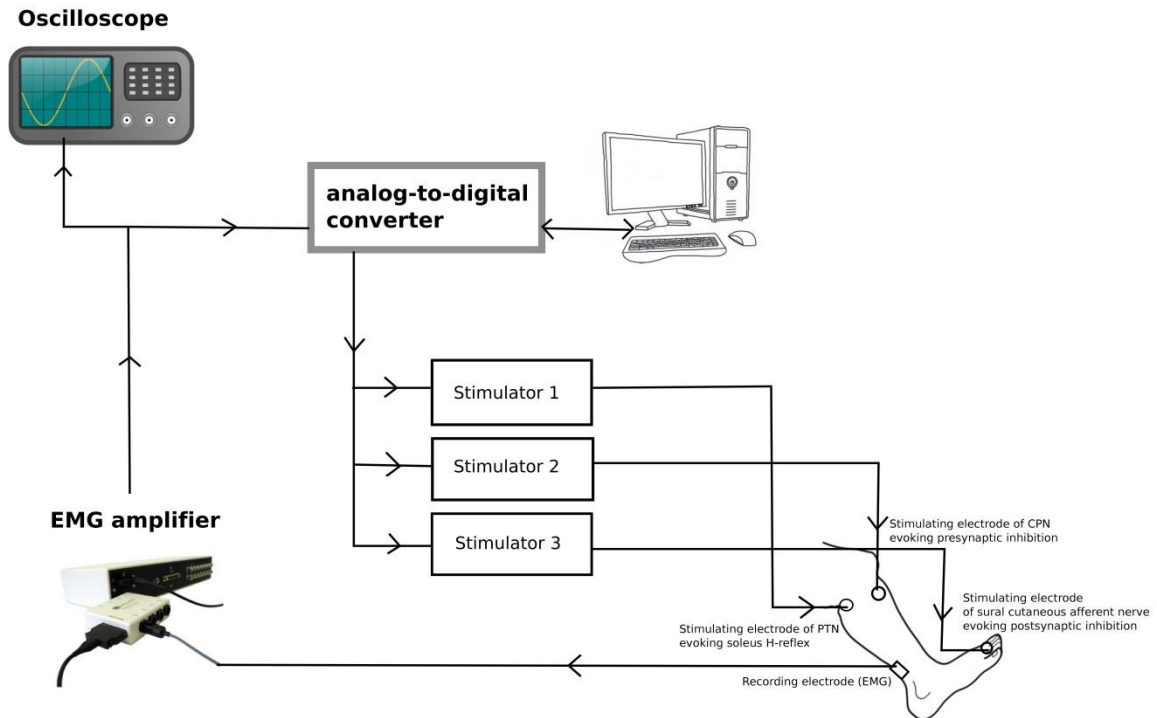


Figure 21 : Example of a recording chain to study the pre- and postsynaptic inhibition in the lower limb with a soleus H-reflex.

The soleus H-reflex is evoked by a stimulus test applied over the posterior tibial nerve at the popliteal fossa using stimulator 1. To evoke presynaptic inhibition, the soleus H-reflex was conditioned with a preceding stimulation of the common peroneal nerve through electrodes placed below the neck of the fibula using stimulator 2. To evoke postsynaptic inhibition, the soleus H-reflex was conditioned by electrical stimuli applied to cutaneous afferents of the sural nerve through electrodes placed on the lateral side of the fifth toe using stimulator 3. A computer with the Signal® system triggers the stimulators in a random sequence. The H-reflex was recorded from the EMG signal of the soleus muscle using bipolar surface electrodes positioned on the skin parallel to the muscle belly. The EMG signal is amplified and then transmitted to an oscilloscope for visualization and to a computer for storage and offline analysis.

3.4 Statistical methods

3.4.1 Data processing

To evaluate the amounts of pre- and postsynaptic inhibition, peak-to-peak amplitudes of unconditioned H-reflexes were compared to those of conditioned H-reflexes. The inhibitory effects were quantified by calculation of the inhibition value as $100 - ((\text{conditioned H-reflex}/\text{unconditioned H-reflex}) \times 100)$. Each inhibition value was then normalized to the percentage of its baseline value using the equation: $[\%((\text{Inhibition at } t_x - \text{baseline inhibition})/\text{baseline inhibition})] + 100$.

To evaluate the effects of post-activation depression, the 1/0.16 ratio was normalized to the percentage of its baseline value using the equation: $[\% ((1/0.16 \text{ ratio at } t_x - 1/0.16 \text{ ratio at baseline})/ 1/0.16 \text{ ratio at baseline})] + 100$.

3.4.2 Statistical analysis

The amounts of pre-/postsynaptic inhibition and post-activation depression were averaged among subjects for each time epoch (0-10 min, 11-20 min, 21-30 min, 31-40 min, 41-50 min, 51-60 min, and 61-70 min). For healthy subject experiments, a two-way repeated-measures ANOVA (control vs. furosemide as the within-subject factor) was used to examine the effects of furosemide on pre-/postsynaptic inhibitions or post-activation depression. To compare the effects of furosemide in healthy subjects and paraplegic patients, a two-way ANOVA was employed. A *post-hoc* pairwise comparison was performed using Tukey's HSD test.

A one way repeated-measure ANOVA on time epoch (0-10 min, 11-20 min, 21-30 min, 31-40 min, 41-50 min, 51-60 min, and 61-70 min) was also used to determine the impact of time in the control experiment.

For dose-dependent experiments, an a priori power analysis was first performed to determine the minimal sample size. A posteriori, all our data sets were, as assumed in our power analysis, normally distributed and successfully passed the equality of variance test so parametric tests (one way repeated measures ANOVA) were employed.

Significance was taken at $P < 0.05$. Mean data are provided \pm standard error of the mean (SEM).

4. Methods used in the project 2

4.1 Electrophysiological recordings

4.1.1 Stimulation test: FCR H-reflex

Electromyographic (EMG) activity was recorded from FCR muscle using bipolar surface electrodes positioned on the muscle belly. EMG activity was displayed in a wide analysis window beginning 100 ms before the test stimulus and lasting 300 ms. EMG activity was sampled at 2 kHz, amplified (x 5000 – 10000) and band-pass filtered (250 Hz–3 kHz) using a Digitimer D360 amplifier (DIGITIMER LTD, WELWYN GARDEN CITY, Herts, UK). The FCR H-reflex was evoked by electrical stimuli applied to the median nerve. The maximum direct motor response (Mmax) was first determined and the unconditioned H-reflex was adjusted to 10% – 20% of Mmax in each subject. The amplitude of the unconditioned FCR H-reflex was adjusted to 50% of the maximum amplitude of H-reflex in the majority of cases and it was kept constant throughout the experiment.

4.1.2 Conditioning stimulation

a) *Reciprocal inhibition*

It has been shown that a conditioning stimulus applied to the radial nerve induces an early inhibition of the FCR H-reflex (Day et al., 1983, 1984). The FCR H-reflex was evoked by electrical stimuli applied to the median nerve near the cubital fossa. It was conditioned with conditioning stimulation of the radial nerve through bipolar electrodes placed at the spiral groove. The conditioning intensity was adjusted below the ECR motor threshold (MT) to prevent recurrent inhibition (Aymard et al., 1995) and was between 0.7-0.9 MT depending on the subject. The motor threshold was determined by tendon palpation and by the EMG displaying on the oscilloscope's screen. The ISI was the time interval between test and conditioning stimuli at which the early radial-induced inhibition of the FCR H-reflex was greatest. The ISI was determined using 0.5 ms steps in the range – 3 ms/+ 1 ms (Day et al. 1984).

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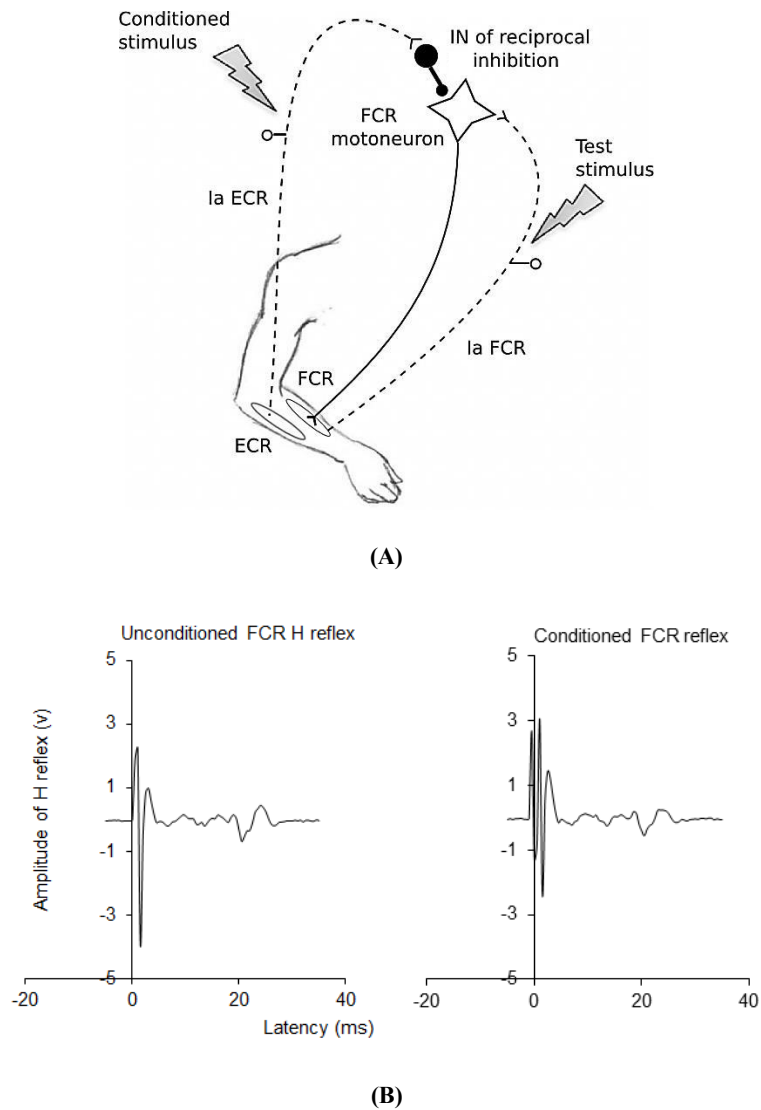


Figure 22 : Schematic diagram of the reciprocal inhibition at the wrist level and example of waveforms of the unconditioned FCR H-reflex, and the FCR conditioned H-reflex induced by conditioning stimulus of radial nerve.

(A) A conditioning stimulus applied to the radial nerve induces the afferent volley in extensor carpi radialis (ECR) (dotted line) activating interneurons (IN) of reciprocal inhibition projecting on flexor carpi radialis (FCR) motoneurons. *Adapted from Pierrot-Deseilligny & Burke, 2012.* (B) Examples of waveforms of the unconditioned H-reflex, and the conditioned H-reflex by electrical stimuli applied to the radial nerve evoking reciprocal inhibition using a rectangular pulse with a duration of 1 ms, with an intensity below the ECR motor threshold (MT).

4.2 Transcranial direct current stimulation

Transcranial direct current stimulation or tDCS is a simple non-invasive cortical modulation technique in which a device sends constant low direct current (DC) delivered to the area of interest through the electrodes. The basic idea design of tDCS, using DC has been used for the treatment of mental and neurologic disorders since Aldini's work in the beginning of 19th century (see Parent, 2004). Since the 1960s, brain polarization has been reintroduced in animals (Creutzfeld et al., 1962; Bindman et al., 1964; Purpura & McMurtry, 1965) and later in humans (Priori et al., 1998; Nitsche & Paulus 2000). There are a number of studies investigating tDCS effects on the central nervous system. Today, transcranial direct current stimulation is beginning to be used more frequently as a new brain stimulation technique and in therapeutic applications in humans.

4.2.1 Animal studies

There was a rise in interest in transcranial direct current stimulation in the 1960s as demonstrated by different researchers (Creutzfeld et al., 1962; Bindman et al., 1964; Purpura & McMurtry, 1965). They showed that stimulation through the scalp could affect brain function by changing the cortical excitability. This change is likely due to the modulation of resting membrane potential of cortical cells. A tDCS device has an anodal electrode (positively charged electrode) and a cathodal electrode (negatively charged electrode). One of the electrodes is placed over the area of interest and the other one (reference electrode) is placed in another area (e.g. the supraorbital region) to complete the circuit. They also demonstrated that positive and negative stimulation had different effects on cortical excitability. Anodal tDCS causes a depolarization of the resting membrane potential that leads to increases in neuronal excitability of the area being stimulated and more spontaneous cell firing, whereas cathodal tDCS causes a hyperpolarization of the resting membrane potential, leading to decreases in neuronal excitability of the area being stimulated and decreased spontaneous cell firing. Purpura & McMurtry (1965) have also demonstrated that tDCS effects on the motor cortex were not only based on what type of stimulation was being used but also on the intensity of stimulation as well as the type of cortical cells being stimulated. The effects of tDCS induced with a given polarity can thus be different between pyramidal cells and non-pyramidal cells. Effects of tDCS beyond the end of stimulation were also reported and these are usually related to the duration and intensity of stimulation. The post-effect of tDCS was also observed and proposed to be due to the plasticity concepts of long-term potentiation (LTP) and long-term depression (LTD) (Bindman et al., 1964; Weiss et al., 1998).

4.2.2 Human studies

a) *Effects on the brain*

The tDCS experiment in humans was first introduced by Priori and colleagues in 1998. They investigated whether direct current could reach and influence the human brain as demonstrated in animal studies. Testing the effect of tDCS on the human motor cortex by assessing the changes in motor potentials evoked by transcranial magnetic brain stimulation (TMS) shows significant modulations in the excitability of the motor cortex. This provides direct evidence that very small electric fields can cross the skull and influence human brain activity. Subsequent experiments by Nitsche and Paulus in 2000 also confirmed the effects of tDCS on the motor cortex with significant changes in the motor evoked potential (MEP) size. They also demonstrated the differentiation effects of anodal and cathodal tDCS on the human brain: anodal increases cortical excitability while cathodal decreases cortical excitability. The authors proposed that these effects are probably due to shifts in neuronal resting membrane potential. The tDCS effects observed in their study also outlasted the duration of stimulation but were reversible. Amplitude and endurance beyond the end of stimulation were reported to be current-intensity and stimulation-duration dependent.

The cortical excitability changes induced by tDCS in humans have been reported to be caused by various mechanisms. In 2003, Nitsche and colleagues proposed that the cortical excitability modulations elicited during tDCS depend on membrane polarization as seen in animals (Purpura & McMurtry, 1965). Blocking sodium and calcium channels eliminates or reduces the excitability enhancement induced by anodal stimulation during and after tDCS, whereas the reduction in excitability caused by cathodal tDCS is not modulated by ion channel blockade. These results suggest that anodal tDCS caused neuronal depolarization, while cathodal tDCS generated neuronal hyperpolarization since neuronal hyperpolarization would inactivate the sodium and calcium channels. Stagg et al (2009) demonstrated using Magnetic Resonance (MR) spectroscopy that the increase of cortical excitability induced by anodal tDCS is due to reduction of cortical inhibitory neurotransmitter (GABA) concentrations while reduction of cortical excitatory neurotransmitter (Glutamate) concentrations (without any modifications of GABA concentration level) causes the decrease of cortical excitability induced by cathodal tDCS.

However, it appears that the mechanisms underlying immediate effects and after-effects of tDCS are different. Mechanisms underlying the post effects induced by tDCS appear to be related to modulations of cerebral plasticity. The long-lasting after-effects of tDCS on the brain are thought to be mediated at the synaptic level by NMDA receptors (Liebetanz et al., 2002; Nitsche et al., 2003; Siebner et al., 2004). The NMDA

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receptor is known to be involved in cortical neuroplastic changes such as long-term potentiation (Bennett, 2000). However, it was subsequently reported that the after-effects of tDCS do not arise from NMDA receptor synaptic involvement alone. Ardolino et al., (2005) proposed that the after-effects of cathodal tDCS in the human brain arising through non-synaptic mechanisms possibly involve alterations in transmembrane proteins and changes in pH. Nitsche et al., (2009) demonstrated in healthy subjects that under placebo medication, anodal tDCS enhanced, and cathodal tDCS reduced, excitability of the motor cortex for about 60-120 min, whereas oral administration of citalopram (a selective serotonin reuptake inhibitor) enhanced and prolonged the facilitation induced by anodal tDCS and turned cathodal tDCS-induced inhibition into facilitation which was prolonged over 120 minutes after tDCS. Serotonin has a prominent impact on tDCS-induced plasticity in humans since the modulation of the serotonergic system affects long-term potentiation (LTP) and long-term depression (LTD).

In addition, it has been shown that the electrode position and the orientation of the electric field could also contribute to the results in the human brain. Current distribution under electrodes over the chin is less tangential to the cerebral cortex than one under electrodes over the frontal bone. Currents with comparable intensities but with different directions can have dramatically different effects on the excitability of the cortex (Priori, 2003).

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b) Effects of tDCS applied over the cerebral cortex on the spinal networks

In 2009, Roche and colleagues have demonstrated the effects of tDCS over the contralateral primary motor cortex area (M1) on spinal networks excitability at the cervical level in humans suggesting that the effects of tDCS should not be considered only on cortical neurons but also on spinal circuits. Anodal tDCS applied continuously for 20 minutes over the contralateral hand motor area increased reciprocal inhibition directed from ECR to FCR motoneurons during the application with no modification of presynaptic inhibition directed to FCR Ia fibres and FCR H-reflex recruitment curves. These authors suggested that anodal tDCS probably modifies the rate of spontaneous discharge of M1 and induces an increase in the efficiency of the descending control acting on spinal neurons. The absence of change in presynaptic inhibition of FCR Ia terminals indicates that tDCS likely mainly increases disynaptic inhibitory interneuron excitability. It was also demonstrated in the same study that cathodal tDCS induces no change in both presynaptic Ia inhibition and reciprocal inhibition. A subsequent study by the same authors showed that anodal tDCS also has effects on spinal networks excitability at the lumbar level: anodal tDCS decreased reciprocal Ia inhibition directed from the tibialis anterior muscle to the soleus motoneuron, increased homonymous soleus recurrent inhibition, and induced no change in both presynaptic inhibition of soleus Ia fibres and soleus H-reflex recruitment curves. The authors proposed that anodal tDCS preferentially increases the spontaneous corticospinal cell firing rate projecting to reciprocal inhibition and Renshaw cells (Roche et al., 2011). It appears that effects of anodal tDCS on reciprocal inhibition between agonist and antagonist muscles are different at cervical spinal and lumbar spinal levels as anodal tDCS over the hand motor cortex increases disynaptic inhibition directed from ECR to FCR while it diminishes reciprocal Ia inhibition directed from the tibialis anterior muscle to the soleus motoneuron. It was proposed that these opposite results may be due to i) the difference in nature of interneurons mediating disynaptic inhibition at wrist and ankle levels, and ii) the differences in cortical structures stimulated by tDCS since tDCS over the leg motor cortex generated an electrical field perpendicular to the target motor cortex, while tDCS over the hand motor cortex generated an electrical field parallel to the stimulated motor cortex. In these series of experiments, tDCS has not been reported induce any after-effects on the spinal networks studied. In a subsequent study in 2012, Roche et al. demonstrated post-effects on the lumbar propriospinal system for at least 20 minutes after the end of anodal tDCS during 20 minutes over the motor cortex (Roche et al., 2012).

4.2.3 Physical parameters of tDCS

a) *Size of electrodes and focality*

tDCS is less focal than other brain stimulation methods such as transcranial magnetic stimulation (TMS) and transcranial electrical stimulation (TES) given its electrode size. Some studies in humans showed that the size of active and reference (cathode) electrodes might complicate tDCS results. A smaller active electrode applied over the M1 showed better increased the corticospinal excitability of the extensor carpi radialis muscle than the larger ones (Bastani & Jaberzadeh., 2013). Anodal tDCS over the supplementary motor area and a reference electrode placed over the contralateral prefrontal cortex impaired complex whole body motor skill learning whereas using a larger reference electrode for the same protocol did not affect the outcome of learning compared to the sham (Kaminski et al., 2013).

A study in animals using functional magnetic resonance imaging (fMRI) to measure the effectiveness of tDCS showed that tDCS over the frontal lobe induced neuronal activation in the target cortex and also in its connected brain region (Takano et al., 2011). The cortical stimulation effect of tDCS on the left primary motor cortex (M1) demonstrated by fMRI in humans show that besides its effects on the left M1, there were also activations in the left supplementary motor cortex (SMA) and the right posterior parietal cortex (Kwon et al., 2008).

b) *Current intensity*

The efficacy of tDCS to induce modification of membrane polarity also depends on current density that determines the induced electrical field strength (Purpura et al., 1965). Nitsche & Paulus (2000) showed the dependency of the size of prolonged motor cortex excitability change after anodal tDCS on different current intensities (0.2-1.0 mA) with larger current densities inducing stronger effects. At 2 mA, anodal or cathodal tDCS over the prefrontal cortex in healthy subjects improved or decreased more verbal fluency compared to the result observed with 1 mA (Iyer et al., 2005).

c) *Duration*

With constant current intensity, duration is an important parameter that influences the occurrence and duration of tDCS post-effects. Nitsche & Paulus (2001) demonstrated in humans the long-lasting excitability of modulations after the end of tDCS. After 5-7 minutes of anodal tDCS over the motor cortical representational field of the abductor minimi muscle (ADM), the MEP amplitudes (measured by TMS) return to baseline within the first few minutes of stimulation, whereas 9 -13 minutes of stimulation results in after-effects remaining for a maximum duration of 1.5 hours after

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the end of stimulation. The same results were also found for the cathode. Cathodal tDCS over the hand motor area of the ADM induced prolonged excitability reduction in humans with duration dependency. 5 and 7 min of stimulation resulted in motor cortical excitability reductions, which lasted for minutes after the end of stimulation, while 9 minutes of stimulation induced after-effects for up to an hour after the end of stimulation (Nitche et al., 2003).

d) Electrode position and polarity

The anode is defined as the positively charged electrode, whereas the cathode is the negatively charged one. Most authors place the anode above the motor cortical representational field of the target muscle, and the cathode above the contralateral orbital to increase cortical excitability, while for decreasing the cortical excitability: the cathode is positioned above the representation of target muscles in the motor cortex, while the anode is placed above the contralateral orbital (Nitche & Paulus 2000, 2001; Nitche et al., 2003).

e) Safety

tDCS is generally regarded as a safe method of brain stimulation. However, it is not advised to use tDCS in people susceptible to seizures and/or people who have metallic implants near the electrodes. In addition, possible side effects of tDCS such as headaches, dizziness, nausea, itching sensations as well as transient skin irritation (i.e. burning) under the electrodes should be made known to subjects (Poreisz et al., 2007; Palm et al., 2008).

f) Comparison to other brain stimulation technique: rTMS

Recently, repetitive transcranial magnetic stimulation (rTMS) and tDCS have come to be used more often as painless and non-invasive brain stimulation techniques in humans. Regarding their effects on the cortex, tDCS greatly differs from rTMS, since rTMS induces action potentials of axons in of the cortex that then release neurotransmitters at their terminal synapses, while tDCS does not cause action potentials in cortical neurons. In general, high-frequency rTMS and anodal tDCS enhance, and low-frequency rTMS and cathodal tDCS decrease, the excitability of neurons of the targeted cortical areas. Both rTMS and tDCS have also been reported to induce post-effects that outlast the period of stimulation. TMS coils come in a variety of different sizes and configurations and it appears that TMS is able to limit stimulation to an area of about 25 mm², while the current flow of tDCS throughout the brain occurs over a wide area. tDCS intensities indicate the current flow between the electrodes with no indication of how much the current enters the brain and polarizes neurons. However,

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the relationship between stimulation intensity and clinical effects has not yet been clearly reported in both rTMS and tDCS. tDCS is easy to sham and relatively simple to apply compared to rTMS. It was reported that tDCS at an intensity below 1.5 mA is generally not perceived by subjects. If low current intensity is used with large area stimulating electrodes and low impedance, subjects would not distinguish between active and sham stimulation. Both rTMS and tDCS are generally considered as safe methods. However, tDCS induces a small or no risk of a seizure compared to rTMS as it does not induce action potentials in axons. tDCS applied for minutes does not increase the markers of neuronal damage such neuron specific enolase or brain N-acetyl-aspartate. However, whether tDCS is safe for long stimulation duration or high intensities is still unknown (Reviewed in Priori et al., 2009).

To conclude, convincing experiments now show that tDCS can pass through the scalp and influence brain and spinal cord activities during their application and sometimes induce post-effects in motor cortex and some spinal circuits (Priori et al., 1998, 2009; Nitsche & Paulus, 2001; Priori, 2003; Roche et al., 2009, 2011, 2012). Together with its advantages such as non-invasiveness, painlessness, and inducing less chance of causing seizure, we therefore chose tDCS to investigate changes in the excitability of spinal network in hemiplegic patients in the present study.

4.2.4 Application of tDCS in the present study

The experimental procedure was identical to that used by Roche et al. (2009). Application of tDCS used a NEUROCONN DC-STIMULATOR (NEUROCONNGMBH COMPANY, Ilmenau, Germany) with two conductive rubber electrodes placed in saline-soaked sponges (5x7 cm). The cathode was placed over the contralateral supra-orbital region. The effects of tDCS were tested in active and sham conditions. In both conditions, current intensity was fixed at 1.75 mA. Current flowed continuously during 20 minutes for active conditions, and only 120 seconds for the sham condition since Nitsche and Paulus (2000) had previously reported that a duration of at least 3 minutes of tDCS was necessary to induce after-effects. The current was ramped up or down over the first and last 8 seconds of stimulation. The minimum delay between the two recording sessions (active and sham tDCS) was about one week. The active and sham conditions were randomly alternated.

5. Recording Chain

The test stimulus evoking FCR H-reflex and the conditioning stimulus were delivered using constant-current stimulators (D7SA Digitimer Ltd., Welwyn Garden City, UK). The stimulations were computer-triggered using a customised script (Signal 4.20, Cambridge Electronic Design, Cambridge, UK). The H-reflex was recorded from the EMG signal of the FCR muscle using bipolar adhesive electrodes (Euro ECG electrodes, 32x36 mm, Vicchio, Florence, Italy) positioned on the skin parallel to the muscle belly. The EMG activity was sampled at 2 kHz, amplified (x 5000 – 10000) and band-pass filtered (250 Hz–3 kHz) using a Digitimer D360 amplifier (DIGITIMER LTD, WELWYN GARDEN CITY, Herts, UK) and then transmitted to an oscilloscope for visualization and to a computer for storage and offline analysis. Anodal tDCS (sham/active) was applied over the motor cortex during 20 minutes. The recording chain in this present study was similar to that in the project 1 (see Figure 21).

6. Statistical methods

6.1 Data processing

The H-reflex size was determined by averaging peak-to-peak amplitudes expressed as a percentage of the maximum motor response (M_{\max}). The amount of reciprocal inhibition was defined as $100 - ((\text{conditioned H-reflex}/\text{unconditioned H-reflex}) \times 100)$. The mean amount of reciprocal inhibition from each period was normalised as a percentage of its baseline inhibition measured over 10 minutes before tDCS (t_0) according to the equation: $[\%((\text{Inhibition at } t_x - \text{inhibition at } t_0) / \text{inhibition at } t_0)] + 100$. Therefore, positive value refers to a larger inhibition than baseline inhibition and *vice versa* for negative value.

6.2 Statistical analysis

For individual data, a two-way ANOVA was performed with time period as the first factor (baseline, period 1 tDCS, period 2 tDCS, and post tDCS) and tDCS condition (sham and active ipsilateral tDCS) as the second factor.

A two-way ANOVA was performed on unconditioned H-reflexes (with time period and condition as factors) to ensure that the modulation of reciprocal inhibition resulted from tDCS not from the variation of the H-reflex over time. Grouped data were analysed using two-way repeated-measures ANOVAs to determine effects of tDCS and time period on reciprocal inhibition. When the F value was significant, *post hoc* pairwise comparisons were performed using the Newman-Keuls test. Significance was taken at $P < 0.05$. Mean data are provided as mean \pm 1 Standard Error of the Mean (SEM). Statistical analysis was performed using the SigmaPlot software 11.0.

Chapter 3

Results

Project 1

1. Subjects

The experiments described in this study conformed to the guidelines issued by the World Medical Association (Declaration of Helsinki) on the ethics of experimentation in humans and the approval of the local ethical committee of the CPP Île-de-France VI - Pitié-Salpêtrière. Written informed consent was obtained from all subjects before participation.

Healthy subjects included in the present study had no history of drug allergies. Patients were selected based on the following criteria : i) the presence of paraplegia/tetraplegia after spinal cord injury (complete or incomplete) ii) the presence of a sizeable H-reflex in soleus muscle iii) the absence of medical history of drug allergies. This randomized controlled study was performed on 19 healthy volunteer subjects (9 females) aged 22-64 years old (mean±SEM 35.1±2.5 years) and 10 SCI patients (1 female) aged 20-67 years old (mean±SEM 36.4±4.8 years). The patients were selected from patients undergoing rehabilitation at Physical Medicine and Rehabilitation department, the Hôpital Raymond Poincaré (Raymond Poincaré Hospital). The average duration after SCI was 6.3±1.5 months. Nine patients present complete cord injuries.

For the recruitment, some patients were excluded from the study because of the absence of soleus H-reflex. Another constraint presented here was that pre- and postsynaptic inhibition could not be always evoked in all included patients; presynaptic inhibition could be demonstrated in 9 of 10 patients and postsynaptic inhibition could be demonstrated in only 4 of 10 patients (see individual inhibition values Table 2). This constraint especially for postsynaptic inhibition has limited our recordings and also the interpretation of the results.

2. Study design

All of healthy subjects participated in two different sessions to investigate the time course of changes in spinal network excitability for a period of 70 minutes (i) after furosemide intake (furosemide experiment), and (ii) without furosemide (control experiment). The two different experiments (furosemide and control) were performed on different days. Only furosemide experiments were performed in SCI patients.

3. Experimental procedure

Medical examinations (blood testing for electrolytes, creatinine, glucose and blood pressure) were performed before the experiment to verify that subjects had no contraindications to furosemide.

All experiments were performed at rest. Subjects sat comfortably with the head, neck, back and arms supported in a slightly reclined armchair. The dominant leg was examined and remained in a stationary supported position with hip semi-flexion (120°), slight knee flexion (160°) and plantar flexion (110°).

Each experiment comprised seven time epochs (0-10 min, 11-20 min, 21-30 min, 31-40 min, 41-50 min, 51-60 min, and 61-70 min). The amount of pre-/postsynaptic inhibition evaluated at the beginning of recording (during 10 minutes before drug administration in the furosemide experiment, or 10 minutes before the test start time in the control experiment) corresponds to baseline inhibition. After the amount of inhibition was determined for the baseline period, 40 mg furosemide was orally administered.

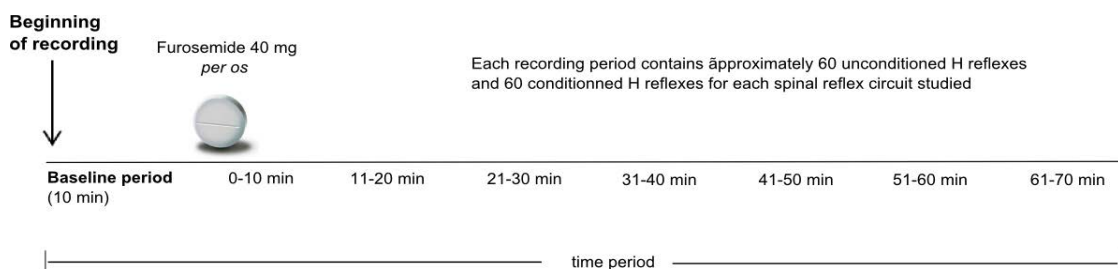


Figure 23 : Schematic diagram of experimental procedure of the control or furosemide experiments.

Once inhibition values are evaluated for baseline period, furosemide is then orally taken. The recording is performed after furosemide intake for 70 minutes. In control experiments, the time period of recording is the same as furosemide experiments.

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The effect of furosemide was also tested on monosynaptic excitatory transmission (synaptic transmission between muscle afferent terminals and soleus motoneurons/post-activation depression of the soleus H-reflex) to explore if furosemide has specific effect on functioning of inhibitory synapses. This experiment was performed in 15 healthy subjects the same way as described previously for pre- and postsynaptic inhibition.

Water intake was recommended to subjects after the experiment. However, apart from the diuretic effect, some side effects of furosemide such as headaches and dizziness were reported from some subjects that participated in the present study.

4. Results from healthy subjects

4.1 Amplitude of H-reflex (as % of M_{\max})

The maximum motor response (M_{\max}) and maximum H-reflex response (H_{\max}) were recorded at the beginning of the each experiment. The stimulus intensity was then adjusted to elicit H-reflex amplitude of 20-35% of M_{\max} , which corresponded to $\sim 50\%$ of H_{\max} . The mean values of H-reflex amplitude are $24.85 \pm 2.8 \% M_{\max}$ in control experiments, and $23.17 \pm 2.2 \% M_{\max}$ in furosemide experiments.

4.2 Effects of time on two spinal inhibitory networks

4.2.1 Presynaptic inhibition of Ia fibres.

Presynaptic inhibition was recorded over 70 minutes in 19 healthy subjects in the control experiments (Figure 24).

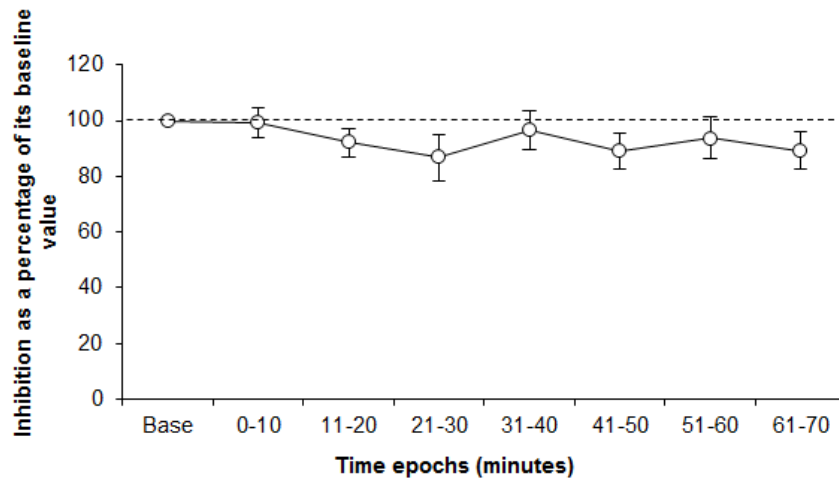


Figure 24 : Time course of presynaptic inhibition without furosemide in healthy subjects.

The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM).

The presynaptic inhibition is not modified over time. A one-way repeated-measures ANOVA failed to detect a significant effect of time on presynaptic inhibition in control experiments ($P > 0.05$).

4.2.2 Postsynaptic inhibition induced by cutaneous stimulation.

The experiments were performed in the all of subjects but this type of spinal synaptic inhibition could not be demonstrated in 4 of them since conditioning stimulus intensity required to evoke postsynaptic inhibition was above that evoking an unbearable painful sensation or triggered the flexor reflex in these subjects. The time course of postsynaptic inhibition was observed in 15 healthy subjects over 70 minutes, illustrating in the Figure 25.

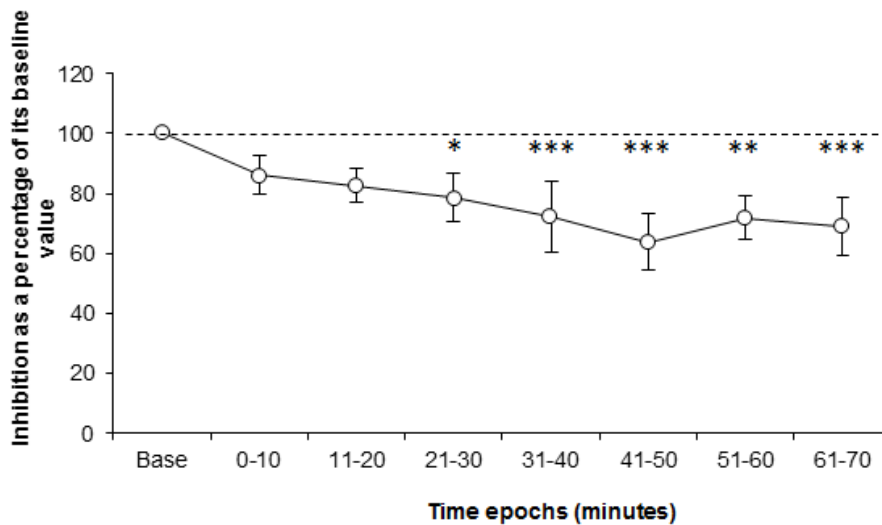


Figure 25 : Time course of postsynaptic inhibition without furosemide in healthy subjects.

The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

There was a gradual decrease of inhibition over time in control experiments ($F_{14,98} = 8.56$, $P < 0.001$, one way repeated-measure ANOVA). The amounts of postsynaptic inhibition is decreased progressively after the onset of the recordings and the decrease is statistically significant from 21-30 minutes up to 61-70 minutes (baseline vs. 21-30 minutes, baseline vs. 31-40 minutes, baseline vs. 41-50 minutes, baseline vs. 51-60 minutes, and baseline vs. 61-70 minutes ($P < 0.05$, $P < 0.001$, $P < 0.001$, $P < 0.01$, and $P < 0.001$ respectively, Tukey test).

4.3 Furosemide effects on the two spinal inhibitory networks.

4.3.1 Presynaptic inhibition of Ia fibres.

Presynaptic inhibition recorded over 70 minutes after oral administration of 40 mg furosemide in 19 healthy subjects is illustrated in Figure 26.

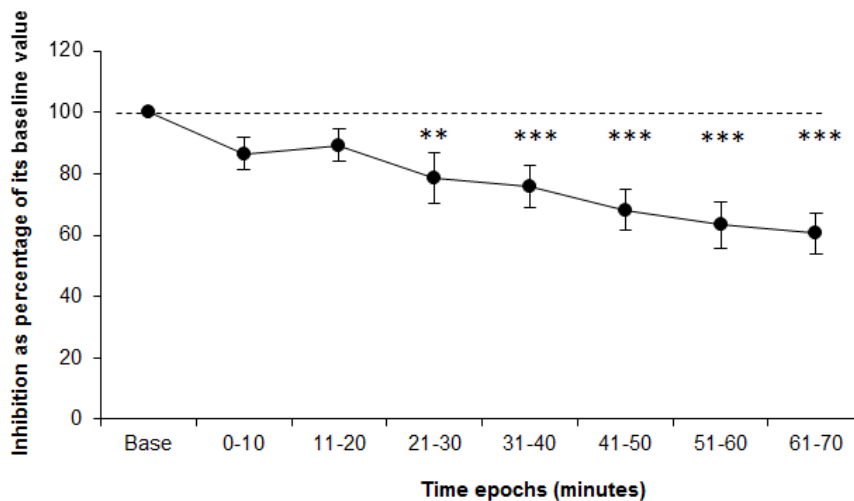


Figure 26 : Time course of presynaptic inhibition with furosemide in healthy subjects.

The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

There is a significant decrease of inhibition after drug administration in furosemide experiments ($F_{15,105} = 9.73$, $P < 0.001$, one way repeated-measure ANOVA). The amounts of presynaptic inhibition is decreased gradually after the 11-20 min following furosemide intake and reached its nadir at 61-70 minutes (60.66 % of its baseline value). The decrease is statistically significant from 21-30 minutes up to 61-70 minutes (baseline vs. 21-30 minutes, baseline vs. 31-40 minutes, baseline vs. 41-50 minutes, baseline vs. 51-60 minutes, and baseline vs. 61-70 minutes ($P < 0.01$, $P < 0.001$, $P < 0.001$, and $P < 0.001$ respectively, Tukey test).

CHAPTER 3 RESULTS : PROJECT 1

a) Furosemide vs. control : a representative individual result

Presynaptic inhibition recorded over 90 minutes in control and furosemide experiments in a representative subject is shown in Figure 27 (The recording is prolonged in this case (>70 minutes) for the reason of high diuretic resistance of the representative subject).

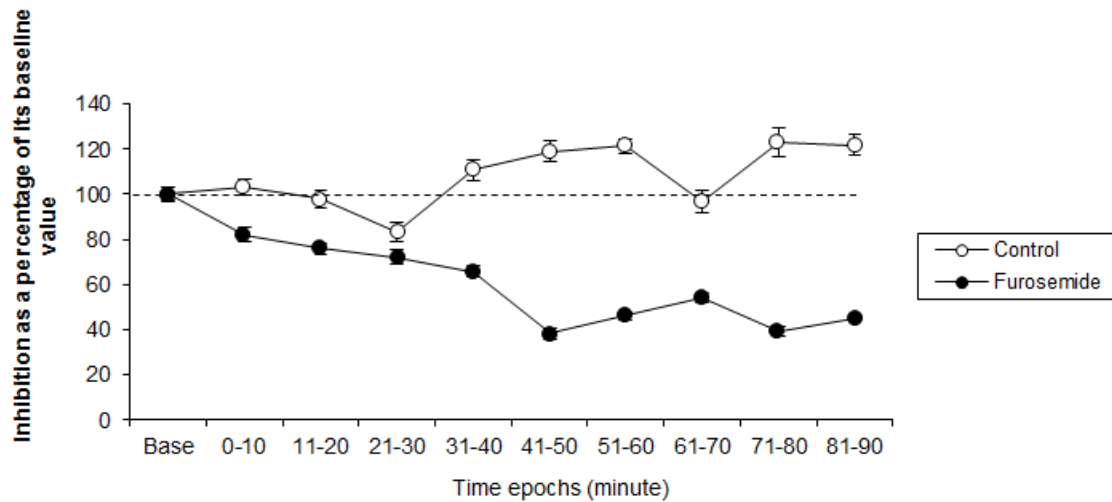


Figure 27 : Time course of presynaptic inhibition with and without furosemide in a healthy individual subject.

The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM).

The amount of presynaptic inhibition in control experiments is constantly at the baseline level over time, while there is a gradual decrease of presynaptic inhibition immediately after furosemide intake until reaching its nadir at 41-50 minutes (38.16 ± 2.36 % of its baseline value) and being continuously at this level up to 90 minutes.

CHAPTER 3 RESULTS : PROJECT 1

b) Furosemide vs. control : Group results

The comparison between variations of presynaptic inhibition in control and furosemide experiments in 19 subjects is shown in Figure 28.

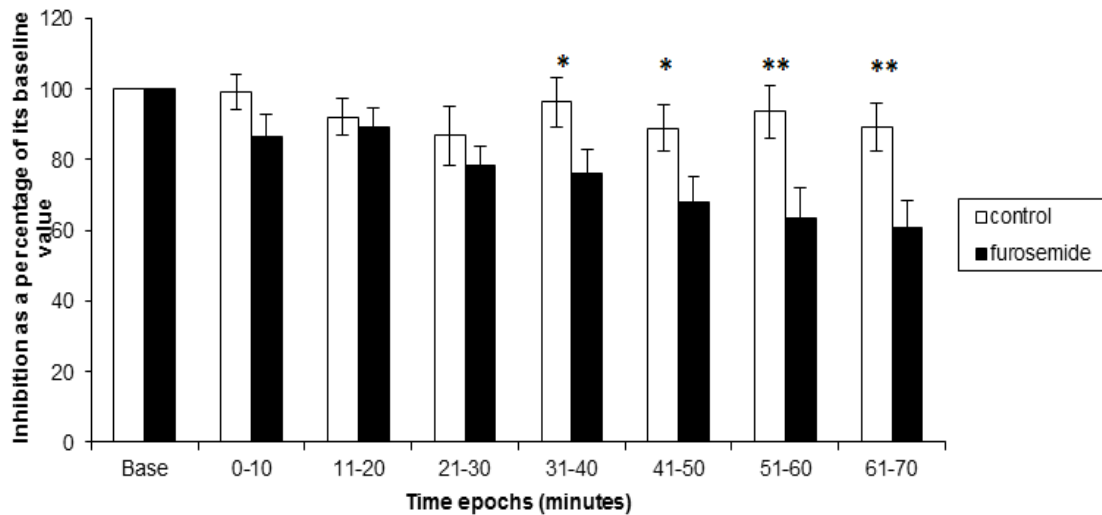


Figure 28 : Histogram of time course of presynaptic inhibition with and without furosemide in all healthy subjects.

The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. Vertical bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Presynaptic inhibition is not modified over 70 minutes in the control experiment while there is a gradually decrease of inhibition following furosemide administration. The amounts of presynaptic inhibition in the furosemide experiments start to be significantly different from that observed in control situation at 31-40 minutes after drug administration. Two-way repeated-measures ANOVA detected a significant interaction of condition (control vs. furosemide), time epochs, and condition \times time epochs ($F_{18,108} = 2.40$, $P_{\text{condition}} = 0.037$, $P_{\text{time epochs}} < 0.001$, $P_{\text{condition} \times \text{time epochs}} = 0.032$). Post-hoc analysis showed that furosemide significantly reduced presynaptic inhibition at 31-40 minutes, 41-50 minutes, 51-60 minutes, and 61-70 minutes ($P < 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.01$ respectively, Tukey test).

4.3.2 Postsynaptic inhibition

Postsynaptic inhibition recorded over 70 minutes after oral administration of 40 mg furosemide in 15 healthy subjects is illustrated in Figure 29.

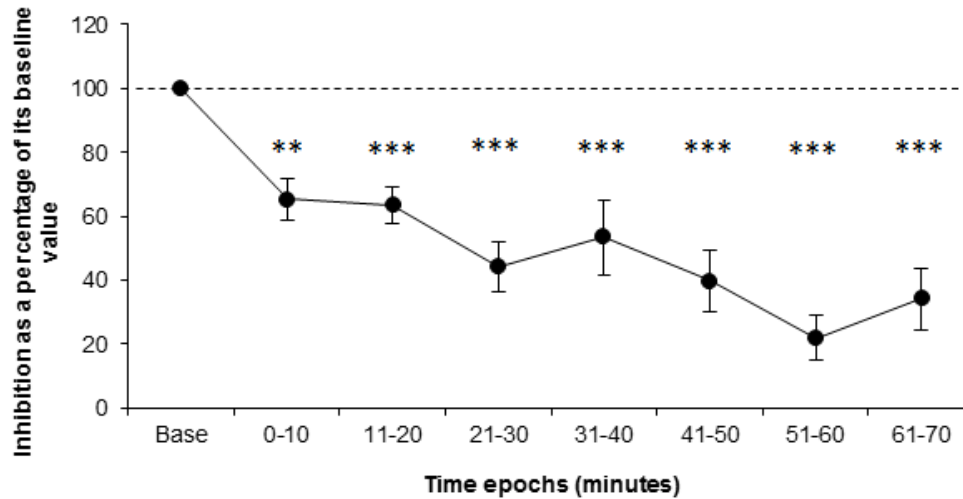


Figure 29 : Time course of postsynaptic inhibition with furosemide in healthy subjects.

The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

There is a significantly decrease of postsynaptic inhibition after drug administration in furosemide experiments ($F_{14,98} = 17.67$, $P < 0.001$, one way repeated-measure ANOVA). The amounts of postsynaptic inhibition is decreased immediately after furosemide intake and progressively decreased until reaching its nadir at 51-60 minutes (21.88 % of its baseline value). The decrease is statistically significant from beginning up to 61-70 minutes (baseline vs. 0-10 minutes, baseline vs. 11-20 minutes, baseline vs. 21-30 minutes, baseline vs. 31-40 minutes, baseline vs. 41-50 minutes, baseline vs. 51-60 minutes, and baseline vs. 61-70 minutes ($P < 0.01$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$ and $P < 0.001$ respectively, Tukey test)).

CHAPTER 3 RESULTS : PROJECT 1

a) *Furosemide vs. control : a representative individual result*

Postsynaptic inhibition recorded over 70 minutes in control and furosemide experiments in a representative subject is illustrated in Figure 30.

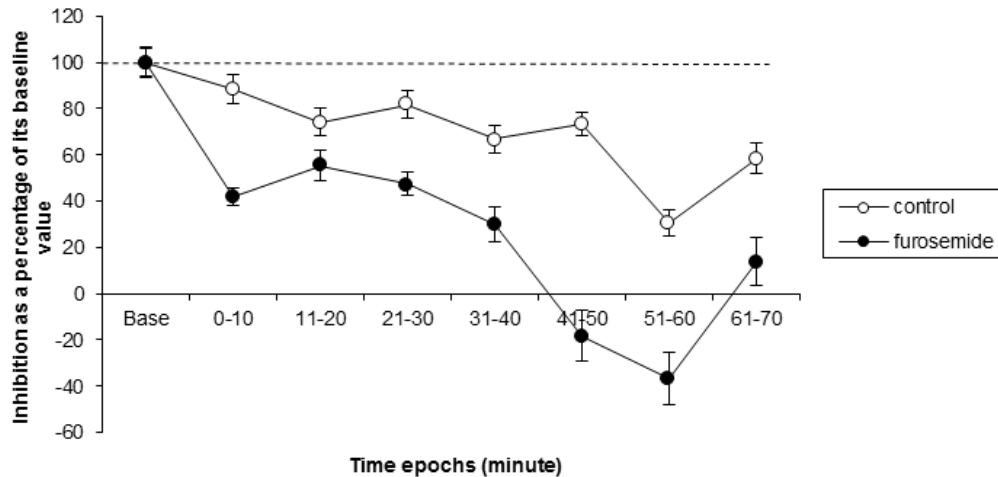


Figure 30 : Time course of postsynaptic inhibition with and without furosemide in a healthy individual subject.

The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM).

The amount of postsynaptic inhibition in control experiments is gradually decreased over time reaching its nadir at 51-60 minutes (30.55 ± 5.89 % of its baseline value). In furosemide experiment, the amount of postsynaptic inhibition is dramatically decreased after furosemide administration until the absence of postsynaptic inhibition at 41-50 minutes and 51-60 minutes (-18.39 ± 10.94 and -36.73 ± 5.89 % of its baseline value, respectively). The inhibition value is calculated using the following equation: $100 - ((\text{conditioned } H \text{ value} / \text{unconditioned } H \text{ value}) \times 100)$, the values in negative means conditioned H value > unconditioned H value, thereby facilitation (the inhibition is not found).

CHAPTER 3 RESULTS : PROJECT 1

b) Furosemide vs. control : Group results

The comparison between variations of postsynaptic inhibition in control and furosemide experiments is presented in Figure 31 (15 subjects).

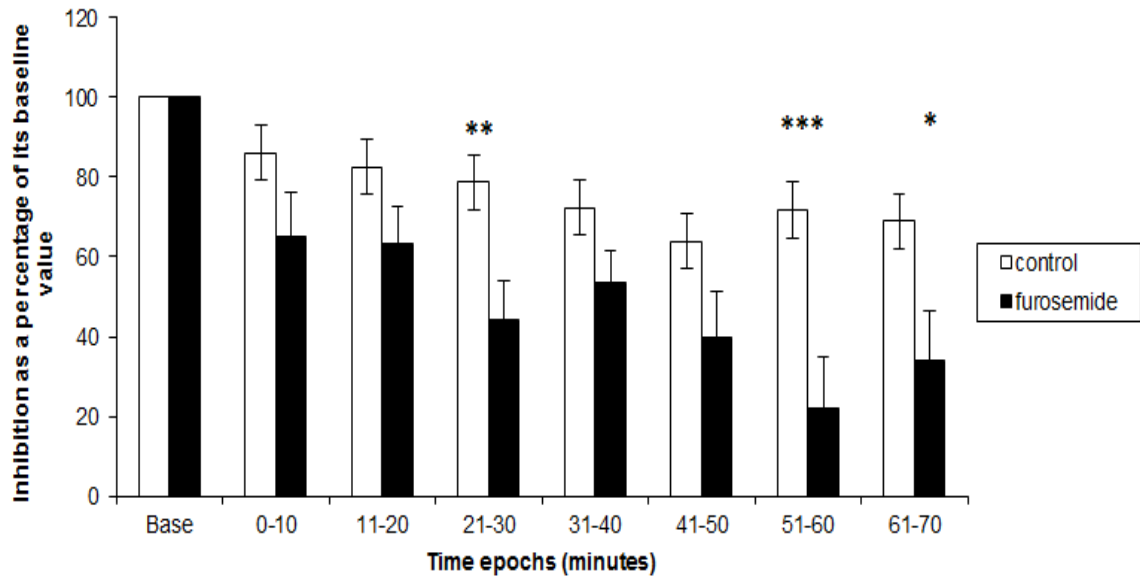


Figure 31 : Histogram of time course of postsynaptic inhibition with and without furosemide in all healthy subjects.

The histogram shows the amounts of postsynaptic inhibition observed from control (□) and furosemide (■) experiment during 70 minutes. The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. Vertical bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

The amounts of postsynaptic inhibition in the furosemide experiments begin to be significantly different from those observed in control experiments at 21-30 minutes after drug administration. Two-way repeated-measures ANOVA detected a significant interaction of condition (control vs. furosemide), time epochs, and condition \times time epochs ($F_{14,84} = 3.43$, $P_{\text{condition}} = 0.034$, $P_{\text{time epochs}} < 0.001$, $P_{\text{condition} \times \text{time epochs}} = 0.004$). Post-hoc analysis showed that furosemide significantly reduced postsynaptic inhibition at 21-30 minutes, 51-60 minutes and 61-70 minutes ($P < 0.01$, $P < 0.001$, and $P < 0.05$ respectively, Tukey test).

4.4 Effects of two different doses of furosemide on the two spinal inhibitory spinal networks

For dose-dependent experiments, an a priori power analysis was performed to determine the minimal sample size. Given that postsynaptic inhibition decreased over time (see Figure 29), the power analysis was performed on presynaptic inhibition only. A sample size of 6 subjects, each having 0, 20 or 40 mg of furosemide provided 80% power to detect a 40% change in presynaptic inhibition (one way repeated measures ANOVA, two-tailed 5%). This calculation was made assuming that raw data are approximately normally distributed with a standard deviation of 10%. One way repeated measures ANOVA was then employed.

4.4.1 Presynaptic inhibition

The effects of two different doses of furosemide were studied on presynaptic inhibition in 6 subjects. Furosemide effects were evaluated during the 40-70 minutes where effects were mostly observed (Figure 32).

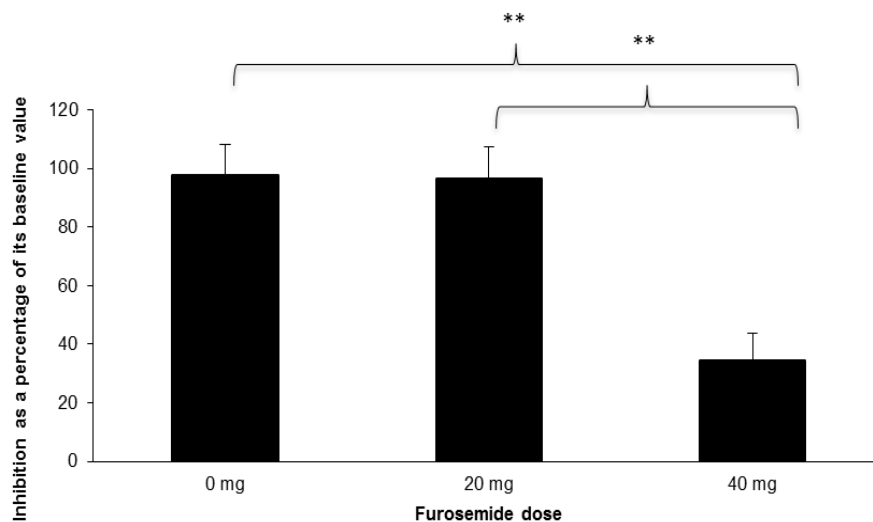


Figure 32 : Effects of two different doses of furosemide on presynaptic inhibition in healthy subjects.

The histogram shows the amount of presynaptic inhibition at different doses (0, 20, and 40 mg) in six healthy subjects, observed over a period of 40-70 minutes after furosemide administration where the effects were mostly observed. Vertical bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Furosemide 0 and 20 mg induce no changes in presynaptic inhibition (97.53 \pm 10.69% and, 96.39 \pm 10.79% of its baseline value, respectively), while presynaptic inhibition is decreased up to 34.61 \pm 9.22% of its baseline value following

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administration of furosemide 40 mg. A one-way repeated-measures ANOVA detected a significant dose-effect ($F_{5,10} = 13.67$, $P = 0.001$). A Post-hoc Turkey test revealed significant difference between 0 mg vs. 40 mg, and 20 mg vs. 40 mg ($P < 0.01$ and $P < 0.01$, respectively).

4.4.2 Postsynaptic inhibition

The effects of two different doses of furosemide administration were studied on postsynaptic inhibition in 6 subjects. The amounts of postsynaptic inhibition recorded at 40-70 min after drug administration are illustrated in Figure 33.

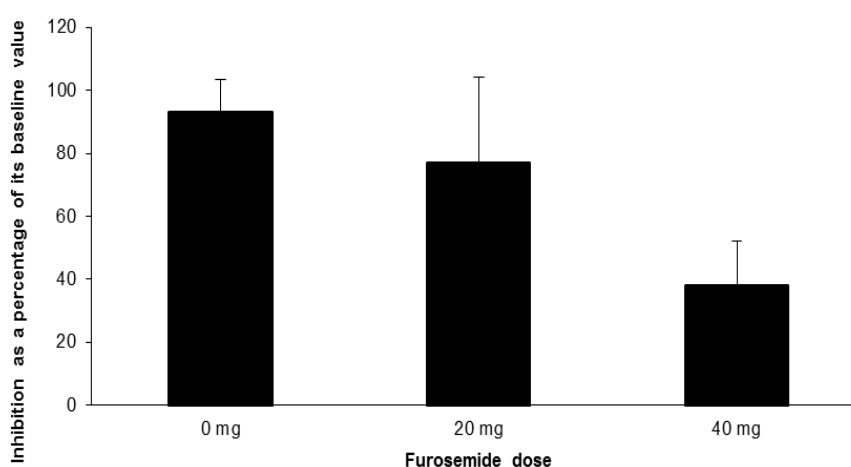


Figure 33 : Effects of two different doses of furosemide on postsynaptic inhibition in healthy subjects.

The histogram shows the amount of postsynaptic inhibition at different doses (0, 20, and 40 mg) in six healthy subjects, observed over a period of 40-70 minutes after furosemide administration where the effects were mostly observed. Vertical bars represent the standard error of the mean (± 1 SEM).

As a percentage of its baseline value, the mean levels of inhibition are: furosemide 0 mg (control) = $92.98 \pm 10.60\%$, 20 mg = $77.12 \pm 27.23\%$, and 40 mg = $38.13 \pm 14.11\%$. There is a trend for a dose-effect in postsynaptic inhibition as observed in presynaptic inhibition. However, a one-way repeated-measures ANOVA failed to detect a significant dose-effect on postsynaptic inhibition: $P > 0.05$. Since time has an influence on postsynaptic inhibition, the effect of furosemide evaluated at 40-70 minutes could be confounded with this time effect.

4.5 Furosemide effects on monosynaptic excitatory transmission.

Post-activation depression

This experiment was performed after the study of pre- and postsynaptic inhibitions was completed. Fifteen subjects from the previous recordings participated in this session (see Table 1). The comparison between variations of post-activation depression determined using 1/0.16 ratio observed from 15 healthy subjects in control and furosemide experiments is shown in Figure 34.

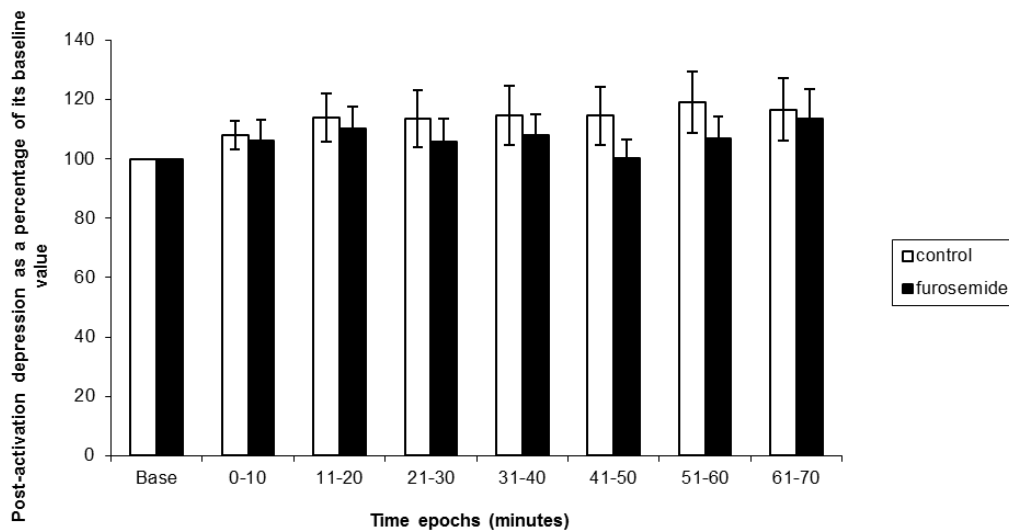


Figure 34 : Histogram of time course of monosynaptic excitatory transmission (post-activation depression) with and without furosemide in all healthy subjects.

The histogram shows post-activation depression value determined using 1/0.16 ratio observed from control (□) and furosemide (■) experiment during 70 minutes. The post-activation depression value was normalized to the percentage of its baseline value and plotted against the recording time. Vertical bars represent the standard error of the mean (± 1 SEM).

Post-activation depression is not modified over 70 minutes of recording in both control and furosemide conditions. A two-way repeated-measure ANOVA failed to detect a significant interaction of condition \times time epochs: $P > 0.05$. There is no significant difference between two groups (control vs. furosemide).

4.6 Individual raw data of healthy subjects

Individual values (not-normalised) of pre- and postsynaptic inhibition reported as a percentage of amplitude of unconditioned H-reflex, and values of post activation depression (not-normalised) reported as 1/0.16 ratio recorded at baseline and during the 40-70 minutes (grouped all the three subpools data (41-50 minutes, 51-60 minutes, and 61-70 minutes) into one) where effects were mostly observed, are shown in Table 1.

	Sex (F/M)	Age (years)	Side (L/R)	Presynaptic (control)		Presynaptic (furosemide)		Postsynaptic (control)		Postsynaptic (furosemide)		Post-activation depression (control)		Post-activation depression (furosemide)	
				Base-line	40-70 min	Base-line	40-70 min	Base-line	40-70 min	Base-line	40-70 min	Base-line	40-70 min	Base-line	40-70 min
1	F	64	L	29.60	15.09	22.42	0.01	28.71	12.87	19.5	-5.73	56.23	32.94	72.05	49.33
2	F	32	R	23.53	24.26	19.68	4.82	21.95	21.75	14.68	4.47	37.84	63.70	26.77	36.77
3	M	26	R	36.33	38.67	44.26	45.52	25.85	10.26	17.30	6.37	48.13	43.46	49.01	55.15
4	F	50	R	47.38	39.89	23.54	14.27	14.10	19.50	26.12	19.04	31.36	35.51	31.74	42.56
5	M	34	R	18.90	8.08	25.63	14.31	23.38	9.69	24.34	4.73	13.54	21.68	22.31	21.18
6	F	40	R	30.69	6.12	24.30	21.46	1.50	-6.01	12.50	-8.48	40.93	43.36	39.19	54.87
7	F	45	R	24.21	26.54	16.67	3.76	-	-	-	-	56.30	41.62	41.47	39.25
8	M	52	R	37.79	40.80	28.70	19.01	14.64	10.31	18.79	10.33	-	-	-	-
9	M	29	R	19.44	12.70	38.74	39.15	33.67	18.47	31.03	28.82	42.15	56.59	46.23	52.07
10	F	31	R	29.34	32.73	41.95	39.27	30.89	21.18	28.95	26.18	72.27	87.81	65.03	66.54
11	M	32	R	31.29	28.18	41.22	19.18	-	-	-	-	-	-	-	-
12	M	37	R	12.79	15.00	30.20	16.80	-	-	-	-	42.87	42.68	46.58	39.65
13	M	22	R	14.54	7.33	14.89	16.18	32.28	18.42	19.96	11.33	64.66	74.56	74.45	66.94
14	M	39	R	49.62	36.27	32.22	35.66	55.53	55.15	32.22	35.66	29.15	49.85	41.84	45.99
15	F	25	R	31.14	25.23	15.57	7.02	1.52	-1.63	6.91	1.20	74.54	51.55	41.93	51.36
16	F	25	R	22.78	27.38	33.23	14.00	54.34	57.42	29.78	16.90	-	-	-	-
17	F	25	R	17.48	17.47	11.06	5.36	21.28	6.55	6.85	-0.08	47.82	62.05	61.98	67.75
18	M	23	R	33.17	34.87	28.50	31.87	-	-	-	-	69.01	82.12	65.54	80.57
19	M	35	R	46.75	45.06	14.74	7.13	30.34	14.57	27.33	4.38	-	-	-	-

Table 1 : Individual values (not-normalised) of pre- and postsynaptic inhibition, and post-activation depression in healthy subjects.

The inhibition value was calculated using the following equation: $100 - ((H \text{ conditioned}/H \text{ unconditioned}) \times 100)$. The values in negative means H conditioned > H unconditioned, thereby facilitation (the inhibition is not found). The sign "-" means not recorded.

5. Results from SCI patients

5.1 Amplitude of H-reflex (as % of M_{max})

The maximum motor response (M_{max}) and maximum H-reflex response (H_{max}) were recorded at the beginning of the each experiment. The stimulus intensity was then adjusted to elicit H-reflex amplitude of 20-35% of M_{max} , which corresponded to ~ 50% of H_{max} . The mean values of H-reflex amplitude in SCI patients is $27.27 \pm 4.7 \% M_{max}$ in furosemide experiments (normal subjects = $23.17 \pm 2.2 \% M_{max}$).

5.2 Degree of pre- and postsynaptic inhibition

5.2.1 Presynaptic inhibition

The degree of presynaptic inhibition evaluated before drug administration (baseline) in all healthy subjects and SCI patients is illustrated in Figure 35.

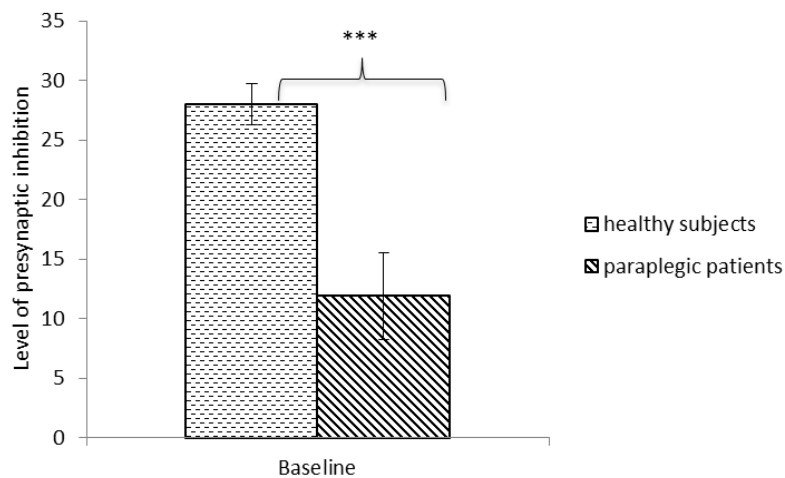


Figure 35 : The average degree of presynaptic inhibition evaluated before drug administration (baseline) in healthy subjects and SCI patients.

The histogram shows the degree of presynaptic inhibition observed at the beginning of recording in 19 healthy subjects and 10 patients with SCI. The presynaptic inhibition values presented in the histogram are defined using a following equation: $100 - ((\text{conditioned H reflex} / \text{unconditioned H reflex}) \times 100)$. Vertical bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

The degree of presynaptic inhibition in SCI patients is smaller than those observed from healthy subjects. The degree of presynaptic inhibition at the beginning of recording (baseline) is 28.00 ± 1.7 in 19 healthy subjects, and 11.88 ± 3.7 in 10 patients.

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A t-test reveals a statistically difference between the degree of inhibition in healthy subjects and in SCI patients ($p < 0.001$).

5.2.2 Postsynaptic inhibition

The degree of postsynaptic inhibition evaluated before drug administration (baseline) in all healthy subjects and SCI patients is illustrated in Figure 36.

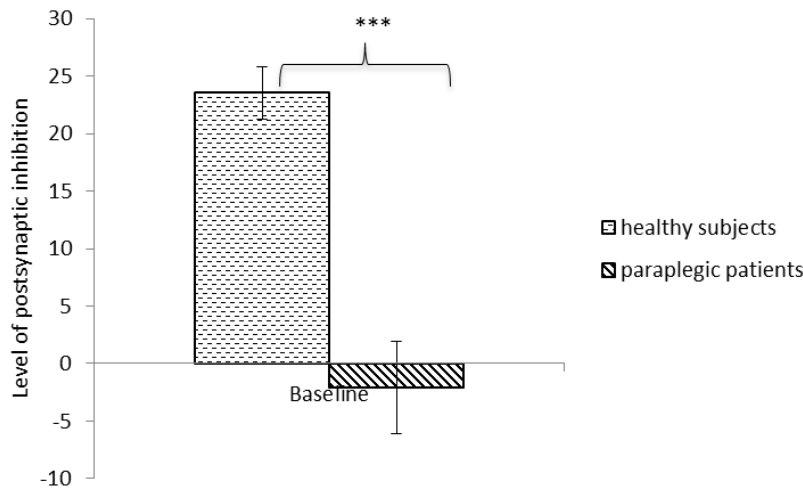


Figure 36 : The average degree of postsynaptic inhibition evaluated before drug administration (baseline) in healthy subjects and SCI patients.

The histogram shows the degree of postsynaptic inhibition observed at the beginning of recording (baseline) in 15 healthy subjects and 10 patients with SCI. Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

The results show that the degree of postsynaptic inhibition is on average abolished in SCI patients. Some of them present facilitation instead of inhibition (see individual data in table X). The inhibition value is calculated using the following equation: $100 - ((\text{conditioned } H \text{ value} / \text{unconditioned } H \text{ value}) \times 100)$, the values in negative means conditioned H value > unconditioned H value, thereby facilitation (the inhibition is not found). The degree of postsynaptic inhibition at baseline is 23.54 ± 2.3 in 15 healthy subjects, and -2.01 ± 4.0 in 10 patients with SCI. A t-test reveals a statistically reliable difference between the degree of inhibition in healthy subjects and in SCI patients ($p < 0.001$).

5.3 Effects of furosemide on the two spinal inhibitory networks.

5.3.1 Presynaptic inhibition of Ia fibres.

Presynaptic inhibition recorded over 70 minutes after furosemide administration in 9 SCI patients is illustrated in Figure 37.

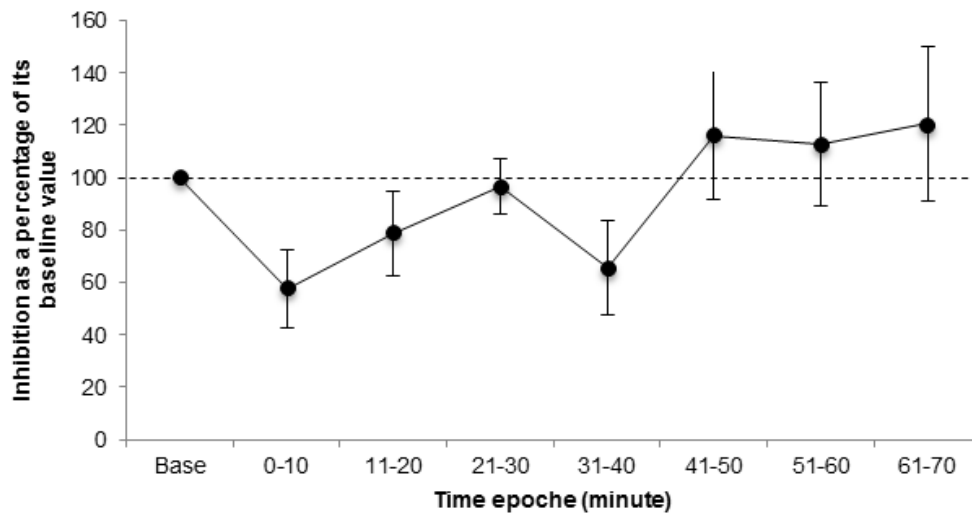


Figure 37 : Time course of presynaptic inhibition with furosemide in SCI patients.

The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM).

The presynaptic inhibition following furosemide administration is highly fluctuated during 0-40 minutes after furosemide intake, and subsequently become stable at 41-70 minutes where the inhibition values are above the baseline. A one-way repeated-measures ANOVA failed to detect a significant effect of time on presynaptic inhibition in control experiments ($P > 0.05$).

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a) SCI patients vs. healthy subjects

The comparison between variations of presynaptic inhibition in 19 healthy subjects and 9 SCI patients is presented in Figure 38.

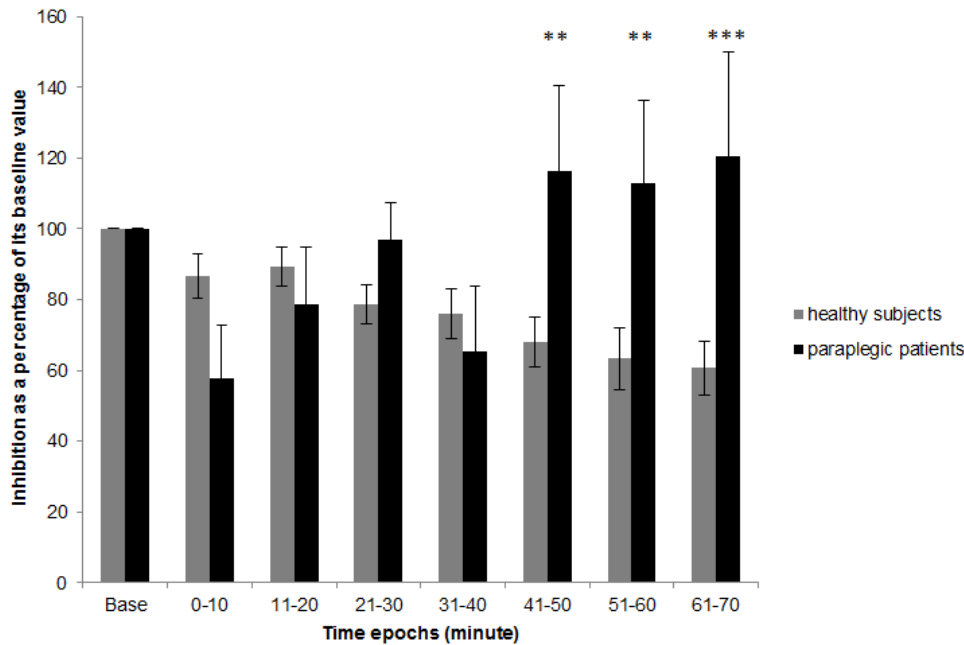


Figure 38 : Histogram of time course of presynaptic inhibition with and without furosemide in all healthy subjects and SCI patients.

The presynaptic inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. Vertical bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

The amount of presynaptic inhibition is gradually reduced after furosemide administration up to 70 minutes in healthy subject, while after furosemide intake in SCI patients; presynaptic inhibition is fluctuated at the beginning and stays over baseline value at 41-50 minutes until the end of recording. Two-way repeated-measures ANOVA detected a significant interaction of subjects (healthy subjects vs. SCI patient), and subjects \times time epochs ($F_{6,182} = 5.19$, $P_{\text{subject}} = 0.003$, $P_{\text{subject} \times \text{time epochs}} < 0.001$). Post-hoc analysis showed that presynaptic inhibition of healthy subjects vs. presynaptic inhibition of SCI patients significantly different at, 41-50 minutes, 51-60 minutes, and 61-70 minutes ($P < 0.01$, $P < 0.01$ and $P < 0.001$ respectively, Tukey test). At these time epochs, furosemide does not decrease presynaptic inhibition contrastively to what observed in healthy subjects.

5.3.2 Postsynaptic inhibition induced by cutaneous stimulation

This type of inhibition could be detected only in 4 of 10 patients. The rest of subjects showed a tendency to facilitation instead of inhibition (see individual raw data in Table 2). However, the amounts of postsynaptic inhibition recorded before furosemide administration (baseline) in the 4 patients is very small. Figure x. represents the postsynaptic inhibition recorded over 70 minutes after furosemide administration in the 4 patients.

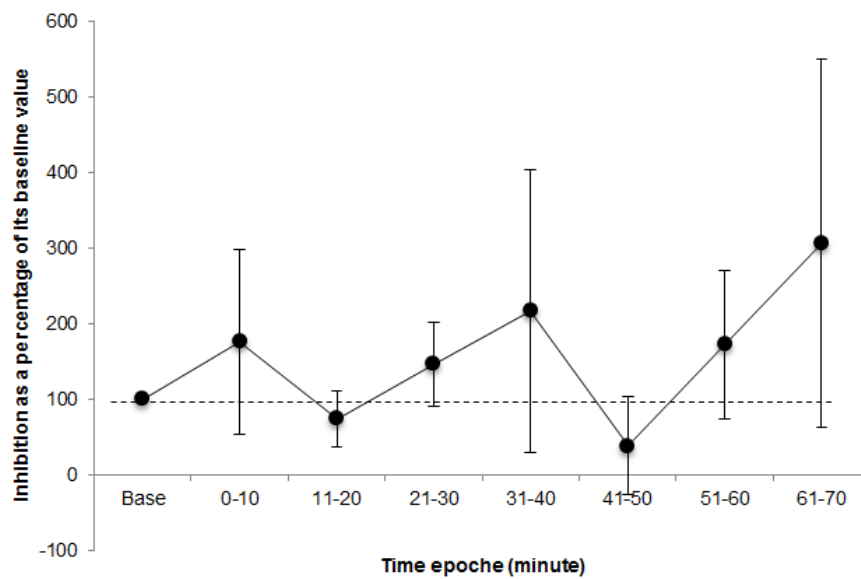


Figure 39 : Time course of postsynaptic inhibition with furosemide in SCI patients.

The inhibition value was normalized to the percentage of its baseline value and plotted against the recording time. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM).

The data are not normally distributed, the variances are heterogeneous and the sample size is too small for statistical analysis. However, the results observed from the 4 patients suggest that there no tendency of decrease of the postsynaptic over recording as observed in healthy subjects.

The comparison between postsynaptic inhibition in healthy subjects and SCI patients is not presented here because the sample of patients is very small and the patient's data have too high variation.

5.4 Individual raw data of SCI patients

Individual values (not normalised to baseline) of pre- and postsynaptic inhibition and of post-activation depression recorded at baseline and during the 40-70 minutes period are shown in Table 2. Note that three subpools at the intervals with the most prominent effects (41-50 minutes, 51-60 minutes, and 61-70 minutes) were pooled. The inhibition value was calculated using the following equation: $100 - ((H \text{ conditioned}/H \text{ unconditioned}) \times 100)$. The values in negative means $H \text{ conditioned} > H \text{ unconditioned}$, thereby facilitation (the inhibition is not found, indicated in red). These values were excluded from the calculation. The sign "-" means not record.

	Sex (FM)	Age (years)	Side (L/R)	Lesion	Onset duration (month)	Presynaptic (furosemide)		Postsynaptic (furosemide)	
						Base-line	40-70 min	Base-line	40-70 min
1	M	32	R	T6	6	17.99	19.44	-12.73	-11.56
2	M	22	R	C5-C6	16	11.30	4.02	-0.72	2.02
3	M	28	R	C5	2	11.38	9.74	-3.83	-1.56
4	M	48	G	T4-T5	3	38.81	29.01	-1.68	-0.42
5	M	67	R	C8	14	6.81	4.45	2.58	-0.54
6	M	29	R	T7	4	18.33	16.73	8.92	4.33
7	M	27	R	T9	6	-3.42	-4.84	-1.17	-3.92
8	F	20	R	C5	2	5.85	10.04	-30.53	-22.20
9	M	54	R	C7 (incomplete)	3	1.76	4.38	1.61	6.51
10	M	37	R	C5	7	10.07	15.68	16.72	10.29

Table 2 : Individual values (not-normalised) of pre- and postsynaptic inhibition in SCI patients

Project 2

1. Subjects

Healthy volunteer subjects included in the present study had no history of intracranial metal implantation, cochlea implant, or cardiac pacemaker. Patients were selected based on the following criteria: i) the presence of hemiparesis after unilateral stroke ii) the absence of pain and severe clonus following the electrical stimulation in the tested limb iii) the presence of a sizable H-reflex in flexor carpi radialis (FCR) muscle; iv) the absence of medical history of intracranial metal implantation, cochlea implant, or cardiac pacemaker; v) the ability to sit upright in a wheelchair or armchair during at least one hour; and vi) the ability to understand simple explanation of the study. A randomised, sham-controlled tDCS study was performed on 33 healthy volunteer subjects (20 females, 8-left handed) aged 20-59 years old (mean \pm SEM 30.7 \pm 1.8 years), 9 hemiplegic patients (4 females) aged 32-69 years old (mean \pm SEM 53.6 \pm 4.0 years). The patients were selected from patients undergoing rehabilitation at Physical Medicine and Rehabilitation service, the Hôpital de la Pitié-Salpêtrière (Pitié-Salpêtrière Hospital). The average duration after stroke was 4.7 \pm 1.7 months.

2. Study design

Contra- and ipsilateral anodal tDCS effects on reciprocal inhibition between forearm muscles were tested in healthy subjects and hemiplegic stroke patients in the present study.

Contralateral study: anodal tDCS is applied over the hand motor cortex of the non-dominant right hemisphere, and reciprocal inhibition is recorded in the non-dominant upper limb in right-handed healthy subjects. This result is then compared to the previous results in the dominant upper limb observed by Roche et al., 2009. In stroke patients, anodal tDCS is applied over the unaffected hemisphere, and reciprocal inhibition is recorded in the non-paretic upper limb.

Ipsilateral study: study in healthy subjects is performed in right-handers and left-handers. Anodal tDCS is applied over the hand motor cortex of the hemisphere that is ipsilateral to their dominant limbs, thus reciprocal inhibition is recorded on the right side in right-handed subjects and on the left side in left-handed subjects. In stroke patient, anodal tDCS is applied over the unaffected hemisphere, and reciprocal inhibition is recorded in the paretic upper limb.

The subjects were blind to the conditions of tDCS. The active and sham tDCS were randomly alternated. Each condition was performed on the subjects on different days to avoid carry-over effects.

3. Experimental procedure

Subjects were seated in a comfortable reclining armchair with the shoulder slightly abducted at about 60°, elbow semi-flexed and slight pronation of the forearm. The distal part of the upper limb was supported by an armrest in order to exclude any active maintenance of wrist posture, and the subjects were asked to keep the recording arm at rest.

Once all parameters (unconditioned H reflex amplitude, conditioning stimulation intensity and ISI) were set the baseline inhibition (without tDCS) was determined over the first 10 minutes, being defined as the baseline period. The tDCS electrodes were attached. The anode was placed over the hand motor cortex for 20 minutes in both active and sham conditions. These 20 minutes were divided into two “10 minute” periods: period 1 and period 2. The tDCS electrodes were removed immediately after the end of the stimulation. The amount of reciprocal inhibition was also measured for 10 minutes after the end of stimulation to evaluate post-effects. In each period (baseline, period 1, period 2 and post), the amount of inhibition was assessed with three series of 40 H reflexes (20 conditioned H reflexes and 20 unconditioned H reflexes). Conditioned and unconditioned H- reflexes were evoked every 3 s and randomly alternated.

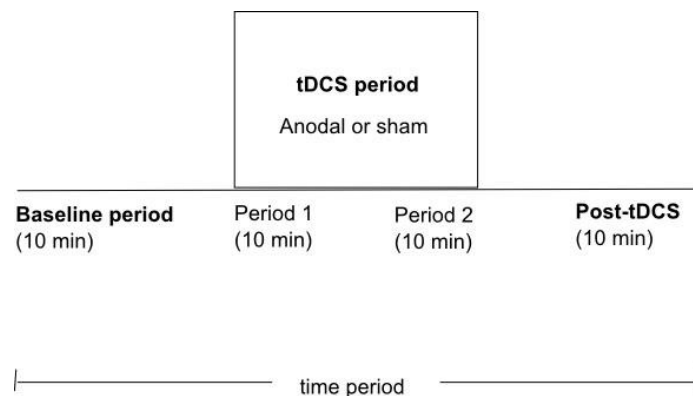


Figure 40 : Diagram of recording of tDCS study.

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Before application of tDCS, the amount of reciprocal inhibition is recorded during 10 minutes, corresponding to baseline period. tDCS period of 20 minutes is divided into 2 session of 10 minutes; period 1 and period 2. The amount of reciprocal inhibition is recorded during 10 minutes after application of tDCS, corresponding to post-tDCS period.

4. Results from healthy subjects

4.1 Contralateral study

4.1.1 Non-dominant upper limb

Effect of anodal tDCS of the non-dominant right hemisphere on reciprocal inhibition in the non-dominant upper limb is observed in right-handed healthy subjects. Figure 41 represents the results obtained from 11 right-handed subjects in active condition, and 7 right-handed subjects in sham condition.

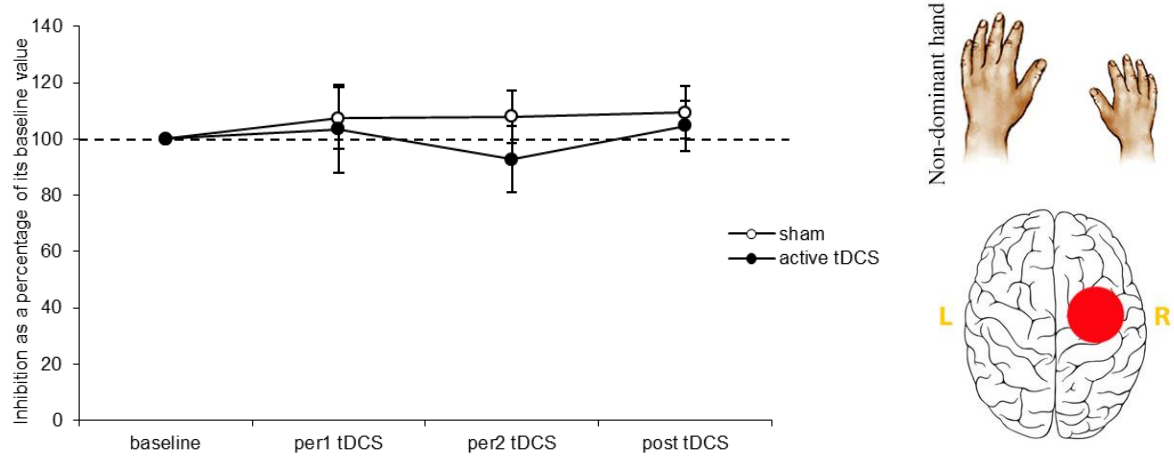


Figure 41 : Effect of contralateral anodal tDCS on reciprocal inhibition in the non-dominant upper limbs of right-handed subjects.

The inhibition value was normalized as a percentage of its baseline value and plotted against the time period. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). The figure in the right shows the side (larger one), which the reciprocal inhibition was recorded and the hemisphere that was stimulated (red point).

Results obtained from active tDCS resemble with those from sham condition. Both of results from active and sham conditions observed during and after tDCS are very close to baseline value. A two-way measure ANOVA failed to detect a significant interaction of condition \times time period: $P > 0.05$. There was no significant difference between two groups (sham vs. active tDCS).

4.1.2 Dominant limb vs. Non-dominant limb: Group results

Roche et al., (2009) have shown in healthy right-handed subjects that tDCS of the dominant left motor cortex induced net increase of reciprocal inhibition of the dominant upper limb. In the present study, we demonstrated effect of tDCS of the non-dominant right motor cortex on reciprocal inhibition recorded in the non-dominant upper limb in 11 healthy right-handed subjects. The present observations are compared with the results from Roche et al., 2009 performed in 13 healthy right-handed subjects (Figure 42).

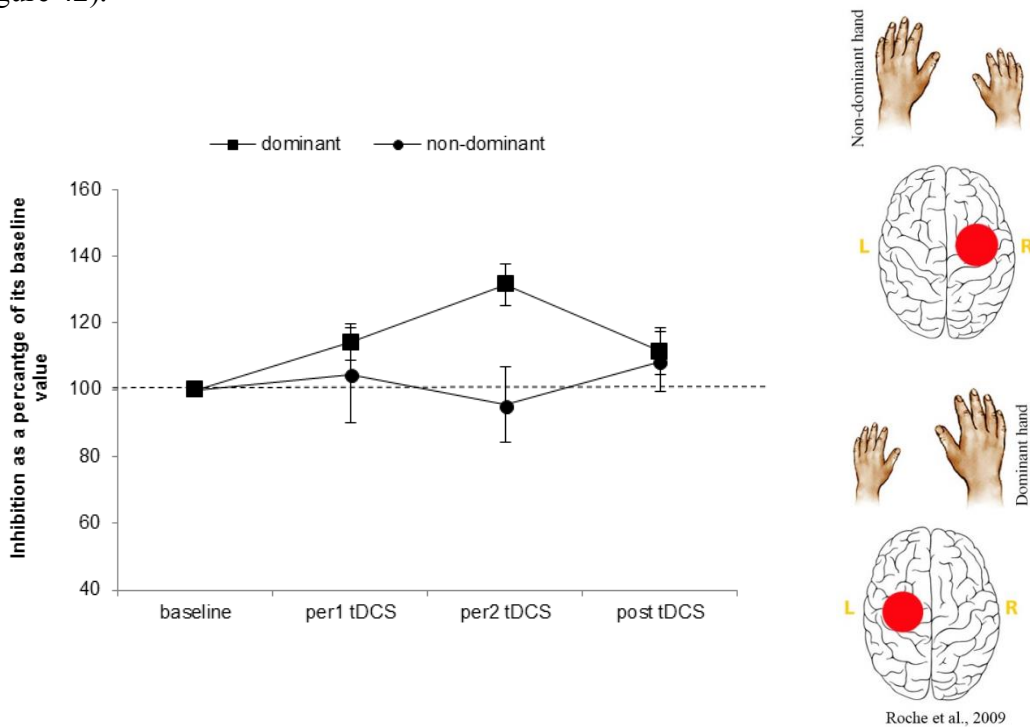


Figure 42 : Effect of contralesional anodal tDCS on reciprocal inhibition in the dominant and the non-dominant upper limbs in right-handed subjects.

The inhibition value was normalized as a percentage of its baseline value and plotted against the time period. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). The figure in the right shows the side (larger one), which the reciprocal inhibition was recorded and the hemisphere that was stimulated (red point).

Reciprocal inhibition recorded in the dominant upper limb is increased during tDCS especially at period 2 and restored to its baseline value after tDCS, while, in the non-dominant limb, reciprocal inhibition remained at the baseline level throughout the recording. Two-way measures ANOVA detected a significant difference between two groups (dominant vs. non-dominant) ($F_{1,88} = 5.90$, $P = 0.017$).

4.1.3 Dominant limb vs. Non-dominant limb: a representative individual result

The Figure 43 represents results of an individual subject who participated in the study of Roche et al., 2009 and in our present study.

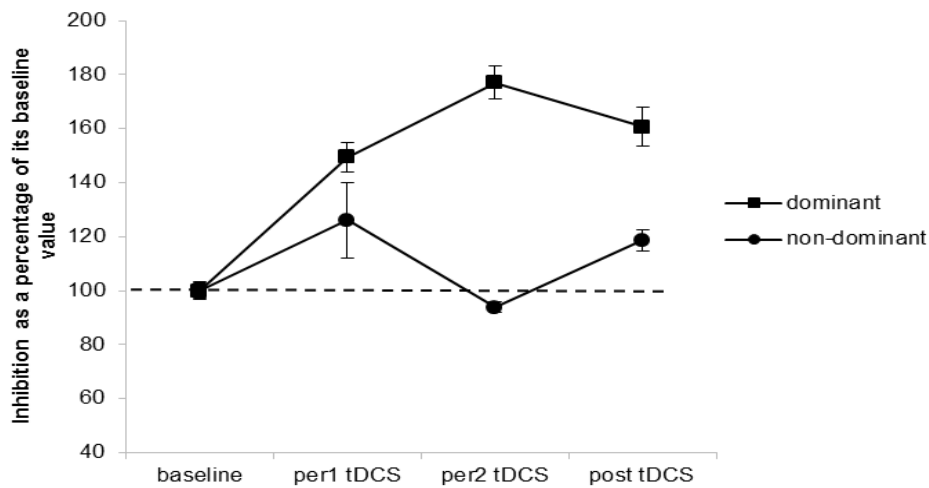


Figure 43 : Effect of contralesional anodal tDCS on reciprocal inhibition in the dominant and the non-dominant upper limbs in an individual subject.

The inhibition value was normalized as a percentage of its baseline value and plotted against the time period. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM).

Results from an individual subject show that reciprocal inhibition recorded in the dominant limb is rapidly increased during tDCS especially at the period 2, while in the non-dominant limb, reciprocal inhibition is initially increased at the period 1 but restores to the baseline level during the period 2 and remained at this level until post tDCS.

4.2 Ipsilateral study

Effects of anodal tDCS over ipsilateral motor area on reciprocal inhibition were firstly demonstrated in right-handed subjects. The experiments were performed in active tDCS and sham conditions. Secondly, results from right-hander were compared to those observed from left-hander, only in active condition.

4.2.1 Right-handed subjects

Effect of ipsilateral anodal tDCS on reciprocal inhibition in the dominant upper limb is observed in healthy right-handed subjects. The reciprocal inhibition values are recorded in both active and sham conditions in 19 healthy right-handed subjects, illustrating in Figure 44.

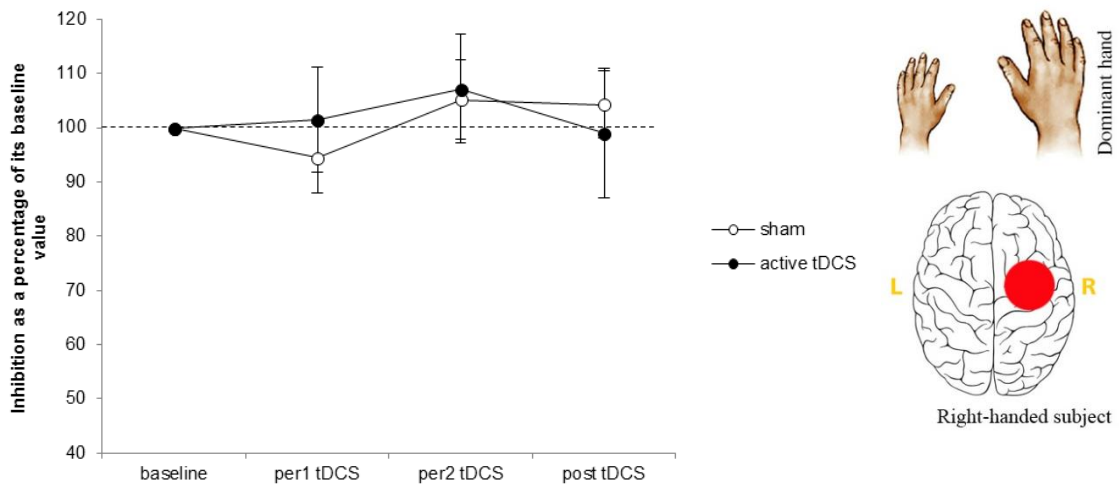


Figure 44 : Effect of ipsilateral anodal tDCS on reciprocal inhibition in the dominant upper limbs in right-handed subjects.

The inhibition value was normalized as a percentage of its baseline value and plotted against the time period. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). The figure in the upper right shows the side (larger one), which the reciprocal inhibition was recorded and the hemisphere that was stimulated (red point).

Results obtained from active tDCS experiment strongly resemble with those from sham experiments. Both of results from active and sham conditions observed during and after tDCS are very close to baseline value. A two-way repeated-measure ANOVA failed to detect a significant interaction of condition \times time period: $P > 0.05$. There was no significant difference between two groups (sham vs. active tDCS).

4.2.2 Right-handed subjects vs. left-handed subjects

As it has shown previously that sham tDCS induced no changes in reciprocal inhibition (see result above, Figure 44), examining the effect of ipsilateral tDCS in right-handed and left-handed subjects was only focused in active tDCS condition. Figure 45 represents the results obtained in 24 right-handed subjects and 8 left-handed subjects.

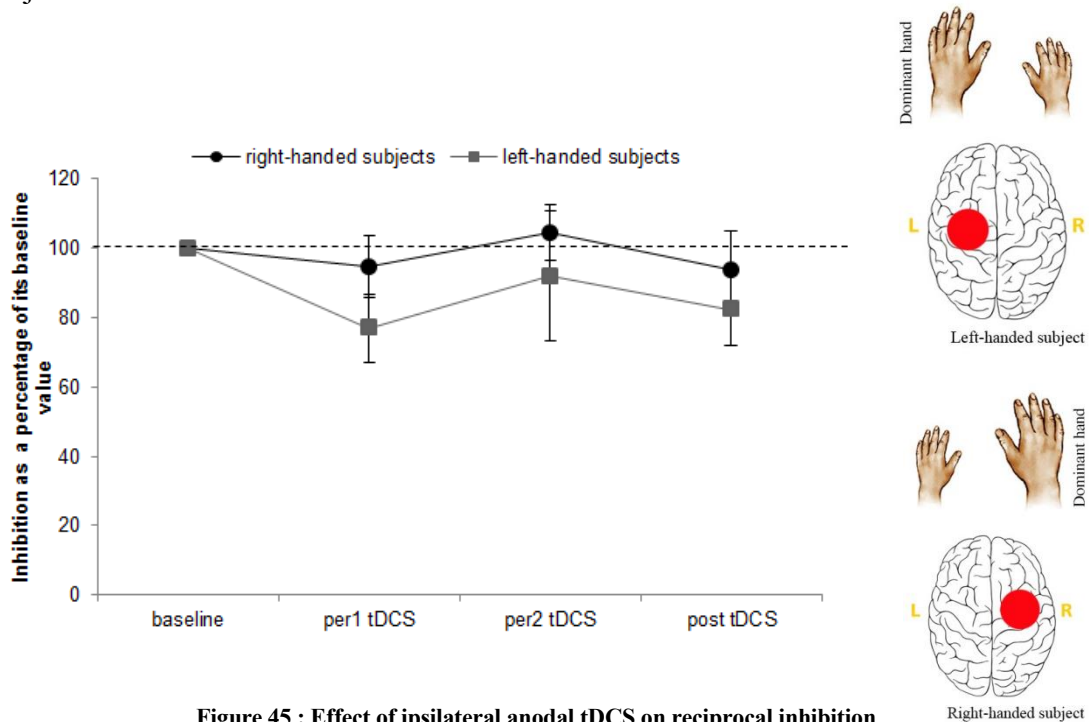


Figure 45 : Effect of ipsilateral anodal tDCS on reciprocal inhibition in the dominant upper limbs in right-handed and left-handed subjects.

The inhibition value was normalized as a percentage of its baseline value and plotted against the time period. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). The figure in the upper right shows the side (larger one), which the reciprocal inhibition was recorded and the hemisphere that was stimulated (red point).

Reciprocal inhibition is not modified in right-handed subjects, while, little decreased of the inhibition is observed in left-handed subjects. However, a two-way measure ANOVA failed to detect a significant interaction of condition \times time period: $P > 0.05$. There was no significant difference between two groups (right handers vs. left handers).

4.3 Individual raw data of healthy subjects

Individual values (not-normalised) of reciprocal inhibition at wrist level reported as a percentage of amplitude of unconditioned H-reflex, recorded at baseline, period 1 tDCS, period 2 tDCS and post-tDCs in non-dominant and/or dominant upper limbs of 33 healthy subjects, are shown in Table 6. The inhibition value was calculated using the following equation: $100 - ((H \text{ conditioned value} / H \text{ unconditioned value}) \times 100)$. The values in negative means H conditioned > H unconditioned, thereby facilitation (the inhibition is not found). The sign "-" means not record.

Subjects	Sex (F/M)	Age (year)	Dominant hand	IPSILATERAL								CONTRALATERAL							
				ACTIVE				SHAM				ACTIVE				SHAM			
				baseline	per1 tDCS	per2 tDCS	post tDCS	baseline	per1 tDCS	per2 tDCS	post tDCS	baseline	per1 tDCS	per2 tDCS	post tDCS	baseline	per1 tDCS	per2 tDCS	post tDCS
1	F	32	R	19,63	31,95	28,78	15,17	35,90	38,76	45,02	42,05	23,53	29,54	23,81	30,23	33,84	29,89	23,30	41,05
2	F	21	R	20,58	18,65	28,96	29,12	48,50	54,94	64,31	45,28	-	-	-	-	-	-	-	-
3	M	22	R	39,77	45,75	41,13	46,37	28,16	34,65	33,19	38,16	-	-	-	-	-	-	-	-
4	F	30	R	14,46	17,46	17,64	9,79	18,05	4,06	1,28	22,70	-	-	-	-	-	-	-	-
5	M	38	R	16,92	17,81	17,27	7,78	30,18	31,18	30,50	33,47	-	-	-	-	-	-	-	-
6	M	22	R	28,71	35,43	35,50	38,29	35,41	37,68	38,40	37,51	-	-	-	-	-	-	-	-
7	F	28	R	27,90	25,33	31,28	36,99	26,68	23,68	34,65	23,37	-	-	-	-	-	-	-	-
8	M	35	R	14,09	2,61	5,80	12,53	42,69	40,69	37,91	33,66	-	-	-	-	-	-	-	-
9	F	28	R	39,54	47,22	41,05	31,24	26,68	23,68	34,65	23,37	-	-	-	-	-	-	-	-
10	M	32	R	43,81	32,10	43,84	44,40	35,94	32,49	37,65	32,17	-	-	-	-	-	-	-	-
11	M	27	R	24,98	19,31	17,47	20,32	16,25	11,71	17,36	19,89	18,29	40,09	33,22	26,72	18,81	19,38	20,41	26,90
12	F	51	R	30,37	18,43	21,48	26,73	36,40	40,56	36,83	19,69	54,86	42,16	51,86	58,28	52,85	61,13	56,61	56,38
13	M	21	R	18,61	9,71	4,93	5,93	28,73	37,93	44,58	39,24	47,46	47,65	53,16	44,55	21,08	34,82	24,09	14,65
14	F	47	R	19,28	29,22	19,46	25,00	26,49	40,03	40,23	32,99	-	-	-	-	-	-	-	-
15	M	40	R	6,41	2,74	13,13	2,97	24,02	19,45	18,65	38,37	-	-	-	-	-	-	-	-
16	F	22	R	8,70	7,20	1,26	14,47	24,04	15,40	28,24	20,20	13,68	7,53	3,85	8,77	37,79	32,24	40,80	32,42
17	F	22	R	9,52	12,35	13,86	1,19	35,94	32,49	37,65	32,17	10,96	3,76	12,28	16,30	-	-	-	-
18	F	26	L	39,88	44,84	59,23	37,75	46,54	47,36	45,14	47,11	-	-	-	-	-	-	-	-
19	F	26	L	26,01	20,69	21,21	29,47	53,73	66,22	59,23	46,86	38,14	43,17	47,55	57,33	-	-	-	-
20	F	23	L	35,85	32,60	18,25	29,13	52,90	55,41	58,62	59,16	-	-	-	-	-	-	-	-
21	F	57	L	15,65	11,93	15,09	5,83	-	-	-	-	-	-	-	-	-	-	-	-
22	F	20	L	22,30	12,13	31,39	27,64	-	-	-	-	-	-	-	-	-	-	-	-
23	F	30	L	16,98	4,07	-0,01	10,72	-	-	-	-	-	-	-	-	-	-	-	-
24	F	24	L	29,97	20,91	16,30	15,68	-	-	-	-	-	-	-	-	-	-	-	-
25	F	39	L	41,11	40,52	44,65	47,19	-	-	-	-	-	-	-	-	-	-	-	-
26	F	31	R	40,99	36,71	43,98	49,24	-	-	-	-	-	-	-	-	-	-	-	-
27	M	42	R	31,77	33,01	30,78	34,23	-	-	-	-	-	-	-	-	-	-	-	-
28	F	26	R	38,08	30,28	32,77	22,79	-	-	-	-	-	-	-	-	-	-	-	-
29	F	59	R	33,27	29,21	38,20	42,79	-	-	-	-	7,26	10,29	7,61	15,39	-	-	-	-
30	M	21	R	11,51	-1,30	7,50	-3,39	-	-	-	-	25,00	11,42	15,47	26,18	-	-	-	-
31	M	27	R	20,27	17,26	22,99	22,28	-	-	-	-	9,91	11,59	5,65	10,26	-	-	-	-
32	M	20	R	-	-	-	-	-	-	-	-	27,95	35,24	26,22	33,15	-	-	-	-
33	M	25	R	23,87	22,86	19,08	16,02	-	-	-	-	49,77	48,37	36,80	31,82	-	-	-	-

Table 3 Individual raw data of reciprocal inhibition in all healthy subjects

5. Results from stroke patients

5.1 Degree of reciprocal inhibition in stroke patients

The levels of reciprocal inhibitions between wrist muscles were evaluated before application of tDCS (baseline) and recorded in 32 dominant limbs and 12 non-dominant limbs of healthy subjects, and 7 paretic limbs and 5 non-paretic limbs of hemiplegic patients, illustrating in Figure 46.

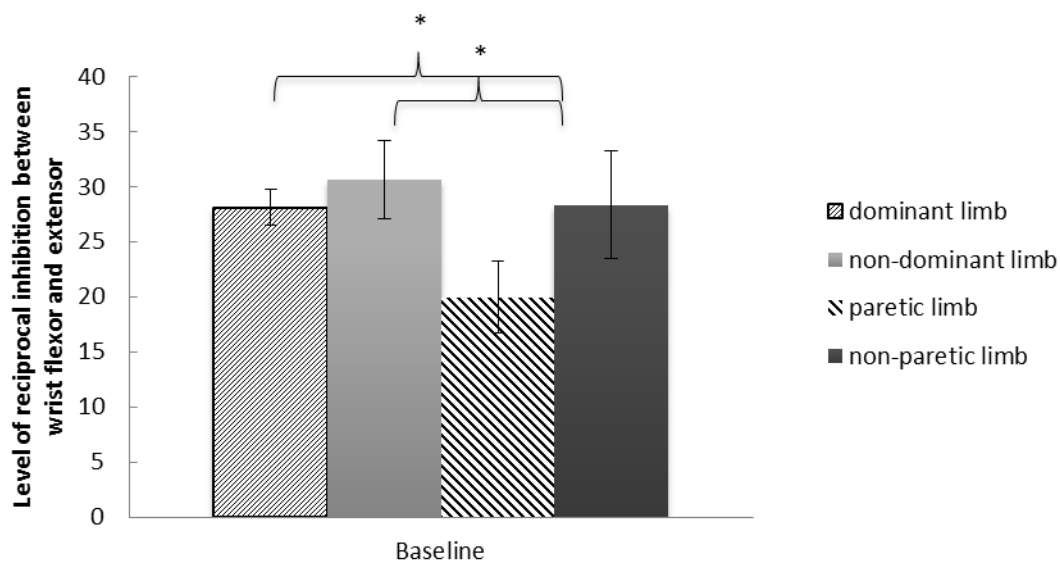


Figure 46 : The degree of reciprocal inhibition in healthy subjects and stroke patients.

The reciprocal inhibition values presented in the histogram are defined using a following equation: $100 - ((\text{conditioned H reflex}/\text{unconditioned H reflex}) \times 100)$. Vertical bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent significant values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

The degree of reciprocal inhibition observed in the dominant and the non-dominant upper limbs in healthy subjects and in the non-paretic limb in hemiplegic patients are similar, while the levels of inhibition in paretic upper limb is relatively small compared to the others. The levels of reciprocal inhibition are 28.10 ± 1.6 in the dominant limbs, 30.62 ± 3.6 in the non-dominant limbs, 19.93 ± 3.6 in the paretic limbs, and 28.34 ± 4.9 in the non-paretic limbs. A t-test detected a significant different between the dominant limb vs. the paretic limb ($P = 0.032$) and the non-dominant limb vs. the paretic limb ($P = 0.049$).

5.2 Ipsilateral study

5.2.1 Stroke patients

Effects of anodal tDCS of the unaffected motor cortex on reciprocal inhibition in the paretic upper limb were observed in 7 stroke patients (active n=6, sham n=6), illustrating in Figure 47.

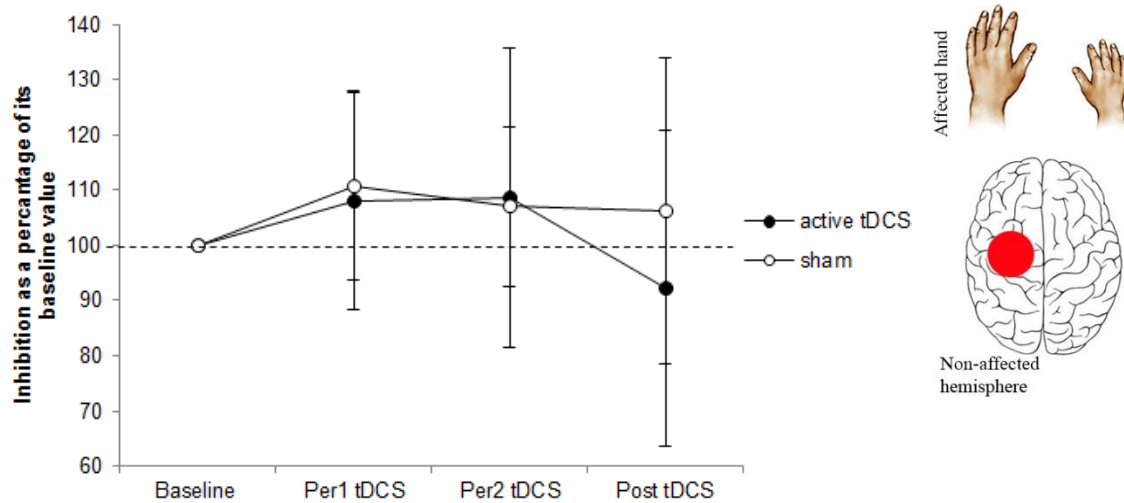


Figure 47 : Effect of ipsilateral anodal tDCS on reciprocal inhibition in the paretic upper limbs in stroke patients.

The inhibition value was normalized as a percentage of its baseline value and plotted against the time period. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). The figure in the upper right shows the side (larger one), which the reciprocal inhibition was recorded and the hemisphere that was stimulated (red point).

Reciprocal inhibition is slightly increased during tDCS in active and sham condition. Nevertheless, the data have a high variation. A two-way measure ANOVA failed to detect a significant interaction of condition \times time period: $P > 0.05$. There was no significant difference between conditions (sham vs. active tDCS).

5.2.2 Stroke patients vs. Healthy subjects

The comparison between variations of reciprocal inhibition demonstrated by ipsilateral tDCS in healthy subjects and hemiplegic patients are presented in Figure 48. The reciprocal inhibitions are recorded in 32 healthy subjects and 6 hemiplegic patients in active tDCS condition.

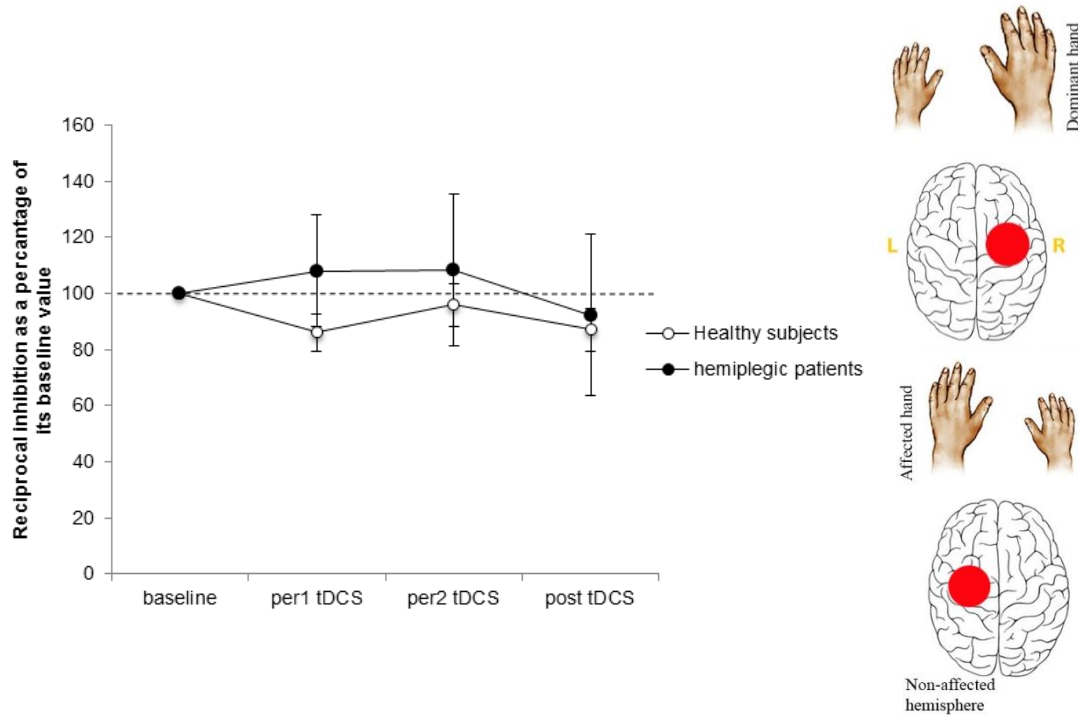


Figure 48 : Effect of ipsilateral anodal tDCS on reciprocal inhibition in the dominant upper limb in healthy subjects and in the paretic upper limbs in stroke patients.

The inhibition value was normalized as a percentage of its baseline value and plotted against the time period. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). The figure in the upper right shows the side (larger one), which the reciprocal inhibition was recorded and the hemisphere that was stimulated (red point).

The reciprocal inhibition is not modified during ipsilateral tDCS in healthy subjects and in stroke patients. The amount of reciprocal inhibition is close to baseline value throughout the experiment in the both groups. A two-way measure ANOVA failed to detect a significant interaction of condition \times time period: $P > 0.05$. There was no significant difference between the two groups (healthy subjects vs. stroke patients).

5.3 Contralateral study

5.3.1 Stroke patients

Effects of contralateral anodal tDCS of the unaffected motor cortex on reciprocal inhibition in the non-paretic upper limb were recorded in 5 stroke patients (active n=5, sham n=4), illustrating in Figure 49.

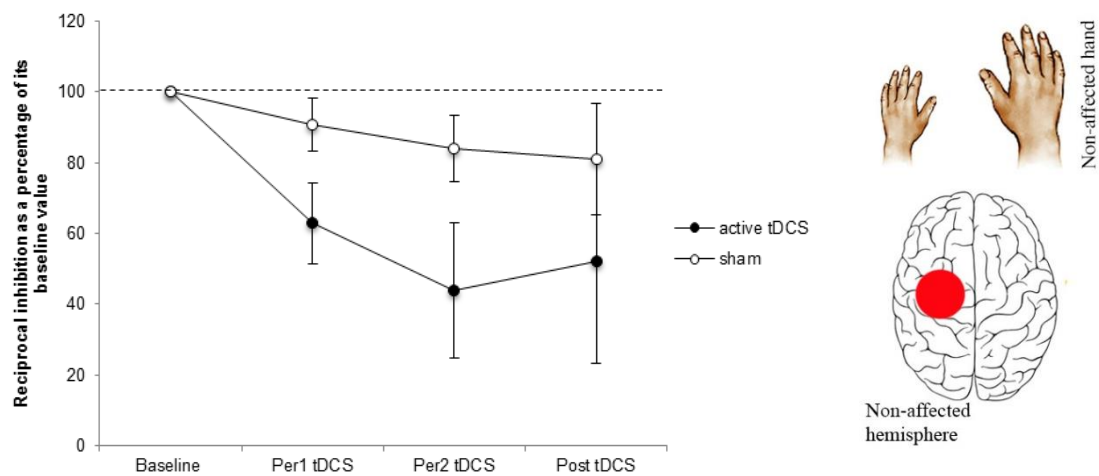


Figure 49 : Effect of contralateral anodal tDCS on reciprocal inhibition in the non-paretic upper limb in stroke patients.

The inhibition value was normalized as a percentage of its baseline value and plotted against the time period. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). The figure in the upper right shows the side (larger one), which the reciprocal inhibition was recorded and the hemisphere that was stimulated (red point).

There is a slightly decreased of reciprocal inhibition in sham condition while, in active condition, the inhibition is extremely decreased during tDCS especially during period 2 and remain reduced after tDCS. However, the sample of subjects is too small for statistical analysis.

5.3.2 Stroke patients vs. Healthy subjects

The comparison between variations of reciprocal inhibition demonstrated by contralateral tDCS in healthy subjects and hemiplegic patients are presented in Figure 50. The reciprocal inhibitions recorded in the dominant limb in 13 healthy subjects (results from Roche et al., 2009), the non-dominant limb in 11 healthy subjects and the non-paretic limbs in 5 stroke patients (the dominant upper limb n=2, the non-dominant upper limb n=3).

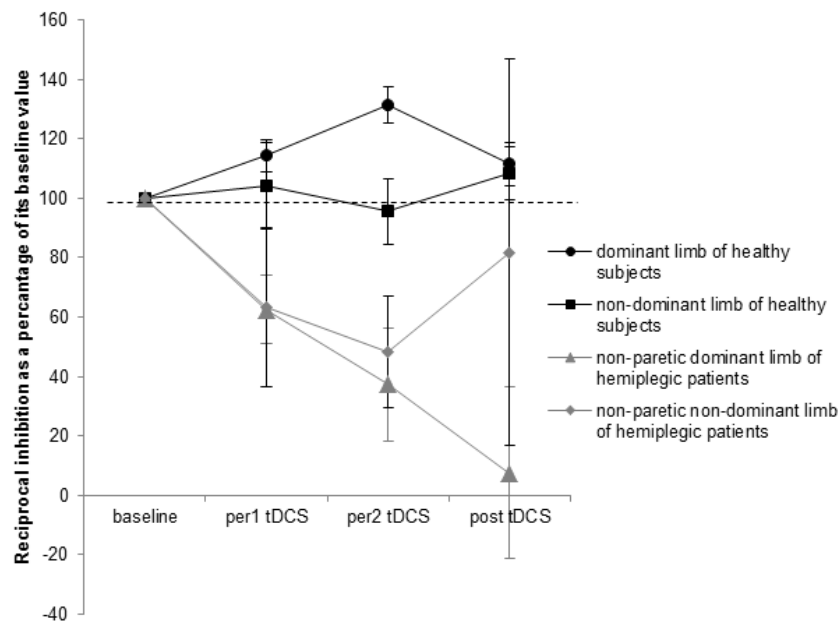


Figure 50 : Effect of contraetral anodal tDCS on reciprocal inhibition observed in the dominant and the non-dominant upper limb in healthy subjects and in the non-paretic upper limb in stroke patients.

The inhibition value was normalized as a percentage of its baseline value and plotted against the time period. The dotted line represents the level of baseline value. Vertical bars represent the standard error of the mean (± 1 SEM). The figure in the upper right shows the side, which the reciprocal inhibition was recorded and the hemisphere that was stimulated (red point).

In healthy subjects, reciprocal inhibition recorded in the dominant limb is increased during contralateral tDCS especially at period 2 and restored to its baseline value after tDCS, while, in the non-dominant limb, reciprocal inhibition remained at the baseline level throughout the recording. In hemiplegic patients, the inhibition is extremely decreased during tDCS, even after tDCS the inhibition is remain reduced. However, statistical analysis is not employed to compare the results in the two groups since the sample of patients is very small.

5.4 Individual raw data of stroke patients

Individual values (not-normalised) of reciprocal inhibition at wrist level reported as a percentage of amplitude of unconditioned H-reflex, recorded at different periods (baseline, period 1 tDCS, period 2 tDCS and post-tDCS) in paretic and non-paretic upper limbs of nine stroke patients, are shown in Table x. The inhibition value was calculated using the following equation: $100 - ((H \text{ conditioned value} / H \text{ unconditioned value}) \times 100)$. The values in negative means H conditioned > H unconditioned, thereby facilitation (the inhibition is not found). The values in red indicate a very small value of inhibition that were excluded from the calculation. The sign "-" means not record.

Subject	Sex (F/M)	Age (years)	Side of hemiplegia	Dominant hancter	stroke onset (mont	IPSILATERAL								CONTRALATERAL							
						ACTIVE				SHAM				ACTIVE				SHAM			
						Baseline	Per1 tDCS	Per2 tDCS	Post tDCS	Baseline	Per1 tDCS	Per2 tDCS	Post tDCS	Baseline	Per1 tDCS	Per2 tDCS	Post tDCS	Baseline	Per1 tDCS	Per2 tDCS	Post tDCS
1	F	55	R	R	4	12,60	21,32	17,77	23,93	14,21	18,23	15,66	25,31	27,01	15,78	10,55	13,88	43,08	29,7815	24,98	22,23
2	M	63	R	R	9	1,46	4,48	1,52	1,73	-	-	-	-	-	-	-	-	-	-	-	-
3	F	32	R	R	2	16,71	22,72	23,71	21,10	11,29	12,08	11,14	7,38	38,22	34,51	41,55	37,57	24,91	22,8239	23,77	25,42
4	M	47	L	R	2	11,94	17,28	19,84	5,68	7,45	13,66	12,54	29,80	20,29	18,05	3,73	-11,65	-	-	-	-
5	F	69	R	R	2	10,85	5,54	-1,61	-1,62	23,68	21,39	23,28	9,56	17,60	7,22	-0,50	16,85	56,19	57,1138	47,21	63,99
6	M	60	L	R	0,5	25,65	21,77	21,50	28,69	-	-	-	-	-	-	-	-	-	-	-	-
7	M	62	L	R	6	38,15	23,92	50,98	35,57	43,06	40,02	46,13	22,61	9,32	3,35	5,23	6,77	-	-	-	-
8	F	55	L	R	1	-	-	-	-	23,58	14,67	13,96	23,81	-	-	-	-	-	-	-	-
9	M	39	L	R	16	-	-	-	-	0,28	2,17	10,60	23,26	-	-	-	-	18,49	18,6004	18,29	10,41

Table 4 Individual raw data of reciprocal inhibition in all stroke patients.

Chapter 4

Discussion

Project 1

The main finding of the present study is that oral administration of furosemide at the usual clinical dose (40 mg) decreases the function of inhibitory synapses in the spinal cord without affecting monosynaptic excitatory transmission in healthy subjects. The presynaptic inhibition is not modified over 70 minutes of recording without furosemide while there is a gradual decrease of inhibition after furosemide orally-administered until the end of recording in the same participants. For postsynaptic inhibition, the amount of inhibition is decreased over time in control experiments (without furosemide). However, after furosemide intake, the amount of postsynaptic inhibition is also decreased and the decrease is more marked than that without furosemide. The reduction of inhibition is also dose-dependent. To verify if furosemide has specific effects on the functioning of inhibitory synapses, effects of furosemide were also tested on post-activation depression at the Ia fibre-motoneuron synapse (an excitatory synaptic transmission). The results reveal no changes of excitatory synaptic transmission observed in both control and furosemide experiments.

In SCI patients, the effect of furosemide was also tested on pre- and postsynaptic inhibitions. First, the levels of pre- and postsynaptic inhibition were evaluated before drug administration (baseline) revealing that presynaptic inhibition is decreased in patients with spinal cord lesion compared to healthy subjects and postsynaptic inhibition induced by cutaneous stimulation is extremely decreased or completely abolished in these patients. The results obtained in SCI patients reveal that the action of furosemide on presynaptic inhibition is less important than in healthy subjects, and the same results likely occur for postsynaptic inhibition but the data had a very high variation and the sample size of patients is too small to conclude.

1. Methodological considerations

In humans, available methods to study spinal cord networks are indirect and rely on EMG recordings that operate as a *window* on motoneuron and interneuron excitability (Pierrot-Deseilligny & Burke, 2012). Since the pioneering experiments in humans by Magladery et al. (1951) and Paillard (1955), electrophysiological non-invasive but selective methods have been developed to study the excitability of spinal networks. Recent results obtained in animals show that lesion of the spinal cord induces a down-regulation of KCC2 co-transporter, which reverses the inhibitory pattern of GABAergic and glycinergic neurons back towards their immature and excitatory state (Jean-Xavier et al. 2006) and also results in reduction of inhibitory synaptic strength (Boulenguez et al. 2010). These findings raise the possibility of setting up a technique, which would allow us to determine if such a reversal also exists in patients with spinal cord injury (SCI). Furosemide, a loop diuretic, has been shown to selectively and reversibly block inhibitory postsynaptic potentials (IPSPs) without affecting excitatory postsynaptic potentials (EPSPs) in animal spinal motoneurons (Nicoll, 1978) and has been used as a cation-chloride co-transporter blocker (reviewed in Kahle et al. 2008). Here, we used furosemide to demonstrate changes in spinal inhibitory networks in healthy subjects and patients with spinal cord lesion. The effect of furosemide was also tested on an example of spinal excitatory synapses in healthy subjects to verify whether furosemide affects both inhibitory and excitatory synapses. These spinal circuits were studied using non-invasive electrophysiological techniques based on the H-reflex evoked in soleus muscle at rest. The specific effects of furosemide on spinal inhibitory synapses as demonstrated here provide a window on the intrinsic functioning of inhibitory synapses mediated through cation-chloride cotransporters.

1.1 The choice of furosemide

Experiments relying on motoneuron intracellular recordings in the frog demonstrated that furosemide selectively and reversibly blocks IPSPs mediated by GABA and glycine without alteration of EPSPs (Nicoll, 1978). Furosemide has been used as a KCC2 blocker in animal experiments (Gillen et al. 1996; Russell, 2000; Thompson & Gähwiler 1989). However, since furosemide is also capable of inhibiting NKCC1, the question may arise as to how we can interpret the results from this non-specific KCC2 blocker. NKCC1 is known to establish an increase of $[Cl^-]_i$ that underlies the excitatory effect of GABAergic and glycinergic neurons in the immature state (Ben Ari, 2002; Dzhala et al., 2005; Yamada et al., 2004), while KCC2 activity is crucial for synaptic inhibition via GABA and glycine (Hübner et al., 2001). If, in our study, furosemide dominantly blocks NKCC1 or equally antagonises NKCC1 and KCC2, this

would lead to an increase or unchanged in inhibitory synapses excitability in healthy subjects instead of reduction. Moreover, Boulenguez et al. (2010) demonstrated that the level of NKCC1 is not modified after spinal cord transection in the rat. If this is the case in SCI patients, even though furosemide blocks both NKCC1 and KCC2 co-transporters, its effects on NKCC1 would likely be the same in normal subjects and in SCI patients. The action of GABA and glycine is due to the distribution of Cl⁻ across the membrane regulated by cation-chloride co-transporters. Exploring the effects of a NKCC1 antagonist (i.e. bumetanide) on the inhibitory synaptic activity would be interesting to test especially in SCI patients. However, given that strength of synaptic inhibition is known to be regulated by KCC2 (Boulenguez et al., 2010), and that the aim of our study is to test the excitability of inhibitory synapses, furosemide, which is a safe and widely used diuretic was chosen in the study.

2. Results in healthy subjects

2.1 Changes in inhibitory synapses over time

Habituation is a gradual decrease in responsiveness following repetitive stimulation and is a well-known phenomenon in the nervous system (Harris, 1943; Hernández-Peón et al. 1956). Most studies report habituation as a reduction in excitatory responses. Spontaneously active spinal neurons are progressively inhibited (inhibitory build-up) by repeated stimulation (Haber & Wagam, 1974). The characteristics of inhibitory build-up regarding stimulus intensities are similar to the frequency effects on inhibitory synapses observed in *Aplysia* (Waziri et al., 1969) and in the mammalian spinal cord (Kuno & Weakly, 1972). Habituation of the flexor reflex has been reported in isolated frog spinal cord showing that habituation is due to a reduction in the efficacy of synapses within the pathway (Farel et al., 1973). It has been shown in decerebrate animals that prolonged stimulation of the sural nerve at 0.5 Hz or higher produced a complete habituation or failure of the C-fibre evoked response in a dorsal horn neuron (Woolf, 1983). However, habituation has also been referred to as a reduction in inhibitory responses. In the decerebrate cat, intracellular recordings of motoneurons participating in the flexion reflex revealed that there was a marked and significant decrease of PSP amplitude during habituation which contains not only the EPSP's habituate, but also decrements of IPSP components. The IPSPs of polysynaptic responses decrease rather than increase during habituation suggesting that the amount of postsynaptic inhibition on the motoneuron is also "habituating" (i.e., decreasing). Besides, the decrease in response during habituation was proposed as a result of reduced input to motoneurons occurring in interneurons (Brazier, 1961). Habituation

CHAPTER 4 DISCUSSION : PROJECT 1

has also been observed with the flexor reflex in normal human subjects and patients with complete spinal cord lesion. Regular repeated stimuli delivered to the skin on the medial side of the plantar surface of the foot induce a significant decrease of flexor reflex response in both normal subjects and patients. As habituation can be observed in patients with complete spinal cord injury, it was suggested that this phenomenon may occur partly or totally at the spinal cord level (Dimitrijevic & Nathan, 1970; Dimitrijevic et al., 1972). Recently, studies in normal subjects reported the degree of habituation of the nociceptive flexion reflex evoked by stimulation of the sural nerve is dependent on both interstimulus interval (ISI) and stimulus intensities. Lower stimulus intensities and a shorter ISI lead to strong habituation. The authors proposed that the mechanisms underlying habituation in humans are still unclear; however, psychological adaptation of subjects and also the effects of sensory saturation could be taken into account (see Dincklage et al., 2013).

The experiments performed in this study lasted up to 70 minutes and the same electrical stimuli were repeated at 0.33 Hz throughout. It could thus be argued that changes in inhibitory spinal network excitability were due to a habituation effect, not furosemide. To exclude this hypothesis, we performed control experiments without furosemide in the same healthy subjects. No changes were observed over time for presynaptic inhibition of Ia fibres without furosemide. This finding demonstrates that changes in presynaptic inhibition following furosemide intake in healthy subjects are most likely due to the effects of furosemide. In the case of postsynaptic inhibition, there was a gradual decrease of inhibition over time in control experiments revealing that habituation may involve the function of cutaneous receptors or synapses interposed between afferent fibres and soleus motoneurons. The diminution of postsynaptic inhibition after furosemide administration is more pronounced compared with controls, which is likely caused by furosemide even though the difference was not statistically significant for all epochs. Put together, our results in healthy subjects suggest that furosemide is at least partly responsible for the decrease of the efficacy of pre- and postsynaptic inhibition of soleus motoneurons. This point of view is also supported by the significant dose effect relationship for presynaptic inhibition and by the trend of the dose effect relationship for postsynaptic inhibition.

2.2 Effects of furosemide on spinal inhibitory networks in healthy subjects

2.2.1 Absence of furosemide effect on monosynaptic excitatory transmission

Although furosemide is known from animal experiments to block cation-chloride co-transporters, its effects on the human spinal network have never been tested before. The question could be asked as to whether the effects demonstrated for inhibitory synapses are specific to GABAergic and glycinergic synapses, or if they are non-specific, affecting both inhibitory and excitatory synapses. Our results revealed no significant difference observed in post-activation depression at the Ia fibre-motoneuron synapse, a pure excitatory synaptic transmission, between control and furosemide experiments in healthy subjects. These results allow one to exclude the hypothesis that furosemide also acts on excitatory synapses.

2.2.2 Specific effect of furosemide on pre- and postsynaptic inhibitions in healthy subjects

Orally-administered furosemide, at doses commonly used in the clinic (40 mg), significantly reduced spinal inhibitory activity (pre- and postsynaptic inhibitions) for at least 70 minutes from intake compared to control experiments in the same healthy subjects. Although furosemide decreases both pre- and postsynaptic inhibitions in healthy subjects, the effect of furosemide seems to be more important for postsynaptic than presynaptic inhibition. The most likely explanation for the difference between the time courses of pre- and postsynaptic inhibition is that in the control condition (without furosemide), there was no systematic change over time for presynaptic inhibition while the amount of postsynaptic inhibition is progressively decreased over time. This effect of time by itself on postsynaptic inhibition is interpreted above with respect to habituation. Thus, for postsynaptic inhibition, the inhibition following furosemide intake is the sum of the habituation effect and the effect of furosemide itself, while the changes in presynaptic inhibition following furosemide intake are only due to the furosemide effect. Another explanation may be that the kinematics of pre- and postsynaptic inhibitions are different; however, to our knowledge, there is no evidence to support this hypothesis. Altogether with the absence of furosemide action on excitatory synapses, these observations strongly suggest that furosemide selectively decreases the functioning of spinal inhibitory synapses and thus has potential to detect changes in the intrinsic functioning of inhibitory synapses in humans.

2.2.3 Does furosemide affects all the structure of the CNS?

Our results in healthy subjects show that furosemide induces changes in spinal inhibitory circuits. Since the neurons that compose these networks are controlled by descending (supraspinal) input as well, any change induced by furosemide via changing the efficacy of supraspinal input onto the neurons inhibiting soleus motoneurons pre- or postsynaptically must also be considered. It has been shown in animals that furosemide decreases IPSPs without modifying EPSPs (Nicoll, 1978), and we also demonstrated here that furosemide decreases the excitability of inhibitory synapses without affecting excitatory synapses. The main supraspinal control input acting on presynaptic inhibition is inhibitory (for references see Pierrot-Deseilligny & Burke, 2012). Thus, at its level, the effect of furosemide is likely, leading to a decrease in the supraspinal inhibitory input acting on presynaptic (PAD) interneurons. This disinhibition of PAD interneurons would likely result in an increase of the amount of presynaptic inhibition. However, we observed here in healthy subjects a reduction of presynaptic inhibition after furosemide intake; this cannot be explained by the act of furosemide on supraspinal control. Logigian et al. (1999) have presented data obtained in human experiments suggesting that the descending control acting on interneurons fed by cutaneous afferents is facilitatory. Since furosemide has no effect on excitatory synapses, the reduction of postsynaptic inhibition following furosemide administration in healthy subjects would not be due to changes in supraspinal control acting on cutaneous interneurons.

3. Results in SCI patients

3.1 Limitations of the study

3.1.1 Difficulty of recruitment

To be compatible with animal data, we initially planned to conduct our research only in patients with complete cord injury since down-regulation of KCC2 was reported after complete spinal cord transection in the rat (Boulenguez et al., 2010). However, it was difficult to find patients with complete spinal cord injury. Given that animal experiments showed afterwards that this phenomenon is also present in the case of incomplete spinal cord lesion, we have subsequently requested a research authorization to perform our experiments in both patients with complete and incomplete spinal cord injuries. However, since we received the authorization at the end of 2013, only one patient with incomplete spinal cord injury was recruited in this study.

For the recruitment, 4 of 14 SCI patients were excluded from our study since the amplitude of their soleus H-reflex was too small or disappeared. Modifications of H-reflex amplitude after SCI are still under debate. The H-reflex has been reported to be absent or markedly decreased below the lesion in early human spinal shock and the degree and duration of H-reflex suppression reflects the severity of the injury (Leis et al., 1996). This loss of reflex is proposed to be due to reduced excitability of motoneurons caused by the sudden loss of supraspinal inputs. However, the H-reflex starts to rapidly recover after SCI shock (Hiersemenzel et al., 2000). An increase of the soleus H-reflex amplitude in patients with chronic complete cord lesion suggests an increase in central synaptic excitability that may contribute to the appearance of hyperreflexia after SCI (Hiersemenzel et al., 2000; Little & Halar, 1985). However, a study has reported an unchanged soleus H-reflex threshold, gain and amplitude in chronically complete SCI patients compared to normal subjects (Schindler-Ivens & Shields, 2004). Soleus H/M ratio is less than 0.7 in normal subjects (Delwaide, 1984). In our study, the average soleus H/M ratio found in SCI patients was 0.56 ± 0.08 .

3.1.2 Decrease or absence of presynaptic inhibition of Ia fibres and postsynaptic inhibition induced by cutaneous stimulation in SCI patients

Another constraint present in the present study was that pre- and postsynaptic inhibition could not be always be evoked in all included patients. Presynaptic inhibition could be demonstrated in 9 of 10 patients; however, the degree of inhibition observed in these SCI patients is considerably reduced compared to normal subjects, but still detectable. Postsynaptic inhibition induced by cutaneous stimulation could be detected in only 4 of 10 patients. The modifications of postsynaptic inhibition in soleus motoneurons induced by cutaneous stimulation after spinal cord lesion have not been well documented. In our study, we found that this inhibition is extremely reduced after SCI. This unexpected constraint has limited our recordings and the interpretation of results since the sample size of participants is small and the degree of cutaneous inhibition found in this small sample is also small. A recent study has also reported a decrease of flexor reflex response in soleus motoneurons in paraplegic patients during the spastic state following tibial nerve stimulation at the malleolus medialis level. This decrease was stable even with an increase of stimulation intensity (Hiersemenzel et al., 2000). Similarly as we observed here, even with an increase of the conditioning sural stimulus intensity until triggering movement of flexors of the foot, the degree of postsynaptic inhibition in patients was no longer increased. In addition, as this cutaneous inhibition could not easily be demonstrated in SCI patients, another type of postsynaptic inhibition, crossed inhibitory responses in soleus motoneurons (Aggelopoulos et al., 1996; Stubbs & Mrachacz-Kersting, 2009) was tested in the present study. However, we failed to evoke this crossed inhibition in SCI patients. Alternative possibilities to test postsynaptic inhibition should be either reciprocal inhibition or recurrent inhibition. Ia-reciprocal inhibition has been shown to be reduced (Boorman et al., 1996) or replaced by reciprocal excitation of antagonistic muscle after SCI (Crone et al., 2003). Abnormal of recurrent inhibition was reported in SCI patients (Shefner et al., 1992) and the method used to assess recurrent inhibition in humans is difficult to perform (Katz & Pierrot-Deseilligny, 1998). Together with these constraints, the study of the effects of furosemide in SCI patients is mainly considered on presynaptic inhibition.

3.1.3 Effects of furosemide on spinal inhibitory networks in SCI patients

The action of furosemide is likely reduced in SCI patients. Changes in presynaptic inhibition of Ia fibres following furosemide were significantly different between healthy subjects and SCI patients. In healthy subjects, the amount of presynaptic inhibition is significantly gradually decreased from baseline at every time epoch after 21-30 minutes following furosemide administration (see Figure 26). In contrast, no significant difference is found for the time course of presynaptic inhibition with furosemide in SCI patients. At the intervals (41-70 minutes) where the most prominent effects were found in normal subjects, the amount of presynaptic inhibition is stable around the baseline in SCI patients (see Figure 37). For postsynaptic inhibition, as the sample size of patients is too small, it is very difficult to interpret the results. However, considering the individual raw data of postsynaptic inhibition, there is a marked decrease of inhibition at 41-70 minutes following furosemide in all individual healthy subjects (see Table 3.), while non-significant changes of postsynaptic inhibition were observed from the 4 patients at this time interval (see Table 4).

To sum up, even if the sample of patients is relatively small, the comparison reveals clearly differences of furosemide action between normal subjects and SCI patients. These differences suggest that the effect of furosemide is likely reduced in SCI patients. However, a larger number of SCI patients is still required to confirm the results.

Cation-chloride co-transporters are regulators of the chloride gradient in neurons. Activation of co-transporter KCC2 (a chloride extruder) during development reduces $[Cl^-]_i$; thus resulting in hyperpolarizing effects of GABA and glycine (Ben Ari, 2002; Takahashi, 1984; Wu et al. 1992; Ziskind-Conhaim, 1998). Several studies in animals have shown that loss or reduction of co-transporter KCC2 function results in the development of central nervous system (CNS) hyper-excitability (Woo et al. 2002; Zhu et al. 2008). In the presence of furosemide, the functioning of inhibitory synapses is markedly decreased in healthy subjects, while there is likely no tendency of decrease in spinal inhibitory synapses in SCI patients. These results lead us to hypothesise that the expression of KCC2 is probably decreased after SCI in humans. This hypothesis is consistent with the data obtained in animals showing that KCC2 co-transporter is down-regulated in motoneurons after spinal cord transection (Lu et al., 2008; Boulenguez et al., 2010; Modol et al., 2014). A down-regulation of KCC2 induces an accumulation of $[Cl^-]_i$ that reverses GABA/glycine response polarity to excitation (Nabekura et al., 2002; Vinay & Jean-Xavier, 2008) and thus results in reduction of inhibitory synaptic efficiency (Boulenguez et al., 2010). To sum up, the preliminary results observed in this study suggest that the intrinsic functioning of inhibitory synapses is reduced in SCI patients.

4. Applications of physiopathology related to results obtained in SCI patients

Presynaptic inhibition of Ia fibres is decreased in lower limbs in patients with spinal cord lesions, whatever the nature of the lesion (Faist et al., 1994; Nielsen et al., 1995; Pierrot-Deseilligny, 1990). As the main descending controls on PAD interneurons mediating presynaptic inhibition of Ia fibres is depressive, the interruption of the corticospinal tract could not explain the reduction of the presynaptic inhibition of Ia fibres in the lower limb in patients with spinal cord lesions (if anything, it would produce an increase of presynaptic inhibition). Thus, the interruption of other descending pathways that normally maintain a tonic level of presynaptic inhibition of Ia fibres under resting conditions in normal subjects has been proposed. The inhibitory action of the cutaneous and articular inputs on first-order PAD interneurons is subjected to a powerful tonic inhibition (see pathway (1) in the Figure 51) via a brainstem pathway that disappears after spinal cord transection in the cat (Rudomin & Schmidt, 1999). Interruption of this strong tonic inhibitory control from the brainstem would consequently liberate the cutaneous and articular inputs to fully exercise their inhibitory actions. This explanation relies on the hypothesis that cutaneous and articular inputs are still generating inhibition in PAD interneurons after spinal cord lesion. However, as observed in our study, cutaneous inhibition to motoneurons is strongly reduced or abolished, and it can be hypothesised that the immobilization of a limb following complete paraplegia reduces the effects of proprioceptive fibres (Lundbye-Jensen & Nielsen., 2008). Therefore, if a decrease in efficacy of cutaneous and proprioceptive fibres is also acting at the PAD interneuron level, one can wonder if the interruption of the supraspinal tonic inhibition really reduces presynaptic inhibition. In such condition, the reduction of intrinsic functioning of inhibitory synapses found here would be a possible explanation for the decrease of presynaptic inhibition in SCI patients. This hypothesis is supported by animal studies suggesting that loss or decrease of KCC2 expression would be a cause of reduced inhibitory neuronal activity after SCI which might also contributes to spasticity (Boulenguez et al., 2010).

CHAPTER 4 DISCUSSION : PROJECT 1

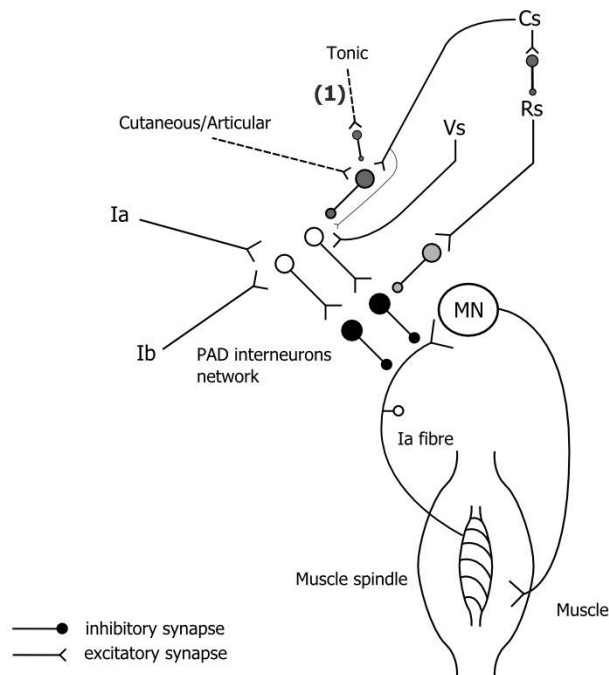


Figure 51 Pathways of presynaptic inhibition with primary afferent depolarisation (PAD) of Ia terminals in the cat. MN = motoneuron, VS = the vestibulospinal tract, CS = the corticospinal tract, RS = reticulospinal tract. Pathway (1) = tonic control from the brainstem. Adapted from Pierrot-Deseilligny & Bruke, 2012.

Spasticity is a frequent complication found in patients with spinal cord lesions. Lance (1980) has stated that “spasticity is a motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes (muscle tone) with exaggerated tendon jerk, resulting from hyperexcitability of the stretch reflex, as one component of the upper motoneuron syndrome”. Transmission is altered in all studied spinal pathways that have been investigated in patients with spinal cord lesions, always in the direction that would exaggerate the stretch reflex (for reference see Pierrot-Deseilligny & Burke, 2012). Our results are in accordance with this evidence since pre- and postsynaptic inhibition is decreased in SCI patients and this decrease likely contributes to the exaggeration of the stretch reflex. The most general mechanism that have been proposed underlying spasticity is that supraspinal inhibitory and excitatory inputs that exert a balanced control on spinal reflex activity in normal condition are interrupted after CNS lesion (Reviewed in Adams & Hicks, 2005; Sheean, 2002). However, to date, the indirect methods used in humans do not allow distinguishing of between changes in supraspinal control acting on neuron and changes in the intrinsic functioning of the synapse itself.

A previous study showed that soleus motoneurons in patients with spinal cord lesion were inhibited by high-intensity cutaneous stimuli of the sural nerve over the same ISI range as normal subjects, but the degree of inhibition is less than in normal subjects (Logigian et al, 1999). This suggests that there is likely a descending

supraspinal control that has a net facilitatory effect on this inhibitory effect of cutaneous stimulation on motoneuron excitability. The interruption of descending control would consequently result in net reduction of the cutaneous inhibition in SCI patients. In addition, the reduction of the intrinsic functioning of the inhibitory synapse suggested in our study would likely also contribute to the reduction of postsynaptic inhibition induced by cutaneous stimulation in SCI patients.

5. Clinical implication

Furosemide decreases spinal inhibitory synapse excitability in healthy subjects. The question may arise whether prolonged intake of furosemide would affect daily life or physical activity of people. In patients who have spasticity (i.e. SCI patients) the prolonged treatment of furosemide probably requires careful observation since furosemide could reduce the functioning of inhibitory synapses which may result in an increment of spasticity. However, exaggerated reflexes or increase in muscle tone have never been reported as a side effect of furosemide. No systematic study of exaggerated reflexes and muscle tone has been performed during prolonged furosemide treatment. However, none of the healthy volunteers who participated in our experiments reported exaggerated reflexes and changes in muscle tone following a single dose of furosemide administration. We may thus assume that in people with normal functioning of inhibitory synapses, the effects of furosemide have no clinical consequences. However, it would be interesting to further explore the possible muscle tone changes following prolonged furosemide administration, especially in patients with spasticity.

6. Therapeutic implication

Our observations provided indirect evidence in humans that the KCC2 is probably down-regulated in SCI patients as demonstrated in the spinal rat. Given that KCC2 plays an important role in regulating the strength of inhibitory synapses in SCI (Boulenguez et al., 2010), it is interesting for the therapy if increases of KCC2 expression would help SCI patients to reduce spasticity. A recent study has demonstrated that restoring chloride homeostasis by activation of the serotonin receptor in spinal motoneurons after SCI (hyperpolarizing the reversal potential of inhibitory postsynaptic potentials (IPSPs), E_{IPSP}) induces up-regulation of KCC2. This results in the restoration endogenous inhibition in the neonatal spinal rat (Bos et al., 2013). The authors have proposed that up-regulation of KCC2 function has therapeutic potential in the treatment of neurological disorders involving altered chloride homeostasis. This would be a novel therapeutic strategy for spasticity in SCI patients.

7. Conclusion

Our results suggest that furosemide, a safe and widely used diuretic, at doses commonly used in the clinic, has the potential to test the intrinsic functioning of inhibitory synapses in humans. The difference in furosemide effects on spinal inhibitory synapse excitability in healthy subjects and SCI patients favours the hypothesis of a decrease in inhibitory neuronal activity induced by down-regulation of KCC2 after SCI in humans that likely contributes to spasticity.

Project 2

The main finding of the present study is that contralateral anodal tDCS induces no change in reciprocal inhibition at the wrist level in the non-dominant upper limb of healthy subjects, while an increase of reciprocal inhibition was reported in their dominant-limb (Roche et al., 2009). Ipsilateral anodal tDCS induces no change in reciprocal inhibition in the dominant upper limb of right-handed healthy subjects. Even though there is a small decrease of inhibition in left-handed subjects, no significant difference between the two groups was observed. Preliminary results in hemiplegic patients show no changes in reciprocal inhibition of the paretic upper limb during ipsilateral anodal tDCS of non-lesioned hemisphere in both active and sham conditions. In contralateral study, anodal tDCS of the unaffected motor cortex dramatically decreases reciprocal inhibition in the non-paretic upper limb.

In addition, the degree of reciprocal inhibition at baseline (before tDCS) is reduced in the paretic upper limb in stroke patients compared to healthy subjects. In the non-paretic upper limb, the degree of reciprocal inhibition is not different from healthy subjects. However, statistical analysis did not reveal any difference between the paretic and the non-paretic upper limbs. In normal subjects, there is no difference of the degree of reciprocal inhibition between the dominant and the non-dominant upper limbs.

1. Methodological considerations

Transcranial direct current stimulation

Repetitive TMS and tDCS are two tools currently available to non-invasively modify excitability of the motor cortex (Priori et al., 2009). Apart from its simplicity, the major argument in favour of tDCS over rTMS is that rTMS produces effects on cortical excitability that may occur not only at the stimulation site but also at distant connected sites. For example, Wassermann et al. (1998) showed that 1 Hz rTMS over the primary motor cortex (M1) changes interhemispheric and corticospinal excitability in the contralateral homologous M1 as the authors found a small decrease in the MEPs indicating a reduced cortical excitability in the contralateral homologous cortex. By contrast, tDCS of the left M1 can facilitate or suppress MEPs triggered from the same hemisphere depending on the direction of current flow but has no effect on MEPs evoked from the opposite M1 (Lang et al., 2004). Thus, despite the fact that tDCS is applied through large electrodes and thus, is less focal, we assume that it is more suitable for the comparison of the effects of ipsi- and contralateral tDCS. However, to distinguish or selectively activate the muscle area target is not possible since the size of the active electrode applied over the motor cortex is large (35 cm²), the cortical areas of agonist muscle (FCR) and antagonist muscle (ECR) were thus simultaneously stimulated. The second argument in favour of tDCS is related to sham stimulation. TMS produces a loud click when each stimulus is delivered and electric current is induced in the scalp as well as in the brain, leading to local activation of sensory nerves and muscles which is readily perceived by subjects. Moreover, even if the click is indistinguishable between real and sham TMS, the lack of induced electric current means that there is no accompanying scalp sensation. In contrast with tDCS, subjects only feel the current intensity variation, so they can be entirely unaware of the difference between real and sham stimulation (Priori et al., 2009), even though here, for simplicity's sake the experimenter was not blind to the intervention.

Since Roche and colleagues (2009) have demonstrated the effect of contralateral anodal tDCS applied over the motor cortex on reciprocal inhibition in healthy subjects, this opens up the possibility of using tDCS as a tool to investigate changes in supraspinal descending control on spinal circuitry in humans. Nowadays, tDCS has begun to be used as a rehabilitation tool in patients with CNS lesions. However, knowledge of tDCS effects on spinal networks in normal subjects and patients with CNS lesions is still required.

2. Effects of anodal tDCS in healthy subjects

2.1 Contralateral study

Roche et al. (2009) previously demonstrated in our laboratory that anodal tDCS over the contralateral cortex induced an increase of reciprocal inhibition between forearm muscles in the dominant upper limb of healthy subjects. The authors proposed that tDCS acts by modifying the rate of spontaneous discharge of M1 neurons and thus induces an increase in the efficiency of the descending control on spinal neurons involved in reciprocal inhibition. Our present findings, using the same protocol as Roche's experiments, show that anodal tDCS induces no change in reciprocal inhibition of non-dominant upper limb. The results from an individual healthy subject who participated in the study of Roche et al., (2009) and in the present study also confirm the difference of reciprocal inhibition between dominant and non-dominant upper limbs during tDCS application.

Interhemispheric transcallosal interactions between both homologous cortical areas have been studied in humans using paired-pulse TMS (Ferbert et al, 1992; Hanajima et al., 2001). These studies show that stimulation of one hemisphere can induce facilitatory, but mostly inhibitory effects in the opposite hemisphere known as inter-hemispheric inhibition (IHI) (Ferbert et al, 1992). Recording of MEPs of the first dorsal interosseous muscle elicited by TMS in right-handed subjects showed that the IHI originating in the "dominant" left hemisphere is more marked than after stimulation of the 'non-dominant' right hemisphere. In contrast, left-handed subjects show inhomogeneous findings with either right- or left-sided predominant IHI. It was proposed that not handedness, but hemispheric dominance contributes to the laterality of inhibition between the two hemispheres (Netz et al., 1995). Other studies have also reported similarly that the IHI between motor cortices is asymmetric, with prominent IHI projections originating in the "dominant" left hemisphere (Baümer et al., 2007; Gorsler et al., 2003; Giovannelli et al., 2009; Wassermann et al., 1998; Ziemaan & Hallett, 2001). As in primates, interhemispheric interaction in human has been considered to be mediated by transcallosal connections (Boroojerdi et al., 1996, 1998; Meyer et al., 1995). There is some evidence from developmental studies showing that the transcallosal connection of the hand muscle area plays a role in execution of unilateral movement. The mirror movements of the hand are common in children, who have an immature transcallosal connection, but these movements are rarely seen after transcallosal fibres are completely myelinated (Connonlly & Stratton, 1968; Danek et al., 1992; Stern et al., 1976). This transcallosal connection enables healthy adults to perform strictly unilateral tasks even though some mirror movements can be observed in the non-dominant hand if the task is complex (Ziemaan & Hallett, 2008). It was

proposed that the increase in IHI originating from the active M1 to the M1 of the opposite hemisphere is responsible for the inhibition of undesired mirror movement (Hübers et al., 2008). Transcallosal connections between motor cortices have been shown to contribute to control of the corticospinal output from the M1 on forearm muscles (i.e. flexor carpi radialis) (Perez et al., 2008). Note that all subjects that participated in our contralateral study and in study of Roche et al., (2009) were right-handed subjects. Together with this evidence, it can probably be assumed that asymmetrical IHI with stronger inhibition from “dominant” left to “non-dominant” right hemisphere plays a role in the difference of reciprocal inhibition in dominant and non-dominant upper limbs in right-handed subjects. Another explanation would be that the sensitivity of the two hemispheres to tDCS is not similar. However, the motor threshold for induction of contralateral MEPs in hand muscles was reported to be the same in both hemispheres (Kobayashi et al., 2003). The last possible explanation would be the excitability of corticospinal control on reciprocal inhibition is different between non-dominant and dominant limbs. If this is the case, the degree of inhibition should be different in the both sides. However, the degree of reciprocal inhibition observed at baseline (before tDCS) in this study is similar between dominant and non-dominant upper limbs (see Figure 46). Overall, the hypothesis of asymmetrical IHI seems to mostly favour our results of asymmetrical control on reciprocal inhibition in right-handed subjects.

2.2 Ipsilateral study

The objective of an ipsilateral study in healthy subjects is to compare the results with stroke patients. Our results revealed no changes of reciprocal inhibition in the dominant upper limb of right-handed subjects during ipsilateral anodal tDCS over the motor cortex. An anatomical study suggests that ipsilateral control from hand primary motor cortex may be different in left-handed and right-handed subjects (Hervé et al. 2005). The volume of transcallosal in left-handers was found to be larger compared to right-handers (Clarke & Zaidel, 1994; Luders et al., 2003; Preuss et al., 2002) suggesting an association between handedness and inter-hemispheric connectivity. To our knowledge, there is no evidence for the difference of ipsilateral corticospinal control between left-handers and right-handers. We thus explored the ipsilateral effects of tDCS on reciprocal inhibition in left-handed subjects. The comparison between the two different groups of handedness revealed no significant difference of results. If anything, it seems likely that there is a small decrease of reciprocal inhibition in left-handed subjects compared to right-handed subjects. However, the sample of left-handed subjects was small compared to right-handers (8 left-handers vs. 24 right-handers).

The existence of uncrossed corticospinal tracts from the motor cortex to spinal motoneurons has been established in healthy humans and higher primates. It contains only 8-10% of the pyramidal tract (Kuypers 1981), and the majority of them finally cross at the segmental spinal cord level (Porter & Lemon, 1993). However, such ipsilateral corticospinal fibres preferentially reach proximal rather than distal muscles (Colebatch & Gandevia, 1989). The ipsilateral motor projection to distal upper limb muscles in normal subjects is still under debate. At rest, ipsilateral MEPs evoked by TMS in distal muscles (i.e. the forearm and hand muscles) are rarely found in healthy subjects (Bawa et al., 2004; Carr et al., 1994; Wassermann et al. 1991). A few studies have reported ipsilateral responses in distal muscles in healthy subjects, but under strong voluntary contractions and high intensity TMS (Wassermann et al. 1991; Ziemann et al. 1999). However, the ipsilateral responses in distal muscles are usually small and inconstant (Alagona et al., 2001). In functional magnetic resonance imaging (fMRI) studies, ipsilateral activation of the primary motor area can be observed during unilateral motor tasks in some healthy subjects, but not all of them (Allison et al., 2000; Cramer et al., 1999; Rao et al., 1993; Singh et al., 1998). Such ipsilateral activation is more frequently observed with the non-dominant hand (Kobayashi et al., 2003). Increasing the excitability of the ipsilateral motor cortex has no effect on reciprocal inhibition particular right-handed subjects. This can probably be explained by the few ipsilateral motor projections to spinal motoneurons or interneurons of distal muscles in the upper limbs.

3. Effects of anodal tDCS in stroke patients

3.1 Degree of reciprocal inhibition in stroke patients (before tDCS)

The degree of reciprocal inhibition of the paretic upper limb evaluated in 9 stroke patients is significantly reduced compared to the dominant and non-dominant upper limb of normal subjects (see Figure 45). These results are consistent with studies of Artieda et al., 1999; Nakashima et al., 1989 and Raoul, 2001. The decrease in reciprocal inhibition is frequently found in patients with spasticity, but not in patients with muscle tone or flaccid hemiplegia (Nakashima et al., 1989). The most likely explanation of this phenomenon is that the excitability of inhibitory interneurons is reduced as a result of cortical lesion. Reduced activity of reciprocal inhibition interneurons could be partly responsible for motor deficits including abnormalities of contraction of antagonist muscles in these patients. The hypothesis is supported by the fact that in normal subjects anodal tDCS does not modify presynaptic inhibition of Ia fibres (Roche et al., 2009).

3.2 Ipsilateral study

Ipsilateral corticospinal control on reciprocal inhibition at the wrist level was tested by anodal tDCS of unaffected motor cortex in 6 patients with unilateral stroke. No significant difference is found between tDCS and sham groups in these patients as observed in normal subjects.

Some TMS studies report that MEPs in paretic hand muscles of stroke patients can be obtained more frequently than in healthy subjects by TMS of the ipsilateral, unaffected motor cortex (Netz et al., 1997; Trompetto et al., 2000), with some characteristics that differ from those observed in normal subjects (i.e. shorter latency) (Alagona et al., 2001). However, it has been reported that ipsilateral MEPs in the paretic hand are inconsistently found (Bastings et al., 2002; Caramia et al., 2000), and that they were more often observed in patients with poor recovery than with good recovery (Netz et al., 1997; Turton et al., 1996). Here, we observed no changes in reciprocal inhibition in paretic limb during ipsilateral tDCS of unaffected motor cortex. A possible hypothesis would be that the unilateral stroke does not induce much changes in the ipsilateral projections to the distal muscles in stroke patients. However, our sample of patients is small. A larger number of patients is required to confirm the results.

3.3 Contralateral study

Anodal tDCS of the unaffected motor cortex induces a strong decrease in reciprocal inhibition in the non-paretic upper limb. All hemiparetic patients (n=5) participating in this contralateral study are right-handed subjects (3 right hemiplegia, 2 left hemiplegia). The amounts of reciprocal inhibition in the unaffected side of patients are decreased during contralateral anodal tDCS in all cases (dominant and non-dominant side). In all hemiparetic subjects, the clinical disability was presented only in affected side. Nevertheless, a marked depression of reciprocal inhibition during contralateral anodal tDCS was seen in the unaffected side in 4 of 5 patients.

After unilateral stroke, clinical symptoms are visible in the affected side. However, abnormal transmission in some spinal pathways in the upper limb has been described in the unaffected side. For example, presynaptic inhibition of Ia fibres in the upper limb is reduced in the non-paretic upper limb, though to a less extent than on the affected side (Aymard et al., 2000; Lamy et al., 2009). It was shown in the paretic side of hemiparetic patients that the response to stretch in biceps muscle is increased and that in triceps muscle is diminished compared to normal subjects. In contrast, the opposite results are found in the unaffected side, decreased response in biceps muscle and increase the response in triceps muscle that not seen in normal subjects (Thilmann et al., 1990). The muscle strength is also reduced on the unaffected side compared to normal

CHAPTER 4 DISCUSSION : PROJECT 2

subjects; 21% decrease of the muscle strength was reported for the upper limb muscles (reviewed in Gandevia, 1993). The preliminary results observed here are likely in accordance with these findings indicating that the non-affected upper limb of stroke patients is not similar to the normal upper limbs of healthy subjects.

To discuss the results in terms of reciprocal inhibition response to contralateral tDCS in normal subjects would be unsuitable to clarify our findings since contralateral anodal tDCS induces either increase of reciprocal inhibition in the dominant limb or no change in the non-dominant upper limb, while the inhibition in the unaffected side is decreased in the both cases (dominant and non-dominant). Moreover, the degree of reciprocal inhibition in the non-paretic upper limb before tDCS is not different from normal subjects. The changes that may occur at cortical level would also be unlikely to explain the results. Indeed, it was reported that the excitability of unaffected hemisphere is increased after unilateral stroke (Leipert et al., 2000; Shimizu et al., 2002). Anodal tDCS effect is known to increase cortical excitability. If anything, anodal tDCS of the unaffected motor area would produce a strong, even stronger increase of reciprocal inhibition compared to the results in normal subjects.

To date, there is no evident explanation for this finding and this result is still required to be confirmed with a larger number of patients.

4. Clinical implication

Contralateral anodal tDCS can induce an increase of reciprocal inhibition in the dominant upper limb, but not in the non-dominant upper limb in healthy subjects. As it is known, and we also found in the present study that the reciprocal inhibition between forearm muscles is reduced in paretic hand of stroke patients. Anodal tDCS could probably be used as an intervention to enhance reciprocal inhibition in the paretic upper limb. However, ipsilateral tDCS of the non-lesioned hemisphere seems to have no influence on reciprocal inhibition in the paretic upper limb. If we use contralateral tDCS of the lesioned hemisphere, it should be noted that this would probably work only when the paretic side is the dominant limb.

Studies in stroke patients have reported an increase in the excitability of unaffected hemisphere (Leipert et al., 2000; Shimizu et al., 2002) and an abnormally high IHI drive from intact to lesioned hemisphere during execution of voluntary movement by the paretic hand (Murase et al., 2004). To reduce the inhibition exerted by the unaffected hemisphere on the affected hemisphere and restore the normal balance of IHI, dual-hemisphere tDCS (upregulated excitability in M1 of affected hemisphere by anodal and downregulated excitability in the intact hemisphere M1 by cathodal) has

been described to enhance motor learning in healthy subjects (Vines et al., 2008) and to facilitate motor recovery in stroke patients (Bolognini et al., 2011; Lefebvre et al., 2013, 2014). Our results suggest that during increased cortical excitability by anodal tDCS of the unaffected hemisphere, a decrease of reciprocal inhibition in the unaffected upper limb is observed. The question may arise that what cathodal tDCS of the unaffected hemisphere would do on reciprocal inhibition in the non-paretic upper limb? However, no study has reported changes in non-paretic limb following dual or unilateral cathodal tDCS of the non-affected motor cortex (reviewed in Ayache et al., 2012). Nevertheless, evaluating changes in the non-paretic upper limb following the intervention would be needed.

5. Conclusion

It appears that interhemisphere inhibition between motor areas exerted via transcallosal connections plays an important role in the lateralization of neural activity during execution of unimanual movement in healthy subjects (Hübers et al., 2008; Ziemaan & Hallett, 2001). Several studies reveal stronger drive of IHI from the dominant to non-dominant hemisphere in normal subjects (especially in right-handed subjects: Bäumer et al., 2007; Gorsler et al., 2003; Giovannelli et al., 2009; Netz et al., 1995; Wassermann et al., 1998). Asymmetrical reciprocal inhibition control on the both sides of upper limb observed in right-handed subjects is likely related to asymmetrical IHI drive. The constraint-induced movement therapy (CIMT) is an intervention often used to improve affected upper limb function in patients with unilateral hemiparesis under the concept of decreasing activity of one hemisphere to release activity of the opposite hemisphere. Recently, it was suggested in healthy subjects that unilateral motor training of the non-dominant hand and contralateral dominant hand restraint (CIMT's concept) induce a decrease of cortical excitability in the dominant hemisphere and decrease in IHI from dominant to non-dominant hemisphere, thus improving performance in the non-dominant hand (Williams et al., 2010). Reciprocal inhibition is important in the process of movement (i.e. stabilizing joint, allowing agonist and antagonist muscles to work in synchrony for smooth movement). Using this type of training to increase reciprocal inhibition in the non-dominant upper limb would be of benefit for performing movements of the non-dominant upper limb in normal subjects.

The results of ipsilateral tDCS in left-handed subjects suggest that they might be different from those observed in right-handers. It would be interesting to confirm this result by increasing the numbers of subjects. This would provide a better understanding of the role of corticospinal control in handedness.

CHAPTER 4 DISCUSSION : PROJECT 2

It is still a matter of debate in TMS studies whether ipsilateral activation exists in stroke patients. To our knowledge, the effects of ipsilateral anodal tDCS on spinal networks in stroke patients have not yet been well documented (see Ayache et al., 2012). An increase in the number of stroke patients in our ipsilateral study is still required for further investigation. Changing some parameters of tDCS in the study in patients (i.e. increased intensity, reduced electrode size) would also be interesting for further research in motor recovery.

Finally, our results suggest that the unaffected upper limb does not respond to contralateral anodal tDCS with the 'normal' pattern. A larger number of patients is still required to confirm our results and since there is only little knowledge about unaffected side, more research on unaffected side of patients with hemiparesis should be required too. However, the hypothesis that there are also some changes on the 'unaffected side' after unilateral stroke is also supported by our study.

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List of Abbreviations

Acronym	Description
ADM	Abductor minimi muscle
CCC	Cation chloride cotransporter
CIMT	Constraint-induced movement therapy
CS	Corticospinal control
CNS	Central nervous system
CPN	Common peroneal nerve
E_{Cl}	Chloride equilibrium potential
ECR	Extensor carpi radialis
ECS	Extracellular space
EMG	Electromyographic
EPSP	Excitatory postsynaptic potentials
FCR	Flexor carpi radialis
fMRI	Functional magnetic resonance imaging
GABA	Gamma aminobutyric acid
H_{max}	Maximum H-reflex response
IHI	Inter-hemispheric inhibition
IN	Interneuron
IPSI	Inhibitory postsynaptic potentials

List of Abbreviations

ISI	Interstimulus interval
KCC	Na ⁺ -Cl ⁻ cotransporter
LTD	Long-term depression
LTP	Long-term potentiation
M1	Primary motor cortex
M _{max}	Maximum motor response
MEP	Motor evoked potential
MN	Motoneuron
MT	Motor threshold
NKCC	Na ⁺ -K ⁺ -2Cl ⁻ cotransporter
PAD	Primary afferent depolarization
PSTH	Post-stimulus time histogram
PT	Perception threshold
PTN	Posterior tibial nerve
rTMS	Repetitive transcranial magnetic stimulation
RS	Reticulospinal pathways
Rw	Renshaw cell
SMA	Supplementary motor cortex
SCI	Spinal cord injury
TA	Tibialis anterior
TEN	Transcranial electrical stimulation
TMS	Transcranial magnetic brain stimulation
TVR	Tonic vibration reflex
tDCS	Transcranial direct current stimulation
PAD	Primary afferent depolarization
VS	vestibulospinal pathway

Ma thèse est consacrée à l'étude des réseaux neuronaux spinaux impliqués dans la motricité chez l'Homme est comprend deux chapitres.

1. Modifications du fonctionnement des réseaux neuronaux inhibiteurs de la moelle épinière, induites par un diurétique (furosémide) chez l'Homme

Des travaux récents effectués sur la moelle épinière du rat ont mis en évidence qu'au cours du développement chez les mammifères, les synapses GABAergiques et glycinergiques sont tout d'abord excitatrices avant de devenir inhibitrices et qu'une section de la moelle épinière ne permet pas cette transformation. Cette transition développementale semble due à l'action d'un transporteur transmembranaire (KCC2) au cours de développement qui diminue après section de la moelle épinière. La diminution de l'expression du KCC2 dépolarise l'action du GABA et de la glycine, ce qui conduit donc à une réduction de l'efficacité de synapse inhibitrice. Le but de ce projet est d'explorer si chez l'Homme une section traumatique de la moelle épinière qui prive les neurones inhibiteurs de leur contrôle suprasegmentaire a pour conséquence de modifier leur comportement synaptique, voire de les ramener à un fonctionnement « immature », c'est-à-dire de transformer des synapses inhibitrices en synapses facilitatrices. Pour tester cette hypothèse, nous avons étudié l'effet sur des synapses inhibitrices de la moelle épinière d'une prise per os de furosémide, un antagoniste de KCC2, et comparé ses effets chez des sujets sains et chez des patients porteurs d'une section de la moelle épinière. L'étude sur les sujets sains suggère que le furosémide (40 mg) a pour effet une réduction du fonctionnement des synapses inhibitrices. Cet effet du furosémide sur les synapses inhibitrices semble être réduit chez des patients. Les résultats obtenus chez les sujets sains indiquent que furosémide administré per os à des dose largement utilisé en clinique humain modifie sélectivement le fonctionnement des synapses inhibitrices et permet donc de disposer d'une mesure non-invasive de fonctionnement intrinsèque de la synapse inhibitrice. Les résultats préliminaires obtenus chez les patients porteurs d'une section de la moelle épinière suggèrent une réduction de l'efficacité de synapses inhibitrices qui probablement contribue à la spasticité.

2. Contrôle ipsi et contralatéral de l'inhibition réciproque au niveau du poignet chez des sujets sains et des patients atteints d'accident vasculaire cérébral (AVC) démontré par la stimulation transcrânienne de courant continu

La stimulation électrique transcrânienne de courant continu encore appelée « transcranial direct current stimulation (tDCS) » par les anglo-saxons, a connu un essor considérable et constitue aujourd'hui une technique de référence pour moduler l'excitabilité du cortex chez l'Homme. En 2009, Roche et al. ont montré dans notre laboratoire, que la tDCS anodale appliquée sur l'hémisphère contralatéral pouvait également modifier l'excitabilité des réseaux neuronaux spinaux (i.e. l'inhibition réciproque au niveau du poignet) enregistrée sur le côté dominant chez les sujets sains. L'existence de projection corticale ipsilatérale sur les réseaux neuronaux spinaux de la moelle épinière et leurs éventuelles modifications après lésion cortico-sous-corticale reste très controversée. Dans ce projet, nous avons testé les effets de la tDCS ipsi- et contralatérale du cortex non-lésé sur l'inhibition réciproque chez des patients AVC. La tDCS ipsilatérale n'induit pas de modifications de l'inhibition réciproque chez les sujets sains. Des résultats similaires enregistrés sur le membre supérieur lésé ont été observés chez des patients AVC, mais ces résultats mériteraient d'être confortés avec un plus grand nombre de sujets. La tDCS contralatérale chez les sujets sains n'induit pas de modifications de l'inhibition réciproque enregistrées sur le membre supérieur non-dominant. Ce résultat est différent de celui observé sur le membre supérieur dominant par Roche et al. (2009). Ce contrôle asymétrique sur l'inhibition réciproque est argument en faveur de l'hypothèse que l'inhibition inter-hémisphérique (IHI) entre les deux cortex moteurs est asymétrique. L'IHI à partir de l'hémisphère « dominant » est probablement plus importante. Les effets de la tDCS contralatérale sur l'inhibition réciproque du membre supérieur non-lésé est différent des résultats observés chez des sujets sains, indiquant que la commande motrice contralatérale sur le membre supérieur non-lésé n'est pas indemne. Néanmoins, un plus grand nombre de patients est encore nécessaire pour confirmer le résultat.

Mots clés : réseaux neuronaux spinaux, synapses inhibitrices, furosémide, tDCS, sujets sains, patients blessé médullaire, patient hémiplegique.

My thesis is devoted to the study of the spinal circuitry involved in motor functions using non-invasive electrophysiological methods in humans. It comprises two research projects.

1. Changes in spinal inhibitory networks induced by a diuretic (furosemide) in humans

Studies in animals have shown that during neural development, GABAergic and glycinergic neurons are first excitatory, and then become inhibitory during maturation. This developmental transition is mainly due to the activation of co-transporter KCC2 at the mature state. A down-regulation of KCC2 was reported after spinal cord transection in the rat that leads to the depolarising (excitatory) action of GABA and glycine and thus results in a reduction of inhibitory synaptic efficiency. The aim of this project was to explore if spinal cord injury (SCI) in human reverses the pattern of GABAergic and glycinergic neurons back towards the immature state (primarily excitatory). To test this hypothesis, we studied the effects of furosemide (a KCC2 antagonist) on spinal inhibitory synaptic function, and compared the results obtained in healthy subjects and SCI patients. Results in healthy subjects suggest that furosemide (40 mg, orally-administrated) induces a reduction of inhibitory synapse functions. This effect of furosemide on inhibitory synapses seems to be reduced in SCI patients. Our results suggest that furosemide has the potential to test functions of inhibitory synapses in humans. The difference of furosemide effects on spinal inhibitory synapse excitability in healthy subjects and SCI patients favours the hypothesis of a decrease in inhibitory neuronal activity induced by down-regulation of KCC2 after SCI in humans that likely contributes to spasticity.

2. Ipsi- and contralateral corticospinal control on reciprocal inhibition between forearm muscles in healthy subjects and hemiplegic stroke patients as demonstrated by transcranial direct current stimulation.

Transcranial direct current stimulation (tDCS) has emerged as a method for exploring cortex excitability in humans. Roche et al. (2009) have shown in our laboratory that using anodal tDCS over contralateral motor cortex can also induce changes in spinal network excitability (i.e. reciprocal inhibition between forearm muscles) in the dominant limb in healthy subjects. It is unknown whether motor activity from the unaffected cerebral hemisphere could be employed after semi-brain damage in patients with hemiplegia. Moreover, little is known about the non-affected limb if it always functions like 'normal' after unilateral stroke. In this project, the ipsi- and contralateral corticospinal controls on reciprocal inhibition between forearm muscles were explored using anodal tDCS applied over the unaffected motor cortex of stroke patients and then compared to the results obtained in healthy subjects. Ipsilateral tDCS induces no change in reciprocal inhibition in healthy subjects. Similar results recorded on the affected upper limb are observed in stroke patients. However a larger number of patients is required to confirm the results. Contralateral anodal tDCS in healthy subjects shows no changes of reciprocal inhibition recorded in the non-dominant upper limb. This result is different from that observed in the dominant upper limb by Roche et al. (2009). This asymmetrical control on reciprocal inhibition would favour the hypothesis that the inter-hemispheric inhibition (IHI) between both motor cortices is asymmetric, with prominent IHI projections originating in the "dominant" left hemisphere. Contralateral anodal tDCS of the unaffected motor cortex induces a strong decrease in reciprocal inhibition in non-affected upper limb in stroke patients. This is different from that observed in both dominant and non-dominant upper limb in healthy subjects suggesting that the pathophysiological changes after unilateral stroke would probably not occur only on the hemiparesis side, but may also the non-affected side. A larger number of patients is still required to confirm the results.

Key words: spinal network, inhibitory synapses, furosemide, tDCS, healthy subjects, SCI patients, stroke patients.

Changes in spinal inhibitory networks induced by furosemide in humans

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Key Point Summary

- It has already been demonstrated in humans that furosemide crosses the blood-brain barrier and blocks activity in the epileptic brain.
- In this study, we demonstrated using non-invasive electrophysiological techniques in healthy human subjects that furosemide, a cation-chloride co-transporter blocker, orally-administered at doses commonly used in the clinic (40 mg), reduces the efficacy of pre- and postsynaptic inhibition of soleus motoneurons in the spinal cord.
- Furosemide can be a useful tool to detect the intrinsic functioning of inhibitory synapses and to explore in the future if the reduced inhibitory interneuronal activity which likely contributes to spasticity, also exists in humans with spinal cord injury.

Word count: 103

Abstract

During neural development in animals, GABAergic and glycinergic neurons are first excitatory, and then become inhibitory in the mature state. This developmental shift is mainly due to strong expression of the cation-chloride KCC2 and down-regulation of co-transporters NKCC1 during maturation. The down-regulation of co-transporter KCC2 after spinal cord transection in animals leads to the depolarising (excitatory) action of GABA and glycine and thus results in a reduction of inhibitory synaptic efficiency. Furosemide, a loop diuretic, has been shown to selectively and reversibly block inhibitory postsynaptic potentials (IPSPs) without affecting excitatory postsynaptic potentials (EPSPs) in animal spinal neurons. Moreover, this diuretic has been also demonstrated to block the cation-chloride co-transporters. Here, we used furosemide to demonstrate changes in spinal inhibitory networks in healthy human subjects. Non-invasive electrophysiological techniques were used to assess presynaptic inhibition, postsynaptic inhibition and the efficacy of synaptic transmission between muscle afferent terminals and soleus motoneurons in the spinal cord. Orally-administered furosemide, at doses commonly used in the clinic (40 mg), significantly reduced spinal inhibitory interneuronal activity for at least 70 minutes from intake compared to control experiments in the same subjects while no changes were observed in the efficacy of synaptic transmission between muscle afferent terminals and soleus motoneurons. The reduction of inhibition was dose-dependent. Our results provide indirect evidence that reversible changes in the cation-chloride transport system induce modulations of inhibitory neuronal activity at spinal cord level in humans.

Abbreviations GABA, γ -Aminobutyric acid; NKCC1, Na-K-Cl cotransporter1; KCC2, K-Cl cotransporter2; IPSP, inhibitory postsynaptic potential; EPSP, excitatory postsynaptic potential; CNS, central nervous system; PTN, posterior tibial nerve; ISI, interstimulus interval; M_{\max} , maximum motor response; H_{\max} , maximum H-reflex response; CPN, common

peroneal nerve; MT, motor threshold; PT, perception threshold; PAD, primary afferent depolarization; SCI, spinal cord injury; TA, tibialis anterior; SEM, Standard error of the mean.

Introduction

GABA and glycine are depolarising and excitatory in the immature spinal cord, becoming inhibitory in the mature state (Takahashi, 1984; Wu et al. 1992; Ziskind-Conhaim, 1998, Ben Ari, 2002). This developmental transition is generated by the differential expression of cation-chloride transporters, the chloride inward co-transporter (NKCC1) being highly expressed in immature neurons whereas the chloride outward co-transporter (KCC2) is dominant in mature neurons. The abundant KCC2 expression on mature neurons lowers the intracellular chloride ion concentrations. When GABA and glycine neurotransmitters contact the neuron, there will subsequently be an inward flux of chloride ions, thus lowering the membrane potential (i.e. inhibition).

In the animal spinal cord, the lack of KCC2 expression leads to depolarising action of GABA and glycine (Vinay & Jean-Xavier, 2008), and also results in reduction of inhibitory synaptic efficiency (Boulenguez et al. 2010). In KCC2 knockout animals, sciatic nerve recordings revealed abnormal spontaneous electrical activity and altered lumbar motoneurons responses to sciatic nerve stimulation (Hübner et al. 2001). Several studies in animals have shown that loss or reduction of co-transporter KCC2 function results in the development of central nervous system (CNS) hyper-excitability (Woo et al. 2002; Zhu et al. 2008).

The loop diuretics, furosemide and bumetanide, are thought to modulate inhibitory synapses through their antagonist effect on cation-chloride co-transporters (Gillen et al. 1996; Payne et al. 2003; Yamada et al. 2004). It has been suggested that furosemide antagonises the KCC2 co-transporter but it is also used as a NKCC1 blocker. Nevertheless, its effect on the NKCC1 co-transporter is less potent than bumetanide (Gillen et al. 1996; Russell, 2000). To date, no

substance has been identified that selectively inhibits KCC2 (Kahle et al. 2008; Löscher & Schmidt, 2011), and some of the KCC2 blockers used in animal studies require very high concentrations (Payne, 1997) that are likely to be toxic in humans. Nicoll (1978) demonstrated in the frog that furosemide selectively and reversibly blocks inhibitory postsynaptic potentials (IPSPs) elicited in spinal motoneurons by GABA and glycine without affecting excitatory postsynaptic potentials (EPSPs) elicited in spinal motoneurons by glutamate. Since furosemide has been shown to cross the blood-brain barrier after intravenous injection in humans (Haglund & Hochman, 2005) and is a widely used diuretic which has been employed with a good safety record for many years in humans, we have chosen furosemide to investigate the functioning of inhibitory synapses in this study.

The aim of the present study was to investigate the possibility that orally-administered furosemide at normal clinical doses could modify the functioning of spinal inhibitory synapses in healthy human subjects. We studied two types of inhibitory spinal networks: i) presynaptic inhibition of Ia fibres which is likely mediated *via* GABAergic synapses, and ii) postsynaptic inhibition produced by cutaneous stimulation which is probably mediated via glycinergic synapses (Jankowska, 1992). In addition, we explored the effects of furosemide on the efficacy of synaptic transmission between muscle afferent terminals and soleus motoneurons by studying post-activation depression of the H-reflex. The results obtained in healthy subjects reveal that orally-administered furosemide (40 mg) reduces the efficacy of spinal inhibitory interneurons without affecting the efficacy of synaptic transmission between muscle afferent terminals and soleus motoneurons.

Materials and Methods

Ethical approval.

The experiments described in this study conformed to the guidelines issued by the World Medical Association (Declaration of Helsinki) on the ethics of experimentation in humans and

the approval of the local ethical committee of the CPP Île-de-France VI - Pitié-Salpêtrière. Written informed consent was obtained from all subjects before participation.

Study design.

This randomized controlled study was performed on 19 healthy subjects (9 females) aged 22-64 years old (mean±SEM 35.1±2.5 years). All subjects participated in two different sessions to investigate the time course of changes in spinal network excitability for a period of 70 minutes (i) after furosemide intake (furosemide experiment) and, (ii) without furosemide (control experiment). The two different experiments (furosemide and control) were performed on different days.

Experimental procedure

Medical examinations (blood testing for electrolytes, creatinine, glucose and blood pressure) were performed before the experiment to verify that subjects had no contraindications to furosemide.

All experiments were performed at rest. Subjects sat comfortably with the head, neck, back and arms supported in a slightly reclined armchair. The dominant leg was examined and remained in a stationary supported position with hip semi-flexion (120°), slight knee flexion (160°) and plantar flexion (110°).

Each experiment comprised seven time epochs (0-10 min, 11-20 min, 21-30 min, 31-40 min, 41-50 min, 51-60 min, and 61-70 min). The amount of pre-/postsynaptic inhibition evaluated at the beginning of recording (during 10 minutes before drug administration in the furosemide experiment, or 10 minutes before the test start time in the control experiment) corresponds to baseline inhibition. After the amount of inhibition was determined for the baseline period, 40 mg furosemide was orally administered. For the subject's convenience, due to its diuretic effect, the amount inhibition was monitored through repetitive measurement over a time period limited to 70 minutes after administration of drug. Furosemide pharmacokinetics and

pharmacodynamics in healthy subjects show that furosemide (40-80 mg) appears in the serum within 10 minutes and peaks from 60 to 90 minutes after oral administration (Branch et al. 1976, 1977; Tilstone & Fine, 1978).

The effects of varying doses of furosemide (0 mg, 20 mg and 40 mg) were also investigated on pre-/postsynaptic inhibition in 6 subjects using the same experiment design.

The experiments testing the effects of furosemide on the efficacy of synaptic transmission between muscle afferent terminals and soleus motoneurons (post-activation depression of the H-reflex) were performed in 15 subjects the same way as described previously for pre- and postsynaptic inhibition.

Water intake was recommended to subjects after the experiment. However, apart from the diuretic effect, some side effects of furosemide such as headaches and dizziness were reported by some of the subjects that participated in the present study.

Electrophysiological recordings

Method of assessing spinal network excitability

Soleus H-reflex. Spinal inhibitory circuits were studied using non-invasive electrophysiological techniques based on the H-reflex. The H-reflex was evoked in the soleus muscle by percutaneous stimulation of the posterior tibial nerve (PTN) at the popliteal fossa using a constant-current stimulator (D7SA Digitimer Ltd., Welwyn Garden City, UK). A rectangular 1 ms duration stimulus, delivered at 0.33 Hz to the PTN, was computer-triggered using a customised script (Signal 4.20, Cambridge Electronic Design, Cambridge, UK). The H-reflex was recorded from the EMG signal of the soleus muscle using bipolar surface electrodes (Delsys Inc., Boston, USA, Ag electrodes DE-2.1) positioned on the skin parallel to the muscle belly. The EMG signals were amplified ($\times 1000$), band pass filtered at 20-450 Hz (Delsys, Inc., Boston, MA, US), digitised at 1 kHz (Power 1401 A/D board, Cambridge Electronic Design, Cambridge, UK) and then stored on computer for offline analysis.

The maximum motor response (M_{\max}) and maximum H-reflex response (H_{\max}) were recorded at the beginning of the each experiment. The stimulus intensity was then adjusted to elicit H-reflex amplitude of 20-35% of M_{\max} , which corresponded to ~ 50%, of H_{\max} [mean value of H-reflex amplitude $24.85 \pm 2.8 \% M_{\max}$ (control experiments); $23.17 \pm 2.2 \% M_{\max}$ (furosemide experiments)]. The unconditioned H-reflexes were set to have similar size across subjects and kept constant throughout the experiment, since the sensitivity of the H-reflex to facilitation or inhibition conditioning effects depends on its non-conditioned size (Crone et al. 1990).

Inhibitory conditioning stimulations. (see Figure 1)

Presynaptic inhibition of Ia fibres. The D1 inhibition of the H-reflex was used to assess presynaptic inhibition of Ia fibres (Mizuno et al. 1971). The soleus H-reflex was conditioned with preceding stimulation of the common peroneal nerve (CPN) through hemispherical bipolar electrodes placed 2 cm below the neck of the fibula. The stimulus consisted of a train of 3 rectangular pulses, each of 1 ms duration, with an interpulse interval of 3 ms, with an intensity 1.2 times the tibialis anterior (TA) motor threshold (MT), delivered 21 ms before the test stimulation (Faist et al. 1996). The motor threshold was determined by tendon palpation and by the oscilloscope EMG display [MT mean value 15.00 ± 0.31 mA (control experiment); 11.60 ± 0.13 mA (furosemide experiment)]. At this inter-stimulus interval (21 ms), the CPN conditioning volley evokes an inhibition of the soleus H-reflex that is probably caused by presynaptic inhibition of soleus Ia afferents (Faist et al. 1996).

Postsynaptic inhibition produced by cutaneous stimulation. It has been shown that stimulation of the sural nerve (a purely cutaneous nerve) induces an inhibition of the soleus H-reflex (Hugon & Bathien, 1967). The soleus H-reflex was conditioned by electrical stimuli applied to cutaneous afferents of the sural nerve through bipolar adhesive electrodes placed on the lateral side of the fifth toe. The stimuli consisted of trains of 17 pulses, each of duration 1 ms,

with an interpulse interval of 3 ms, delivered 50 ms before the test stimulus. In such conditions, the induced inhibition is probably caused by postsynaptic inhibition of soleus motoneurons (Pierrot-Deseilligny & Burke, 2012). The intensity of stimulation was based on the subject's perception threshold (PT) defined as the lowest stimulus intensity at which the subject reported sensation of the stimulation using a single pulse [PT mean value 1.73 ± 0.02 mA (control experiment); 1.61 ± 0.1 mA (furosemide experiment)]. The intensity of the stimulus train was slowly increased to 3-5 times the perceptual threshold in order to induce a clear-cut inhibition of the soleus H-reflex [intensity mean value 4.33 ± 0.3 PT (control experiment); 3.79 ± 0.3 PT (furosemide experiment)].

Each stimulation sequence comprised three different conditions of 20 stimuli each: 1) test stimulus alone, 2) paired stimulation (test stimulus + conditioning stimulus to CPN evoking presynaptic inhibition), 3) paired stimulation (test stimulus + conditioning stimulus to the sural nerve inducing postsynaptic inhibition). Consequently, 40 conditioned and 20 unconditioned H-reflexes, evoked at 0.33 Hz, were determined during each time epoch. A complete sequence took about 3 minutes. Conditioned and unconditioned H-reflexes were randomly alternated.

Post-activation depression. To test the possible effect of furosemide on spinal excitatory synapses, we used post-activation depression of soleus H-reflexes. It has been shown in both animals (Eccles, 1964) and humans (Magladery et al. 1951; Paillard, 1955; Crone & Nielsen, 1989; Hultborn & Nielsen, 1998) that post-activation depression at the Ia fibre-motoneuron synapse reflects the efficiency of the monosynaptic excitatory transmission between Ia fibre and motoneuron and is thus a simple and reliable tool to assess the functioning of excitatory synaptic transmission without contamination of other synaptic mechanisms (Meunier et al. 2007). Post-activation depression can be demonstrated by the depressive effect of increasing the stimulus rate on the size of H-reflexes. H-reflex is dramatically depressed when the

interval between the two consecutive stimuli is 1-2 s with gradual recovery as the interval increase over 10 s (Crone & Nielsen, 1989). In the present study, the H-reflex evoked every 3 s (0.33 Hz) was initially adjusted at $H_{\max}/2$. The soleus H-reflex evoked at low frequency (every 6s or 0.16 Hz) and high frequency (every 1s or 1 Hz) was measured in each case (see Figure 4B). Twenty soleus H-reflexes were evoked for each stimulus rate (1 Hz and 0.16 Hz). The amount of post-activation depression of the H-reflex was evaluated as the size of the H-reflex elicited every 1 Hz (high stimulus rate) expressed as a percentage of its value when elicited every 0.16 Hz (low stimulus rate). This is referred to as the 1/0.16 ratio. This ratio was used to determine the amount of post-activation depression of the H-reflex (Aymard et al. 2000).

Statistical methods

Data processing

To evaluate the amounts of pre- and postsynaptic inhibition, peak-to-peak amplitudes of unconditioned H-reflexes were compared to those of conditioned H-reflexes. The inhibitory effects were quantified by calculation of the inhibition value as $100 - ((\text{conditioned } H \text{ value} / \text{unconditioned } H \text{ value}) \times 100)$. Each inhibition value was then normalized to the percentage of its baseline value using the equation: $[\%((\text{Inhibition at } t_x - \text{baseline inhibition}) / \text{baseline inhibition})] + 100$.

To evaluate the effects of post-activation depression, the 1/0.16 ratio value was normalized to the percentage of its baseline value using the equation: $[\%((1/0.16 \text{ ratio at } t_x - 1/0.16 \text{ ratio at baseline}) / 1/0.16 \text{ ratio at baseline})] + 100$.

Statistical analysis

The amounts of pre-/postsynaptic inhibition and post-activation depression were averaged among subjects for each time epoch (0-10 min, 11-20 min, 21-30 min, 31-40 min, 41-50 min, 51-60 min, and 61-70 min). A two-way repeated-measures ANOVA (control vs. furosemide

as the within-subject factor) was used to examine the effects of furosemide on pre-/postsynaptic inhibitions or post-activation depression. A *post-hoc* pairwise comparison was performed using Tukey's HSD test.

A one way repeated-measure ANOVA on time epoch (0-10 min, 11-20 min, 21-30 min, 31-40 min, 41-50 min, 51-60 min, and 61-70 min) was also used to determine the impact of time in the control experiment.

For dose-dependent experiments, an a priori power analysis was first performed to determine the minimal sample size. A posteriori, all our data sets were, as assumed in our power analysis, normally distributed and successfully passed the equality of variance test so parametric tests (one way repeated measures ANOVA) was employed.

Significance was taken at $P < 0.05$. Mean data are provided \pm standard error of the mean (SEM).

Results

Effects of time on two examples of spinal inhibitory networks.

Presynaptic inhibition of Ia fibres.

The amount of presynaptic inhibition in the control experiments (without furosemide) observed over 70 minutes is shown in Figure 2A (19 subjects). A one-way repeated-measures ANOVA failed to detect a significant effect of time on presynaptic inhibition in control experiments ($P > 0.05$).

Postsynaptic inhibition induced by cutaneous stimulation.

The experiments were performed in all 19 subjects but this type of spinal synaptic inhibition could not be demonstrated in four of them since the conditioning stimulus intensity required to evoke postsynaptic inhibition was above that evoking an unbearable painful sensation and/or triggered the flexor reflex in these subjects. The results from postsynaptic inhibition

were thus observed in only 15 subjects. The amount of postsynaptic inhibition in the control experiments observed over 70 minutes is shown in Figure 2B. There was a gradual decrease of inhibition over time in control experiments ($F_{14,98} = 8.56$, $P < 0.001$, one way repeated-measure ANOVA). The amounts of postsynaptic inhibition decreased progressively after the onset of the recordings and the decrease is statistically significant from 21-30 minutes up to 61-70 minutes (baseline vs. 21-30 minutes, baseline vs. 31-40 minutes, baseline vs. 41-50 minutes, baseline vs. 51-60 minutes, and baseline vs. 61-70 minutes ($P < 0.05$, $P < 0.001$, $P < 0.001$, $P < 0.01$, and $P < 0.001$ respectively, Tukey test)).

Furosemide effects on the two spinal inhibitory networks.

Presynaptic inhibition

The comparison between variations of the amount of presynaptic inhibition (expressed as a percentage of its baseline value) in control (□) and furosemide (■) experiments is shown in Figure 3A (19 subjects). The amounts of presynaptic inhibition in the furosemide experiments start to be significantly different from that observed in control situation at 31-40 minutes after drug administration. The amount of presynaptic inhibition following furosemide decreased progressively and reached its nadir at 61-70 minutes (60.66 % of its baseline value). Two-way repeated-measures ANOVA detected a significant interaction of condition (control vs. furosemide), time epochs, and condition × time epochs ($F_{18,108} = 2.40$, $P_{\text{condition}} = 0.037$, $P_{\text{time epochs}} < 0.001$, $P_{\text{condition} \times \text{time epochs}} = 0.032$). Post-hoc analysis showed that furosemide significantly reduced presynaptic inhibition at 31-40 minutes, 41-50 minutes, 51-60 minutes, and 61-70 minutes ($P < 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.01$ respectively, Tukey test).

Postsynaptic inhibition

The comparison between variations of postsynaptic inhibition (expressed as a percentage of its baseline value) in control and furosemide experiments are presented in Figure 3B (15

subjects). The strength of postsynaptic inhibition decreased progressively after furosemide administration. The amounts of postsynaptic inhibition in the furosemide experiments began to be significantly different from those observed in control experiments 21-30 minutes after drug administration. Then amount of postsynaptic inhibition decreased continuously and reached its nadir at 51-60 minutes (21.88 % of its baseline value). Two-way repeated-measures ANOVA detected a significant interaction of condition (control vs. furosemide), time epochs, and condition \times time epochs ($F_{14,84} = 3.43$, $P_{\text{condition}} = 0.034$, $P_{\text{time epochs}} < 0.001$, $P_{\text{condition} \times \text{time epochs}} = 0.004$). Post-hoc analysis showed that furosemide significantly reduced postsynaptic inhibition at 21-30 minutes, 51-60 minutes and 61-70 minutes ($P < 0.01$, $P < 0.001$, and $P < 0.05$ respectively, Tukey test).

Furosemide effects on monosynaptic excitatory transmission.

Post-activation depression.

This supplementary experiment was performed after the study of pre- and postsynaptic inhibitions was completed. Fifteen subjects of the previous recording participated in this session (see Table 1). The comparison between variations of post-activation depression (expressed as a percentage of its baseline value) in control and furosemide experiments, observed in 15 subjects is shown in Figure 4A. A two-way repeated-measure ANOVA failed to detect a significant interaction of condition \times time epochs: $P > 0.05$. There was no significant difference between two groups (control vs. furosemide).

Individual values (not normalised to baseline) of pre- and postsynaptic inhibition and of post-activation depression recorded at baseline and during the 40-70 minutes period are shown in Table 1. Note that three subpools at the intervals with the most prominent effects (41-50 minutes, 51-60 minutes, and 61-70 minutes) were pooled in Table 1.

Effects of two different doses of furosemide

For dose-dependent experiments, an a priori power analysis was performed to determine the minimal sample size. Given that postsynaptic inhibition decreased over time (i.e., habituation, see above), the power analysis was performed on presynaptic inhibition only. A sample size of 6 subjects, each having 0, 20 or 40 mg of furosemide provided 80% power to detect a 40% change in presynaptic inhibition (one way repeated-measures ANOVA, two-tailed 5%). This calculation was made assuming that raw data are approximately normally distributed with a standard deviation of 10%. One way repeated measures ANOVA was then employed.

Presynaptic inhibition

The effects of two different doses of furosemide were studied on presynaptic inhibition in six subjects (Figure 5A). The histogram shows the amount of presynaptic inhibition at different doses observed over a period of 40-70 minutes after furosemide administration where the effects were mostly observed (see Figure 3A). A one-way repeated-measures ANOVA detected a significant dose-effect ($F_{5,10} = 13.67$, $P = 0.001$). A *Post-hoc* Tukey test revealed significant difference between 0 mg vs. 40 mg, and 20 mg vs. 40 mg ($P < 0.01$ and $P < 0.01$, respectively).

Postsynaptic inhibition

The effects of two different doses of furosemide administration were studied on postsynaptic inhibition in six subjects (Figure 5B). The histogram shows the amount of postsynaptic inhibition at different doses observed over 40-70 minutes after furosemide administration. There was a trend for a dose-effect in postsynaptic inhibition as observed in presynaptic inhibition. However, a one-way repeated-measures ANOVA failed to detect a significant dose-effect on postsynaptic inhibition: $P > 0.05$. Since time has an influence on postsynaptic inhibition, the effect of furosemide evaluated at 40-70 minutes could be confounded with this time effect.

Discussion

The main finding of the present study is that oral administration of furosemide at the usual clinical dose (40 mg) decreases the function of inhibitory synapses in the spinal cord without affecting muscle afferent-evoked excitation of motoneurons.

Spinal networks involved in the present study

We have chosen to assess presynaptic inhibition directed to soleus Ia fibres using D1 inhibition. Presynaptic inhibition of Ia fibres was first described by Frank & Fuortes (1957) since a depression of a monosynaptic EPSP occurs without any change in motoneuron membrane potential. It was then extensively studied by Eccles and Colleagues (1964) and Rudomin and Colleagues (for references, see Rudomin & Schmidt, 1999). There is a general agreement that the pathways mediating presynaptic Ia inhibition involve two interneurons, where the last order interneuron is GABAergic. The D1 inhibition was first described in humans by Mizuno et al. (1971). Berardelli et al. (1987) and Faist et al. (1996) subsequently showed in both upper and lower limbs that the antagonistic conditioning volley eliciting the D1 inhibition was not depressing the motor evoked potential elicited in the target muscle by cortical stimulation. It is therefore likely that the D1 inhibition is presynaptic in origin since postsynaptic inhibition should depress the monosynaptic H-reflex and also the motor evoked potential. The time interval between conditioning and test stimuli used to evoke the D1 inhibition was proposed by Faist et al. (1996) who demonstrated that at this specific time interval (21 ms), the inhibition of the soleus H reflex is only due to presynaptic inhibition acting on soleus Ia afferents.

Among the postsynaptic effects induced in the soleus motoneurons, the inhibition elicited by electrical stimulation of sural cutaneous afferents, which is a part of the withdrawal reflex (for reference see, Pierrot-Deseilligny & Burke, 2012), is one of the most robust reflexes in animals and humans. In humans, the amount of this inhibition is marked (around 40 % of the

unconditioned reflex (Pierrot-Deseilligny et al., 1973). Neurotransmitter involvement in this type of spinal inhibitory network has never been directly demonstrated; however, postsynaptic inhibition has been shown to be mediated by glycine in the cat spinal motoneurons (Curtis et al., 1973).

To study postsynaptic inhibition induced by cutaneous stimulation, we have used the protocol originally described by Hugon & Bathien (1967). The conditioning stimulus was a train of 17 shocks, since it has been shown that cutaneous stimulation induced responses require temporal summation. Later, Burke et al. (1991) and Nielsen et al. (1997) presented the evidence that the cutaneous effect induced in the lower limb with latency shorter than 70 ms, is spinal in origin. Their results are also supported by the fact that the cutaneous effect exists in spinal animals and complete spinal cord lesions in humans (Longchamp & Chanelet, 1968; Logigian et al, 1999). Put together, although the exact pattern of the spinal reflexes fed by cutaneous afferents is not completely known, we favour the hypothesis that the effects are spinal in origin. Post-activation depression was chosen to test the possible effect of furosemide on excitatory effects since it has been thought to reflect the efficiency of the monosynaptic excitatory transmission between Ia fibre and motoneuron that is an example of pure excitatory effect. Alternative possibilities to test pure excitatory effects should be either homonymous or heteronymous monosynaptic facilitation of the soleus H-reflex. However, homonymous monosynaptic facilitation cannot be used in humans to assess purely excitatory effects, due to several factors, for example the mixed excitatory and inhibitory actions and duration of after-hyperpolarisation. Heteronymous monosynaptic Ia facilitation from the quadriceps to the soleus requires an accurate determination of the time interval between the conditioning and test stimulus to ensure its monosynaptic origin (for references, see Pierrot-Deseilligny & Burke, 2012). For other excitatory pathways, for example group II disynaptic excitatory pathways, the indirect method we used cannot eliminate possible contamination by

monosynaptic afferent projections or by inhibitory projections involved in the pathway. Moreover, testing group II excitatory pathways at rest relying on the use of the quadriceps H-reflex is not as easy as evoking the H-reflex in the soleus muscle and group II excitatory effects are much larger in the standing than in the sitting position.

Changes in inhibitory synapses over time

Habituation is a gradual decrease in responsiveness following repetitive stimulation and is a well-known phenomenon in the nervous system (Harris, 1943; Hernández-Peón et al. 1956). Most studies report habituation as a reduction in excitatory response, for example a decrease in the flexor reflex (Farel et al., 1973) or failure of the C-fibre evoked response in a dorsal horn neuron following prolonged stimulation (Woolf, 1983). However, it has also been referred to as a reduction in inhibition response. In the decerebrate cat, intracellular recording of motoneurons participating in the flexion reflex revealed that there was a marked and significant decrease of PSP amplitude during habituation which contains not only the EPSP's habituate, but also decrements of IPSP components. The IPSPs of polysynaptic responses decrease rather than increase during habituation suggesting that the amount of postsynaptic inhibition on the motoneuron is also "habituating" (i.e., decreasing). Besides, the decrease in response during habituation was proposed as a result of reduced input to motoneurons occurring in interneurons (Brazier, 1961). Habituation can be observed in patients with complete spinal cord injury, suggesting that this phenomenon may occur partly or totally at the spinal cord level (Dimitrijevic & Nathan, 1970; Dimitrijevic et al., 1972). Mechanisms underlying habituation in humans are still unclear; however, psychological adaptation of subjects, and also the effects of sensory saturation, could be taken into account (see Dincklage et al., 2013). The experiments performed in this study lasted up to 70 minutes and the same electrical stimuli were repeated at 0.33 Hz throughout. It could thus be argued that changes in inhibitory spinal network excitability were due to a habituation effect, not furosemide. To

exclude this hypothesis, we performed control experiments without furosemide in the same participants. No changes were observed over time for presynaptic inhibition of Ia fibres without furosemide (see Figure 2A). This finding demonstrates that changes in presynaptic inhibition following furosemide intake is most likely due to the effects of furosemide. In the case of postsynaptic inhibition, there was a gradual decrease of inhibition over time in control experiments revealing that habituation may involve the function of cutaneous receptors or synapses interposed between afferent fibres and soleus motoneurons. The diminution of postsynaptic inhibition after furosemide administration is more pronounced compared with control (see Figure 3B), which is likely caused by furosemide even though the difference is not statistically significant for all epochs.

Put together, our results suggest that furosemide is at least partly responsible for the decrease of the efficacy of pre- and postsynaptic inhibition of soleus motoneurons. This point of view is also supported by the significant dose effect relationship for presynaptic inhibition and by the tendency of dose related changes in the postsynaptic inhibition (Figure 5).

Specific effects of furosemide onto spinal inhibitory synapses

Although furosemide is known from animal experiments to block cation-chloride co-transporters, its effects on the human spinal network have never been tested before. The question could be asked as to whether the effects demonstrated for inhibitory synapses are specific to GABAergic and glycinergic synapses, or if they are non-specific, affecting both inhibitory and excitatory synapses. The results obtained from the experiments testing the effects of furosemide on post-activation depression at the Ia fibre-motoneuron (an excitatory synaptic transmission) allow one to exclude the hypothesis that furosemide also acts on the Ia afferent-motoneuron synapse. There was no significant difference observed in post-activation depression between control and furosemide experiments. Altogether, these observations

strongly suggest that furosemide decreases the functioning of spinal inhibitory synapses. Although furosemide decreases both pre- and postsynaptic inhibition, the effect of furosemide seems to be more important for postsynaptic than presynaptic inhibition. The most likely explanation for the difference between the time courses of pre- and postsynaptic inhibition is that in the control condition (without furosemide), there was no systematic change over time for presynaptic inhibition (Figure 2A) while the amount of postsynaptic inhibition is progressively decreased over time (Figure 2B). This effect of time by itself on postsynaptic inhibition is interpreted above with respect to habituation. Thus, for postsynaptic inhibition, the inhibition following furosemide intake is the sum of habituation effect and the effect of furosemide itself, while the changes of presynaptic inhibition following furosemide intake is only due to furosemide effect. Another explanation may be that the kinematics of pre- and postsynaptic inhibition are different; however, to our knowledge, there is no evidence to support this hypothesis.

Obviously, the possible effects of furosemide are not restricted to the spinal cord, but may affect all the structures of the CNS, thereby the functioning of networks projecting on interneurons mediating pre- and postsynaptic inhibition. However, since it has been shown in animals that furosemide decreases IPSPs without modifying EPSPs (Nicoll, 1978), the possible effect of furosemide on these networks would be a decrease in inhibitory controls. A decrease in inhibitory controls acting on pre- and postsynaptic inhibition would result in an increase in the efficiency of these inhibitory pathways and thus cannot be in all likelihood responsible for the decrease of inhibition we have observed.

Furosemide is known to inhibit in a non-competitive fashion GABA and glycine receptor channels mainly through antagonism of the cation-chloride cotransport system, but this drug also modulates the extracellular space involving, for example, electrical resistance of nervous tissue, extracellular ionic concentration or local ephaptic coupling (Hochman, 2012). Thus, if

furosemide modulates the extracellular space, the possibility exists that it would change the distribution of current applied by the transcutaneous electrical conditioning stimuli applied to the peripheral nerves. Therefore, we have verified that the direct motor response evoked in the TA following the stimulation of the CPN was not modified during the experiments (data not shown) suggesting that there is likely no change in the distribution of current induced by furosemide. Obviously, the site of electrical stimuli are far from the spinal cord but the absence of changes in the TA motor response was the only available data in our experiments to test this hypothesis.

Methodological significance and clinical implications

In humans, available methods to study spinal cord networks are indirect and rely on EMG recordings that operate as a *window* on motoneuron and interneuron excitability (Pierrot-Deseilligny & Burke, 2012). Since the pioneering experiments in humans by Magladery et al. (1951) and Paillard (1955), electrophysiological non-invasive but selective methods have been developed to study the excitability of spinal networks. Recent results obtained in animals show that lesion of the spinal cord induces a down-regulation of KCC2, which reverses the inhibitory pattern of GABAergic and glycinergic neurons back towards their immature and excitatory state (Jean-Xavier et al. 2006). This finding raises the possibility of setting up a technique, which would allow us to determine if such a reversal also exists in patients with spinal cord injury (SCI). The specific effects of furosemide on spinal inhibitory synapses demonstrated here provide a window on the intrinsic functioning of inhibitory synapses mediated through cation-chloride cotransporters. If SCI in humans shows a down-regulation of KCC2 as observed in animal studies, the comparison of furosemide effects on spinal inhibitory synapse excitability in healthy subjects and SCI patients would allow us to study these effects.

Furosemide is observed to decrease presynaptic inhibition directed to soleus Ia fibres and postsynaptic inhibition directed to soleus motoneurons in the present study. The question may arise whether prolonged intake of furosemide would affect daily life or physical activity of people. Exaggerated reflexes or increase in muscle tone have never been reported as a side effect of furosemide. Even though, to our knowledge, no systematic study of exaggerated reflexes or muscle tone has been performed during prolonged furosemide treatment. None of the healthy volunteers who participated in our experiments reported changes in reflexes or muscle tone following a single dose of furosemide administration. We may thus assume that in people with normal functioning of inhibitory synapses, the effects of furosemide have no clinical consequences. However, this may be different in patients with modifications of the functioning of inhibitory synapses. If the down-regulation of KCC2 in spinal rats, (Boulenguez et al. 2010) is also present in patients with SCI, it would be interesting to explore the possible muscle tone changes following prolonged furosemide treatment.

In conclusion, furosemide, which is a safe and widely used diuretic, has an effect on spinal inhibitory synapses in healthy subjects. The use of this loop diuretic would be a very useful tool to explore the possibility as to whether the reduced inhibitory interneuronal activity which likely contributes to spasticity, also exists in humans with spinal cord injury.

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Additional information

Competing interests

None declared.

Author contributions

W.K.: conception and design of experiments, collection, analysis and interpretation of data, preparation of figures, drafting and critical revision of the manuscript. A.L.: conception and design of experiments, collection, analysis and interpretation of data, preparation of figures, drafting and critical revision of the manuscript. R.K.: conception and design of experiments, drafting and critical revision of the manuscript. B.B.: conception and design of experiments,

and critical revision of the manuscript. D.J.: conception and design of electrophysiology experiments. J.L.: conception and design of experiments, interpretation of data, critical revision of the manuscript. N.R.: conception and design of experiments, interpretation of data, critical revision of the manuscript. All authors approved the final version of the manuscript, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Table 1

Individual values (not normalised) of pre- and postsynaptic inhibition reported as a percentage of amplitude of unconditioned H-reflex, and values of post activation depression (not normalised) reported as 1/0.16 ratio recorded at baseline and during the 40-70 minutes are shown in the table.

	Sex (F/M)	Age (years)	Side (L/R)	Presynaptic (control)		Presynaptic (furosemide)		Postsynaptic (control)		Postsynaptic (furosemide)		Post-activation depression (control)		Post-activation depression (furosemide)	
				Base-line	40-70 min	Base-line	40-70 min	Base-line	40-70 min	Base-line	40-70 min	Base-line	40-70 min	Base-line	40-70 min
1	F	64	L	29.60	15.09	22.42	0.01	28.71	12.87	19.5	-5.73	56.23	32.94	72.05	49.33
2	F	32	R	23.53	24.26	19.68	4.82	21.95	21.75	14.68	4.47	37.84	63.70	26.77	36.77
3	M	26	R	36.33	38.67	44.26	45.52	25.85	10.26	17.30	6.37	48.13	43.46	49.01	55.15
4	F	50	R	47.38	39.89	23.54	14.27	14.10	19.50	26.12	19.04	31.36	35.51	31.74	42.56
5	M	34	R	18.90	8.08	25.63	14.31	23.38	9.69	24.34	4.73	13.54	21.68	22.31	21.18
6	F	40	R	30.69	6.12	24.30	21.46	1.50	-6.01	12.50	-8.48	40.93	43.36	39.19	54.87
7	F	45	R	24.21	26.54	16.67	3.76	-	-	-	-	56.30	41.62	41.47	39.25
8	M	52	R	37.79	40.80	28.70	19.01	14.64	10.31	18.79	10.33	-	-	-	-
9	M	29	R	19.44	12.70	38.74	39.15	33.67	18.47	31.03	28.82	42.15	56.59	46.23	52.07
10	F	31	R	29.34	32.73	41.95	39.27	30.89	21.18	28.95	26.18	72.27	87.81	65.03	66.54
11	M	32	R	31.29	28.18	41.22	19.18	-	-	-	-	-	-	-	-
12	M	37	R	12.79	15.00	30.20	16.80	-	-	-	-	42.87	42.68	46.58	39.65
13	M	22	R	14.54	7.33	14.89	16.18	32.28	18.42	19.96	11.33	64.66	74.56	74.45	66.94
14	M	39	R	49.62	36.27	32.22	35.66	55.53	55.15	32.22	35.66	29.15	49.85	41.84	45.99
15	F	25	R	31.14	25.23	15.57	7.02	1.52	-1.63	6.91	1.20	74.54	51.55	41.93	51.36
16	F	25	R	22.78	27.38	33.23	14.00	54.34	57.42	29.78	16.90	-	-	-	-
17	F	25	R	17.48	17.47	11.06	5.36	21.28	6.55	6.85	-0.08	47.82	62.05	61.98	67.75
18	M	23	R	33.17	34.87	28.50	31.87	-	-	-	-	69.01	82.12	65.54	80.57
19	M	35	R	46.75	45.06	14.74	7.13	30.34	14.57	27.33	4.38	-	-	-	-

“ - ” = not recorded

Figure Legends

Figure 1: Schematic diagrams of the techniques used to test spinal inhibitions and the underlying networks

(A) Schematic diagram of the presynaptic inhibition of soleus Ia fibres. The conditioning stimulus induces the afferent volley in the tibialis anterior (TA) Ia afferent fibres and activates primary afferent depolarization (PAD) interneurons responsible for the presynaptic inhibition of Ia soleus fibres before the synapse with the soleus motoneuron. (B) Examples of waveforms of the unconditioned H-reflex (left), and the conditioned H-reflex (right) when preceded by the TA conditioning stimulus. The arrow indicates the trigger of the test stimulation. The conditioning stimulus consists of a train of 3 rectangular pulses, each of 1 ms duration, with an intensity 1.2 times the TA motor threshold (MT), delivered 21 ms before the test stimulation (see the beginning of the trace). (C) Schematic diagram of the postsynaptic inhibition induced by cutaneous stimulation. Conditioning stimulus induces the afferent volley in the cutaneous/nociceptive fibres (dotted line) in the sural nerve from the skin of the lateral side of the fifth toe activating spinal interneurons (INs) projecting on soleus motoneuron. (D) Examples of waveforms of the unconditioned H-reflex, and the conditioned H-reflex by electrical stimuli applied to cutaneous afferents of the sural nerve evoking postsynaptic inhibition. The conditioning stimulus consists of trains of 17 pulses, each of duration 1 ms, with an interpulse interval of 3 ms, delivered 50 ms before the test stimulus. The arrow indicates the triggers of the test stimulus.

Ordinate (B and D): amplitude of H-reflexes expressed in V.

Abscissa (B and D): Latency in ms.

Figure 2: Time courses of the modifications of presynaptic inhibition and postsynaptic inhibition presented over time in the control experiments.

(A) Amount of presynaptic inhibition is plotted against the eight time epochs (baseline, 0-10 minutes, 11-20 minutes, 21-30 minutes, 31-40 minutes, 41-50 minutes, 51-60 minutes, and 61-70 minutes). The dotted line (---) represents the baseline value for presynaptic inhibition.

(B) Amount of postsynaptic inhibition is plotted against the eight time epochs (baseline, 0-10 minutes, 11-20 minutes, 21-30 minutes, 31-40 minutes, 41-50 minutes, 51-60 minutes, and 61-70 minutes). The dotted line (---) represents the baseline value for postsynaptic inhibition.

Asterisks indicate significant differences between inhibition in the baseline and inhibition observed in time epochs. The results were significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). All results are expressed as mean \pm SEM. N = number of participants.

Ordinate: amount of inhibition as a percentage of baseline.

Abscissa: Time epochs from the onset of the experiments (base).

Figure 3: Time courses of the effects of furosemide on presynaptic inhibition of Ia fibres (A) and postsynaptic inhibition (B).

Mean (\pm SEM) presynaptic inhibition values (A) and postsynaptic inhibition values (B) expressed as a percentage of the baseline value over time (baseline, 0-10 minutes, 11-20 minutes, 21-30 minutes, 31-40 minutes, 41-50 minutes, 51-60 minutes, and 61-70 minutes) in control (\square) and furosemide (\blacksquare). Asterisks indicate statistically significant differences between control and furosemide. The results were significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). N = number of participants.

Figure 4: Time courses of furosemide effects on post-activation depression (A) and examples of waveforms of H-reflexes evoked at different frequencies for assessing the post-activation depression (B).

(A) Mean (\pm SEM) postsynaptic activation depression values expressed as a percentage of its baseline value over time (baseline, 0-10 minutes, 11-20 minutes, 21-30 minutes, 31-40 minutes, 41-50 minutes, 51-60 minutes, and 61-70 minutes) in control (\square) and furosemide (\blacksquare).

N = number of participants.

(B) Examples of waveforms of H-reflex evoked at 1 Hz (left), and at 0.16 Hz (right).

Ordinate: amplitude of H-reflex in V.

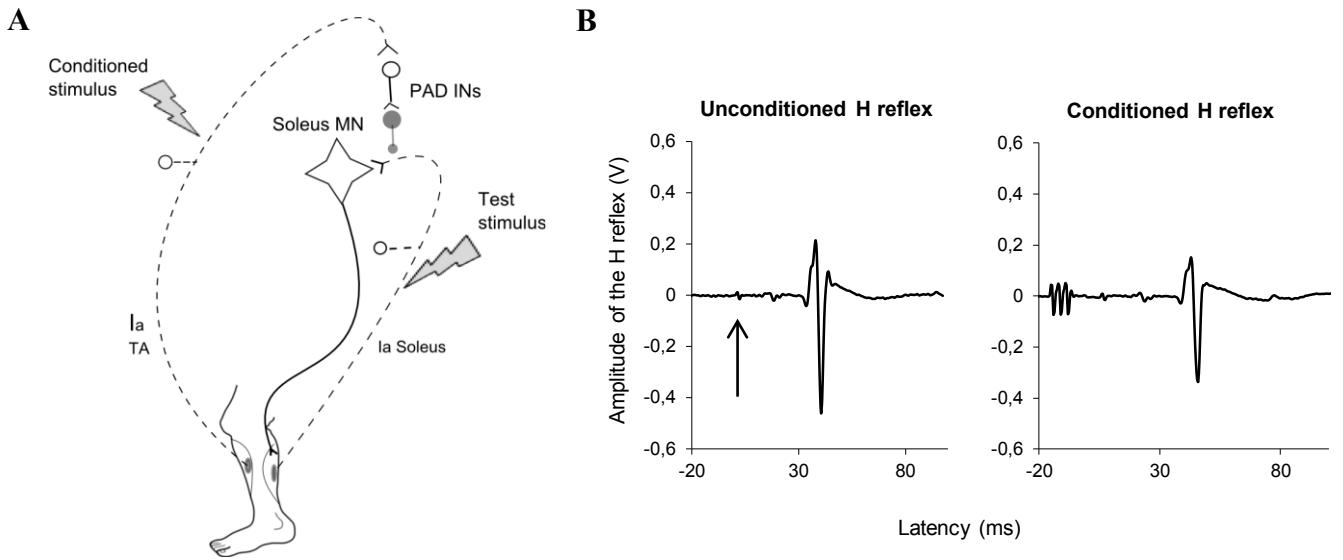
Abscissa: latency (ms)

Figure 5: Dose-effect relationships on presynaptic inhibition of Ia fibres (A) and postsynaptic inhibition (B).

Mean (\pm SEM) relative inhibitions expressed as % of its control value at 40-70 minutes after drug administration in the control group (0 mg furosemide), 20 mg furosemide group, and 40 mg furosemide group. N = number of participants.

Figure 1

Presynaptic inhibition of Ia fibres



Postsynaptic inhibition induced by cutaneous stimulation

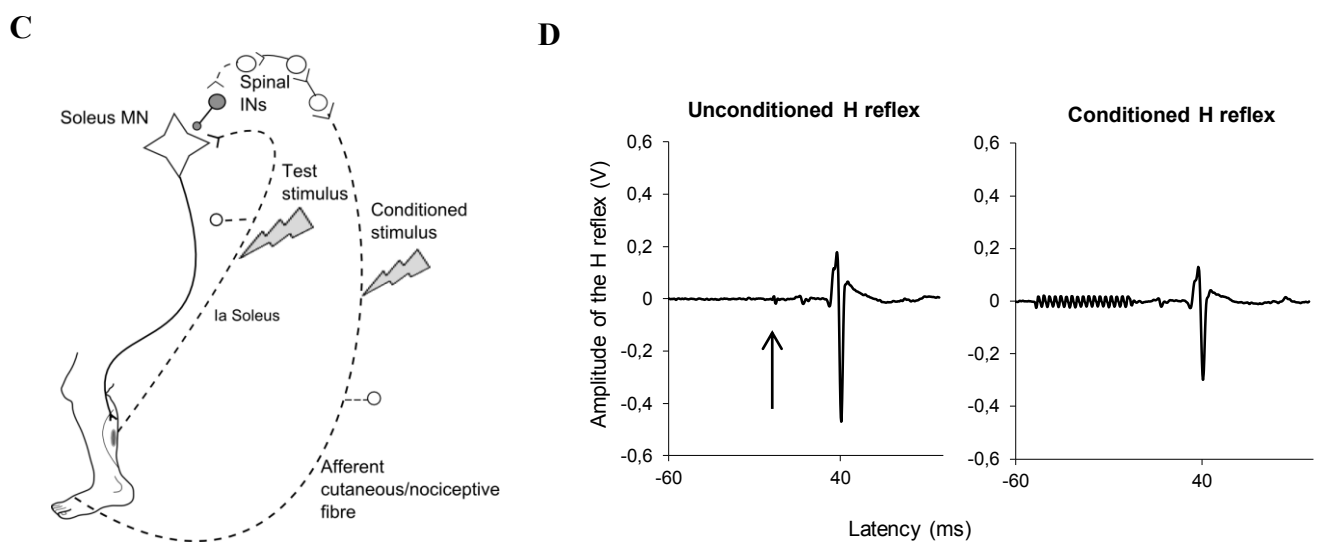
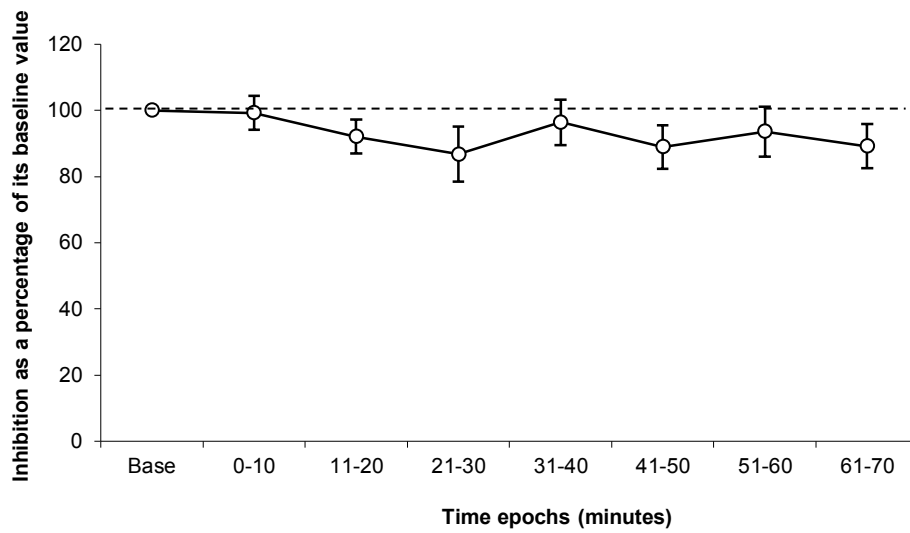


Figure 2

A

Presynaptic inhibition
(N = 19)



B

Postsynaptic inhibition
(N = 15)

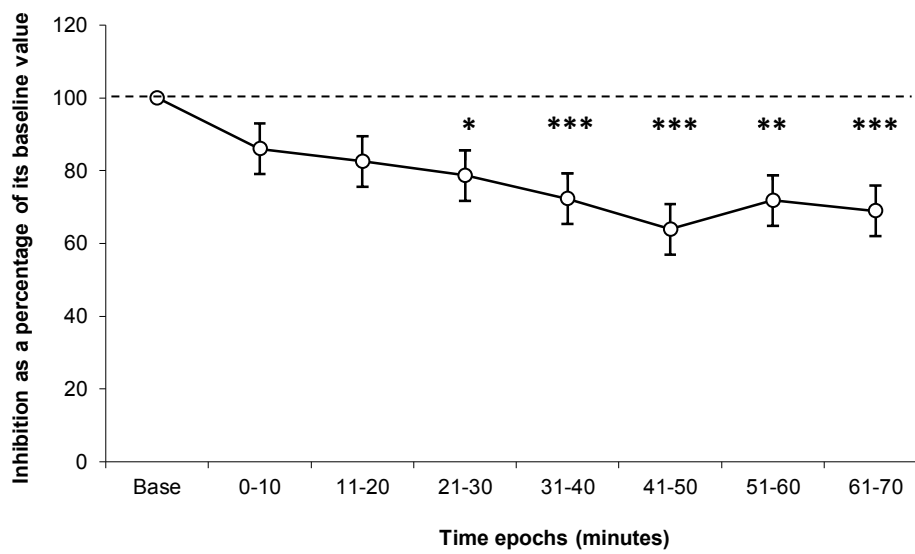
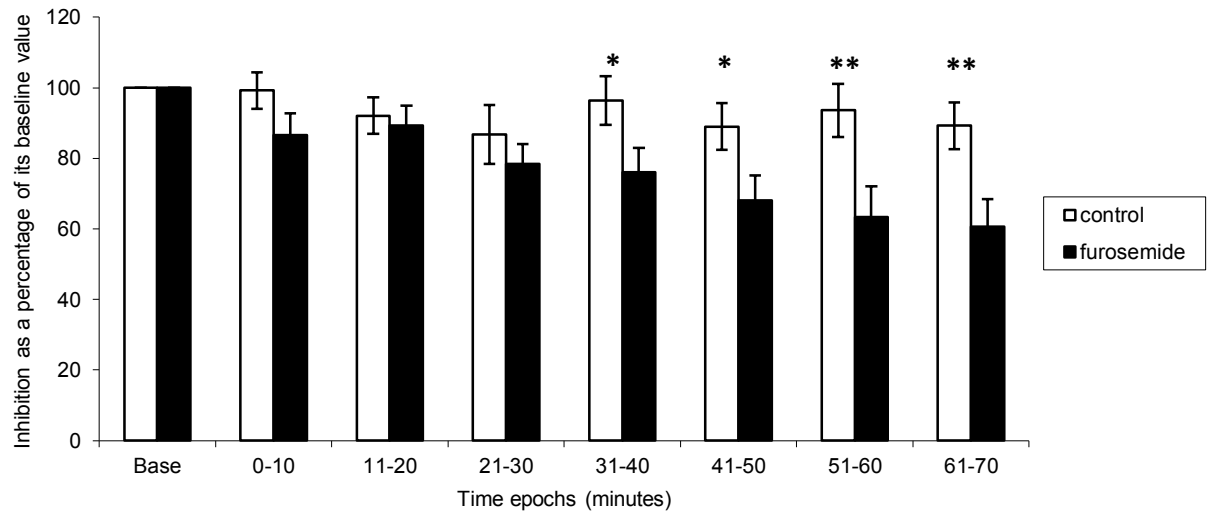


Figure 3

A

**Presynaptic inhibition
(N = 19)**



B

**Postsynaptic inhibition
(N = 15)**

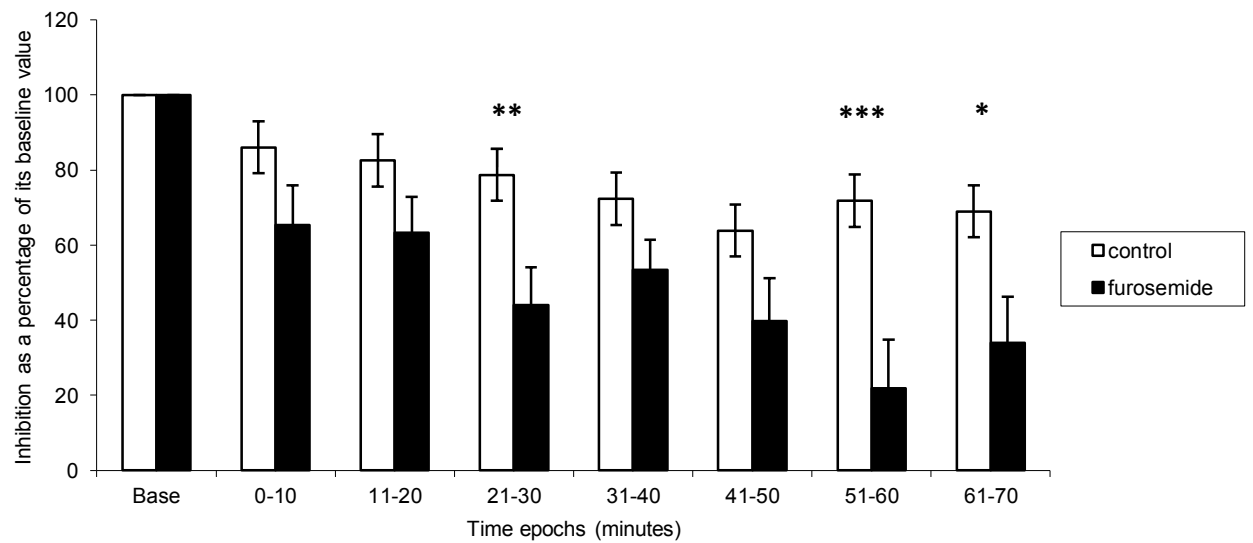
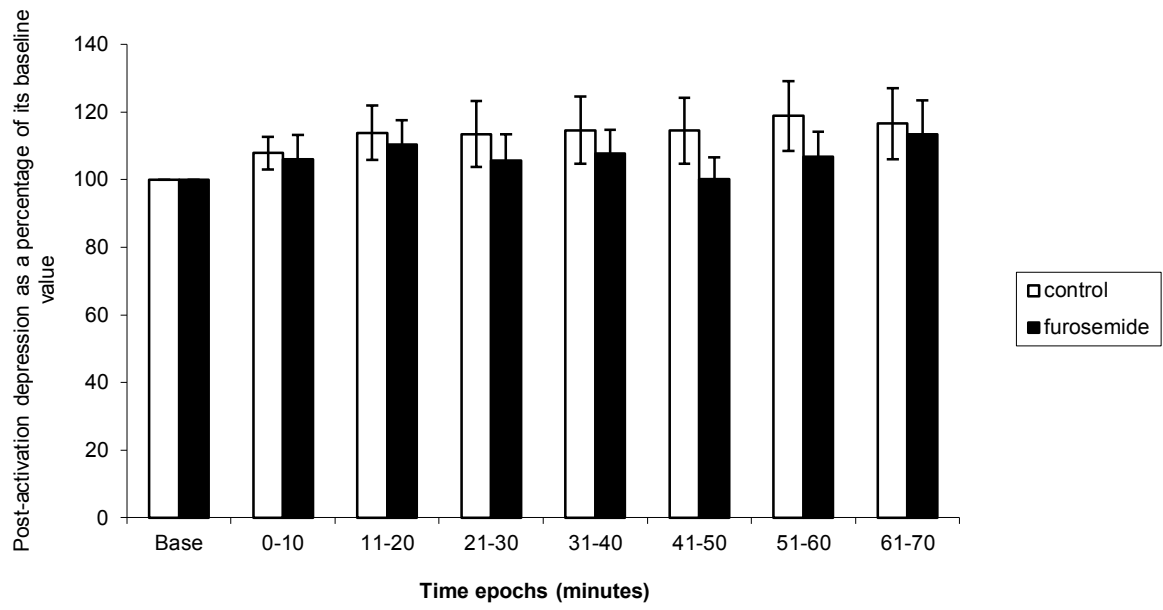


Figure 4

A

**Post-activation depression
(N=15)**



B

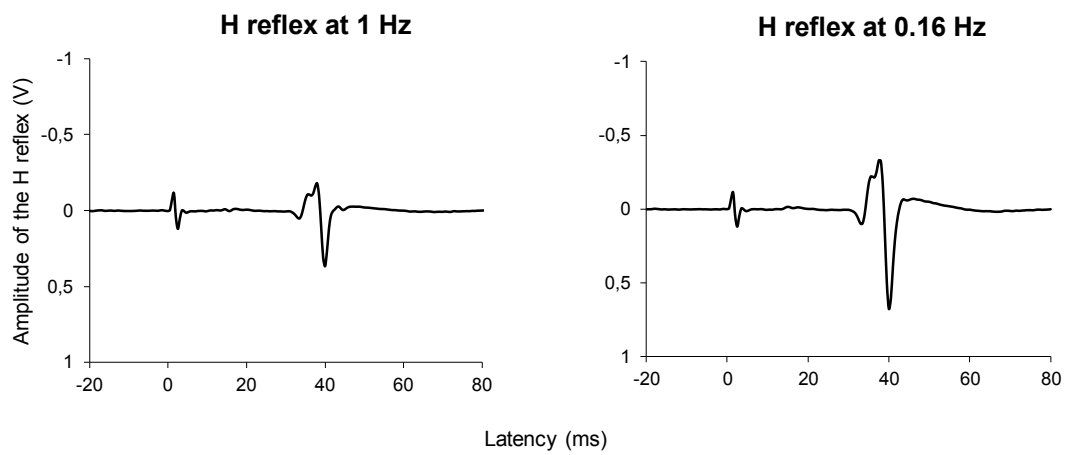
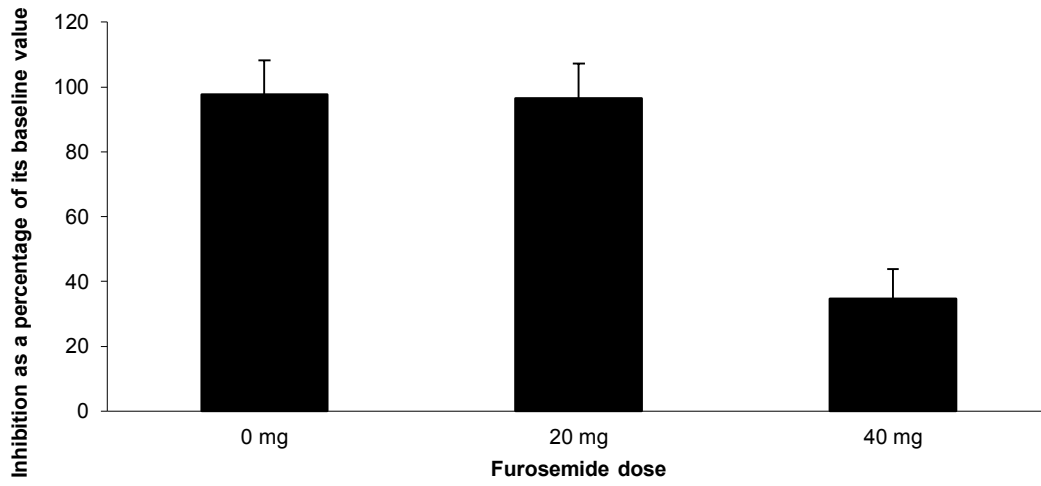


Figure 5

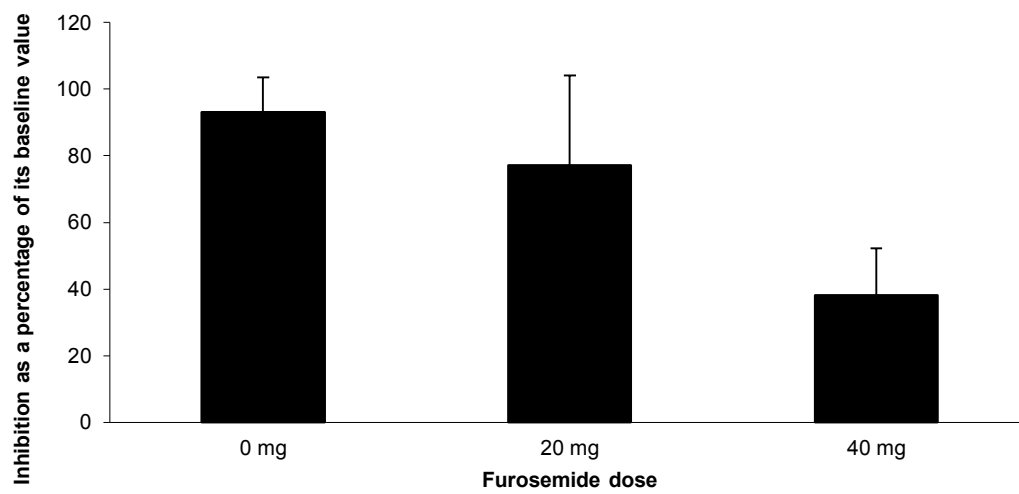
A

**Presynaptic inhibition
(N = 6)**



B

**Postsynaptic inhibition
(N = 6)**



1 **Research article**

2 **Behavioural/Systems/Cognitive**

3 ANODAL TRANSCRANIAL DIRECT CURRENT STIMULATION OF THE MOTOR CORTEX INDUCES
4 OPPOSITE MODULATION OF RECIPROCAL INHIBITION IN WRIST EXTENSOR AND FLEXOR

5 *Asymmetrical control of reciprocal inhibition at wrist level*

6

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23 **Keywords:** Motor cortex, Human, spinal cord

24 **Abbreviations:** transcranial direct current stimulation (tDCS), extensor carpi radialis (ECR),

25 flexor carpi radialis (FCR)

26

27 **Abstract**

28 Transcranial direct current stimulation (tDCS) is used to a non-invasive tool to modulate brain
29 excitability in humans. Recently, several studies have demonstrated that tDCS applied over
30 the motor cortex also modulates spinal neural network excitability and therefore, can be used
31 to explore the corticospinal control acting on spinal neurones. Previously, we showed that
32 reciprocal inhibition directed to wrist flexor motoneurons is enhanced during contralateral
33 anodal tDCS, but it is likely that corticospinal control acting on spinal networks controlling
34 wrist flexors and extensors is not similar. The primary aim of the study was to explore the
35 effects of anodal tDCS on reciprocal inhibition directed to wrist extensor motoneurons. To
36 further examine the supraspinal control acting on the reciprocal inhibition between wrist
37 flexors and extensors, we also explored the effects of the tDCS applied to the ipsilateral hand
38 motor area. In healthy volunteers, we tested the effects induced by sham and anodal tDCS on
39 reciprocal inhibition pathways innervating wrist muscles. Reciprocal inhibition directed from
40 flexor to extensor muscles and the reverse situation, i.e. reciprocal inhibition, directed from
41 extensors to flexors were studied in parallel using the H reflex technique.

42 Our main finding was that contralateral anodal tDCS induces opposing effects on reciprocal
43 inhibition: it decreases reciprocal inhibition directed from flexors to extensors but it increases
44 reciprocal inhibition directed from extensors to flexors. The functional result of these opposite
45 effects on reciprocal inhibition seems to favour wrist extension excitability suggesting an
46 asymmetrical descending control onto the interneurons that mediate reciprocal inhibition.

47 Words: 248

48

49 **Introduction**

50 The control of neural transmission in pathways mediating reciprocal inhibition in antagonist
51 muscles continues to raise questions ever since Sherrington (1906), who first introduced the
52 concept of reciprocal innervation. Intracellular recordings in cat spinal cord reveal a striking
53 similarity in the descending and segmental convergence on agonist α -motoneurons and Ia
54 inhibitory interneurons of the antagonist α -motoneurons (Lundberg 1970, Hultborn 1976).
55 Lundberg (1970) proposed that these connections from the brain to corresponding α -
56 motoneurons and Ia interneurons were also used in parallel during voluntary movement in
57 order to achieve a coordinated contraction and relaxation of the antagonist muscles.
58 Consistent with animal data, it has been shown in humans that pathways mediating reciprocal
59 inhibition in flexor and extensor motoneurons are disynaptic, and that motoneurons and
60 inhibitory interneurons have received similar segmental and descending control (Day et al.
61 1984, Cavallari et al. 1984, Shindo et al. 1984, Rothwell et al. 1984, Cowan et al. 1986). At
62 the level of the elbow and ankle, extensor and flexor muscles operate as *real* antagonists, so
63 interneurons relaying reciprocal inhibition to flexor and to extensor motoneurons are
64 identified as Ia inhibitory interneurons. At the wrist level, extensor carpi radialis (ECR) and
65 flexor carpi radialis (FCR) not only operate as antagonists but also as agonists in wrist
66 abduction so interneurons mediating reciprocal inhibition to α -motoneurons innervating
67 extensor and flexor muscles differ from those mediating Ia inhibition at the ankle and elbow
68 levels. The interneurons mediating reciprocal inhibition between FCR and ECR are not
69 inhibited by Renshaw cells (Aymard et al. 1995) but receive input from both Ia and Ib
70 afferent fibres (Wargon et al. 2006). It has been hypothesised that interneurons mediating
71 reciprocal inhibition between wrist flexors and extensors, probably share more characteristics
72 with Ib than Ia inhibitory interneurons. Regarding their descending control, Ib inhibitory

73 interneurons receive a powerful controlling input from the reticulospinal tract, and thus, are
74 influenced by both contra- and ipsi-lateral descending control (Crosby et al. 1962). Moreover,
75 Illert et al. (1981) have shown that forelimb muscle motoneurons receive a concomitant
76 descending control from both cortices. In humans, the role of the ipsilateral motor cortex is
77 still under debate although recent studies (Bradnam et al. 2011, McCambridge et al. 2011)
78 suggest that ipsilateral tDCS influences arm motor control. In humans, reciprocal inhibition
79 directed from ECR to FCR and its descending control from the contralateral hand motor
80 cortex have been studied following changes in excitability of contralateral hand motor cortex
81 induced by Transcranial Magnetic Stimulation (TMS) (Rothwell et al. 1984; Cowan et al.
82 1986) and by Transcranial Direct Current Stimulation (tDCS) (Roche et al. 2009). To our
83 knowledge, the control of transmission of the reciprocal inhibition from FCR to ECR has not
84 yet been described in humans. This is mainly due to the difficulty of evoking an H reflex in
85 extensor muscles in humans. Previous results suggest that corticospinal control is
86 asymmetrical between flexor and extensor ankle motoneurons in humans (Crone et al. 1987).
87 Although the presence of an ECR H reflex is rarely seen, we were able to recruit a sample of
88 healthy subjects exhibiting an H reflex in ECR at rest with sizeable amplitude allowing the
89 study of reciprocal inhibition directed to ECR motoneurons. The aims of this study were
90 therefore primarily to explore the effects of contralateral anodal tDCS on the reciprocal
91 inhibition directed from FCR to ECR, in order to compare corticospinal control acting on
92 interneurons mediating reciprocal inhibition from flexors to extensors and *vice versa* and
93 secondly to explore the possible effects of anodal tDCS applied over the ipsilateral motor
94 cortex on pathways that mediate reciprocal inhibition in flexor and extensor wrist muscles.

95

96

97 **Materials and Methods**

98 **Subjects**

99 Thirty-two healthy subjects ranging from 22 to 60 years (mean 31.1 ± 9.9 years), 20 females
100 and 12 males (9 left-handed and 23 right-handed), were included in this study. Note all
101 subjects participated in every experiment. Among them only 9 subjects had an H reflex in
102 ECR stable enough at rest to explore reciprocal inhibition directed from ECR to FCR. All
103 subjects gave written informed consent before participating in the experiments. This study
104 was performed according to the Code of Ethics of the World Medical Association
105 (Declaration of Helsinki), and was approved by the local ethical committee (CPP Ile de
106 France 6-Pitié-Salpêtrière).

107 **Transcranial direct current stimulation of the motor cortex**

108 tDCS was applied using a NEUROCONN DC-STIMULATOR (NEUROCONN GMBH
109 COMPANY, Ilmenau, Germany) *via* two conductive rubber electrodes placed in saline-
110 soaked sponges (5x7 cm). Transcranial magnetic stimulation (TMS) elicited by MAGSTIM
111 200 (MAGSTIM DYFED, UK), was used to determine the position of the anode positioned
112 over the hand motor cortex. The cathode was placed over the supra-orbital region. Three
113 conditions of tDCS were tested: i) the active contralateral condition: with the anode placed
114 over the contralateral hand motor cortex and cathode positioned over the ipsilateral supra-
115 orbital area; ii) the active ipsilateral condition; anode over the ipsilateral hand motor cortex
116 and cathode over the contralateral supra-orbital area; iii) the sham condition: the electrodes
117 were placed in the same position as in active conditions but, current was activated for only
118 120 seconds at the beginning and 30 seconds at the end of sham stimulation in order to mimic
119 the sensations of ramp up and ramp down current perceived in the active conditions. In the

120 experiments involving with the ECR H reflex, due to the difficulty of evoking a test H reflex
121 in ECR, the sham condition was performed in only one configuration with anodal tDCS
122 electrode placed over the contralateral motor cortex. We were careful to respect a minimum
123 delay of 48 hours between the different tDCS conditions and on average the delay between
124 two recording sessions was about one week. Moreover, in the present study, active and sham
125 contralateral tDCS was tested only on reciprocal inhibition directed from FCR to ECR.
126 Effects induced by contralateral tDCS on reciprocal inhibition directed from ECR to FCR
127 were obtained from our previous study (Roche et al. 2009).

128 In all conditions, current intensity was fixed at 1.75 mA. Current flowed continuously for 20
129 minutes for both active conditions, and for 150 seconds in the sham condition, since Nitsche
130 and Paulus (2000) had previously reported that a duration of at least 3 minutes of tDCS was
131 necessary to induce after-effects. The current was ramped up or down over the first and last 8
132 seconds of stimulation. The experimental procedure was identical to that used by Roche et al.
133 (2009).

134 **Measurement of spinal network excitability**

135 Electromyogram recordings

136 Subjects were seated in a comfortable reclining armchair with the shoulder slightly abducted
137 at about 60 degrees, the elbow semi-flexed and with slight pronation of the forearm. The
138 distal part of the upper limb was supported by an armrest in order to exclude any active
139 maintenance of wrist posture, and the subjects were asked to relax the arm.
140 Electromyographic (EMG) activity was recorded from FCR and ECR muscles using bipolar
141 surface electrodes positioned on the muscle belly. EMG activity was displayed in a wide
142 analysis window beginning 100 ms before, ending 200 ms after the test stimulus. To ensure

143 that subjects relaxed their wrist muscles, background EMG activity was measured *a*
144 *posteriori*, in the 100 ms pre-trigger windows with rectified EMG activity. EMG activity was
145 sampled at 2 kHz, amplified (x 5000 – 10000) and band-pass filtered (250 Hz–3 kHz) using a
146 Digitimer D360 amplifier (DIGITIMER LTD, WELWYN GARDEN CITY, Herts, UK. In all
147 subjects, experiments were performed on the dominant upper limb based on patient self-
148 reports.

149 Electrical stimuli

150 Transcutaneous electrical stimulation (rectangular pulses of 1 ms duration every 3 s) were
151 applied to radial and median nerves, *via* 3 cm diameter hemi-spherical bipolar electrodes. The
152 electrodes were placed near the cubital fossa to stimulate the median nerve and at the spiral
153 groove to stimulate the radial nerve.

154 *Test stimuli*

155 FCR H reflex

156 The FCR H reflex was evoked by electrical stimuli applied to the median nerve. First, the
157 maximum direct motor response (Mmax) was first determined and the unconditioned H reflex
158 was adjusted to 10% – 20% of Mmax in each subject (mean value $14.17 \pm 2.5\%$ Mmax). The
159 amplitude of the unconditioned FCR H reflex corresponded to 50% of the maximum
160 amplitude of H reflex in the majority of cases. It was kept constant throughout the
161 experiment, since the H reflex sensitivity to facilitation or inhibition can vary with its
162 unconditioned amplitude (Crone et al. 1990).

163 ECR H reflex

164 The ECR H reflex was evoked by radial nerve stimulation. As mentioned previously, it is
165 difficult to elicit the H reflex in ECR (Pierrot-Deseilligny and Burke 2012). Thirty-two
166 subjects participated in this study, but only 9 subjects had an ECR H reflex large enough at

167 rest to show changes in reciprocal inhibition. As above, the Mmax response was determined
168 first and the unconditioned ECR H reflex was adjusted to 5%-15 % of Mmax in each subject
169 and was kept constant throughout the experiment (mean value $6.62 \pm 1.4\%$ Mmax).

170 *Conditioning stimuli*

171 Conditioning stimuli were applied to the nerve innervating the antagonist muscle, i.e. that
172 counteracting the muscle in which the test H reflex was evoked. The intensity of the
173 conditioning stimuli was adjusted to activate group I fibres but was below the activation
174 threshold of Renshaw cells in order to prevent recurrent inhibition (Aymard et al. 1997). This
175 implies that the conditioning stimuli did not evoke either a direct motor response (which may
176 have an influence by antidromic activation of Renshaw cells) or an H reflex (which may have
177 influence by orthodromic activation of Renshaw cells). The motor threshold was determined
178 by the oscilloscope display of EMG. The average intensity of conditioning stimuli was $0.88 \pm$
179 0.02 motor threshold (MT) for the radial nerve, and was 0.66 ± 0.02 MT for the median nerve
180 (Table 1). The interstimulus interval (ISI) was the time interval between test and conditioning
181 stimuli for which the level of reciprocal inhibition was maximal. By convention, a positive ISI
182 corresponds to a conditioning stimulus preceding the test stimulus. The ISI was determined
183 using 0.5 ms steps in the range -3 ms/+ 1 ms for reciprocal inhibition from ECR to FCR (Day
184 et al. 1984) and -1 ms/+ 3 ms for reciprocal inhibition from FCR to ECR (Wargon et al.
185 2006). The mean ISI for reciprocal inhibition from ECR to FCR was 0.12 ± 0.6 ms and for
186 reciprocal inhibition from FCR to ECR was 0.60 ± 0.8 ms. The ISI was kept constant
187 throughout the experiments.

188 **Experimental procedure**

189 A randomised, sham-controlled tDCS study was performed. During the experiments, the
190 subjects were blind to the conditions of tDCS. The active contralateral condition, the active

191 ipsilateral condition, and the sham condition were randomly alternated. Each condition was
192 performed on the subjects on different days to avoid carry-over effects.

193 Once all parameters (unconditioned H reflex amplitude, conditioning stimulation intensity
194 and ISI) were set, the baseline inhibition (without tDCS) was determined over the first 10
195 minutes, being defined as the baseline period. Next, the tDCS electrodes were attached. The
196 anode was placed over the hand motor cortex for 20 minutes for both active (ipsi and
197 contralateral conditions) and sham tDCS. These 20 minutes were divided into two “10
198 minute” periods: period 1 and period 2. The tDCS electrodes were removed immediately after
199 the end of the stimulation. The amount of reciprocal inhibition was also measured for 10
200 minutes following the end of stimulation in order to evaluate post-effects (see diagram on
201 Fig. 1). In each period (baseline, period 1 tDCS, period 2 tDCS and post tDCS), the amount of
202 inhibition was assessed with three series of 40 H reflexes (20 conditioned H reflexes and 20
203 unconditioned H reflexes). Conditioned and unconditioned H reflexes were evoked every 3 s
204 and randomly alternated.

205 **Analysis**

206 Parameter definitions

207 The background EMG activity was evaluated by calculating the pre-trigger root mean square
208 (rms) of rectified EMG activity in the 100 ms window preceding the test stimulation. The
209 average H reflex size was determined from peak-to-peak amplitudes expressed as a
210 percentage of the maximum motor response (Mmax). The amount of reciprocal inhibition was
211 defined as $((\text{unconditioned H value} - \text{conditioned H value}) / \text{unconditioned H value}) \times 100$. The
212 mean amount of reciprocal inhibition from each period was normalised as a percentage of the
213 baseline inhibition measured over the baseline period (t_0) according to the equation:
214 $((\text{inhibition}_t - \text{inhibition}_{t_0}) / \text{inhibition}_{t_0}) \times 100$. Therefore when amount of reciprocal

215 inhibition was normalised to the baseline, a positive value means that inhibition was larger
216 than the baseline and a negative value means the inhibition was weaker than the baseline .

217 The normalised amounts of reciprocal inhibition were only used in the graphics representing
218 group data. Statistical analyses were performed on raw data, *i.e* with amount of reciprocal
219 inhibition expressed as a percentage of unconditioned H value.

220 Statistical analysis

221 For individual data, a two-way ANOVA was performed with time period as the first factor
222 (baseline, period 1 tDCS, period 2 tDCS, and post tDCS) and tDCS condition (sham, active
223 contralateral tDCS and active ipsilateral tDCS) as the second factor. When reciprocal
224 inhibition from FCR to ECR was investigated, we compared effects induced during sham,
225 active contralateral tDCS and active ipsilateral tDCS as subjects were examined in all 3
226 conditions. To evaluate effects induced by active ipsilateral tDCS on reciprocal inhibition
227 from ECR to FCR, we compared the modulations observed from sham and active ipsilateral
228 tDCS conditions, only since active contralateral tDCS had already been tested on another
229 group of subjects in our previous study (Roche et al. 2009).

230 A two-way ANOVA was performed on unconditioned H reflexes (with time period and
231 condition as factors) to ensure that the modulations of reciprocal inhibition resulted from
232 tDCS not from the variation of the H reflex over time. Moreover i) as amplitude of
233 unconditioned H reflex may affect the conditioned H reflex (Crone et al. 1990) and ii) as the
234 mean amplitude of unconditioned H reflex in ECR was smaller than that in FCR (mean value
235 of unconditioned ECR H reflex was $6.62 \pm 1.4\%$ Mmax, compared to mean value of
236 unconditioned FCR H reflex was $14.17 \pm 2.5\%$, *cf* Table 1) a linear regression between the
237 ratio ECR unconditioned H reflex/FCR unconditioned H reflex and the ratio of ECR

238 conditioned H reflex/FCR conditioned H reflex was calculated in order to ensure that
239 modulations induced by tDCS, were not affected by unconditioned H reflex amplitude .
240 Group data were analysed using two-way repeated-measures ANOVAs to determine effects of
241 tDCS and time period on reciprocal inhibition. Moreover, to determine if modulations of
242 reciprocal inhibition from ECR to FCR observed in left-handed subjects during active
243 ipsilateral tDCS, were different to that observed in right-handed subjects, a two-way ANOVA
244 was performed with period (baseline, period 1 tDCS, period 2 tDCS, post tDCS) as the first
245 factor and handedness (left-handed subjects, right-handed subjects) as the second factor. Note
246 both two way ANOVAs were performed on raw data using amount of reciprocal inhibition
247 expressed as a percentage of unconditioned H reflex rather than on normalised data
248 (reciprocal inhibition expressed as a percentage of baseline inhibition). To ensure that
249 background EMG activity was constant all over the time, and whatever the tDCS condition a
250 two-way ANOVA (testing tDCS condition and time period as factors) was performed on rms.
251 When the *F* value was significant, *post hoc* pairwise comparisons were performed using the
252 Newman-Keuls test. Degrees of freedom calculated by statistical analyses are indicated in
253 brackets after the *F* value. Significance was taken at $P < 0.05$. Mean data are shown as mean
254 ± 1 Standard Error of the Mean (SEM). Statistical analysis was performed using the
255 SigmaPlot software 11.0.

256 **Results**

257 Fig. 2 illustrates a representative example of unconditioned and conditioned reflexes obtained
258 over the baseline period in the same subject. Note that reciprocal inhibition induced in the
259 FCR H reflex (upper traces) is larger than that in the ECR H reflex lower traces. Table 1
260 summarises the mean unconditioned ECR and FCR H reflex amplitudes: for each tDCS
261 condition (sham, contralateral, ipsilateral) and period (baseline, during tDCS, after tDCS), no
262 significant change of unconditioned test reflex amplitude was observed.

263 **Background EMG activity**

264 To ensure that all experiments were conducted at rest we assessed the background EMG
265 activity by calculating the rms of rectified EMG activity in the 100 ms pre-trigger window.
266 The mean rms was calculated for experiments testing reciprocal inhibition directed from FCR
267 to ECR (mean rms = 0.103 ± 0.025 mV) and for experiments testing reciprocal inhibition
268 directed from ECR to FCR (mean rms = 0.109 ± 0.0143 mV). Statistical analysis with tDCS
269 condition as first factor (sham, active ipsilateral and active contralateral) and time period as
270 second factors attested that background EMG activity was constant over time and that it was
271 not affected by tDCS: experiments exploring reciprocal inhibition directed from ECR to FCR
272 ($F(2) = 1.868$ $P_{\text{condition}} = 0.177$, $F(3) = 0.853$ $P_{\text{period}} = 0.471$, $F(6) = 0.0198$ $P_{\text{condition} \times \text{period}} =$
273 0.996); experiments testing reciprocal inhibition directed from ECR to FCR : ($F(1) = 0.00561$
274 $P_{\text{condition}} = 0.960$, $F(3) = 0.350$ $P_{\text{period}} = 0.789$, $F(6) = 0.00369$ $P_{\text{condition} \times \text{period}} = 1.000$).

275 **Sham conditions**

276 Fig. 3A shows individual data obtained in 9 subjects in whom it was possible to evoke a
277 stable ECR H reflex at rest (5 right-handers, 4 left-handers). The amount of reciprocal
278 inhibition directed from FCR to ECR was plotted against the four time periods (baseline,
279 period 1 tDCS, period 2 tDCS and post tDCS). The amount of reciprocal inhibition did not
280 change significantly over time. The mean level of inhibition as a percentage of unconditioned
281 H reflex in ECR for each time period was: $17.80\% \pm 2.9$ in baseline period; $15.32\% \pm 2.6$ in
282 period 1 tDCS; $14.43\% \pm 2.3$ in period 2 tDCS and $14.16\% \pm 3.7$ in post tDCS.

283 Fig. 3C shows individual data obtained in 20 subjects (17 right-handers, 3 left-handers). The
284 amount of reciprocal inhibition directed from ECR to FCR did not change significantly over
285 time: the mean level of inhibition as a percentage of unconditioned FCR H reflex for each
286 time period was $34.63\% \pm 2.4$ in baseline period; $35.44\% \pm 3.4$ in period 1 tDCS; $37.59\% \pm$
287 3.2 in period 2 tDCS and $34.63\% \pm 2.3$ in post tDCS. These results confirm that whichever

288 reciprocal inhibition pathway explored (from FCR directed to ECR – Fig. 3A or from ECR
289 directed to FCR – Fig. 3C), sham tDCS never induced a significant change of reciprocal
290 inhibition.

291 **Active tDCS applied over the contralateral hand motor cortex**

292 Reciprocal inhibition from FCR to ECR

293 Fig. 3B shows individual data obtained from 9 subjects (5 right-handers, 4 left-handers). The
294 amount of inhibition of the ECR H reflex is plotted against the four time periods. In 9
295 subjects, the level of inhibition was lower in both periods 1 and 2, compared to baseline.
296 During the post tDCS period, the level of inhibition increased in 7 subjects compared to
297 period 2. The average reciprocal inhibition expressed as a percentage of unconditioned H
298 reflex in the 9 subjects was: 16.55% \pm 1.7 in baseline period; 7.23% \pm 3.1 in period 1 tDCS;
299 3.91% \pm 3.1 in period 2 tDCS and 10.92% \pm 2.4 in post tDCS

300 Fig. 4A shows group data with the amount of reciprocal inhibition from FCR to ECR
301 normalised to a percentage of its baseline value obtained from sham and active contralateral
302 tDCS conditions. The two-way repeated measures ANOVA showed an effect of time period
303 (baseline, period 1 tDCS, period 2 tDCS, post tDCS) and a significant interaction between
304 time period and tDCS condition (active contralateral, active ipsilateral and sham): $F(3) =$
305 2.951 $P_{\text{period}} = 0.05$, $F(2) = 0.993$ $P_{\text{condition}} = 0.395$, $F(2,3) = 3.412$ $P_{\text{condition} \times \text{period}} = 0.009$.
306 These results confirm that the mean values of reciprocal inhibition from the active
307 contralateral tDCS condition were dramatically lower in both periods 1 and 2 compared to
308 those obtained in the baseline period (before tDCS application) and from the sham condition
309 (Newman and Keuls analysis, baseline vs period 1 tDCS $P = 0.009$, baseline vs period 2 tDCS
310 $P < 0.001$, baseline vs post tDCS $P = 0.01$). At the end of stimulation the mean level of
311 reciprocal inhibition was restored to its baseline value (Newman and Keuls analysis; baseline

312 vs post tDCS $P = 0.223$ post tDCS vs period 1 tDCS $P = 0.07$, post tDCS vs period 2 tDCS P
313 $= 0.004$).

314 Note that the mean levels of reciprocal inhibition recorded during the baseline period were
315 similar in sham and active tDCS conditions. Mean inhibition as a percentage of H
316 unconditioned reflex was $17.80\% \pm 3.6$ in the sham condition compared to $16.55\% \pm 2.6$ with
317 contralateral stimulation. (Newman and Keuls analysis $P = 0.879$ (Table 1)). Moreover the
318 mean values of unconditioned H reflex amplitudes were not affected by condition or time
319 period ($F(2) = 0.799$ $P_{\text{condition}} = 0.412$, $F(3) = 1.518$ $P_{\text{period}} = 0.250$, $F(2,3) = 1.55$ $P_{\text{condition} \times}$
320 $\text{period}} = 0.359$). Mean values of unconditioned H reflex amplitude were similar during the
321 baseline period ($7.08 \pm 1.5\%$ Mmax in the sham condition compared to $7.46 \pm 1.3\%$ Mmax
322 with contralateral stimulation) (Table 1).

323 Reciprocal inhibition from ECR to FCR

324 The effects of active contralateral tDCS on reciprocal inhibition directed from ECR to FCR
325 were described in our previous study (Roche et al. 2009). The data presented in Fig. 4B were
326 extracted from our previous findings for comparison with the results obtained from the
327 present study of reciprocal inhibition directed from FCR to ECR (Fig. 4A). Reciprocal
328 inhibition was enhanced in periods 1 and 2 compared to that observed in the baseline period
329 and sham condition (Fig. 4B) (two-way repeated measures ANOVA (4 periods (baseline,
330 period 1 tDCS, period 2 tDCS, post tDCS) and 2 conditions (sham, active contralateral tDCS),
331 $F(3) = 2.062$ $P_{\text{period}} = 0.03$; $F(1) = 2.085$ $P_{\text{condition}} = 0.174$, $F(3,1) = 6.180$ $P_{\text{period} \times \text{condition}} <$
332 0.001). Reciprocal inhibition was restored to near baseline levels after the end of stimulation
333 for the active tDCS condition: Newman and Keuls analysis; baseline vs period 1 tDCS $P =$
334 0.04 , baseline vs period 2 tDCS $P < 0.001$, baseline vs post tDCS $P = 0.38$.

335

336 Influence of the unconditioned H reflex size on the amount of reciprocal inhibition.

337 It can be seen that the size of the unconditioned ECR H reflex was lower than that of the
338 unconditioned FCR H reflex (*cf* Table 1). Therefore, to ensure that the size of the
339 unconditioned H reflex did not impact on the results, two ratios; unconditioned ECR H
340 reflex/unconditioned FCR H reflex and conditioned ECR reflex/conditioned FCR H reflex
341 were calculated. A linear regression was performed to determine whether the difference
342 between ECR and FCR reflexes altered the amount of reciprocal inhibition (figure not
343 shown). The analysis revealed a significant linear correlation ($P < 0.001$; $R^2 = 0.957$)
344 indicating that the level of reciprocal inhibition in ECR and FCR muscles would be equal if
345 unconditioned H reflex evoked in ECR and FCR were similar.

346 **Active tDCS applied over the ipsilateral motor cortex.**

347 Reciprocal inhibition from FCR to ECR

348 Reciprocal inhibition directed from FCR to ECR was tested in 8 subjects as it was not
349 possible to perform the experiments with active ipsilateral tDCS in one subject. The mean
350 levels of inhibition, expressed as a percentage of unconditioned H test reflex were baseline:
351 $17.46\% \pm 3.9$ in baseline period; $15.65\% \pm 4.8$ in period 1 tDCS; $14.28\% \pm 5.1$ in period 2
352 tDCS and $= 14.13\% \pm 5.3$ in post tDCS. The level of inhibition for the group data is
353 illustrated in Fig. 5A which shows that tDCS applied over the ipsilateral motor cortex did not
354 modify reciprocal inhibition directed from FCR to ECR. The results obtained in sham and
355 active tDCS conditions are strikingly similar since the two curves can be superimposed. This
356 was confirmed by the post-hoc test indicating that the time period had no effect on the amount
357 of inhibition in sham and in active ipsilateral tDCS conditions ($P_{\text{period}} > 0.05$ during active
358 ipsilateral tDCS or during sham tDCS).

359 Reciprocal inhibition from ECR to FCR

360 First, reciprocal inhibition directed from ECR to FCR was compared during sham (Fig. 3C)
361 and anodal ipsilateral stimulation (Fig. 3D) in 20 subjects. As a percentage of unconditioned
362 test H reflex, the mean levels of inhibition were: 27.74 ± 2.7 in baseline period; 27.9 ± 3.1 in
363 period 1 tDCS; 29.82 ± 3.4 in period 2 tDCS; 28.08 ± 3.0 in post tDCS. The level of
364 inhibition for group data is illustrated in Fig. 5B which shows that ipsilateral tDCS did not
365 induce any statistical significant modification. Results obtained with anodal stimulation
366 strongly resemble those obtained with sham stimulation. This was confirmed by statistical
367 analysis indicating that the time period had no effect on the amount of inhibition in sham and
368 in active ipsilateral tDCS conditions (two-way ANOVA (2 conditions (sham, active ipsilateral
369 tDCS) X 4 time periods (baseline, period 1, period 2, post tDCS)), $F(3) = 1.829$ $P_{\text{period}} =$
370 0.152 ; $F(1) = 2.492$ $P_{\text{condition}} = 0.071$; $F(3,1) = 0.0868$; $P_{\text{period} \times \text{condition}} = 0.967$). Moreover the
371 mean values of unconditioned H reflex amplitudes were not affected by condition or time
372 period ($F(1) = 2.904$ $P_{\text{condition}} = 0.104$, $F(3) = 2.492$ $P_{\text{period}} = 0.071$, $F(1,3) = 0.119$ $P_{\text{condition} \times}$
373 $\text{period} = 0.948$). Mean values of unconditioned H reflex amplitude were similar during the
374 baseline period ($15.13 \pm 2.3\%$ Mmax in the sham condition compared to $13.00 \pm 1.9\%$ Mmax
375 with ipsilateral stimulation) (Table 1).

376 Subsequently, the number of subjects was increased in order to compare results obtained in
377 right-handed and left-handed subjects. Thirty-one subjects were included, of which 8 were
378 left-handed (individuals data are not shown). In the 23 right-handed subjects we found that
379 the level of inhibition was increased compared to baseline in 9 subjects and decreased in 14
380 subjects in period 1. In period 2, the values were increased compared to baseline in 15
381 subjects and decreased in 8 subjects. Mean levels of inhibition as a percentage of
382 unconditioned test H reflex were $24.02 \% \pm 2.4$ in baseline period $22.19 \% \pm 2.7$ in period 1
383 tDCS; $23.94 \% \pm 2.7$ in period 2 tDCS and $23.62 \% \pm 3.2$ in post tDCS. Moreover for the 8
384 left-handed subjects included in this series of complementary experiments, we found that in 7

385 subjects, the values were decreased compared to baseline in period 1 and in 4 subjects in
386 period 2. Mean level of inhibition as a percentage of unconditioned test H reflex was 26.77 %
387 ± 4.0 in baseline period, 22.01 % ± 5.4 in period 1 tDCS, 25.41 ± 6.6 in period 2 tDCS and
388 25.14 ± 4.9 in post tDCS. Group data are shown in Fig. 5C. Whatever the handedness, no
389 significant modification was seen during or after anodal ipsilateral tDCS (two-way ANOVA
390 $F(3) = 0.252$, $P_{\text{period}} = 0.860$; $F(1) = 0.243$, $P_{\text{handedness}} = 0.623$; $F(3,1) = 0.004$, $P_{\text{period} \times \text{handedness}}$
391 $= 0.987$).

392 **Study limitations**

393 tDCS methodological considerations

394 Repetitive TMS and tDCS are two tools currently available to non-invasively modify
395 excitability of the motor cortex but the two techniques differ mainly with regard to their
396 mechanisms and their spatial resolution (Priori et al. 2009). rTMS triggers action potentials in
397 cortical axons and subcortical white matter whereas tDCS leads to polarisation of neurons, but
398 does not induce axonal action potentials. Apart from its simplicity, the major argument in
399 favour of tDCS over rTMS is that rTMS produces effects on cortical excitability that may
400 occur not only at the stimulation site but also at distant connected sites. For example,
401 Wassermann et al. (1998) showed that 1 Hz rTMS applied over the primary motor cortex
402 (M1) changes interhemispheric and corticospinal excitability in the contralateral homologous
403 M1 as the authors found a small decrease in the MEP recruitment curve indicating a reduced
404 cortical excitability in the contralateral homologous cortex. By contrast, it has been shown
405 that tDCS applied to the left primary motor cortex can facilitate or suppress MEPs triggered
406 from the same hemisphere depending on the direction of current flow but has no effect on
407 MEPs evoked from the right primary motor cortex (Lang et al. 2004). Thus, despite the fact
408 that tDCS is applied through large electrodes and thus, less focal, we assume that it is more

409 suitable for the comparison of the effects of ipsi- and contralateral tDCS. The second
410 argument in favour of tDCS is related to of sham stimulation. TMS produces a loud click
411 when each stimulus is delivered and electric current induced in the scalp as well as in the
412 brain, leads to local activation of sensory nerves and muscle which is readily perceived by
413 subjects. However, even if the click is indistinguishable between real and sham TMS, the lack
414 of induced electric current means that there is no accompanying scalp sensation. In contrast
415 with tDCS, subjects only feel the current intensity variation, so they can be entirely unaware
416 of the difference between real and sham stimulation (Priori et al. 2009), even though here, for
417 simplicity's sake the experimenter was not blind to the intervention.

418 Since the tDCS electrodes applied over the hand motor cortex were always placed at the same
419 position to study the two types of reciprocal inhibition (directed from FCR to ECR and
420 directed from ECR to FCR), it could be argued that cortical structures activated by tDCS were
421 not required for both types of inhibition. However, imaging data have shown that
422 representative map areas of the FCR and ECR are very close or even intermingled
423 (Wassermann et al. 1994; Devanne et al. 2006). As the size of the active electrode applied
424 over the motor cortex is large (35 cm²), we assume that the cortical areas of FCR and ECR
425 were simultaneously stimulated during tDCS. Thus, the hypothesis that tDCS did not
426 stimulate a relevant cortical structure can be ruled out.

427 The main purpose of this study was to test the descending control from the motor cortex to
428 spinal interneurons, so we focussed on effects induced by tDCS on the reciprocal inhibition
429 pathways using only the anodal polarity, which increases cortical activity (Purpura and
430 McMurtry 1965). Moreover, in our previous study (Roche et al. 2009) we showed that
431 cathodal tDCS had no effect on reciprocal inhibition from ECR to FCR.

432 ECR and FCR H reflex methodological considerations

433 Thirty-two healthy subjects were enrolled in this study. Among them, 32 had an FCR H reflex
434 and only 9 of them had an ECR H reflex. This finding is in accordance with previous results
435 (Day et al. 1984; Aymard et al. 1995; Wargon et al. 2006). Furthermore, the amplitude of
436 unconditioned reflexes is usually smaller in ECR than in FCR H. The conditioning stimulus,
437 when applied to the radial nerve, does not usually evoke an H reflex in the ECR, while the
438 conditioning stimulus applied to the median nerve, usually evokes an FCR H reflex. To avoid
439 evoking FCR H reflexes which may induce activation of Renshaw cells between FCR and
440 ECR motoneurons (Aymard et al. 1995), a lower average intensity conditioning stimulus was
441 applied to the median nerve than that applied to the radial nerve (*cf.* Table 1). This lower
442 median nerve conditioning stimulus intensity probably contributes to the lower baseline level
443 of reciprocal inhibition in ECR compared to FCR motoneurons. However, in 1995, Aymard
444 et al. were able to find a subject in which the conditioning stimuli applied to the radial nerve
445 and to the median nerve were identical. In this condition, the amounts of reciprocal inhibition
446 were similar in both ECR and FCR reflexes (Aymard et al. 1995; Fig. 1). However, Crone et
447 al. (1990) emphasised that the amount of inhibition also depends on the unconditioned H
448 reflex amplitude, and the size of the unconditioned ECR H reflex was lower than the FCR H
449 reflex. It could be argued that the differential effects of tDCS on reciprocal inhibition directed
450 from ECR to FCR and from FCR to ECR could be at least partly due to differences in FCR
451 and ECR unconditioned reflex amplitude. However, we found a significant linear correlation
452 between the ratio of unconditioned ECR H reflex/unconditioned FCR H reflex and the ratio of
453 conditioned ECR H reflex/conditioned FCR H reflex indicating that the level of reciprocal
454 inhibition in ECR and FCR would be equal if unconditioned H reflexes evoked in ECR and
455 FCR were similar. This confirms that the effects of anodal tDCS applied over the contralateral
456 hand motor area cannot be explained by the difference in size of conditioned reflexes.
457 Moreover, differences between unconditioned reflex amplitudes may impact on quantitative

458 comparisons but would not influence the qualitative differences (facilitation vs inhibition).
459 Therefore, it is more likely that the reciprocal inhibition behaviour following tDCS strongly
460 reflects differences in the excitability of interneurons.

461 Among the 32 subjects having an FCR H reflex, 23 were right-handed and 9 left-handed,
462 whereas among the 9 subjects having an ECR H reflex, only 4 were left-handed. Therefore,
463 the comparison between left-handed and right-handed subjects, were restricted to reciprocal
464 inhibition directed from ECR to FCR.

465 Discussion

466 The main finding of the present study is that anodal tDCS applied over the contralateral hand
467 motor cortex increases reciprocal inhibition directed from wrist extensors to wrist flexors but
468 decreases reciprocal inhibition from wrist flexors to wrist extensors.

469 **Opposite effects of active tDCS applied to the contralateral hand motor cortex**

470 Since the position and stimulation characteristics of the active tDCS electrode were identical,
471 when we tested reciprocal inhibition from ECR to FCR and from FCR to ECR, hand motor
472 cortex excitability was the same for both directions of reciprocal inhibitions. The most
473 striking finding of the present series of experiments is that increasing the excitability of the
474 same motor cortex area, induces an increase of reciprocal inhibition from ECR to FCR but a
475 decrease in reciprocal inhibition from FCR to ECR. Evidence from both animal and human
476 studies (Purpura and McMurtry 1965; Priori et al. 1998), shows that anodal tDCS applied
477 over the motor cortex decreases the membrane resting potential of cortical cells, which are
478 spontaneously active in M1 (Evarts 1981). In our previous study (Roche et al. 2009), we
479 proposed that the enhancement of the reciprocal inhibition from ECR to FCR, following
480 tDCS, was due to an increase in efficiency of the descending volley reaching the

481 interneurons that mediate reciprocal inhibition from ECR to FCR. To understand the
482 opposing effects of interneurons that mediate reciprocal inhibition from ECR to FCR and
483 *vice-versa*, following the same changes in motor cortex excitability, we propose to take into
484 account the mutual inhibition between opposite-side interneurons that mediate reciprocal
485 inhibition, for example an FCR interneurone which mediates reciprocal inhibition from FCR
486 to ECR also inhibits the interneurone which mediates reciprocal inhibition from ECR to FCR
487 as depicted in Fig. 6. In animal studies, mutual inhibition between opposite-side interneurons
488 has been identified as a general central nervous system mechanism. Baldissera et al. (1987)
489 also demonstrated that mutual inhibition exists in humans at the wrist level. Thus, we could
490 propose two hypothetical mechanisms: i) that the descending control acting on interneurons
491 that mediate reciprocal inhibition is asymmetrical and is concentrated on interneurons that
492 mediate reciprocal inhibition from ECR to FCR (Fig. 6A), ii) the reciprocal inhibition at
493 spinal level between ECR and FCR is asymmetrical (Fig. 6B).

494 The first hypothesis is related to a study by Martens de Noordhout et al. (1999), suggesting
495 that the cortico-motoneuronal synaptic connections are stronger on wrist and finger extensor
496 motoneurons than on flexor motoneurons. Lundberg and collaborators have shown in the
497 cat that there is a corticospinal parallel control of motoneurons and of the corresponding
498 reciprocal Ia interneurons, i.e. the interneurons that mediate reciprocal inhibition to
499 antagonistic motoneurons (Lundberg 1970). Although the interneurone which mediates
500 reciprocal inhibition at the wrist level exhibits characteristics different from that of Ia
501 interneurons, it might thus be hypothesised that there is parallel descending control of ECR
502 motoneurons and of interneurons that mediate reciprocal inhibition from ECR to FCR (and
503 similarly from FCR motoneurons and interneurons that mediate inhibition from FCR to
504 ECR). If the descending control acting on ECR and interneurons that mediate reciprocal
505 inhibition from ECR to FCR, is stronger than the descending control acting on FCR, the

506 excitability of the interneurons that mediate inhibition from ECR to FCR (the **ECR**
507 interneurone in Fig. 6) is increased more than that of the interneurons that mediate reciprocal
508 inhibition from FCR to ECR (the **FCR** interneurone in Fig 6). As a consequence, mutual
509 inhibition between the two opposite-side interneurons is more strongly directed to the **FCR**
510 interneurone than **ECR** interneurone. Thus, the net effect of the descending control and
511 mutual inhibition is an increase in the excitability of the **ECR** interneurone. For the FCR
512 interneurone, its descending control is weaker and leads to the reverse situation: the weaker
513 facilitatory descending control is counteracted by greater mutual inhibition. Hence, this
514 results in a decrease in FCR interneurone excitability.

515 The second hypothesis is shown in Fig. 6B. The descending control from the hand motor
516 cortex projecting to the **ECR** and **FCR** interneurons is similar, but the reciprocal inhibition
517 and the mutual inhibition at the spinal level are asymmetric. The reciprocal inhibition is more
518 strongly directed from **ECR** interneurons to FCR motoneurons than in the reverse situation.
519 This hypothesis is supported by results observed at the lumbar level indicating that reciprocal
520 inhibition is asymmetric: the reciprocal Ia inhibition from extensors to flexors is more
521 powerful than the reciprocal Ia inhibition from flexors to extensors (R. Eccles and Lundberg
522 1958; Crone et al. 1987). Moreover, the activity of spinal circuits is often asymmetric with
523 regard to target motoneurons, for example, the monosynaptic reflex in FCR is stronger than
524 that in ECR. In this case, when the mutual inhibition is more powerful on **FCR** interneurons
525 than on **ECR** interneurons, the net effect on **FCR** interneurons is inhibitory whereas the
526 effect on **ECR** interneurons is facilitatory.

527 However, the indirect methods we used do not allow us to choose between these two
528 hypotheses. Furthermore both asymmetrical descending control and asymmetrical reciprocal
529 inhibition may coexist.

530 **Effects of tDCS applied to the ipsilateral hand motor cortex**

531 The possible effects of tDCS applied to ipsilateral hand motor cortex on spinal networks may
532 originate i) from ipsilateral connections to spinal neurones; or ii) hemispheric connections
533 from ipsilateral to contralateral homologous motor cortex areas.

534 Although the existence of uncrossed corticospinal tracts from motor cortex to spinal
535 motoneurones has been established in healthy humans and higher primates (Kuypers 1981),
536 the effects of activation of the ipsilateral cortex on spinal motoneurones are still under debate.

537 A few papers have reported ipsilateral responses in distal muscles during strong voluntary
538 contractions and high intensity TMS (Wassermann et al. 1991; Ziemann et al. 1999). Bawa et
539 al. (2004) concluded that, in general, forearm and hand muscles in healthy subjects, did not
540 show any ipsilateral motor evoked potentials. However, in post-stroke patients, ipsilateral
541 responses have been recorded (Alagona et al. 2001).

542 Interhemispheric connections between homologous cortical areas are well known and
543 generally considered to be inhibitory although the presence of excitatory interhemispheric
544 connections have also been proposed (Bloom and Hynd 2005). Furthermore, several studies
545 (for example, Hervé et al. 2005) suggest that ipsilateral control may be different in left-
546 handed and right-handed subjects. In the present study, we were unable to find significant
547 effects with ipsilateral anodal tDCS. If anything, it seems that a small decrease of reciprocal
548 inhibition from ECR to FCR may be occurring in the period 1, more marked in left-handed
549 than in right-handed subjects. Even if this decrease is inconsistent or a small decrease, this
550 effect may have therapeutic consequences. Indeed, it might be expected that coupling anodal
551 contralateral tDCS and cathodal ipsilateral tDCS might facilitate the effects of contralateral
552 anodal tDCS by excluding possible inhibitory effects from ipsilateral cortex.

553 **Functional significance and possible therapeutic applications**

554 Our findings have shown that an increase of excitability of contralateral hand motor cortex
555 results in an increase of reciprocal inhibition to FCR motoneurones, and in contrast a decrease

556 of the reciprocal inhibition to ECR motoneurons. In other words, it seems likely that the
557 function of this descending control is to facilitate ECR motoneurone excitability by reducing
558 inhibitory influences and to depress the excitability of FCR motoneurons by enhancing
559 inhibitory influences. Fine motor hand movements in humans (grasping, writing, typing, etc.)
560 require ECR contraction to stabilise the wrist joint. The descending control acting on
561 reciprocal inhibition is likely to contribute to the facilitation of ECR contraction. In this case,
562 anodal tDCS may be useful for the treatment of patients with cortical lesions who show wrist
563 extensor deficit. It might also be used to facilitate ECR contraction and promote control of
564 hand movements. This therapeutic approach is based on papers published since 2008 (for
565 review see Ayache et al. 2012), that indicate that anodal tDCS applied to the lesioned
566 hemisphere may improve motor performance.

567 Recent studies performed in stroke patients suggested that cathodal or dual tDCS may reduce
568 spasticity in upper limb muscles (Vandermeeren et al. 2013, Wu et al. 2013). Cathodal tDCS
569 was not tested in the present study but our findings that reveal an increase of reciprocal
570 inhibition directed from ECR to FCR, suggest that contralateral anodal tDCS may be used to
571 reduce flexor spasticity in stroke patients. Indeed, Nakashima et al. (1989) showed that
572 reciprocal inhibition between wrist muscles is decreased in spastic patients. Therefore, it may
573 be hypothesised that a reinforcement of reciprocal inhibition directed to flexor motoneurons
574 may limit the hyper-excitability of the FCR stretch reflex.

575 Moreover, even though it is inconsistent or small decrease, the ipsilateral effect may also
576 have some therapeutic consequences. Indeed, it could be expected that coupling anodal
577 contralateral tDCS and cathodal ipsilateral tDCS may facilitate the effects of contralateral
578 anodal tDCS by excluding possible inhibitory effects from ipsilateral cortex.

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699 motor-evoked potentials in human hand and arm muscles. *J Physiol* 518: 895-906, 1999.

700

701 **Figure legends**

702 **Figure 1: diagram adapted from Roche et al. (2009) illustrating the experimental**
703 **procedure.**

704 Experiments lasted about 40 minutes divided in 4 periods of 10 minutes: 10 minutes before
705 the beginning of tDCS baseline period, period 1 tDCS, period 2 tDCS and 10 minutes after
706 the end of tDCS (post tDCS). Current flowed continuously for 20 minutes for both active
707 conditions divided into two 10 minute periods (period 1 tDCS and period 2 tDCS), and for
708 120 s at the beginning of period 1 tDCS and 30 sec at the end of period 2 tDCS in the sham
709 condition. Conditioned and unconditioned H reflexes were recorded continuously during each
710 period of 10 minutes. Indeed a sequence of 40 H reflexes (20 conditioned H reflexes and 20
711 unconditioned H reflexes randomly alternated) lasted 3 minutes so 3 sequences of 40 H
712 reflexes were done in each period

713

714 **Figure 2: representative example of unconditioned and conditioned H reflexes in ECR**
715 **and FCR obtained in baseline period in the same subject.**

716 Fig. 2 **AB** illustrates an unconditioned (A) and a conditioned H reflex (B) in ECR.

717 Fig. 2 **CD** illustrates an unconditioned (C) and a conditioned (D) H reflex in FCR. By
718 convention the zero corresponds to the test stimulation represented by an artefact. Latency of
719 the H reflex was symbolized by the dotted line. Peak-to-peak amplitude of the H reflex was
720 expressed in mV.

721

722 **Figure 3: Individual data showing modulation of the two types of reciprocal inhibition**
723 **(from FCR to ECR and from ECR to FCR) induced by tDCS applied over the motor**
724 **cortex.**

725 Fig. **3AB**: modulations of reciprocal inhibition from FCR to ECR when tDCS is applied to
726 contralateral motor cortex: with sham tDCS (**A**) and active contralateral tDCS (**B**).

727 Fig. **3CD**: modulations of reciprocal inhibition from ECR to FCR when tDCS is applied over
728 the ipsilateral motor cortex: with sham tDCS (**C**) and with active ipsilateral tDCS (**D**). The
729 level of reciprocal inhibition is plotted against the four time periods (baseline, period 1 tDCS,
730 period 2 tDCS and post tDCS). Reciprocal inhibition is expressed as a percentage of the
731 unconditioned H reflex elicited in the ECR (**3A** and **3B**) or in the FCR (**3C** and **3D**) and then
732 calculated as follows: $(\text{unconditioned H} - \text{H conditioned})/\text{H test} \times 100$. Variations observed in
733 each subject are represented by different symbols (*cf.* drawing). The mean of amount of
734 reciprocal inhibition obtained in all subjects, represented by the black line.

735

736 **Figure 4: Group data representing modulations of the two types of reciprocal inhibition**
737 **(from FCR to ECR and from ECR to FCR) induced by tDCS applied over the**
738 **contralateral motor cortex.**

739 Fig. **4A**: modulations of reciprocal inhibition from FCR to ECR. Group data are calculated
740 with reference to the amount of inhibition in the baseline period. Amounts of inhibition are
741 plotted against time periods. Data obtained in the sham tDCS condition are represented by the
742 symbol (--○--) whereas those obtained in active contralateral tDCS condition are represented
743 by symbol (—●—). Vertical bars represent the standard error of the mean (± 1 SEM).
744 Asterisks (*) represent values significant at $P < 0.05$.

745 Fig. **4B**: modulations of reciprocal inhibition from FCR to ECR (taken from our previous
746 paper (Roche et al. 2009). Amounts of inhibition are plotted against time periods. Data
747 obtained in the sham tDCS condition are represented by symbol (--□--) whereas those
748 obtained in the active contralateral tDCS condition are represented by symbol (—■—). Vertical

749 bars represent the standard error of the mean (± 1 SEM). Asterisks (*) represent values
750 significant at $P < 0.05$.

751

752 **Figure 5: Group data representing modulations of the two types of reciprocal inhibition**
753 **(from FCR to ECR and from ECR to FCR) induced by tDCS applied over the ipsilateral**
754 **motor cortex.**

755 Fig. **5A**: modulations of reciprocal inhibition from FCR to ECR. Group data are calculated
756 with respect to the amount of inhibition observed in the baseline period. Amounts of
757 inhibition are plotted against time periods. Data obtained in the sham tDCS condition are
758 represented by the symbol (--○--) whereas those obtained in the active ipsilateral tDCS
759 condition are represented by the symbol (—▲—). Vertical bars represent the standard error of
760 the mean (± 1 SEM).

761 Fig. **5B**: modulations of reciprocal inhibition from ECR to FCR. Amounts of inhibition are
762 represented over time. Data obtained in sham tDCS condition are represented by the symbol
763 (—◆) whereas those obtained in the active ipsilateral tDCS condition are represented by the
764 symbol (-◇-). Vertical bars represent the standard error of the mean (± 1 SEM). Standard
765 error of the mean is included in the symbol.

766 **Fig 5C**: Impact of handedness on effects induced by ipsilateral anodal tDCS on reciprocal
767 inhibition directed from ECR to FCR. Group data comparing effects induced by active
768 ipsilateral tDCS in right-handed subjects and in left-handed subjects. Group data are
769 calculated with respect to the amount of inhibition observed in the baseline period. Amounts
770 of inhibition are plotted against time periods. Data obtained in right-handed subjects are
771 represented by the symbol (…△…) whereas those obtained in left-handed subjects condition are
772 represented by the symbol (✱). Vertical bars represent the standard error of the mean (± 1
773 SEM).

774 **Figure 6: schematic diagram of reciprocal inhibition between flexor and extensor:**
775 **cortical control and mutual inhibition**

776 Fig. **6A**: asymmetric descending control from motor cortex projecting onto spinal neurons
777 (interneurons and motoneurons) innervating ECR and FCR.

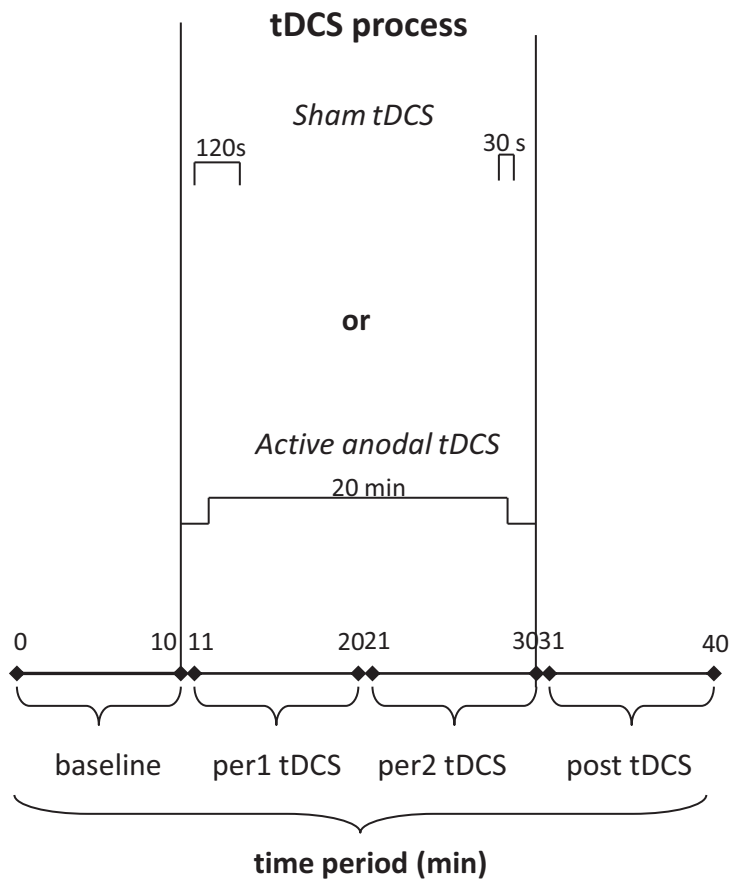
778 Fig. **6B**: asymmetric reciprocal inhibition at the spinal level. The thicker the lines, the
779 stronger the control. Black circles represent inhibitory interneurons (IN) of reciprocal
780 inhibition with their collaterals projecting onto the opposite-side interneurons. Excitatory
781 descending pathways from the motor cortex (contralateral and ipsilateral) projecting to
782 motoneurons (MN) and interneurons (IN) are shown with Y-shape terminals. Motoneurons
783 innervating ECR and FCR are star-like.

784

785 **Table 1:** Two ways repeated measures ANOVA were performed to compare effects of time
786 period (baseline, period 1, period 2 and post tDCS) and effects of tDCS condition (sham,
787 active contralateral and active ipsilateral) on amount of reciprocal inhibition and
788 unconditioned H reflex amplitudes.

789

790



Schema 1: adapted from Roche et al. 2009

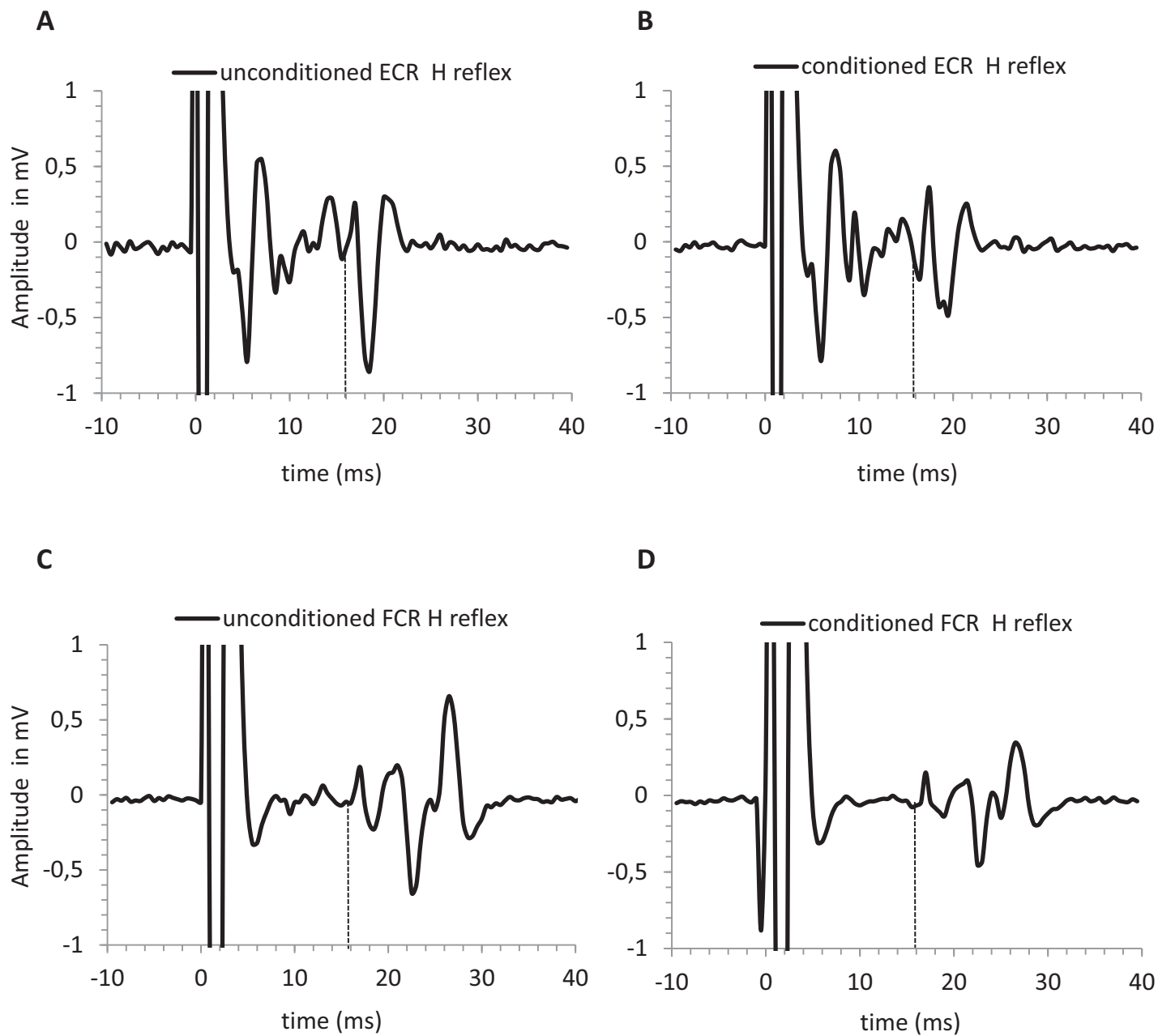
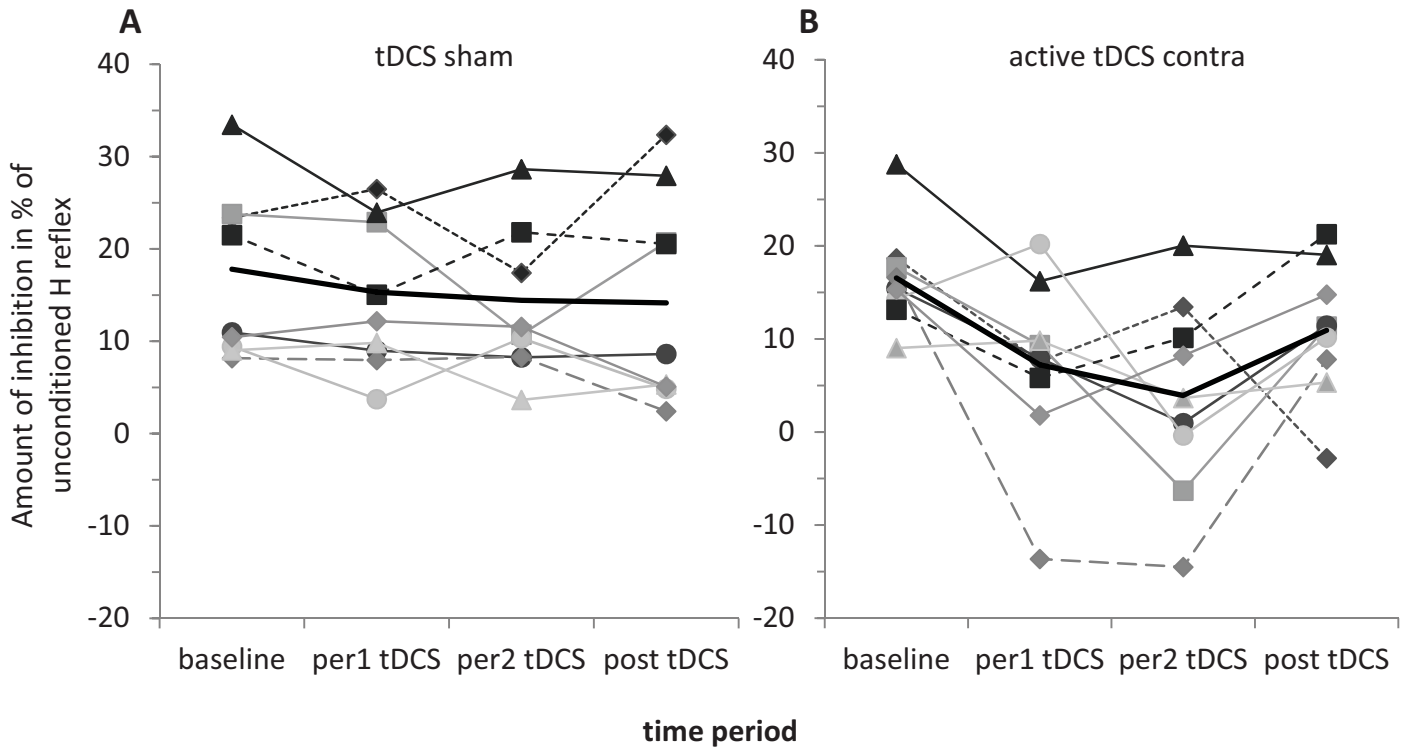


Figure 2

Reciprocal inhibition from FCR to ECR



Reciprocal inhibition from ECR to FCR

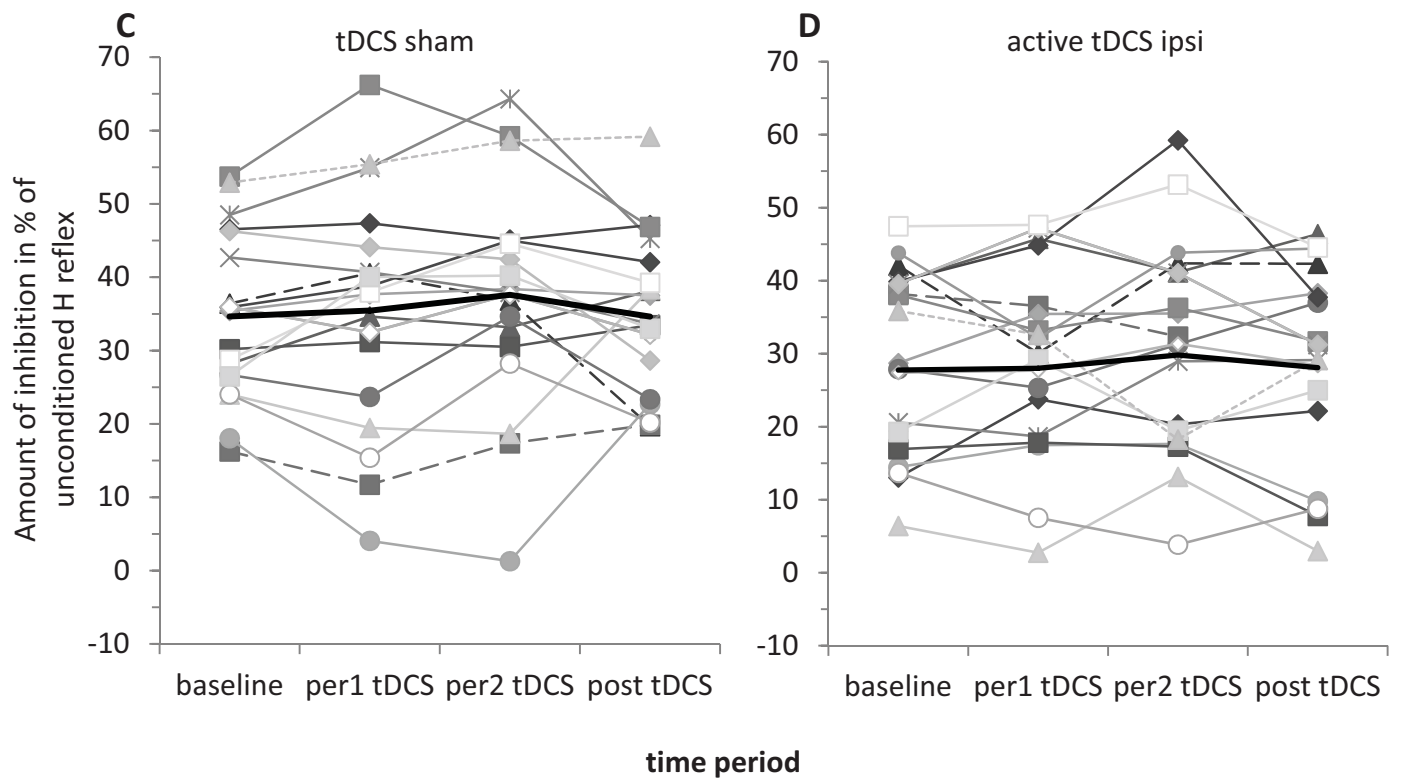
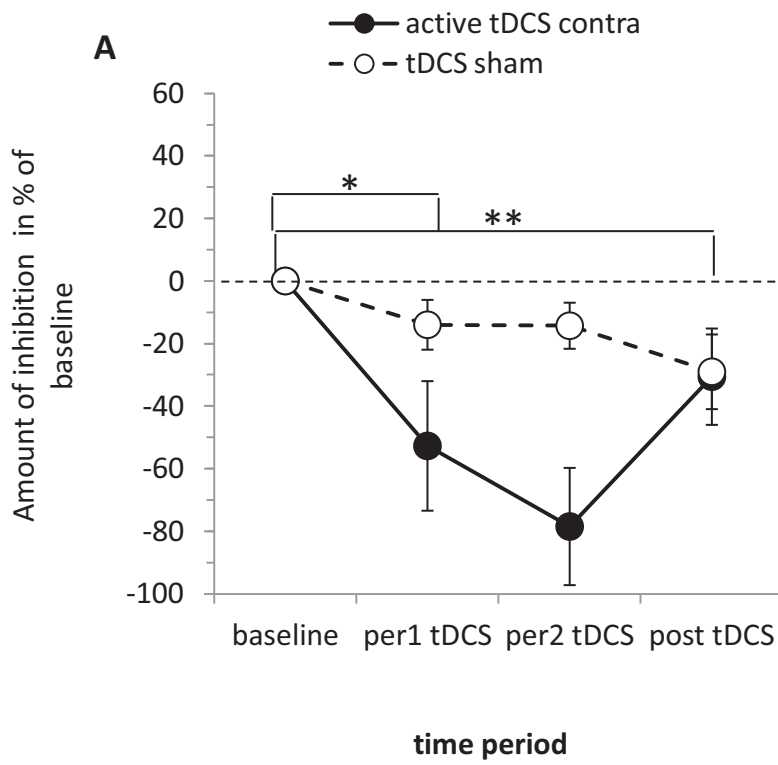


Figure 3

Reciprocal inhibition from FCR to ECR



Reciprocal inhibition from ECR to FCR

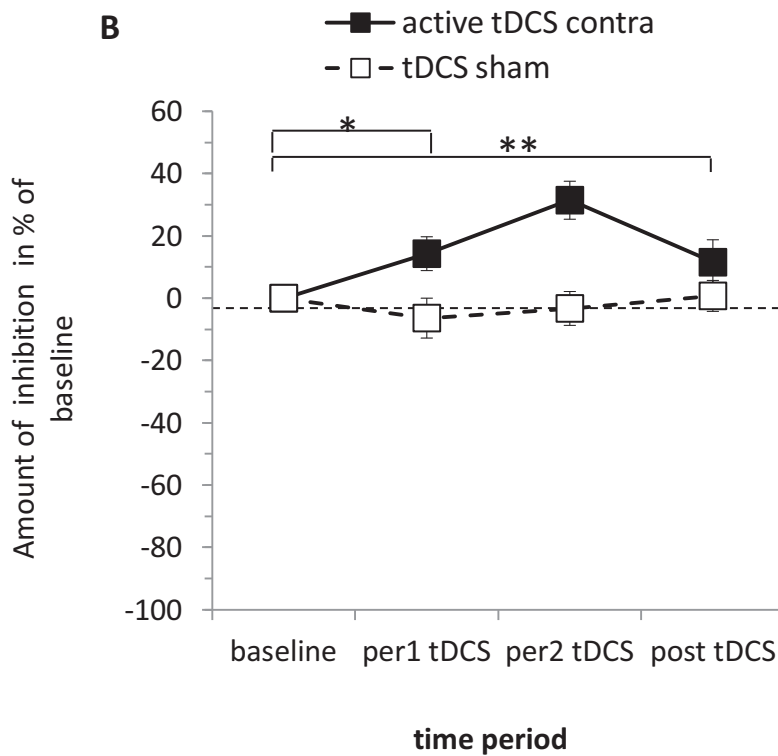
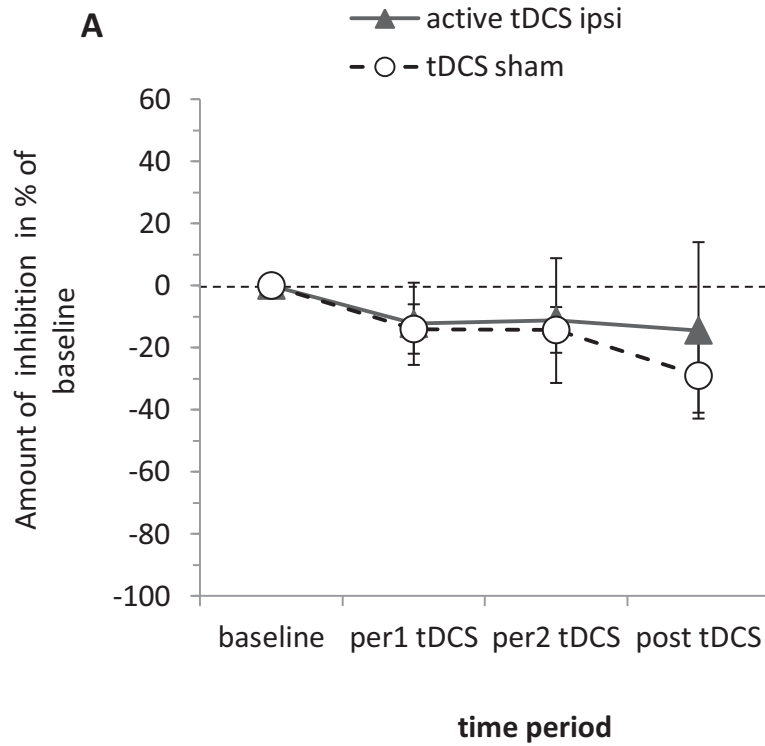


Figure 4

Reciprocal inhibition from FCR to ECR



Reciprocal inhibition from ECR to FCR

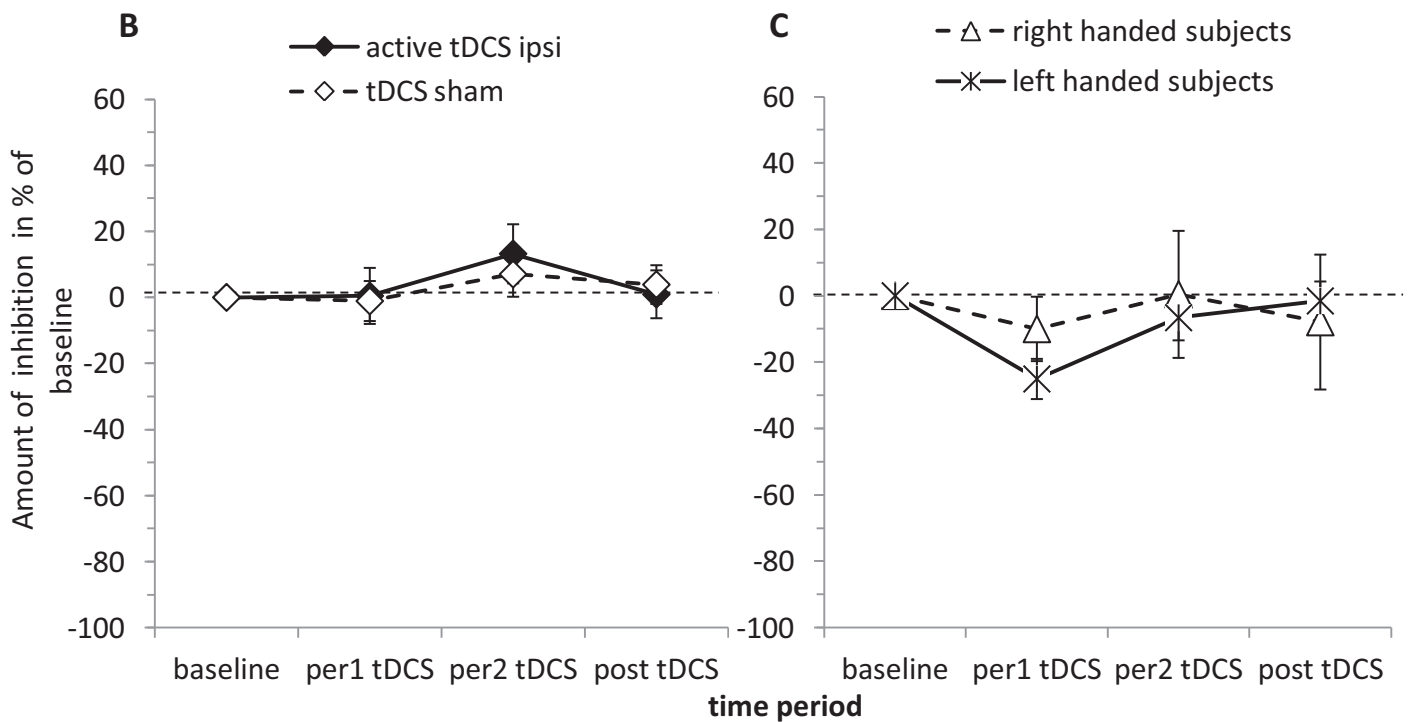


Figure 5

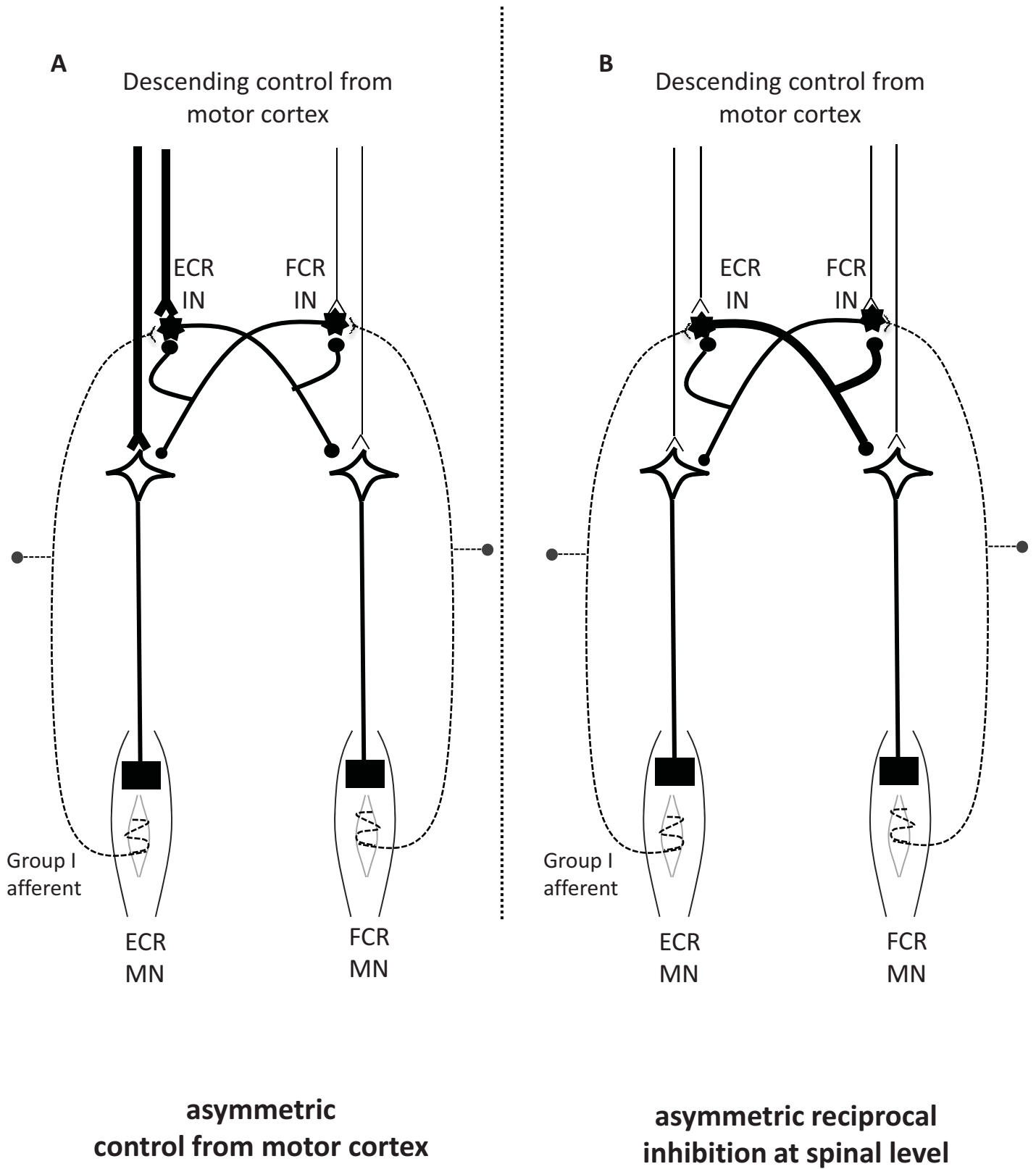


Figure 6

	tDCS conditions	Conditioning stimuli intensities (in % motor threshold)	Mean unconditioned H-reflex amplitudes (in % of Mmax)	Mean RI (in% unconditioned H-reflex) in baseline period
RI from FCR to ECR	<i>sham</i>	0.66 ± 0.02	baseline: 7.08 ± 1.5 period 1: 8.72 ± 2.9 period 2: 7.32 ± 2.1 post tDCS: 7.26 ± 2.3	16.55 ± 1.8
	<i>contralateral</i>		baseline: 7.46 ± 1.3 period 1: 9.01 ± 3.1 period 2: 8.87 ± 2.5 post tDCS: 7.16 ± 1.5	17.80 ± 2.9
	<i>ipsilateral</i>		baseline: 10.9 ± 1.7 period 1: 11.9 ± 1.8 period 2: 13.21 ± 2.8 post tDCS: 11.63 ± 1.8 (P _{period} = 0.189, P _{condition} = 0.603 P _{period X condition} = 0.498)	17.46 ± 3.9 (P _{period} = 0.05, P _{condition} = 0.713 P _{period X condition} = 0.009)
RI from ECR to FCR	<i>sham</i>	0.88 ± 0.02	baseline: 15.13 ± 2.3 period 1: 16.12 ± 3.1 period 2: 15.91 ± 3.1 post tDCS: 15.24 ± 2.6	34.64 ± 2.4
	<i>ipsilateral</i>		baseline: 13.00 ± 1.9 period 1: 12.66 ± 3.8 period 2: 13.05 ± 2.3 post tDCS: 12.31 ± 2.1 (P _{period} = 0.071, P _{condition} = 0.104, P _{period X condition} = 0.948)	27.74 ± 2.7 (P _{period} = 0.152, P _{condition} = 0.071, P _{period X condition} = 0.967)

Table 1

Two ways repeated measures ANOVA were performed to compare effects of time period (baseline, period 1, period 2 and post tDCS) and effects of tDCS condition (sham, active contralateral and active ipsilateral) on amount of reciprocal inhibition and unconditioned H reflex amplitudes.