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► To cite this version:

Wanalee Klomjai, Nicolas Roche, Jean-Charles Lamy, Peter Bede, Alain Giron, et al.. Furosemide unmasks inhibitory dysfunction following spinal cord injury in humans: implications for spasticity. *Journal of Neurotrauma*, 2019, 36 (9), pp.1469-1477. 10.1089/neu.2017.5560 . hal-02274778

HAL Id: hal-02274778

<https://hal.sorbonne-universite.fr/hal-02274778>

Submitted on 30 Aug 2019

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Furosemide unmasks inhibitory dysfunction following spinal cord injury in humans: implications for spasticity

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Running title: furosemide effect in SCI

Key words: spinal cord injury (SCI), spasticity, furosemide, inhibitory dysfunction

Number of pages: 21

Number of figures: 4; **Number of tables:** 2

Abstract: 247 words

Manuscript: 4034 words

Abstract

Spasticity after spinal cord injury has considerable quality of life implications, impacts on rehabilitation efforts and requires long-term multidisciplinary pharmacological and non-pharmacological management. The potassium chloride co-transporter (KCC2) plays a central role in intracellular chloride homeostasis and the inhibitory function of mature neurons. Animal studies have consistently demonstrated a down-regulation of KCC2 activity following spinal cord transection, causing a shift from the inhibitory action of gamma-aminobutyric acid and glycine to an excitatory effect. Furosemide, a recognised KCC2 antagonist in animals, blocks the formation of inhibitory postsynaptic potentials in spinal motoneurons without affecting excitatory postsynaptic potentials.

Based on observations in animals studies, we hypothesized that furosemide may be used to unmask KCC2 down-regulation following spinal cord injury in humans which contributes to reflex hyperexcitability. We have previously shown that furosemide reduces both presynaptic and postsynaptic inhibition in healthy subjects without altering monosynaptic excitatory transmission. These findings provide evidence that furosemide may be used in humans to evaluate inhibitory synapses in the spinal cord.

In this present study, we show that furosemide fails to modulate both pre- and postsynaptic inhibitions relayed to soleus spinal motor neurons in people with spinal cord injury. The lack of furosemide effect following spinal cord injury, suggests KCC2 dysfunction in humans, resulting in reduced inhibitory synaptic transmission in spinal neurons. Our findings suggest that KCC2 dysfunction may be an important aetiological factor in hyperreflexia following spinal cord injury. These observations may pave the way to novel therapeutic strategies against spasticity centred on chloride homeostasis.

Introduction

Our current understanding of the pathophysiology of spasticity following spinal cord injury (SCI) is largely based on landmark animal lesion studies. In animals, the expression of cation chloride co-transporters (CC), such as sodium potassium CC 1 (NKCC1) and potassium CC 2 (KCC2) controls intracellular chloride concentration ($[Cl^-]$). In mature neurons, the predominant role of KCC2 is maintaining low intracellular $[Cl^-]$ levels, which is essential for the hyperpolarizing effect of gamma-aminobutyric acid (GABA) and glycine and thus their well-established inhibitory effect. In immature neurons however, the predominant expression of NKCC1 leads to relatively higher intracellular $[Cl^-]$ concentrations, thus the primary effect of glycine and GABA exposure is often excitatory¹⁻⁴ rather than inhibitory. During neuronal maturation, the incremental expression of KCC2 leads to decreasing $[Cl^-]$ concentrations and GABA and glycine become gradually inhibitory in mature neurons⁴⁻⁶. In rodents, spinal cord injury (SCI) induces a down-regulation of KCC2 function back to its baseline “immature” activity^{7,8} which reduces the inhibitory effect of GABA and glycine⁹. Despite ample evidence from animal models that regression to immature neuronal $[Cl^-]$ homeostasis contributes to spinal reflex abnormalities in SCI, this mechanism has not been comprehensively evaluated in humans to date. The reason for the relative scarcity of human studies is methodological as most animal studies rely on invasive approaches. While non-invasive techniques have been proposed to study inhibitory dysfunction in human SCI¹⁰, these methods assess the activity of entire pathways and do not allow to distinguish between afferent volley dysfunction, altered interneuron excitability and intrinsic synaptic pathology. In animals, furosemide has been shown to selectively block inhibitory postsynaptic potentials (IPSP) in spinal motoneurons¹¹, and is recognised as a potent KCC2 antagonist^{12,13}. We have recently demonstrated that furosemide can also be used to evaluate inhibitory synapses in humans¹⁴. In healthy subjects, furosemide reduces the efficiency of pre- and postsynaptic inhibitions relayed to α -soleus motoneurons without affecting monosynaptic excitatory transmission. In this present study, we use a similar paradigm in people with SCI to evaluate if KCC2 dysfunction contributes to impaired inhibitory transmission in spinal neurons. We hypothesised that if KCC2 down-regulation also exists in humans following SCI, a

decreased furosemide effect will be observed in people with SCI compared to healthy subjects.

Methods

Subjects

The experimental procedures were conducted in accordance with the ethical guidelines of the World Medical Association (Declaration of Helsinki) and were also approved by the local ethics committee; CPP Île-de-France VI - Pitié-Salpêtrière. Written informed consent was obtained from all subjects prior to enrolment. Patients undergoing rehabilitation were recruited from the Physical Medicine and Rehabilitation Department of Raymond Poincaré Hospital. A standardised clinical and biochemical testing was undertaken prior to study-inclusion to identify and exclude subjects with contraindications to furosemide. Blood tests included serum sodium, potassium, bicarbonate chloride, creatinine, and glucose. In accordance to local protocols participants were only enrolled in the study if their serum creatinine values were between 44 and 80 $\mu\text{mol/L}$. The absolute contraindication to furosemide administration included abnormal serum biochemistry screen, sulfamide allergy, hepatic encephalopathy, galactosaemia and lithium therapy. A total of twenty-one adults with SCI were included in this study based on the following criteria: i) presence of paraplegia/tetraplegia following SCI (complete or incomplete) and ii) presence of a readily detectable H-reflex in the soleus muscle. The clinical and demographic profile of the subjects and their pre- and postsynaptic inhibition indices are presented in Tables 1 and 2. The results obtained in adults with SCI were compared to 19 healthy subjects (22-64 years old, 35.1 ± 2.5 years, 9 females) from our previous study¹⁴. The cohort of healthy controls is matched for age to the group of SCI subjects included in this study (Mann Whitney Rank Sum test, $P = 0.228$).

Experimental procedures

The experimental paradigm for evaluating furosemide-effect on pre- and postsynaptic inhibition on spinal soleus motor neurons has been previously described in healthy subjects¹³. In this study, we have meticulously implemented the same experimental protocol in people with SCI.

Study participants were evaluated in a sedentary position in their wheelchairs. We examined the leg with the greater soleus H-reflex, which was stable enough to monitor variations in pre- and postsynaptic inhibition magnitudes. The examined leg was supported in a semi-flexed hip position at 120°, in slight knee flexion at 160° and plantar flexion at 110°. Pre- and postsynaptic inhibitions relayed to soleus motoneurons were assessed using a well-established monosynaptic H-reflex method evoked in soleus (For references see Pierrot-Deseilligny and Burke 2012¹⁰). Our standardised protocol included a run of 60 H-reflexes in the soleus; 20 unconditioned H-reflexes, 20 conditioned H-reflexes to evaluate presynaptic inhibition and 20 conditioned H-reflexes to assess postsynaptic inhibition. Conditioned and unconditioned H-reflexes were evoked every 3 s and randomly alternated. Prior to furosemide administration, pre- and postsynaptic inhibition baselines were documented. Subsequently, 40 mg furosemide was orally administered, and inhibitory function was evaluated by repetitive measurements for 70 minutes. The timeline of the experiment was designed to match the pharmacokinetics of furosemide which typically appears in the serum within 10 minutes of administration and peaks between 60 to 90 minutes^{15–17} after administration. In order to carefully monitor variations in pre- and postsynaptic inhibitions over time, each experiment was divided into 8 time intervals: baseline, 0-10 min, 11-20 min, 21-30 min, 31-40 min, 41-50 min, 51-60 min, and 61-70 min (see **Figure 1A**). Each 10-minute period included 3 runs of 60 H-reflexes as described above. Peak-to-peak amplitudes of unconditioned H-reflexes were compared to those of conditioned H-reflexes (see **Table 1**). Data from 3 runs were averaged within each time period.

Electrophysiological recordings

Test Stimulation: soleus H-reflex

The soleus H-reflex was evoked using a constant-current stimulator (D7SA Digitimer Ltd, Welwyn Garden City, UK) with a rectangular 1 ms duration stimulus, delivered every at 0.33 Hz. Posterior tibial nerve stimulation was applied between a cathode (2.5 cm diameter brass) placed at the popliteal fossa and an anode placed above the patella (4 cm² plate) (custom-made electrodes). Bipolar surface electrodes (Delsys Inc., Boston, MA, USA; Ag electrodes DE-2.1) were positioned on the skin parallel to the soleus muscle belly to record the H-reflexes. EMG signals were amplified (×1000), band pass filtered at 20–450 Hz

(Delsys), digitized at 1 kHz (Power 1401 A/D board, Cambridge Electronic Design) and stored on a computer for offline analyses. At the beginning of each experiment, the maximum motor response ($M_{\max}$) and maximum H-reflex response ($H_{\max}$) were recorded. The stimulus intensity was adjusted to elicit an H-reflex amplitude of about 50% of $H_{\max}$, which corresponded to 20-35% of the $M_{\max}$. We verified that the amplitude of the unconditioned H-reflex was always about 50% of $H_{\max}$ and was constant throughout the experiment as the sensitivity of the H-reflex to facilitation or inhibition depends on its unconditioned amplitude¹⁸.

Inhibitory conditioning stimulations

Presynaptic inhibition

To assess presynaptic inhibition of the soleus Ia afferent fibers, a conditioning stimulus was applied to the nerve innervating the tibialis anterior muscle as described by Mizumo et al.¹⁹ The soleus H-reflex was conditioned by the stimulation of the common peroneal nerve (CPN) using bipolar electrodes (3 cm diameter hemispherical brass electrodes) placed 2 cm below the neck of the fibula. The conditioning stimulus consisted of a series of 3 rectangular pulses, each of 1 ms duration, an inter-pulse interval of 3 ms, an intensity 1.2 times the tibialis anterior (TA) motor threshold (MT), delivered 21 ms before the test stimulation²⁰.

Postsynaptic inhibition

Bipolar adhesive electrodes were placed on the lateral side of the fifth toe, stimuli consisted of 17 pulses, each of 1 ms duration, with an inter-pulse interval of 3 ms, delivered 50 ms before the test stimulus. The average distance from the point of conditioning stimulation on the distal aspect of foot to the mid-popliteal region was 50 cm²¹. In our previous study in healthy subjects, the intensity of electrical stimulation to the sural cutaneous afferents was set to 3-5 times the intensity of the perception threshold. The repetitive electrical stimuli induced a sharp sensation but its intensity was always kept below the triggering threshold of the flexor reflex. Since most people with SCI exhibit considerable sensory impairment (**Table 1**), it was not possible to ascertain the exact perception threshold in each subject. Based on supplementary experiments in healthy subjects, the intensity of the conditioning stimulation of sural cutaneous afferents was set to 8.5 mA in participants with SCI.

Data analyses

Electrophysiological data

The inhibitory effect was quantified for each time period by calculating the inhibition index as (conditioned H value - unconditioned H value) / unconditioned H value x 100. To determine if pre- and postsynaptic inhibitions were present before furosemide administration, one sample-tests were used for each participant. **Table 2** presents the pre- and postsynaptic inhibition profile of the participants.

For each experiment and for each participant, we performed one-way ANOVAs (with time-period as the factor) to determine if pre- and postsynaptic inhibitions changed over time following furosemide administration. For grouped data, Friedman repeated measures ANOVA on ranks were performed, to (1) verify that the unconditioned H-reflex was constant over time to (2) evaluate the modulations of pre- and postsynaptic inhibition over time. If the χ^2 -value reached significance, *post hoc* pairwise comparisons were performed using Tukey's test. χ^2 -values and degrees of freedom were calculated and indicated in brackets before the P-value. Two-way repeated measures ANOVAs were used to compare (1) the amplitude of the M-wave in the three conditions in each time-period and (2) the degree of presynaptic inhibition at baseline and 40-70 minutes period in participants with motor complete and with motor incomplete SCI. If the *F*-value reached significance, *post hoc* pairwise comparisons were performed using Tukey's test. Degrees of freedom were calculated and are indicated in brackets before the *F*-value.

The relationship between different clinical measures and electrophysiological data

First, we verified if an association exists between age, gender, the grade of injury according to the American Spinal Injury Association Impairment Scale (AIS), baclofen therapy, Asworth scale score, and time interval since the SCI. We verified if baclofen therapy, a potent GABA_B agonist interferes with the alteration of pre- and postsynaptic inhibition following furosemide administration. These relationships were explored using the Wilcoxon tests.

Finally, a fixed-effects linear model was used to compare the alteration of pre- and postsynaptic inhibition between healthy subjects and people with SCI. This model

incorporated age as covariate, and was implemented after checking for group-age interaction and homoscedasticity²².

In all of our analyses, statistical significance was set at $P < 0.05$. Analyses were performed using SigmaPlot 12.5. and JMP 14 software.

Results

H-reflex amplitude (as a percentage of $M_{\max}$)

$M_{\max}$ and $H_{\max}$ were recorded at the beginning of each experiment. In participants with SCI, the mean $H_{\max}/M_{\max}$ ratio was $52.46 \pm 5.5 \%$, and did not differ from the mean ratio observed in healthy subjects ($54.28. \pm 5.6 \%$; t -test $P = 0.818$). In people with SCI, the mean unconditioned H-reflex amplitude was $26.34 \pm 2.8 \%$ of $M_{\max}$. During the experiment, we ensured that the unconditioned H-reflex amplitude remained constant over time, which was confirmed *a posteriori* ($\chi^2 = 2.063$, DDL = 7, $P = 0.956$).

M waves

To verify that the experimental conditions were consistent, we evaluated the magnitude of M-waves preceding the H reflexes (**Figure 1B**) in each eight time-period. **Figure 1C** presents the M-wave profile of unconditioned and conditioned H-reflexes at baseline and 70 minutes after furosemide administration. Statistical analyses indicated that M-waves amplitudes of unconditioned and conditioned H-reflexes were similar at baseline. They also revealed that M-waves amplitudes remained constant after furosemide administration ($F_{(2,494)} = 0.171$, $P_{\text{period}} = 0.991$, $F_{(2,494)} = 0.982$, $P_{\text{condition}} = 0.337$, $F_{(14,494)} = 0.0543$, $P_{\text{period} \times \text{condition}} = 1.000$)

The effect of Furosemide on Ia presynaptic inhibition in people with SCI

Figure 2A depicts the mean Ia presynaptic inhibition in people with SCI at baseline and for 70 minutes after furosemide administration. Following furosemide administration, the Ia presynaptic inhibition fluctuated around its baseline value, but these changes were not significant ($\chi^2 = 2.133$, DDL = 7, $P = 0.952$).

As reported by others²³ we observed diminished presynaptic inhibition in people with SCI. Only 12 out of 21 participants exhibited significant inhibition at baseline. Accordingly, in

order to reliably evaluate the effect of furosemide on presynaptic inhibition following SCI, we focussed on these 12 participants. **Figure 2B** depicts the alteration of presynaptic inhibition in this subgroup. We did not observed any significant variation in presynaptic inhibition following furosemide administration ($\chi^2=7.200$, DDL =7, $P=0.408$). In summary, furosemide failed to modulate Ia presynaptic inhibition in participants with SCI, in contrast to healthy subjects where it significantly decreased inhibition 31 minutes after administration.

The group of 12 participants with significant Ia presynaptic inhibition was further stratified into people with motor complete SCI (n=5, AIS A and AIS B) and people with motor incomplete SCI (n=7, AIS C and AIS D). Participants from AIS A and AIS B were pooled given the complete lack of motor functions in both groups. **Figures 2 C and D** represent the inhibitory profile of the two groups at baseline and 40-70 minutes after furosemide administration. We performed a two-way repeated-measures ANOVA with time period and type of SCI as factors. A weak reduction of mean presynaptic inhibition was detected after furosemide administration in people with incomplete SCI, no furosemide-effect was captured in people with complete SCI ($F_{1,23}=5.310$, $P_{\text{period}}=0.044$, $F_{1,23}=0.160$, $P_{\text{SCI}}=0.697$, $F_{1,23}=2.423$, $P_{\text{period} \times \text{SCI}}=0.691$). *Post hoc* analysis: patients with incomplete SCI, $P < 0.05$; patients with complete SCI, $P > 0.05$).

The furosemide-effect on postsynaptic inhibition in people with SCI

Figure 3A shows the estimated postsynaptic inhibition in 21 participants with SCI in the eight time-periods. The degree of postsynaptic inhibition fluctuated around its baseline value following drug administration confirming the lack of furosemide-effect ($\chi^2=4.511$, DDL=7, $P=0.719$). It is important to highlight that postsynaptic inhibition was reduced or absent in participants with SCI.

Only eight participants with SCI exhibited significant inhibition at baseline (**Table 2**). **Figure 3B** represents the alteration of postsynaptic inhibition in these eight participants. Even in this subgroup with baseline inhibition, we did not identify any change in postsynaptic inhibition following furosemide administration ($\chi^2=9.333$, DDL=7, $P=0.230$). In healthy subjects, furosemide induces significantly decreased postsynaptic inhibition (Klomjai et al. 2014¹⁴) while in our cohort of people with SCI no such response could be

detected.

Given the small number of participants with significant postsynaptic inhibition, we cannot further stratify the cohort into those with motor complete and motor incomplete SCI as we have done for presynaptic inhibition.

The relationship of clinical features and electrophysiological measures

Our statistical analyses highlighted a significant interaction between the time elapsed since SCI and baclofen therapy; participants taking baclofen had longer disability duration (Wilcoxon test: $P < 0.01$). Other clinical variables were independent from one another (Wilcoxon test : $P > 0.05$).

We detected a relationship between baclofen therapy and presynaptic inhibition at baseline (Wilcoxon test: $P < 0.04$). The participants taking baclofen were those with the lower levels of presynaptic inhibition at baseline. This association was not more significant after furosemide administration (Wilcoxon test $P > 0.05$). The degree of postsynaptic inhibition however did not correlate with baclofen intake. In summary, these findings suggest that baclofen therapy did not interfere with furosemide-effect on pre- and postsynaptic inhibition.

Comparison between healthy subjects and participants with SCI

In order to assess differences in the modulation of pre- and postsynaptic inhibition after furosemide administration between the two groups, a fixed-effects general linear model was used. The scatter plots in **Figure 4** represent presynaptic inhibition (**Figure 4 A ,C and E**) and postsynaptic inhibition (**Figure 4 B, D and F**) in both groups at baseline and after furosemide intake.

This bi-variate model showed that the degree of pre- and postsynaptic inhibition was consistently lower in people with SCI than in healthy subjects, suggestive of reduced inhibitory synaptic transmission post-SCI. The model did not detect a correlation between age and presynaptic inhibition regardless of the study group examined and the time period considered ($P > 0.05$). In the contrary, it identified a correlation between age and

postsynaptic inhibition at the 31-40 minute interval (**Figure 4B**, $P < 0.04$), 41-50 minute interval (**Figure 4D**, $P < 0.01$) and in the 51-60 minutes period (**Figure 4F**, $P < 0.02$). It seems that 31 minutes after furosemide intake, the older the participant, the higher the amount of postsynaptic inhibition.

Discussion

The main finding of our study is that in contrast to healthy subjects, furosemide fails to modulate pre- and postsynaptic inhibition in people with SCI. The lack of furosemide-effect in people with SCI suggests a down-regulation of KCC2 activity, which leads to decreased inhibitory synaptic transmission in spinal neurons following SCI.

Furosemide-effect on pre- and postsynaptic inhibition in SCI

In this series of experiments, we demonstrate a lack of furosemide-effect in people with SCI in contrast to reduced pre- and postsynaptic inhibition in healthy subjects. In healthy subjects, control experiments without furosemide have been performed during 70 minutes and revealed that postsynaptic inhibition is subject to habituation i.e. reduced postsynaptic inhibition over time. It was not possible to replicate these control experiments in all people with SCI. In participants with significant postsynaptic inhibition at baseline, we observed a slight decrease of inhibition with furosemide which did not reach statistical significance. Moreover, in complementary experiments performed in two subjects with SCI without furosemide, we observed a significant decrease of postsynaptic inhibition over time. It is conceivable that the effect of time on postsynaptic inhibition may persist following SCI. This assumption is supported by the linear pattern of slightly increased postsynaptic inhibition 31 minutes after drug administration in older participants in both SCI and healthy subjects. Since the model did not detect an age-effect on presynaptic inhibition, we favoured the hypothesis that age plays a role on habituation rather than on furosemide-effect. Thus, the older the participant, the lower the habituation and longer the postsynaptic inhibition over time.

In participants with significant presynaptic inhibition at baseline, we observed a lack of furosemide-effect in people with motor complete SCI and a reduced effect in people with motor incomplete SCI. In other words, people with motor incomplete SCI exhibit

decreased presynaptic inhibition, which seems to remain partially susceptible to furosemide. This observation suggests that the remaining descending projections preserved in incomplete SCI may contribute to the maintenance of the functioning of inhibitory synapses.

Relevance to SCI pathophysiology

Similarly to animal studies, we hypothesised that furosemide may be use in humans to unmask KCC2 dysfunction following SCI. The lack of furosemide-effect in people with SCI suggests that a down-regulation of KCC2 activity contributes to reduced inhibitory synaptic transmission in spinal motor neurons. Hyperexcitability of spinal reflexes is one of the key components of the upper motor neuron (UMN) syndrome that invariably occurs following SCI. This has important quality of life and management implications, affects the choice of assistive devices and rehabilitation efforts. UMN syndrome entails hyperexcitability of the stretch reflex, spasticity²⁶, and exaggerated flexor reflexes which become prominent following SCI^{27,28}. Supra-spinal pathways exert a dynamic control (excitatory or inhibitory) on spinal networks and their complete or incomplete interruption is the most common explanation for post-SCI hyperexcitability²⁹⁻³¹. Our data suggest that in addition to supra-spinal control alterations, dysfunction of the KCC2 system and altered chloride homeostasis may also contribute to the UMN syndrome. The characterisation of this mechanism opens novel perspectives and potential pharmacological targets for the management of post-SCI hyperexcitability.

Clinical implications

Furosemide use in SCI patients with spasticity

In people with incomplete motor SCI we observed a slight furosemide-effect of decreased inhibitory transmission in motor neurons. Furosemide administration may thus contribute to reduced residual inhibitory activity and contribute to reflex hyperexcitability post SCI. This raises the question of long-term furosemide therapy in SCI patients with exaggerated spinal reflexes. A prospective study of furosemide could therefore be envisaged in SCI patients with exaggerated spinal reflexes.

Spasticity management

Baclofen is widely used in spasticity management in SCI. It enhances inhibitory effects counteracting the hyperexcitability underlying spasticity. As KCC2 is down-regulated following SCI, intracellular $[Cl^-]$ increases in neurons and GABA becomes depolarising instead of hyperpolarising. Accordingly, baclofen may have a paradoxical effect in this cohort and contribute to an excitatory rather than inhibitory effect.

Our observations provide indirect evidence that KCC2 is down-regulated in people with SCI. This may open novel therapeutic strategies aimed at increasing KCC2 expression. Recent animal studies have shown considerable promise in boosting KCC2 activity and restoring inhibition following SCI^{32,33}. KCC2 and NKCC1 activity in the lumbar cord of rats returned to normal levels after intensive and passive motor training if initiated soon after spinal cord transection, providing the rationale for early rehabilitation to restore inhibitory function³⁴. Animal studies suggest that intensive rehabilitation in the subacute phase after SCI may favour the restoration chloride homeostasis and have unmatched therapeutic potential in the treatment of spasticity.

Future directions

Integrating the findings from our current SCI and previous healthy cohorts, we believe that furosemide can be reliably used to test the cation chloride system in humans. Future studies with larger sample sizes are needed to characterise the inhibitory profile of specific SCI subgroups and the longitudinal evolution of this phenomenon from the initial injury. In both studies, we used furosemide as it has been extensively and safely used in a plethora of clinical indications. We recognise that the diuretic effect of bumetanide is superior to that of furosemide³⁵ but it is more likely to cause electrolyte disturbances and other undesirable effects. Given that alterations of both NKCC1 and KCC2 activity have been observed in a number of neurological conditions, this methodology may also be applied to stroke and primary lateral sclerosis to determine if antagonist of cation-chloride co-transporter may have a neuroprotective role³⁶.

Limitations of the study

Sample of patients.

The method used to explore furosemide-effect on inhibitory spinal circuits relies on the

evaluation of the monosynaptic H-reflex. Therefore, subjects with sizeable soleus H-reflexes were selected with reflexes, which were stable enough to run an experiment for 80 minutes. In order to elicit the H-reflex in the soleus and to study both pre- and postsynaptic pathways, electrical stimuli were delivered through leg and foot electrodes. It is well established that following recovery from spinal shock, cutaneous stimuli applied anywhere in the lower limb can cause an ipsilateral flexion reflex²⁴. This reflex is similar to the flexion withdrawal reflex described by Sherrington²⁵. In humans, the flexion reflex is characterized by a burst activity in flexor muscles which is accompanied by a silent period on the EMG of extensor muscles^{24,25}. This phenomenon makes it harder to elicit the H-reflex in people with SCI and explains why some potential candidates could not be included. Despite these challenges, we succeeded to include 21 subjects with SCI; 13 with motor complete SCI and 9 with motor incomplete SCI.

Our sample is clinically and demographically heterogeneous. Even though our statistical model enabled the necessary adjustments for the heterogeneity of our participants, larger and more homogenous samples may reveal slight differences in pre- and postsynaptic inhibition between baseline and latter time periods. Despite these considerations, our data indicate that our main finding of reduced furosemide-effect in SCI compared to healthy subjects is well established.

Pre- and postsynaptic inhibition at baseline in SCI

In people with SCI, pre- and postsynaptic inhibition at baseline were significantly lower than in healthy subjects. While impaired presynaptic inhibition of Ia fibers has been previously reported in people with SCI²³, to our knowledge, altered postsynaptic inhibition has not previously been documented. Our study highlights that postsynaptic inhibition is significantly reduced and often absent following SCI. This reduction is likely to play central role in hyperreflexia post SCI.

Conclusions

The lack furosemide-effect on spinal inhibitory synapses provides indirect evidence of KCC2 dysfunction in humans following spinal cord injury. The down regulation of KCC2 activity in SCI is likely to contribute to a shift from inhibitory responses to excitation. The down-regulation of KCC2 leads to increased NKCC1 activity, which results in the reversal of GABA effect. The regression to “immature” chloride homoeostasis may be an important aetiological factor in reflex hyperexcitability in people with SCI. These observations may have clinical implications beyond spinal cord injury, as synaptic regression to an “immature” state may also play a role in spasticity associated with other conditions such as spinal cord ischemia, hereditary spastic paraplegia , multiple sclerosis and progressive lateral sclerosis. Finally, the role of altered chloride regulation in spasticity may have implications for the development of novel pharmacological strategies.

Conflict of interest: The authors have no conflicts of interest to declare and have no competing financial interests.

Acknowledgments: The authors wish to express their gratitude to Dr Jacques Rottembourg for supervising furosemide administration and to Dr Nachiket Nadkarni for the revising the manuscript.

Funding: This work was supported by grants from INSERM, MESR, CNRS, ANR, IRME, APHP. Dr Peter Bede is supported by the Health Research Board (HRB-EIA 2017-019 Ireland) and the Iris O'Brien Foundation.

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Dr Wanalee Klomjai : Data collection, data interpretation, experimental design, generation of figures and tables, drafting the manuscript for intellectual content. Dr Nicolas Roche,

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Legends

Table 1. Clinical features of patients.

The grade of injury according to the American Spinal Injury Association Impairment Scale (AIS) relies on the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI). It places motor and sensory impairments of SCI patients into 5 grades defined from A to E. *AIS A = Complete* lesion. No sensory or motor function is preserved in the sacral segment. *AIS B = Sensory Incomplete*. Sensory but not motor function is preserved below the neurological level and induces the sacral segments S4-5 (light touch (LT) or pin prick (PP) at S4-5 or deep anal (DA) pressure) and no motor function is preserved more than three levels below the motor level on either side of the body. *AIS C = Motor Incomplete*. Motor function is preserved at the most caudal sacral segments for voluntary anal contraction (VAC) or the patient meets the criteria for sensory incomplete status (sensory function preserved at the most caudal sacral segments (S4-S5) by LT, PP or DAP) and has some sparing of motor function more than three levels below ipsilateral motor level on either side of the body. *AIS D = Motor Incomplete*. Motor incomplete status defined above, with at least half of key muscle functions below the single neurological level of injury (NLI) having a muscle grade ≥ 3 . *AIS E = Normal*. Motor and sensory functions are normal.

Lower limb spasticity was evaluated using the Ashworth scale, which tests resistance to passive movement with varying degrees of velocity. It is a point scale from 0 to 4. *Score 0* indicates no increase in tone; *score 1* indicates slight increase in tone giving a catch when the limb is moved in flexion or extension; *score 2* means more marked increase in tone but limb easily flexed; *score 3* considerable increase in tone and passive movement difficult and *score 4* means limb rigid in flexion or extension.

Sex : female (F) male (M). Side : left (L), right (R). Soleus muscle (M. Sol)

Table 2 Electrophysiological values at baseline

Individual values (not normalized) of pre- and postsynaptic inhibition were evaluated at baseline and during the 40-70 min. period following furosemide administration. Values were pooled for the time interval between the 40-70 min. period, since the effect of

furosemide peaked in healthy subjects at this interval. The inhibition values were calculated using the following equation: $(\text{conditioned } H\text{-reflex} - \text{unconditioned } H \text{ reflex}) / \text{unconditioned } H\text{-reflex} \times 100$. Negative values mean H conditioned > H unconditioned, so facilitation rather than inhibition. The values with an asterisk indicate a significant value of inhibition at baseline. This was determined by using one-sample t- tests. Significance was set at $P < 0.05$ (*). L Maximum amplitude of the H-reflex (Hmax). Maximal amplitude of the direct motor response (Mmax) The ratio $H_{\max} / M_{\max}$ were also indicated in this table.

Figure 1. Experimental Procedure

A: The overview of the experiments with and without furosemide administration. B: diagram of presynaptic inhibition of soleus Ia fibers (center) and post-synaptic inhibition induced by cutaneous stimulation (right), and illustrative waveforms of conditioned and unconditioned H-reflexes in soleus from a subject with SCI. The arrow indicates the M waveform preceding the unconditioned H-reflex. M waveform is evoked by the stimulation of the motor axons in the posterior tibial nerve. This figure was modified from figure 1A&B, Klomjai et al. 2014. C: The time course of M waveform of unconditioned and conditioned H-reflexes in the soleus.

Figure 2. The effect of furosemide on Ia presynaptic inhibition in people with SCI

A: Histograms representing the mean presynaptic inhibition estimated before and for 70 minutes following furosemide intake in the 21 participants with SCI. B: The time course of mean presynaptic inhibition estimated in the 12 participants with significant inhibition at baseline. C & D: These 12 subjects were separated into two groups: people with motor incomplete (AIS C and D; n=7) and motor complete (AIS A and B); n=5) spinal cord lesion. We have pooled AIS (A) and AIS (B) since in both of them the motor functions are completely abolished. Mean presynaptic inhibition at baseline was compared to that estimated between 40 to 70 minute period after furosemide intake, since in healthy subjects furosemide effects peaked at this interval. Inhibitions were calculated using a following equation: $(\text{conditioned } H\text{-reflex} - \text{unconditioned } H\text{-reflex}) / \text{unconditioned } H\text{-reflex} \times 100$. Vertical bars represent the standard error of the mean (± 1 SEM). Group statistical comparisons were done with Repeated Measures Anova; asterisks represent significant differences of $P < 0.05$ (*), ns = non-significant.

Figure 3. The effect of furosemide on postsynaptic inhibition in people with SCI

A: Histograms representing the mean postsynaptic inhibition estimated before and for 70 minutes following furosemide intake in the 21 participants with SCI. B: The time course of mean postsynaptic inhibition estimated in the 8 participants with significant inhibition at baseline. Inhibitions were calculated using a following equation: $(\text{conditioned } H\text{-reflex} - \text{unconditioned } H\text{-reflex}) / \text{unconditioned } H\text{-reflex} \times 100$. Vertical bars represent the standard error of the mean (± 1 SEM). Group statistical comparisons were done with Repeated Measures Anova.

Figure 4. Comparison between healthy subjects and participants with SCI

Scatter plots depict the results of the fixed-effects general linear model testing the difference between the degrees of both inhibitions estimated in 19 healthy subjects and 21 people with SCI enrolled in the study. Mean inhibitions are plotted against the age of the subjects. Inhibitions were calculated using a following equation: $(\text{conditioned } H\text{-reflex} - \text{unconditioned } H\text{-reflex}) / \text{unconditioned } H\text{-reflex} \times 100$. Healthy subjects are representing by white dots and people with SCI by black dots. P-values were calculated and indicated at the bottom of the graph. When P-value reached significance, the R-squared (R^2) value has been indicated at the top of the graph.

Figure 1

Figure 2

Figure 3

Presynaptic inhibition

Postsynaptic inhibition

Figure 4

	Sex (F/M)	Age (years)	Side (L/R)	Lesion	Medication	AIS	Time elapsed since SCI (month)	M Sol Ashworth score
1	M	32	R	T6	baclofen pregabalin	AIS A	6	1
2	M	22	R	C5-C6	baclofen	AIS A	16	2
3	M	28	R	C5	amitriptylin	AIS A	2	1
4	M	48	G	T4-T5	tramadol	AIS A	3	1
5	M	67	R	C8	baclofen pregabalin	AIS A	14	2
6	M	29	R	T7	pregabalin	AIS A	4	0
7	M	27	R	T9	baclofen	AIS A	6	1
8	F	20	R	C5	clonazepam amitriptylin duloxetine	AIS A	2	1
9	M	54	R	C7	pregabalin baclofen	AIS B	3	1
10	M	37	R	C5	none	AIS B	7	1
11	M	47	R	C4-C5	gabapentin	AIS C	3	1
12	M	34	R	T4	baclofen gabapentin	AIS B	6	1
13	M	44	R	C6	none	AIS C	41	2
14	M	51	R	C4	gabapentin	AIS C	6	1
15	M	28	R	L1	pregabalin	AIS D	4	1
16	M	62	R	C6-C7	baclofen	AIS B	540	5
17	M	45	R	C7	baclofen	AIS C	31	1

18	F	31	R	C6	dantrolen bromapezam	AIS C	22	4
19	M	46	L	C6	baclofen clonazepam gabapentin	AIS C	92	5
20	M	40	R	T4	baclofen	AIS C	8	3
21	M	41	R	C6	baclofen clonazepam gabapentin	AIS B	54	2

Table 1. Clinical features

	Side (L/R)	$H_{\max}/M_{\max}$	Presynaptic Ia inhibition furosemide		Postsynaptic inhibition furosemide	
			baseline	40-70 min	baseline	40-70 min
1	R	52.37	17.99*	19.44	-12.73	-11.56
2	R	75.50	11.30*	4.02	-0.72	2.02
3	R	31.93	11.38*	9.74	3.67	-1.56
4	L	20.14	38.81*	29.01	-1.68	-0.42
5	R	67.68	6.81	4.45	2.58	-0.54
6	R	89.87	18.33*	16.73	8.92*	4.33
7	R	84.12	-3.42	-4.84	-1.17	-3.92
8	R	22.72	5.85	10.04	-30.53	-22.20
9	R	48.71	1.76	4.38	-1.61	6.51
10	R	64.94	10.07*	15.68	16.72*	10.29
11	R	20.46	12.57*	3.73	7.14*	4.23
12	R	62.94	11.65*	12.20	-0.89	-0.01
13	R	89.32	4.91*	2.17	4.61*	-0.25
14	R	92.16	24.45*	14.94	11.56*	14.75
15	R	34.31	9.72	20.73	13.51	-16.96
16	R	17.78	-11.53	-12.10	5.29*	2.57
17	R	74.11	9.49*	11.63	-2.88	2.79
18	R	39.68	42.14*	14.17	15.61*	5.95
19	L	45.07	-2.43	-0.98	-2.29	-2.08
20	R	43.89	-7.07	15.73	6.39	1.04
21	R	24.00	-18.04	-15.60	24.74*	17.34

Table 2. Electrophysiological values