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Alteration of glycinergic receptor expression in lumbar spinal motoneurons is involved in the mechanisms underlying spasticity after spinal cord injury

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ABSTRACT

Spasticity is a disabling motor disorder affecting 70% of people with brain and spinal cord injury. The rate-dependent depression (RDD) of the H reflex is the only electrophysiological measurement correlated with the degree of spasticity assessed clinically in spastic patients. Several lines of evidence suggest that the mechanism underlying the H reflex RDD depends on the strength of synaptic inhibition through GABA_A (GABA_AR) and glycine receptors (GlyR). In adult rats with spinal cord transection (SCT), we studied the time course of the expression of GABA_AR and GlyR at the membrane of retrogradely identified Gastrocnemius and Tibialis anterior motoneurons (MNs) 3, 8 and 16 weeks after injury, and measured the RDD of the H reflex at similar post lesion times. Three weeks after SCT, a significant decrease in the expression of GABA_A and GlyR was observed compared to intact rats, and the H-reflex RDD was much less pronounced than in controls. Eight weeks after SCT, GlyR values returned to normal. Simultaneously, we observed a tendency to recover normal RDD of the H reflex at higher frequencies. We tested whether an anti-inflammatory treatment using methylprednisolone performed immediately after SCT could prevent alterations in GABA_A/glycine receptors and/or the development of spasticity observed 3 weeks after injury. This treatment restored control levels of GlyR but not the expression of GABA_AR, and it completely prevented the attenuation of RDD. These data strongly suggest that alteration of glycinergic inhibition of lumbar MNs is involved in the mechanisms underlying spasticity after SCI.

1. Introduction

Spasticity is a common motor disorder affecting 70% of individuals with brain or spinal cord injury (SCI), which negatively influences the quality of life by restricting daily activities, causing pain and fatigue, disturbing sleep, participating in the development of contractures, and impeding rehabilitation efforts (Adams and Hicks, 2005). Spasticity is characterized by a velocity-dependent increase in muscle tone resulting from hyperexcitable stretch reflexes (Lance, 1980) and spasms. This can result from hyperexcitability of MNs due to changes in their intrinsic properties (Brocard et al., 2016) and/or altered endogenous inhibitory regulation of MNs excitability (Boulenguez et al., 2010). In the spinal sub-lesional neuronal networks of spastic patients, endogenous

inhibition, including presynaptic, recurrent or reciprocal inhibition between antagonist muscles, was shown to be altered (Boorman et al., 1996; Crone et al., 2003; Crone et al., 2007; Katz, 1999; Mazzocchio and Rossi, 1997; Nielsen et al., 2007). The Hoffmann (H) reflex, which is the electrophysiological equivalent of the monosynaptic stretch reflex, has long been used to study the excitability of MNs induced by the activation of Ia afferents. The H reflex is usually found to be increased in spastic patients. In healthy subjects, the amplitude of the H reflex is gradually depressed when the frequency of the nerve stimulation increases between 0.2 and 5 Hz. In spastic patients, this rate-dependent depression (RDD) is altered (i.e. the H reflex amplitude is less depressed at high stimulation frequencies), which is the only electrophysiological measurement that correlates with the degree of spasticity assessed

Abbreviations: ip, intra peritoneal; MP, methylprednisolone (solumedrol); MN, motoneuron; PI, post-injury; SCT, spinal cord transection; SCI, spinal cord injury; RDD, rate-dependent depression; GlyR, glycine receptor; GABA_AR, GABA_A receptor; H reflex, Hoffmann reflex

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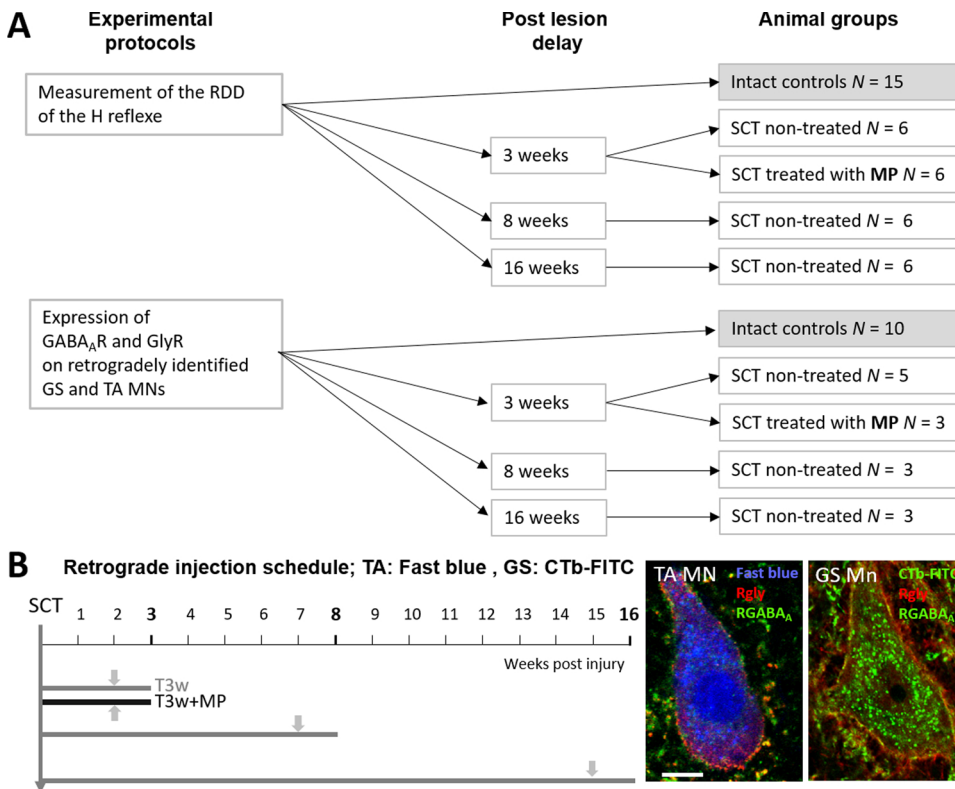


Fig. 1. A) experimental design of the two experimental procedures. The measurement of the RDD of the H reflex and the expression of GlyR and GABA_AR on GS and TA MNs were performed in intact control rats and in SCT animals, at 3, 8 and 16 weeks post injury (number N of animals used indicated in each animal group). In both experimental procedures, the effects of early treatment with methylprednisolone (MP) was tested in two other groups of SCT rats, 3 weeks post-injury. B) Schedule of the injection of retrograde dyes (arrows) in tibialis anterior and gastrocnemius muscles along the longitudinal studies. Representative figures of TA MN fast blue positive, and GS MN CTb-FITC positive are shown in the right part. Scale bar: 10 μ m for both.

clinically using the Ashworth scale (Biering-Sorensen et al., 2006; Katz and Rymer, 1989; Nielsen et al., 2007). Altered RDD of the H reflex results in increased synaptic efficacy of action potential trains from Ia afferents, thereby contributing to the exaggerated stretch reflex, and could be one of the main spinal mechanisms underlying spasticity (Hultborn et al., 1996; Hultborn and Nielsen, 1996). The mechanism underlying the H reflex RDD was described as a presynaptic mechanism which reduces the probability of neurotransmitter release at high stimulation frequency (Hultborn et al., 1996; Hultborn and Nielsen, 1996; Lev-Tov and Pinco, 1992). However, several studies in rats showed that RDD was also dependent on - or counterbalanced by - the strength of synaptic inhibition through GABA_A and glycine receptors (Barriere et al., 2008; Boulenguez et al., 2010; Jolivald et al., 2008; Lee-Kubli and Calcutt, 2014).

Altered synaptic inhibition after SCI results both from the loss of descending pathways which normally regulate spinal neuronal networks, but also from different post-lesional plastic changes within these networks below the lesion. Downregulation of membrane chloride ion cotransporters KCC2 after SCI was shown to alter the strength of inhibition through GABA_A and glycine receptors which are associated with chloride channels. This participates in the development of neuropathic pain (Cramer et al., 2008; De, 2007; Hasbargen et al., 2010; Lu et al., 2008) and spasticity (Bos et al., 2013; Boulenguez et al., 2010). A loss of GABAergic interneurons is also involved in the development of spasticity after transient spinal cord ischemia (Kakinohana et al., 2006). There is strong evidence that complete thoracic spinal cord transection (SCT) in neonates (Edgerton et al., 2001; Khristy et al., 2009; Sadlaoud et al., 2010) or adult rats (Esmaili and Zaker, 2011) produces noteworthy alterations in the levels of GABA_A and/or glycine membrane receptors on sublesional lumbar motoneurons (MNs). However, the variability of the experimental procedures and particularly the onset of the lesion and the post lesional delays prevents a clear overview of the time course of this alteration after SCI. Nevertheless, we previously showed that neonatal SCT alters GABA_A receptor expression in the sublesional spinal cord, without any change in glycine receptors (Sadlaoud et al., 2010). However, there is a shift from GABAergic to

glycinergic inhibition during development so both receptors may be differentially altered in adults.

Inflammatory processes following SCI have also been shown to trigger deleterious postlesional plastic changes in the sublesional spinal networks, including microglia activation and KCC2 downregulation (Coull et al., 2005; Ferrini et al., 2013; Oudega, 2013). Among neuroprotective treatments aimed at reducing some of these secondary lesioning processes following SCI, the corticosteroid methylprednisolone (MP) has shown some efficacy in outcome motor functions, sensitivity to pain and tactile sensitivity. Pharmacological trials have reported that this anti-inflammatory agent improved neurological recovery in humans (Bracken et al., 1998) and animals after SCI (Ates et al., 2006; Kaptanoglu et al., 2000).

The first objective of this study was to perform a long term analysis (at 3, 8 and 16 weeks post injury) of the expression of GABA_A and glycine receptors at the membrane of lumbar MNs. As previously shown, extensor and flexor MNs can respond differently to spinal transection, in terms of pre and post signaling (Chopek et al., 2014; Chopek et al., 2015; Khalki et al., 2018; Khristy et al., 2009; Skup et al., 2012). We differentially identified hindlimb extensor (Gastrocnemius) and flexor (Tibialis anterior) MNs with two retrograde fluorescent tracers. In parallel, we tested the hypothesis that the post lesional development of spasticity, assessed by the measurement of RDD of the H reflex, was correlated with the time course of the expression of glycine and/or the GABA_AR. Then, we tested whether an early post-lesional anti-inflammatory treatment using MP could prevent GABA_A/glycine receptor alterations and/or the development of spasticity.

2. Material and Methods

2.1. Animals

In total, 63 adult female Wistar rats (220-280 g, Charles River Laboratories) were housed in a temperature-controlled animal care facility with a 12 h light-dark cycle. All experimental procedures were conducted according to French and European regulations for animal

experimentation and were approved by the local ethics committee [Comité d'Éthique en Neurosciences INT-Marseille (CEEA N°71), authorization A90113]. The experimental design and the number of animals used for the different experiments are given in Fig. 1.

2.2. Surgery and postoperative care

The lesion procedure has been described previously (Boulenguez et al., 2010; Khalki et al., 2018). For spinal cord injury, the rats were deeply anesthetized using ketamine (Imalgen, Merial, 50 mg/kg ip) and medetomidine (Domitor, Janssen, 0.25 mg/kg ip). They received an injection of a long acting antibiotic (amoxicillin, Duphamox LA, Pfizer, 100 mg/kg s-c) and were then kept on a thermoregulated plate during the surgical procedure. Their skin was incised at T8-T10 vertebral bones. Paravertebral muscles were retracted to expose vertebral bones and a laminectomy of the dorsal part of the T9 vertebra was performed carefully. The dura was cut transversally and procaine was applied for 5 min before transsecting the spinal cord with small scissors. The paravertebral muscles and skin were then sutured and disinfected. The rats were rehydrated with 5 ml of 0.9% NaCl s-c and kept warm until awakening. They were injected with a morphinic analgesic (buprenorphine, Vetergesic, Sogeval, 0.05 mg/kg s-c every 8 h for 24 h) and placed in individual cages with easily accessible food and water containing 1.33 g/l of aspirin (Aspégic nourisson 100 mg, Sanofi Aventis, for 3 days). Their bladder was emptied twice a day until recovery of autonomy and their water intake, temperature and weight were checked every day until they recovered an ascending weight curve.

2.3. Measurement of Rate-dependent depression (RDD) of the H reflex

For H reflex RDD measurement previously described (Boulenguez et al., 2010), rats were sedated with ketamine (100 mg/kg ip, supplemented with half of the dose after 1 h when applicable) and their temperature was maintained at around 37 °C. The hindpaw was fixed with tape and a pair of stainless steel needle electrodes were transcutaneously inserted into the vicinity of the tibial nerve for stimulation. The recording electrode was placed in the flexor digitorum brevis muscle (FDB) beneath the ankle and the reference electrode subcutaneously in the sole of the foot. A ground electrode was placed subcutaneously at the base of the tail. We first stimulated the tibial nerve for 0.2 ms at 0.2 Hz with increasing current intensities until maximal contraction of the muscle (Mmax), and determined the intensity to get a maximal H reflex response. We then used this intensity for trains of 20 stimulations at 0.2, 0.5, 1, 2 and 5 Hz with 2 min intervals between each train of stimulation. For quantification, traces were rectified and areas under the curve of the M wave and the first main 2 peaks of the H reflex were quantified. These values were then reported to the mean response at 0.2 Hz in the same series of measurements in order to determine the depression of the H reflex as a function of increasing stimulation frequencies (RDD). We averaged two series of measurements per animal and nonlinear regression was used to compare the distributions of the percentages of H reflex amplitudes at different frequencies between groups of rats. They included 15 intact control rats and 24 SCT non-treated rats. These latter include three groups of un-treated rats at 3 weeks, 8 weeks and 16 weeks post SCT ($n = 6$ at each time point), and one group of 6 rats treated with methylprednisolone immediately after SCT, which were tested for RDD 3 weeks post-injury (See Fig. 1). The maximal H reflex was reported as the maximal M wave of each rat in order to better compare animals with different muscle capacities and variable electrode locations. Activation thresholds for the H reflex and latencies between the M and H waves in the different groups were also compared to controls using Mann and Whitney tests. All values were expressed as means \pm SEM.

2.4. Retrograde labeling of MNs

The expression of GlyR and GABA_AR was analyzed in specific motor pools, the tibialis anterior (TA: ankle flexor) and gastrocnemius motoneurons (GS, ankle extensor), labeled by intramuscular injections of retrogradely transported fluorescent dyes. The animals were anaesthetized by i.p. administration of a mixture of ketamine (Imalgene, Merial, 60 mg/kg) and xylazine (Rompun, Bayer, 10 mg/kg). Then they received bilateral injections of Fast blue (FB, 0.5% in NaCl 0.9%, F-5756, Sigma, 10 μ L) in the TA muscles and Cholera Toxin-FITC β subunit (CT β 1/100 in H₂O; Sigma Aldrich, 20 μ L) in the lateral GS muscles. An optimal labeling with these dyes was obtained after 7 days of retrograde transport. As previously described (Khalki et al., 2018), retrograde labeling with Fast Blue and Cholera toxin- β subunit-FITC resulted, respectively, in the presence of blue or green fluorescent dots distributed with varying densities in the cytoplasm of GS or TA MNs (representative figures in Fig. 1B).

2.5. Perfusion and Immunohistochemistry

The animals were anaesthetized with sodium pentobarbitone (Nembutal CEVA, 80 mg/Kg) and perfused intracardially with 100 ml of phosphate buffer saline (PBS 0.1 M, pH 7.4) followed by 50 ml of 1% paraformaldehyde (in PB 0.1 M). Lumbar spinal cords were cryoprotected for 24 h in 30% sucrose and frozen at -80 °C. The lumbar segments were then serially cut in 25 μ m transverse sections using a cryostat (Microm) and collected in 48 wells plates filled with cryoprotective solution at -20 °C. The dual immunodetection of glycine and GABA_AR was performed using a mixture of rabbit anti GlyR α 1 AB (1:100, AB5052, Chemicon) and monoclonal AB anti GABA_A β _{2,3} (1:400, bd-17 antibody US Biological). This antibody directed against both β 2 and β 3 subunits recognizes the major GABA_AR subtypes in the CNS. The secondary antibodies were Cy3-donkey anti rabbit (1:500 Jackson) and Alexa 488-donkey anti mouse (1:800, Molecular Probes), respectively. As negative controls, we omitted each primary antibody and observed a complete absence of labeling with the appropriate secondary antibody.

2.6. Quantification of the expression of GABAA and glycine receptors on GS and TA MNs

Retrogradely labeled TA (Fast blue) or GS (Cholera Toxin-FITC β subunit) MNs were first detected in immunofluorescence with appropriate filters, and then scanned in stacks of 0.2 μ m-thick optical sections 0.5 μ m apart, using laser scanning confocal microscopy (LSM ZEISS 510 Meta) with a \times 63 oil objective, an argon laser (488) and Helium/Neon (543) for the detection of GABA_ARc and GlyRc, respectively. Only MNs with a visible nucleus were scanned, which allows the average diameter of the cell to be measured. Only somata, excluding dendrites were considered, and the distinction between gamma and alpha MNs was not made. Two adjacent sections of each MN were selected for analysis (software: Zen 2009). During each confocal acquisition, sections of one control animal and one or more injured animals (all mounted on the same glass slide) were scanned with the same laser parameters. Then, for the quantitative analysis of the GlyRc or GABA_ARc labeling on each MN, we overlaid the internal and external borders of the immunolabeled membrane and measured the fluorescence membrane intensity within this area (Sadlaoud et al., 2010). This value was divided by the mean diameter of the soma. Each confocal acquisition session gave rise to a separate analysis of the data in which values of fluorescent intensity obtained for SCT-groups were normalized to intact control values to further compare results from the 4 groups of animals. In total, 24 rats were used, including 10 control rats and 14 SCT rats (see Fig. 1). Quantifications were performed on 345 TA and 337 GS MNs. The nonparametric one-way ANOVA (Kruskal–Wallis) was performed with a Dunn's post test for multiple comparisons between control animals and SCT rats. Data were expressed as medians (Box and

whiskers with min to max values).

2.7. Pharmacological treatment with methylprednisolone (MP)

On the basis of the efficiency of early administration of Methylprednisolone sodium succinate (MP) on patients with SCI (Bracken et al., 1998), the rats received injection of MP (30 mg/kg, ip) immediately after the SCT and 12 h later ($n = 3$ rats for immunohistochemical studies, and $n = 6$ rats for RDD measurement). The American Federal Drug Administration (FDA) Guidance for Industry (2005) provided an equation that enables to identify the human equivalent dose (HED) from animal data. Translation of our data from rats weighting 250 g to humans with a weight of 70 kg is given by the following injection equation:

$$\text{HED} = \text{Animal Dose (mg/kg)} \times (\text{animal wt/human wt in kg})^{0.33} = 30 \text{ mg/kg} \times (0.25 \text{ kg}/70 \text{ kg})^{0.33}$$

Consequently, the dose used here in rats is comparable to a dose of 4.67 mg/kg in human³⁹. A Mann and Whitney test was used for comparison of the two groups of SCT rats 3 weeks post injury with or without pharmacological treatment with MP.

3. Results

3.1. Time course of expression of GlyR and GABA_AR in TA and GS MNs of SCT rats

We examined the time course of expression of GlyR and GABA_AR in TA versus GS MNs at 3 weeks, 8 weeks and 16 weeks post injury (later referred to as T3 w, T8 w and T16 w). Note that transected rats were paraplegic, with a paralysis of the hind limbs persisting at all three stages of the experiments and no recovery of hind limb function was noted after pharmacological treatment of the animals.

3.1.1. GlyR

In control rats, GlyR α 1 was intensely and brightly expressed at the membrane of TA and GS MNs (Fig. 2A). Three weeks after SCT, the expression of GlyR had declined in both TA and GS MNs (Fig. 2A) as proven by quantitative analysis showing significant decrease in membrane fluorescence intensity compared to controls (Fig. 2B, $***p < 0.001$ in both TA and GS MNs). This down regulation was transitory, since at T8 w the median fluorescent intensity of GlyR recovered control values in both TA and GS MNs (Fig. 2B). At late post injury chronic phase (T16 w) the levels of GlyR expression were significantly lower than in controls, on both TA and GS MNs (Fig. 2A, B, $***P < 0.001$ in both cases).

3.1.2. GABA_AR

In control animals GABA_AR displayed a punctiform fluorescent expression in the plasma membrane of TA and GS MNs (Fig. 2A). At T3W, the fluorescent intensity of GABA_AR significantly decreased on the membrane of both TA and GS MNs as compared to intact control rats ($***p < 0.001$ for both TA and GS). Unlike GlyR, there was no rebound of expression of GABA_AR 8 weeks after SCT. Indeed, in TA MNs, the levels of expression of GABA_AR remained significantly lower than in controls at Tw8 and Tw16 (Fig. 2B). Similarly, in GS MNs the expression of GABA_AR remained low in Tw8 and Tw16 animals with levels of fluorescent intensity similar to Tw3 rats (Fig. 2B).

3.2. Differential effects of early post lesion treatment with MP on the expression of GlyR and GABA_AR

We then wanted to know if early anti-inflammatory treatment could reduce the post lesional down regulation of GlyR and GABA_AR observed 3 weeks post injury. SCT rats were treated with MP within 24 hours of surgery. At T3 w, in both TA and GS MNs, the levels of expression of GlyR were significantly higher than in non-treated T3 w animals ($***P$

< 0.001 for both TA and GS), and restored control values in TA MNs (Fig. 2B).

On the contrary, the anti-inflammatory treatment had no effect on the post-lesional down regulation of GABA_AR observed at T3 w. On both TA and GS MNs, T3 w-MP rats displayed similar levels of GABA_AR than T3 w non-treated rats (Fig. 2B).

3.3. Electrophysiological correlates of the development of spasticity after SCT

As expected, in control rats, the H reflex magnitudes gradually attenuated by repeated stimulation at frequencies over 0.2 Hz (Fig. 3A). At T3 w, the H-reflex attenuations at 0.5, 1, 2 and 5 Hz were much less pronounced, as observed in spastic patients. We observed a tendency to recover normal rate-dependent depression of the H reflex at the higher frequencies (2 and mostly 5 Hz) at T8 w and T16 w compared to 3 weeks (Fig. 3A). However, nonlinear regression fits were all significantly different from controls ($p < 0.001$ at 3 weeks and $p < 0.01$ at 8 and 16 weeks).

The analysis of different parameters of the electromyograms showed that the index Hmax/Mmax clearly increased at T3 w ($p < 0.001$) and then returned to control levels at T8w (Fig. 3B). At T16 w, the Hmax/Mmax ratio increased again, although less significantly than T3 w ($P < 0.01$). H reflex activation thresholds and latencies significantly decreased only at T8 w and T16 w (Fig. 3C, D; $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$) although the tendency to decrease was already present at T3w. Correlation analyses showed that the degree of RDD is not dependent on the Hmax size between animals at any given post-operative time (eg. Fig. 3E, F). However, the mean Hmax/Mmax index at different times post-SCT was significantly correlated with the degree of alteration of the H reflex RDD at 2 and 5 Hz (Fig. 3G; $*p < 0.05$).

3.4. The early post lesion treatment with MP completely prevented the attenuation of RDD as measured 3 weeks after SCT

After treatment with MP, the H-reflex attenuations at 0.5, 1, 2 and 5 Hz observed at T3 w were prevented (Fig. 3A). Hmax/Mmax was still slightly but significantly increased compared to controls (Fig. 3B), whereas activation threshold and latencies were significantly decreased (Fig. 3C, D). Moreover, the H reflex was maximal at stimulation intensity where the M wave was 65% of Mmax, whereas it occurred at around 40% of Mmax in controls (Fig. 3E).

4. Discussion

We provide a long-term study of the expression of GlyR and GABA_AR in TA and GS MNs of adult rats after SCT and a parallel investigation of the development of the H-reflex RDD. Although this work does not provide direct evidence of a causal effect between the post-lesion alteration of inhibitory receptors at the membrane of lumbar MNs and the development of spasticity, several data point to significant correlations: 1) Three weeks after SCT, a significant decrease in the expression of GABA_AR and GlyR was observed compared to intact control rats, and the RDD of the H-reflex was much less pronounced than in controls. 2) Eight weeks after SCT, the levels of GlyR returned to normal values whereas a recovery of normal RDD of the H reflex was observed at the higher frequencies. 3) The anti-inflammatory treatment using methylprednisolone restored GlyR to control levels without affecting the expression of GABA_AR, and completely prevented the attenuation of RDD.

4.1. Development of spasticity after SCT

Considering that the RDD alteration of the H reflex is the only electrophysiological parameter identified so far that correlates with the degree of clinically assessed spasticity in humans, this method was used

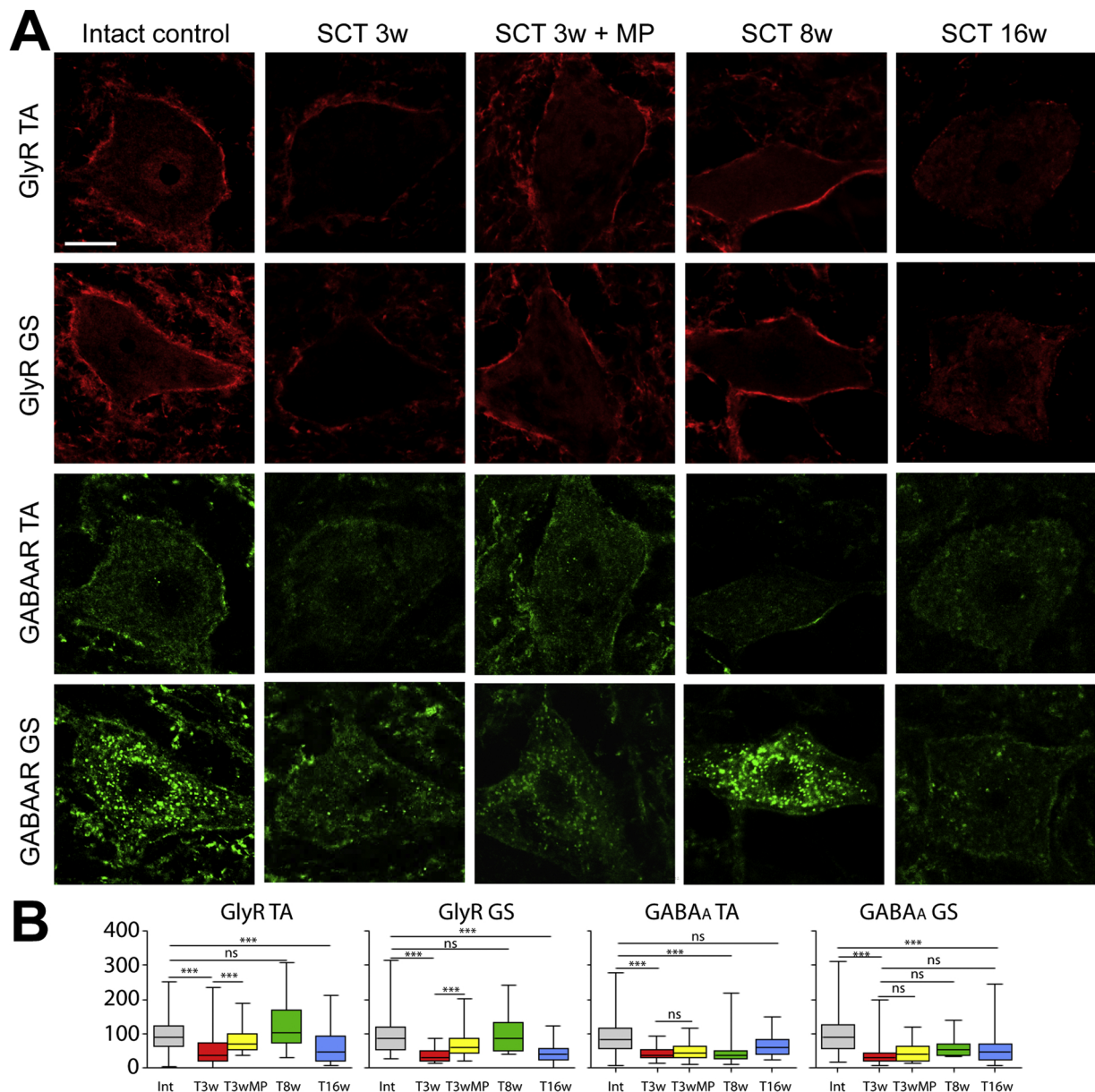


Fig. 2. Longitudinal quantitative immunohistological study of the expression of GlyR and GABA_AR at the membrane of tibialis anterior (TA) and gastrocnemius (GS) MNs in intact control and SCT adult rats. (A) double labeling of GlyR (red) and GABA_AR (green) on TA and GS MNs in intact rats, and in SCT rats 3 weeks, 8 weeks and 16 weeks post injury, and 3 weeks post injury after treatment with MP. (B) Quantitative study of the membrane fluorescent intensities at corresponding post lesion times. The values of the experimental SCT groups are normalized to control values. Int: intact control (grey), T3 w: SCT 3 w post injury (red), T3 wMP: SCT 3 w post injury + methylprednisolone (yellow), T8 w: SCT 8 w post injury (green), T16 w: SCT 16 w post injury (blue).

to quantify spasticity in rats after SCT. Our long term longitudinal study in rats shows that the degree of alteration and the mechanisms underlying the alteration seems to evolve with time since injury. An attenuation of RDD alteration between 1 and 2 months post SCT had already been observed (Lee et al., 2005) although these authors found restoration at low stimulation frequencies, whereas in our study there was a complete and reproducible restoration of RDD at 5 Hz at T8 w and T16 w compared to T3 w. However, our results are in line with an earlier clinical study on the alteration of low frequency depression of the H reflex in individuals with acute and chronic SCI compared with able-bodied controls (Schindler-Ivens and Shields, 2000). They showed that the amplitude of the H reflex decreased with an increasing stimulation rate in all three groups, but the H amplitude was less decreased in the chronic group (8 weeks in our study), particularly at low frequencies. A closer analysis of the parameters of the electromyograms revealed that the main alteration at 3 weeks post SCT is an increase in

the Hmax/Mmax ratio, whereas later a significant increase in activation threshold and latency of the H reflex becomes prominent. Consequently, there is a clear progressive increase in MN excitability which is not yet fully expressed at 3 weeks post SCT when the alteration of RDD is maximal. The restoration of RDD at high stimulation frequency at T8 w is correlated with a restoration of the Hmax/Mmax index, although at this time, activation threshold and latency of the H reflex have become significantly reduced (Fig. 3C, D). When rats were treated with the anti-inflammatory corticoid MP, RDD was completely restored and although Hmax/Mmax was still slightly increased at T3 w, this came almost exclusively from a reduced Mmax (data not shown). These data suggest that RDD attenuation at 5 Hz is correlated with increased Hmax, independently of the degree of excitability of MNs evidenced by decreased activation threshold and latency. These conclusions are reinforced by the significant correlation coefficient between RDD attenuation at 2 and 5 Hz and the size of the Hmax/Mmax index at

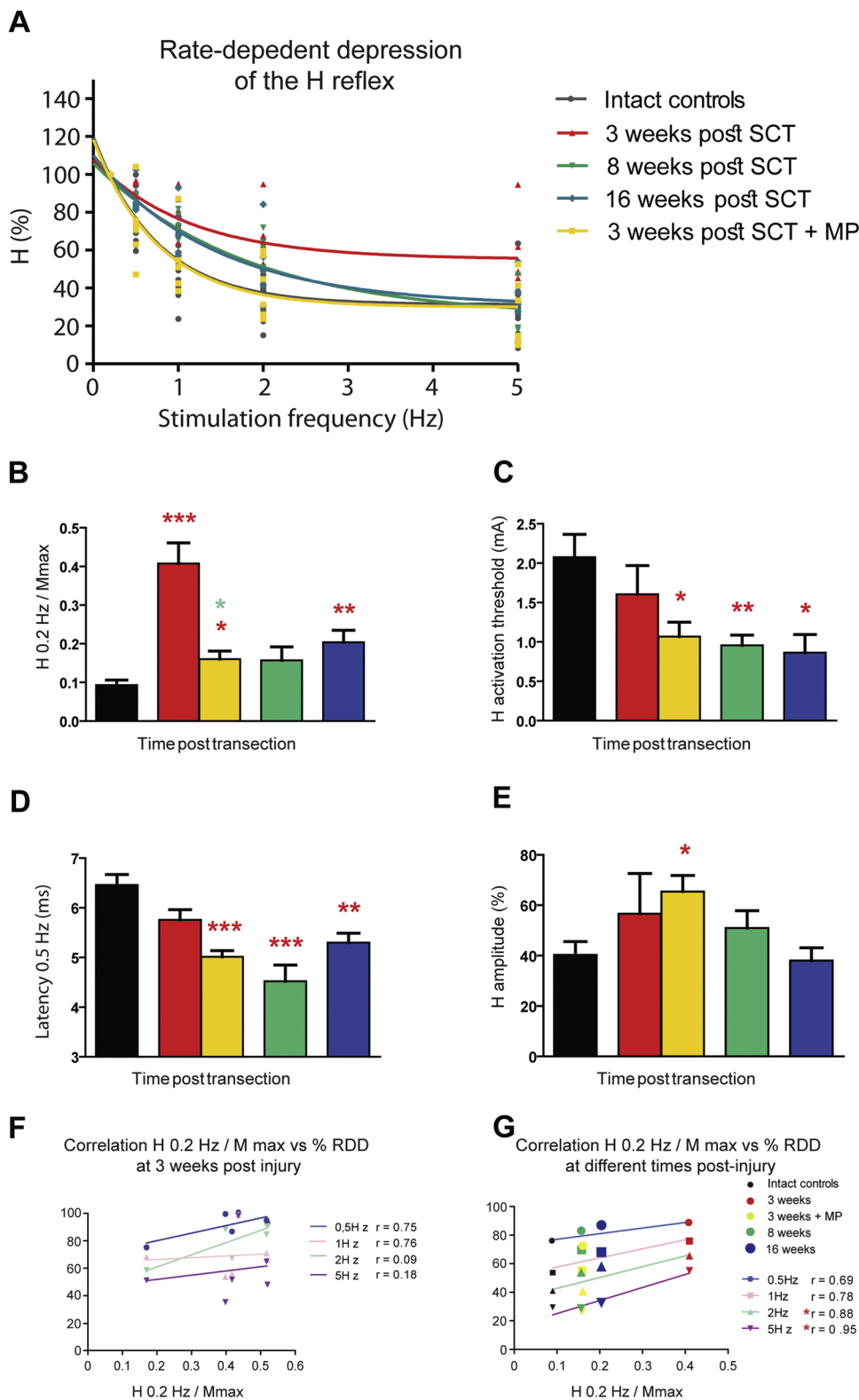


Fig. 3. Longitudinal electrophysiological study of the development of spasticity after SCT. (A) Mean relative amplitudes of the H reflex at frequencies of 0.5, 1, 2 and 5 Hz in intact control rats and in SCT rats at 3 weeks (red), 8 weeks (green) and 12 weeks (blue) after SCT and 3 weeks post-SCT, with early treatment with methylprednisolone (MP; yellow). The data of all experiments (dots) are represented together with the one-phase exponential decay fit of these data. Different parameters of the electromyograms were analyzed: (B) Hmax/Mmax at 0.2 Hz, (C and D) H reflex activation threshold and latencies, (E) amplitude of M wave (percentage of Mmax) at maximal H reflex. Correlation between the degree of RDD and the amplitude of Hmax at 3 weeks post injury (F) or at different post lesion times (DPO) (G).

different times post-SCT.

4.2. GlyR and RDD

Interestingly, the comparison between the time-course of H reflex alterations and GlyR expressions in lumbar MNs shows that the Hmax/Mmax index follows the evolution of GlyR expression, with a maximal increase when GlyR is very low at T3 w, and a restoration of both at T8

w or T3 w after MP treatment, and a significant alteration at T16 w, when GlyR levels are significantly smaller than in controls (Fig. 3B). This indicates that Hmax amplitude in adult rats greatly depends on the level of glycinergic inhibitory influence on MNs. Of course, the loss of GABAergic influences is also important, but the GABAergic axon terminals represent only 20% of the inhibitory inputs (Glycine + GABA) on lumbar MNs in intact adult rats (Khalki et al., 2018) and therefore, the restoration of GlyR expression alone at T8 w or after MP

treatment must be enough to restore a normal Hmax/Mmax. Considering the correlation between alterations of RDD at high stimulation frequency and the size of the Hmax/Mmax index, we suspect that RDD alteration at 5 Hz is also mainly influenced by the degree of glycinergic inhibition on MNs. It has been shown earlier that administration of glycine or closely related compounds reduces spasticity after spinal cord injury (Simpson et al., 1995). Lumbar MNs receive direct glycinergic innervation from different sources, including Ia, Ib and commissural interneurons (Coull et al., 2005; Simpson et al., 1995), as well as Renshaw cells, responsible for recurrent inhibition (Alvarez and Fyffe, 2007). Although Renshaw cells are known to receive inputs from MN collaterals and primary afferents (Alvarez and Fyffe, 2007), their excitability is largely set via reticulo-spinal cholinergic afferents (Jordan et al., 2014). After SCT, the suppression of this source of tonic descending facilitator inputs reduces Renshaw cell activity. Thereby, the decrease of recurrent inhibition activity on MNs results in an increased motoneuronal excitability, which is also due to alteration in intrinsic properties (Brocard et al., 2016). Interestingly, this corroborates early clinical studies showing that, in spastic patients with SCI, a reduction of Renshaw cell activity results from an interruption of the reticulo-spinal system (Mazzocchio and Rossi, 1997). In all, these data suggest a major influence of glycinergic recurrent inhibition in spasticity.

4.3. GABA_AR, RDD and anti-inflammatory treatment

RDD of the H-reflex amplitude has been used as an index of spinal GABA-mediated physiological response in diabetic or BDNF-treated rat models of neuropathic pain (Jolivalt et al., 2008; Lee-Kubli and Calcutt, 2014). However, it seems that the inhibitory mechanisms involved in depressed RDD are different in these pain models from SCT models of spasticity. 1) In pain models, spinal KCC2 expression is significantly reduced in the dorsal but not ventral spinal cord (Jolivalt et al., 2008; Lee-Kubli and Calcutt, 2014), whereas after SCT the levels of KCC2 in lumbar MNs are significantly reduced (Bos et al., 2013; Boulenguez et al., 2010; Khalki et al., 2018). 2) While in pain models the levels of GABA_AR were unchanged (Jolivalt et al., 2008; Lee-Kubli and Calcutt, 2014), our SCT model showed a significant decrease in the post synaptic expression of GABA_AR in lumbar MNs at different times post injury. Interestingly, in line with our findings, a loss of GABAergic inhibitory systems associated with diminished RDD and spasticity have been reported in models of spinal cord injury induced by transient ischemia in rats (Kakinohana et al., 2006). Our results also point to the lack of effect of the anti-inflammatory treatment on the expression of GABA_AR whereas it restored the control levels of GlyR concomitantly with a completely prevented attenuation of RDD. In this regard, in vitro experiments in the spinal cord of intact mice have demonstrated that MP increases the frequency of IPSCs and decreases the amplitude of inhibitory postsynaptic currents (IPSCs), presumably reducing responsiveness of the postsynaptic GABA_AR (Foroutan et al., 1996). This has been attributed to an increase in the presynaptic GABA release and a diminished responsiveness of the postsynaptic GABA_AR, respectively (Foroutan et al., 1996). Interestingly, the SCT by itself has the effect of significantly increasing the density of presynaptic GABAergic axon terminal on lumbar MNs (Khalki et al., 2018) and of down regulating the GABA_AR in the motoneuronal membrane (present work). Therefore, also the relationship between morphological parameters (afferent input vs receptor densities) and their efficiency cannot be established on the basis of anatomical data alone, it cannot be ruled out that an effect of MP treatment on GABAergic transmission could be masked by SCT itself.

4.4. Functional considerations

Our results show that the time-course of GlyR expression is very similar in TA and GS MNs. In the same way, the temporal expression

pattern of GABA_AR is close in both groups of MNs. According to some authors (Khristy et al., 2009), the similarities between fast flexor and fast extensor motors were predictable given the similarities in the synaptic organization of afferent inputs to the antagonist MNs (amplitudes of IPSPs and EPSPs from group 1 afferents, EPSPs from medio-lateral funiculus or vestibulospinal tract). With regard to synaptic glycinergic transmission, after the acute post-operative down-regulation, we observed, at T8 w, a rebound in GlyR expression to normal values, in both TA and GS MNs. In this post-injury delay window, the densities and size of pre-synaptic glycinergic axon terminals apposed on GS and TA MNs displayed normal values (Khalki et al., 2018). This suggests a matching of presynaptic axon terminals and postsynaptic membrane receptors, which is a prerequisite for efficient synaptic transmission (Lardi-Studler and Fritschy, 2007). In contrast, several weeks after SCI, the strength of postsynaptic inhibition is reduced due to the down regulation of both GlyR and the co-transporter KCC2 in the motoneuronal membrane (Khalki et al., 2018), thereby contributing to spasticity (Boulenguez et al., 2010). In this regard, it is important to mention recent studies suggesting that in patients with SCI, KCC2 dysfunction may be an important aetiological factor in hyperreflexia (Klömjai et al., 2019).

5. Conclusions

This study demonstrates differential effects of SCT on the expression of GABA_AR and GlyR on lumbar MNs in adult rats, in line with results reported after neonatal transection (Sadlaoud et al., 2010). Furthermore, our results strongly suggest that the RDD of the H-reflex, correlated with the degree of spasticity, is influenced by alteration of post lesional glycinergic transmission and could particularly involve recurrent inhibition. We also show that anti-inflammatory treatment using Methylprednisolone restored normal levels of GlyR expression and prevented the attenuation of RDD, but has no effect on the expression of GABA_AR. Our study points out some of the multifactorial causes of spasticity and underlies relationships between several aspects of the alterations of the sub-lesional networks in the chronic phases after SCT.

CRedit authorship contribution statement

Karina Sadlaoud: Methodology, Investigation, Resources. **Loubna Khalki:** Methodology, Investigation, Resources. **Fredéric Brocard:** Conceptualization, Funding acquisition. **Laurent Vinay:** Supervisor, Funding acquisition. **Pascale Boulenguez:** Conceptualization, Methodology, Writing - original draft, Visualization. **Hélène Bras:** Conceptualization, Methodology, Funding acquisition, Writing - original draft, Visualization.

Declaration of Competing Interest

No competing financial interest exist

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