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Differential effects of spinal cord transection on glycinergic and GABAergic synaptic signaling in sub-lesional lumbar motoneurons.

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Abstract

This review takes stock on the impact of complete spinal cord transection (SCT) on the plasticity of the inhibitory synaptic transmission on sub-lesional lumbar motoneurons (MNs), differentiating between studies in neonate and adult rats.

After neonatal SCT, normal maturational up-regulation of glycine receptors was observed. On the other hand, the developmental downregulation of the GABA_A receptors, as well as the up-regulation of the co-transporter KCC2, but not the normal decrease of NKCC1, were prevented. In adult SCT rats, glycinergic synaptic transmission, which is the major contributor to spinal MNs inhibition in adulthood, had normal control levels 2 months post-injury. On the other hand, the GABAergic transmission was altered through an up-regulation of the pre-signaling levels and a down-regulation in the density of post synaptic receptors. KCC2 membrane expression was down-regulated at all post-injury times tested (24h to 4 months), thereby depolarizing the Cl⁻ equilibrium potential and reducing the strength of postsynaptic inhibition.

The preservation of glycinergic pre- and post signaling, is probably a key factor in the success of locomotor rehabilitation programs in adult SCT rats. However, these data highlight the need to develop strategies to restore KCC2 levels in lumbar MNs, to stabilize the excitation/inhibition balance, which is essential to the effective control of skeletal muscle activity.

Highlights

- Rats with complete spinal cord transection (SCT) are unambiguous models of paraplegia.
- We review the plasticity of inhibition on spinal MNs in neonate *vs* adult SCT rats.
- In both models, glycinergic pre- and post-synaptic signalings were normal.

- On the contrary, the levels GABA_AR and KCC2 were dysregulated.
- After SCT, KCC2 levels must be restored to increase glycinergic inhibition strength.

Introduction

More than 53% of patients with traumatic spinal cord injury are paraplegic. This justifies the use of rats with low thoracic spinal cord transection (SCT) rats, unambiguous models of paraplegia, to study the plasticity of the neural networks below the lesion (Edgerton *et al.*, 2001; Rospignol, 2006). Among the changes resulting from the loss of supra-spinal descending inputs in SCT rats, alterations in inhibitory synaptic transmission to lumbar motoneurons (MNs), mediated by glycine receptors (GlyR) and γ -aminobutyric acid receptors (GABA_AR) (for reviews see (Fritschy, 2015; Legendre, 2001)), have been the subject of several studies over the last 20 years. They include changes in size and density of pre-synaptic inhibitory inputs (glycinergic and/or GABAergic) (Edgerton *et al.*, 2001; Ichiyama *et al.*, 2011; Khalki *et al.*, 2018), post-synaptic expression of GlyR and GABA_AR subunits (Edgerton *et al.*, 2001; Khristy *et al.*, 2009; Sadlaoud *et al.*, 2020; Sadlaoud *et al.*, 2010), anchoring protein gephyrin (Edgerton *et al.*, 2001; Sadlaoud *et al.*, 2010), and cation chloride co-transporters KCC2 (and/or NKCC1) that regulate intracellular chloride levels required for inhibition (Bos *et al.*, 2013; Boulenguez *et al.*, 2010; Chopek *et al.*, 2015; Cote *et al.*, 2014; Jean-Xavier *et al.*, 2006; Khalki *et al.*, 2018; Liabeuf *et al.*, 2017). However, the variability of the experimental procedures and particularly of the onset of the lesion (cord-transected neonates *vs* adults) and the post-lesional delays, prevent a clear overview of the time course of this alteration. The purpose of the present work is to take stock of these data, based on the works in the literature listed in Table 1. Although some early studies have been done in SCT cats (Tillakaratne *et al.*, 2002; Tillakaratne *et al.*, 2000), only those done in SCT rats, which are the majority, have been considered here.

Developmental membrane expression of GlyR and GABA_AR in spinal MNs: Glycinergic ionotropic receptors form pentameric transmembrane quaternary structure. In fetal rat spinal cord neurons, GlyRs have a dominant homomeric form composed of 5 α 2 subunits. Expression of α 2 subunits decreases after birth, in favour of α 1 expression, and GlyR becomes heteromeric with an invariant stoichiometry of 3 α /2 β (Legendre, 2001). During development, the density of GlyR increased from P1 to P7 (See Fig.1) to reach a plateau that remains stable at P12 (Sadlaoud *et al.*, 2010). In adults, glycine and strychnine bind to α subunits only (Legendre, 2001).

GABA_ARs form pentameric complexes assembled from a family of at least 15 subunits (α 1-6, β 1-3, γ 1-3, δ , ρ 1-2, (Bohlhalter *et al.*, 1996)). GABA_ARs are expressed early during ontogeny (E13), and α 1, α 2, α 3 and α 5 mRNAs are detected in the embryonic spinal cord (Ma *et al.*, 1993). At P1, only the GABA_AR α 2 and GABA_AR α 3 remain expressed on the spinal motoneuronal membrane (Sadlaoud *et al.*, 2010). During the first postnatal week, the expression of both subunits decreases but they are still observed in juvenile animals (P12), with a ratio of 5 GlyR α 1 to 1 GABA_AR α 2, α 3 (Sadlaoud *et al.*, 2010). In the mature spinal cord, the α 2 β 3 γ 2 and α 3 β 3 γ 2 combinations are preponderant (Fritschy and Brunig, 2003). The α and β subunits of the GABA_AR are required for GABA binding, and the α subunits determines the kinetics of deactivation and/or desensitization of the channel properties (Gingrich *et al.*, 1995). The co-assembly of γ with α sub-units is required for benzodiazepine binding (Khristy *et al.*, 2009).

Membrane expression of GlyR and GABA_AR in spinal MNs after neonatal SCT: After neonatal SCT (P0), normal densities of GlyR α 1 were observed in the plasma membrane of triceps surae (TS) MNs at P7 (Sadlaoud *et al.*, 2010). On the contrary, the developmental downregulation of the α 2 and α 3 subunits of the GABA_AR was prevented (Sadlaoud *et al.*, 2010) (see Fig.1), whereas the GABA_AR γ 2 was down-regulated in soleus MNs and up-regulated in tibialis anterior (TA) MNs (Khristy *et al.*, 2009). Moreover, the up-regulation of KCC2 normally observed from P0 to P7 was prevented after neonatal SCT in the region of MNs (Jean-Xavier *et al.*, 2006) as well as on TS MNs (Bos *et al.*, 2013). SCT eliminates the major supra spinal source of 5HT, and only 2–15% of the 5-HT levels remain below the lesion, partly due to intrinsic spinal 5-HT neurons (Kong *et al.* 2010). 5-HT_{2A} receptors expression increases after SCI (Kong *et al.* 2010) and their activation with the specific agonist TCB2 leads to the restoration of KCC2 expression (Bos *et al.* 2013). In neonatal SCT rats treated with DOI, a non-selective agonist of 5-HT_{2A}, B and C receptors, GlyR α 1 expression remained stable, while that of GABA_AR (Sadlaoud *et al.*, 2010) as well as KCC2 (Bos *et al.*, 2013) returned to control values. Interestingly, 5-HT_{2B}Rs and 5-HT_{2C}Rs, but not 5-HT_{2A}Rs become constitutively active (spontaneously active without 5-HT) after SCI (Murray *et al.*, 2010) and this constitutive activity may be partly responsible for the depolarizing shift of the chloride equilibrium potential after SCI (Bos *et al.*, 2013). This suggested that the plasticity of GlyR is independent of supraspinal influences whereas that of GABA_AR and KCC2 is markedly influenced by descending pathways, in particular serotonergic projections (Jean-Xavier *et al.*, 2006; Sadlaoud *et al.*, 2010).

Membrane expression of GlyR and GABA_AR in spinal MNs of adult SCT rats: In rats transected as adults, the expression of glycinergic and GABAergic synaptic signaling followed different time courses. The acute post-lesion phase was marked by an unstable temporal and spatial pattern of alterations in GlyR1-3 mRNA expression with different levels of up or down regulation occurring within 6h to 10 days after lesion (Esmaeili and Zaker, 2011). In the early chronic phase, 3 weeks post-injury, the levels of expression of GlyR α 1 were significantly lower than in controls (Sadlaoud *et al.*, 2020). This is related to inflammatory responses, as early treatment with the corticosteroid methylprednisolone allows a return to normal values. This down-regulation of the levels of GlyR α 1 was transitory, since at later times (8 weeks post-lesion), GlyR α 1 expression had normal control values in both TA and gastrocnemius (GS) MNs (Sadlaoud *et al.*, 2020). In this post-injury period, the density and size of pre-synaptic glycinergic inputs apposed on GS and TA MNs also displayed normal values (Khalki *et al.*, 2018), suggesting a matching between pre-synaptic inputs and post-synaptic membrane receptors, which is a prerequisite for efficient synaptic transmission (Lardi-Studler and Fritschy, 2007). Note that after very long post-injury periods (16 weeks), the expression of GlyR α 1 was significantly lower than in controls, in both TA and GS MNs (Sadlaoud *et al.*, 2020).

In adult SCT rats, the sub-lesional spinal GABAergic synaptic system was differently affected than the glycinergic one. Three weeks after SCT, a down-regulation of GABA_AR occurred, and could not be reversed by treatment with the anti-inflammatory methylprednisolone (Sadlaoud *et al.*, 2020). Then, at longer times post-injury (8 and 16 weeks) the levels of GABA_AR at the membrane of GS and TA MNs remained significantly lower than in intact controls (Sadlaoud *et al.*, 2020). In this time window (11 weeks post-injury) an increase in the densities of GABAergic axon terminals apposed on lumbar MNs has been reported (Khalki *et al.*, 2018). Therefore, we are in the presence of a drastic mismatch between pre-synaptic and post-synaptic specializations, which suggests an impairment of the GABAergic synaptic transmission to sub-lesional MNs, from early to late chronic phase post-injury.

In summary, these data suggest that SCT differentially affects the two inhibitory neurotransmissions to lumbar MNs caudal to the lesion, whether the lesion is done at birth or in adults. The glycinergic system remains relatively stable, at least up to 2 months post-injury, whereas the GABAergic transmission is strongly altered. In the other hand, SCT prevents the maturational up-regulation of KCC2 expression in neonate rats (Bos *et al.*, 2013; Jean-Xavier *et al.*, 2006) and results in a down-regulation of KCC2 levels in MNs of adult rats (Boulenguez

et al., 2010; Chopek *et al.*, 2015; Cote *et al.*, 2014; Khalki *et al.*, 2018), thereby depolarizing the Cl⁻ equilibrium potential and reducing the strength of post-synaptic inhibition (see Figure 1) and contributing to spasticity (Boulenguez *et al.*, 2010).

Functional considerations: Since GlyR expression returns to control levels by 2 months post-lesion, one would expect improvement of the hindlimb function at this stage. However, no spontaneous locomotor recovery could be observed in adult SCT rats (Alluin *et al.*, 2015). At all post-injury times tested (1, 7, 15, 21, 28 and 45 days, 8 and 11 weeks, 3 and 4 months, see Table 1) there was a down regulation of the levels of KCC2 in sub-lesional MNs. Note that the expression rates of NKCC1, which decreases from P5 to low levels in adult spinal MNs (Still *et al.* 2009), are not significantly affected by SCI in adult rats (Boulenguez *et al.*, 2010; Cote *et al.*, 2012). A restoration of KCC2 levels has been observed in SCT adult rats using sensorimotor strategies (Chopek *et al.*, 2015; Cote *et al.*, 2014; Khalki *et al.*, 2018) or pharmacological treatments with the KCC2 activator prochlorperazine (Liabeuf *et al.*, 2017) or the calpain inhibitor MDL28170 (Plantier *et al.*, 2019). The effect of this up-regulation is twofold. It allows 1) a strengthening of inhibition through the preserved GlyR and chloride homeostasis and 2) a decrease of MNs hyperexcitability which resulted from the up-regulation of the persistent sodium current (INap) responsible for spasms (Brocard *et al.*, 2016; Plantier *et al.*, 2019). Therefore, the post lesional preservation of the glycinergic synaptic system, which is the major contributor to spinal MNs inhibition in adult (Gao *et al.*, 2001; Khalki *et al.*, 2018), is undoubtedly one of the factors that makes the locomotor rehabilitation possible in SCT adult rats. However, the search for strategies for restoring KCC2 levels, to maintain the chloride homeostasis and reduce the imbalance between excitation and inhibition, is a major issue to alleviate motor disorders affecting individuals with spinal cord injury, such as spasticity.

Study	Animal	Detection	Lesion	Lesion onset	Post lesional delay	studied regions (targets)	pre-synaptic signaling (inputs) onto MNs	post synaptic signaling (MN)	Expression/levels % controls
Edgerton et al., 2001	rat	WesternBlot	SCT	P7	2 weeks			GlyRa1	up-regulated
								Gephyrin	up-regulated
Khristy et al., 2009	rat	IHF	SCT	P5	3 month	Soleus MNs		GABA _A Rγ2	down-regulated
						TA MNs		GABA _A Rγ2	up-regulated
Jean-Xavier et al. 2006	rat	IHF	SCT	P0	7 days	MNs pool (L4 segment)		KCC2	down-regulated
Sadlaoud et al. 2010	rat	IHF	SCT	P0	7 days	TS MNs		GlyRa1	no change
								GABA _A Rα2	up-regulated
				P5	7 days	TS MNs		GlyRa1	no change
								GABA _A Rα2	up-regulated
				GABA _A Rα3	up-regulated				
Ichijama et al., 2011	rat	electron microscopy	SCT	P5	16 days	α and γ TA MNs	S-type boutons F-type boutons C-type boutons M-type boutons		Proportion of inhibitory to excitatory boutons increased in both MNs
Bos et al., 2013	rat	IHF, WB	SCT	P0	7 days	TS MNs		KCC2	down-regulated
Tillakaratne et al. 2000	cat	IHF, ISH, WB	SCT	adult	3, 6, 9 months	lumbar grey matter		GAD67	up-regulated
								GAD65	no change
Tillakaratne et al 2002	cat	IHC, ISH, WB	SCT (T12)	adult	3 to 25 months	LIX neurons (L5-L7 segments)		GAD67	up-regulated
Boulenguez et al., 2010	rat	IHF, WB	SCT	adult	24h 7 days 15 days 45 days	lumbar MNs		KCC2	8% decrease
									10% decrease
									19% decrease
									22% decrease
Esmaili and Zaker 2011	rat	real time reverse transcription PCR	SCT	adult	6h, 24h, 3j, 7j, 10j	spinal cord 2,5 cm pre and post lesion site		GlyR1 mRNA, GlyR2 mRNA, GlyR3 mRNA	up regulate 7 day PL, down regulated, up regulated 3 days PL
Coté et al., 2014	rat	WB	SCT	adult	28 days	lumbar segment		NKCC1	no change
								KCC2	down-regulated
Chopek et al. 2015	rat	qRT-PCR mRNA	SCT	adult	3 months	GS, Soleus MNs		KCC2	no significant change
						TA, EDL MNs		KCC2	20% decrease
Liabeuf et al. 2017	rat	IHF	SCT	adult	3 weeks	lumbar MNs		KCC2	20% decrease
Khalki et al., 2018	rat	IHF	SCT	adult	11 weeks	GS MNs	GABA boutons		up-regulated
						TA MNs			up-regulated
						GS MNs	Glycine boutons		no change
						TA MNs			no change
				KCC2	30% decrease				
Sadlaoud et al. 2020 (in press)	rat	IHF	SCT	adult	3 weeks	TA MNs		GlyRa1	down-regulated
								GABA _A Rβ2,3	down-regulated
						GS MNs		GlyRa1	down-regulated
								GABA _A Rβ2,3	down-regulated
					8 weeks	TA MNs		GlyRa1	no change
								GABA _A Rβ2,3	down-regulated
					GS MNs			GlyRa1	no change
								GABA _A Rβ2,3	down-regulated
16 weeks	TA MNs		GlyRa1	down-regulated					
			GABA _A Rβ2,3	down-regulated					
	GS Mns		GlyRa1	down-regulated					
			GABA _A Rβ2,3	down-regulated					
Table 1									

Table 1: Summary of literature data on the effect of SCT on the inhibitory pre- and post-synaptic signaling. IHF: Immunohistofluorescence; IHC: immunohistochemistry; ISH: In situ hybridization; WB: Western blot; SCT: Complete spinal cord transection; MN: Motoneuron; TA: Tibialis anterior; GS: Gastrocnemius; TS: Triceps surae

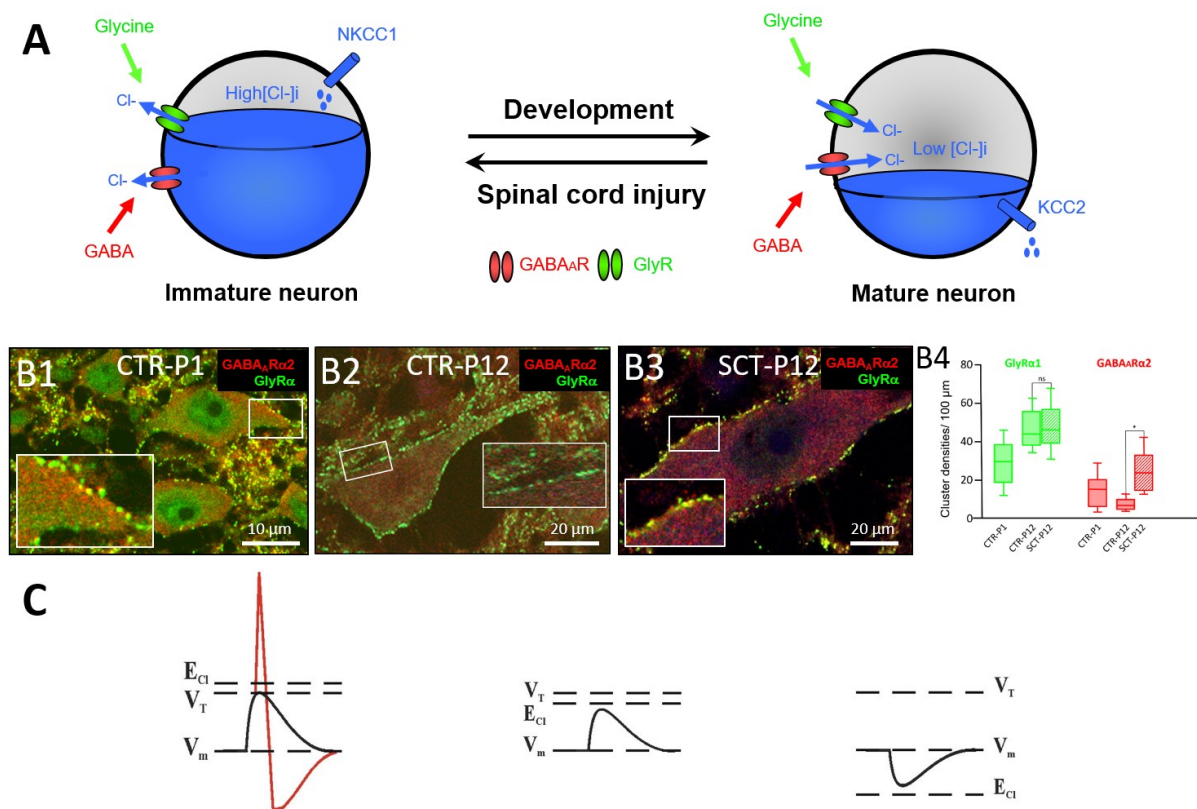


Figure 1: Regulation of intracellular Cl^- concentration during development and after spinal cord injury. A) The two inhibitory neurotransmitters glycine and GABA act through glycine and GABA_A receptors (GlyR and GABA_AR), respectively. Cation–chloride (Cl^-) cotransporters NKCC1 (Cl^- - intruder) and KCC2 (Cl^- - extruder) are responsible for the regulation of intracellular chloride concentration ($[Cl^-]_i$). B) Dual immunohistochemistry of GlyR α 1 and GABA_AR α 2 on identified TA MNs in control (CTR) rats at P1 (B1), during development and on juvenile control rats (P12; B2), and P12 rats transected at P7 (SCT-12; B3). B4: Histograms of the densities of GlyR α 1 and GABA_AR α 2 per 100 μm of somatic motoneuronal membrane, quantified in CTR-P1, CTR-P12 and SCT-P12, respectively. Adapted from (Sadlaoud *et al.*, 2010). C) In immature neurons, $[Cl^-]_i$ is high and the equilibrium potential of Cl^- (E_{Cl}) is above the resting membrane potential (V_m). Activation of GlyR and GABA_AR leads to depolarization and may even trigger cell firing when E_{Cl} is above action potential threshold (V_T). During development, because of the down-regulation of NKCC1 and up-regulation of KCC2, $[Cl^-]_i$ decreases, E_{Cl} shifts to values below V_m . As a result, the strength of post-synaptic inhibition increases. Spinal cord transection rapidly transforms the actions of GABA and glycine from inhibition to excitation, by increasing the $[Cl^-]_i$ via downregulation of KCC2. Adapted from (Vinay and Jean-Xavier, 2008).

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