Alteration of the H-reflex after compression spinal cord injury in mice (*Mus musculus*, Linné 1758)

Dissertation

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Abstract

Alterations in spinal reflexes and functional improvements occur after incomplete spinal cord injury but the relationship between these phenomena is not understood. Here we show that spontaneous functional recovery after compression injury of the spinal cord at low-thoracic level (Th10-12) in C57BL/6J mice is associated with a progressively increasing, over 3 months, excitability of the plantar H-reflex. The stimulation rate-sensitive H-reflex depression, already strongly reduced at 1 week after injury, when compared with non-injured mice, decreased further during the observation time period. Twelve weeks after injury, the degree of motor recovery estimated by single-frame motion analysis in individual animals correlated positively with their H-reflex responses at 2-Hz stimulation. Functional recovery and reflex alterations were accompanied by an increase in glycine/GABAergic and glutamatergic terminals around motoneuron cell bodies between 6 and 12 weeks after injury. Enhanced H-reflex responses at frequencies between 0.1 and 5 Hz were also observed in mice deficient in the extracellular matrix glycoprotein tenascin-R and the adhesion molecule close homolog of L1, mice previously shown to have better motor recovery after spinal cord injury than wild-type littermates. In contrast, attenuated H-reflex and worse functional recovery, as compared with wild-type littermates, was found in mice deficient in the extracellular glycoprotein tenascin-C. These results indicate that better functional outcome of compression spinal cord injury in mice is associated

with alterations of the monosynaptic reflex pathway which facilitate motoneuron recruitment. Our observations support the view that plasticity of spinal circuitries underlies specific aspects of motor recovery and demonstrate the usefulness of H-reflex analyses in studies on spinal cord injury in mice.

I Introduction

Although the mammalian central nervous system has a limited ability for regeneration, patients with spinal cord injuries gradually recover some motor and sensory functions with time after injury (Barbeau and Rossignol, 1987; Belanger et al., 1996; Rossignol, 2000, 2002). This limited recovery is attributed to reorganizations of spinal circuitries, a process known as plasticity and including adaptive changes in physiological, morphological and chemical properties of the injured spinal cord. This work is focused on the relationships between motor outcome and physiological alterations in motoneuron reflex pathways, as well as synaptic plasticity after spinal cord injury in mice.

1.1 Spinal cord injury and reflexes

1.1.1 Hyperreflexia after spinal cord injury

Acute spinal cord injury in humans and experimental animals like monkeys, cats and rats causes spinal shock, a syndrome consisting of loss of sensory and motor functions, loss of tendon (stretch) reflexes and flaccid muscle tone (Dietz and Colombo, 2004; Frigon and Rossignol, 2006). Over weeks and months after injury, a spasticity syndrome progressively develops. This syndrome is characterized by hyperreflexia (enhanced tendon reflexes), increased muscle tone, clonus (repetitive, rhythmic contractions of muscles upon stretching them) and muscle spasms (Little et al., 1989). Initially, development of spasticity is paralleled by recovery of some motor and sensory functions whereby the degree of

spontaneous restoration is inversely related to the lesion severity and is generally limited. In the chronic phase of spinal cord injury, spasticity and functional recovery occur at lower rates and are no longer simultaneous. Spasticity is debilitating in that it causes pain, sleep disturbances and affects preserved motor functions, and it is believed that it limits functional restoration (Little et al., 1989; Little et al., 1999). However, the latter view is contradicted by the fact that muscle hypertonia aids walking in many patients with central nervous system (CNS) lesions and observations showing that antispastic drugs can worsen pareses (Dietz and Sinkjaer, 2007). The symptoms of spasticity have been considered to be consequences of exaggerated monosynaptic (tendon) and polysynaptic (cutaneous/flexor) reflexes (Abbruzzese, 2002; Sheean, 2002). It is now obvious that the role of hyperreflexia has been overestimated and a current view is that spastic movement disorders arise from loss of supraspinal control, defective use of afferent information in the context of altered spinal reflexes, and alterations in the paralyzed muscles (Dietz and Sinkjaer, 2007). It is thus evident that the relationships between functional recovery, spinal reflex properties and spasticity after spinal cord injury are not understood.

1.1.2 Motoneuron excitability and H-reflex

Spinal reflex excitability is regulated by two physiological mechanisms, excitatory initiation of reflexes and inhibitory regulation. A balanced sum of these two processes is required for normal reflex excitability which is critical for locomotor outcomes. After spinal cord injury, both excitatory inputs to motoneurons and inhibitory regulation are disturbed. Various experimental models

have shown that the excitatory postsynaptic potentials (EPSPs) from muscle afferents to motoneurons increase after, for example, spinal cord transection (Nelson et al., 1979; Cope et al., 1988) and acute dorsal hemisection (Carp and Rymer, 1986). In addition to the excitatory potential changes in the reflex pathway, decreased inhibition contributes to the hyperreflexia observed after spinal cord injury (Ishikawa et al., 1966). Reflex excitability in neurological disorders, including spinal cord injury, can be assessed by the H-reflex. The H- (Hoffmann) reflex, first described by the German physiologist Paul Hoffmann (Hoffmann, 1910), is an analog of the spinal stretch reflex elicited by electrical stimulation of afferent fibers in a mixed nerve with subsequent recruitment of motor neurons through monosynaptic Ia connections in the spinal cord (Magladery and McDougal, 1950). The H-reflex provides information on the functional properties of Ia afferents and homonymous alpha-motoneurons under physiological and pathological conditions (Magladery et al., 1951; Gozariu et al., 1998; Pierrot-Deseilligny and Mazevet, 2000). The amplitude of the H-reflex, as a compound muscle action potential, reflects the amount of activated motor units in the reflex pathway under given conditions of stimulation. Conditioning stimuli and different stimulation frequencies are used to investigate changes in the excitability of spinal reflexes. For example, tendon vibration reduces the amplitude of H- and tendon reflexes (De Gail et al., 1966; Gillies et al., 1969; Van Boxtel, 1986), changes in muscle length modulate the H-reflex amplitude (Hultborn et al., 1996; Pinniger et al., 2001) and trains of electrical stimuli reduce the H-wave amplitude as a result of presynaptic depression in Ia terminals (Kohn et al., 1997; Morita et al., 1998; Enriquez-Denton et al., 2002).

Several parameters of the H-reflex are crucial for evaluating reflex properties in clinical conditions such as CNS injuries and neuropathies, and under normal physiological conditions, for example during walking and standing in humans (Capaday and Stein, 1986; Crenna and Frigo, 1987; Simonsen and Dyhre-Poulsen, 1999; Hopkins et al., 2000; Rossignol et al., 2006). Threshold and recruitment curve during the H-reflex initiation are used as critical measures for the sensitivity of the H-reflex analysis (Thompson et al., 1992; Valero-Cabre et al., 2004; Ginanneschi et al., 2006). It has been demonstrated that threshold and recruitment of the soleus H-reflex are not altered in patients, even with spasticity, after complete spinal cord injury as compared with intact individuals (Boorman et al., 1996; Schindler-Ivens and Shields, 2004). The amplitude of M- and H-wave is considered as an useful measure of maximum voluntary contractions, especially following injuries (Hopkins and Wagie, 2003). The magnitude of the H-reflex is proportional to the number of activated motoneurons (Magladery and McDougal, 1950) and depends on the neurotransmission efficacy at the Ia afferent – motoneuron synapses (Magladery et al., 1951; Burke et al., 1984; Wolpaw, 1987).

One interesting property of the H-reflex is the rate-dependent depression of its amplitude which decreases gradually as stimulation frequency increases. The range of frequencies used to analyze the rate depression is between 1 and 10 Hz in humans (Lloyd and Wilson, 1957; Schindler-Ivens and Shields, 2000) and between 0.3 and 5 Hz in rats (Meinck, 1976; Thompson et al., 1992). Because the high-frequency stimulation does not affect the magnitude of the M-wave and motoneuron excitability modulations elicited by conditioning stimuli, it is assumed that presynaptic inhibition at Ia – motoneuron synapses plays a major role in the rate depression of the H-reflex (Lloyd, 1957; Curtis and Eccles, 1960; Thompson

et al., 1992; Schindler-Ivens and Shields, 2000). Gamma-aminobutyric acid (GABA)-mediated inhibition is involved in presynaptic inhibition (Rudomin, 1999). For example, the type B GABA receptor agonist L-baclofen reduces the voltage dependent Ca^{2+} conductance and influx evoked by presynaptic action potentials with consequently impaired transmitter release in Ia synaptic terminals leading to rate-dependent depression of spinal monosynaptic reflexes (Lev-Tov et al., 1988).

1.1.3 Alteration of the H-reflex after spinal cord injury

During the period of spinal shock after acute spinal cord injury in human, stretch reflexes are lost but H-reflex responses with reduced amplitudes can be usually recorded (Cadilhac et al., 1977; Little and Halar, 1985; Leis et al., 1996). The H-reflex is rapidly restored after the spinal shock and increases further as spasticity develops (Hiersemenzel et al., 2000). After spinal cord transection in rats, the M-wave slightly decreases immediately after the injury and barely recovers thereafter, whereas the H-reflex amplitude is increased, reaching significantly higher values compared with intact animals at 2 weeks after injury (Valero-Cabre et al., 2004). In the chronic phase of spinal cord injury in humans and laboratory animals, the H-reflex is facilitated upon single and repetitive low-frequency stimulation (Thompson et al., 1992; Little et al., 1999; Valero-Cabre et al., 2004; Lee et al., 2005). This is an objective measure of reflex hyperexcitability which, however, has barely been exploited to correlate reflex pathway properties with functional outcome in controlled animal experiments (Lee et al., 2005).

After spinal cord injury, the rate depression of the H-reflex is reduced in both human subjects with spasticity (Ishikawa et al., 1966; Calancie et al., 1993)

and in experimental animals such as cats (Murayama and Smith, 1965), and dogs (Gelfan, 1966). Also in the rat, 3 months after spinal transection, the H-reflex is decreased compared with control animals at stimulation frequencies of 1, 5 and 10 Hz (Skinner et al., 1996). Reduction of the rate depression has also been observed after contusion spinal cord injury in rats at 28 and 60 days, but not at 6 days after injury (Thompson et al., 1992). Rate depression is enhanced in patients under spinal shock and is reduced with time after injury (Calancie et al., 1993). It seems that reduced presynaptic inhibition is responsible for the alteration of rate depression after spinal cord injury. Histological studies have revealed that only 25% of the intact interneuronal population survive after temporary lumbosacral ischemia, a procedure leading to hind limb rigidity (Gelfan, 1966). In general, it is assumed that inhibitory interneurons are critical for the physiological expression of rate depression in the spinal monosynaptic reflex (Eccles et al., 1961; Jankowska et al., 1981).

Although the role of presynaptic inhibition is emphasized, changes in motoneuron properties apparently also contribute to alterations of rate depression after spinal cord injury. For example, high frequency stimulation of Ia afferents evokes excitatory postsynaptic potentials (EPSPs) in motoneurons which depend on motoneuron properties such as rheobase (Koerber and Mendell, 1991).

1.2 The close homologue of L1 (CHL1)

1.2.1 The neural cell adhesion molecule CHL1

Cell adhesion molecules (CAMs) are proteins mediating homo- and heterophilic binding which are crucial for appropriate cell-cell interactions. The CAMs are subdivided into three major groups: the integrins, the cadherins and the immunoglobulin superfamily. The CAMs are involved in intracellular signaling pathways (Panicker et al., 2003) and are essential for cellular processes such as cell migration, proliferation and differentiation (Walsh et al., 1997; Kiryushko et al., 2004). In the nervous system, CAMs mediate neural cell proliferation, migration, differentiation, neurite outgrowth and polarization, neural survival, and formation of synapses, processes of crucial importance for neural development and synaptic plasticity, as well as regeneration after injuries (Loers and Schachner, 2007).

The close homologue of L1 (CHL1) belongs to the cell adhesion molecules of the immunoglobulin superfamily. CHL1 was discovered by screening of the cell adhesion molecule L1 in the lambda gt11 expression cDNA library with a polyclonal anti-L1 antibody (Tacke et al., 1987) and cloned with the entire coding region (Holm et al., 1996). CHL1, like L1, promotes neurite outgrowth and neuronal survival *in vitro* (Chen et al., 1999) and enhances cell migration in cooperation with integrins (Holm et al., 1996; Hillenbrand et al., 1999; Buhusi et al., 2003).

1.2.2 Structure and gene expression of the CHL1

CHL1 is a transmembrane protein with molecular weight of 185 kDa and can be cleaved to 165 kDa or 125 kDa fragments (Holm et al., 1996). CHL1 is composed of structural elements common for the other members of the L1 family and shares 60% homology in the extracellular domain and 40% homology in the cytoplasmic domain with L1. Typical structural components of the L1 family are six immunoglobulin-like domains, four to five fibronectin type III repeats, a single transmembrane fragment and a highly conserved intracellular domain. CHL1 is composed of 1081 amino acids containing a transmembrane segment composed of 23 amino acids, an intracellular fragment of 105 amino acids, an immunoglobulin part of the extracellular domain of 585 amino acids, and fibronectin-type III repeats of 472 amino acids.

CHL1 expression occurs mainly at the early stages of development of the nervous system (Holm et al., 1996; Hillenbrand et al., 1999). In mice and rats, CHL1 is expressed already at embryonic day 13. The level of CHL1 expression increases to reach a maximum around birth and declines thereafter to low levels in the mature nervous system except for areas of persistent synaptic plasticity such as the hippocampus (Hillenbrand et al., 1999). Although the expression pattern of CHL1 coincides with that of L1 in most types of neuronal cells, there are some differences between the two related molecules. For example, granular cells in the cerebellum express only L1, whereas astrocytes express, in contrast, CHL1 but no L1. CHL1 is expressed in immature oligodendrocytes and this expression decreases upon oligodendrocyte maturation (Hillenbrand et al., 1999). CHL1 is also expressed by non-myelinating Schwann cells and some neurons in the peripheral nervous system (Hillenbrand et al., 1999).

1.2.3 Functions of CHL1

In humans, mutations in the CHL1 gene appear to be associated with mental retardation (Angeloni et al., 1999; Frints et al., 2003) and schizophrenia (Sakurai et al., 2002; Chen et al., 2005). CHL1-deficient mice are vital and fertile, normal in appearance and motor abilities, but have altered social and exploratory behavior, reactivity to novelty, and impaired ability to gate sensorimotor information (Montag-Sallaz et al., 2002; Irintchev et al., 2004; Morellini et al., 2007). The behavior of CHL1–/– mice in the open field, the elevated plus maze, and the Morris water maze indicates abnormalities in information processing in the brain probably caused by developmental deficits such as misguided axon projections of hippocampal mossy fibers and olfactory axons (Montag-Sallaz et al., 2002), and positioning and dendrite orientation of pyramidal cells in the cortex (Demyanenko et al., 2004). CHL1 is involved in clathrin-mediated recycling of synaptic vesicles (Leshchyns'ka et al., 2006) and is essential for inhibitory synaptic transmission and long-term potentiation in CA1 pyramidal cells of the hippocampus (Nikonenko et al., 2006).

After traumatic injury in the adult central and peripheral nervous system, CHL1 expression is strikingly upregulated in neurons and astrocytes (Chaisuksunt et al., 2000; Zhang et al., 2000; Rolf et al., 2003). Recently, it has been demonstrated that upregulation of CHL1 expression induced by basic fibroblast growth factor (FGF-2) in glial scar astrocytes inhibits motor recovery after spinal cord injury in mice (Jakovcevski et al., 2007). These findings suggest that CHL1 has a negative impact on axonal regeneration after CNS injury.

1.3 The extracellular matrix molecules tenascin-R and tenascin-C

1.3.1 The extracellular matrix glycoprotein tenascin-C (TNC)

The extracellular matrix (ECM) is a complex system containing various types of molecules such as collagens, proteoglycans and glycoproteins. Interactions of ECM molecules with different binding partners activate signal transduction pathways thus initiating cellular processes such as proliferation, differentiation, migration and survival.

A major component of the ECM is the glycoprotein tenascin-C (TNC) which is expressed by mature and immature astrocytes, radial glia, meningeal fibroblasts, subsets of neurons and Schwann cells (Bartsch et al., 1992; Bartsch et al., 1994; Steindler et al., 1995; Zhang et al., 1995; Jones and Jones, 2000; Camand et al., 2004). Tenascin-C was discovered as a protein enriched in the stroma of gliomas, initially called glial/mesenchymal extracellular matrix protein (Bourdon et al., 1983) and also known as hexabrachion (Erickson and Inglesias, 1984), cytotactin (Grumet et al., 1985), J1 220/200 (Kruse et al., 1985) and neureonectin (Rettig et al., 1989).

Like all other tenascins, TNC is composed of a common set of protein modules and has four domains: a cysteine-rich amino-terminal or tenascin assembly domain (TA) containing three to four alpha-helical heptad repeats, epidermal growth factor-like repeats (EGFL), fibronectin type III domains (FN-III) and a C-terminal globular domain shared with fibrinogens (fibrinogen globe). All members of the tenascin family except tenascin-W, have alternatively spliced FN-III domains (Jones and Jones, 2000). Mouse TNC molecules are basically composed of 14.5 EGFL repeats and eight FN-III domains and additional nine

distinctive repeats depending on the splice variant. In the developing mouse brain, 27 isoforms with mRNA variants have been identified (Joester and Faissner, 1999). TNC is able to form hexamer, known as hexabranchion, which is stabilized by disulfide bonds between cysteine-residues in the subunits (Kammerer et al., 1998). The human TNC is a large oligomeric molecule of 1900 kDa and composed of six identical subunits with variable numbers of repeated domains (Taylor et al., 1989).

1.3.2 Expression and functions of TNC

TNC is expressed early, at embryonic day 10, in the developing mouse nervous system (Kawano et al., 1995). It is transiently expressed during organogenesis and reduced dramatically in the developed organs (Chiquet-Ehrismann and Chiquet, 2003). TNC is mainly secreted by immature, reactive astrocytes and radial glia cells. In addition, some immature neurons including granule cells in the hippocampus and motoneurons of the spinal cord as well as neurons of the developing retina produce TNC (Bartsch, 1996). Although TNC expression is low in the normal adult brain, the molecule persists in areas where synaptic plasticity is persistently high, including hypothalamic nuclei implicated in endocrine regulation (Theodosis et al., 1997). High expression level of TNC is maintained in the olfactory bulb which is likely related to the continuous neuronal regeneration and axonal growth throughout life in this system (Gonzalez and Silver, 1994).

TNC enhances neurite outgrowth and polarity of some neurons *in vitro*, but it inhibits other neuronal types (Husmann et al., 1992; Lochter and Schachner, 1993; Dorries et al., 1996). TNC inhibits outgrowth when offered as a sharp

substrate boundary, in conjunction with a neurite outgrowth conducive environment (Faissner and Kruse, 1990; Taylor et al., 1993). Recently, these dual properties have been assigned to different splice variants of TNC (Meiners and Geller, 1997) and molecular epitopes within those splice variants (H. Y. Liu, M. L. Mercado, A. Nur-e-kamal, M. Schachner and S. Meiners, unpublished observations). TNC expression is upreguated by glial cells following CNS trauma (Zhang et al., 1997) as well as in neurons after exposure to excitotoxic agents or induction of long-term potentiation (Mitrovic et al., 1996; Nakic et al., 1996; Nakic et al., 1998). However, the functional significance of this upregulation is unknown.

1.3.3 Structure and expression of tenascin-R (TNR)

Tenascin-R (TNR) is an extracellular matrix glycoprotein expressed primarily by oligodendrocytes and subpopulations of neurons in the adult CNS of vertebrates (Bartsch et al., 1993; Erickson, 1993; Schachner et al., 1994). TNR has homologous structure with overall identity of 47 % to TNC in the mouse. TNR has a cysteine-rich N-terminal region, followed by 4.5 epidermal growth factor like (EGFL) domains, 8 fibronectin type III (FNIII) like repeats and a fibronogen-homologous C-terminal (Jones and Jones, 2000). Two isoforms with molecular weight of 160 kDa and 180 kDa are formed by alternative splicing of the sixth FNIII domain (Pesheva et al., 1989).

TNR is temporally expressed by oligodendrocytes during development (Bartsch et al., 1993; Wintergerst et al., 1993) and its expression seems to be restricted exclusively to the CNS, although a cultured cell line from peripheral nervous system (PNS) produced the molecule *in vitro* (Probstmeier et al., 2001). It

is also expressed by oligodendrocyte progenitors and type-2 astrocytes and most abundant during the phase of active myelination. When myelination has ceased, TNR levels are downregulated to lower levels in the adult (Bartsch et al., 1993; Wintergerst et al., 1993). TNR is also expressed by small subsets of CNS neurons, such as interneurons and motoneurons in spinal cord, retina, cerebellum and hippocampus (Fuss et al., 1993; Wintergerst et al., 1993; Weber et al., 1999). TNR is accumulated at the nodes of Ranvier (Bartsch et al., 1993) and in perineuronal nets (often in association with TNC) that surround inhibitory interneurons (Celio and Chiquet-Ehrismann, 1993; Wintergerst et al., 1996). In addition, TNR protein is localized in distinct zones of the olfactory bulb (Saghatelyan et al., 2004).

1.3.4 Function of TNR

TNR regulates inhibitory perisomatic inhibition through interactions of its HNK-1 (human natural killer cell) carbohydrate epitope with $GABA_B$ receptors and thus influences synaptic transmission and plasticity in the hippocampus (Saghatelyan et al., 2000; Bukalo et al., 2001; Saghatelyan et al., 2001; Dityatev and Schachner, 2003; Brenneke et al., 2004). TNR is a functional modulator of the beta subunit of voltage-gated sodium channels in myelinated CNS axons (Srinivasan et al., 1998; Xiao et al., 1999). *In vitro* study revealed that neurites of retinal and dorsal root ganglion cells and cerebellar neurons show repulsion at the border of a TNR substrate (Pesheva et al., 1993; Taylor et al., 1993; Becker et al., 2000). Further *in vivo* studies in zebrafish have demonstrated inhibitory functions of TNR during outgrowth and guidance of optic axons (Becker et al., 1999; Becker et al., 2003; Becker et al., 2004).

After spinal cord lesion, TNR mRNA is temporally upregulated in the lesion area (Deckner et al., 2000). As for other oligodendrocyte-derived molecules, TNR has been considered as an inhibitor of axonal regeneration (Pesheva and Probstmeier, 2000; Sandvig et al., 2004). In line with this notion, a recent study showed that TNR-deficient mice recover better than wild-type mice after compression spinal cord injury (Apostolova et al., 2006).

II Aims of the study

This study addressed the question of whether the functional outcome of spinal cord injury in mice is influenced by alterations in motoneuron excitability. The experimental design included analyses of C57BL/6J mice subjected to compression spinal cord injury at low thoracic level using simultaneously two approaches: assessment of motor functions by means of a classical open-field locomotion rating and numerical parameters (single-frame motion analysis, Apostolova et al., 2006), on the one hand, and recording of H-reflexes, on the other. To further elucidate relationships between reflex properties and functional recovery, H-reflex analyses were performed on mice deficient in CHL1 and TNR, mice previously shown to recover better from spinal cord injury than wild-type littermates (Jakovcevski et al., 2007; Apostolova et al., 2006). Finally, the role of TNC in spinal cord injury regeneration was addressed by analyses of motor functions, H-reflex and chemically defined synapses.

III Materials and methods

3.1 Animals

The animals used in this study were adult female C57BL/6J mice, CHL1-deficient (CHL1–/–) mice and wild-type (CHL1+/+) littermates from heterozygous breeding [mixed C57BL/6J-129Ola genetic background, subsequent 6 backcrosses into C57BL/6J (Montag-Sallaz et al., 2002)], TNR-deficient (TNR–/–) mice and wild-type (TNR+/+) littermates [mixed C57BL/6J-129Ola genetic background, 5 backcrosses into C57BL/6J genetic background (Weber et al., 1999)], and TNC-deficient (TNC–/–) mice and wild-type (TNC+/+) littermates [mixed C57BL/6J-129Ola genetic background, backcrosses into C57BL/6J genetic background (Evers et al., 2002)]. All mice were obtained from the animal facility of the Universitätsklinikum Hamburg-Eppendorf (Hamburg, Germany) at the age of 3 months. Genotyping of the mice from the CHL1, TNR, and TNC stocks was performed by PCR assays. The animals were kept under standard laboratory conditions. All experiments were conducted in accordance with the German and European Community laws on protection of experimental animals. Numbers of animals studied in different experimental groups are given in the text and figures. Data acquisition and analyses were performed in a blinded manner, i.e. without knowledge of genotype or performance of individual animals in different tests.

3.2 Spinal cord injury

The mice were anesthetized by intraperitoneal injection of a ketamin-xylazin mixture (100 mg/kg ketamin, Ketanest, Parke-Davis/Pfizer, Karlsruhe, Germany; 10 mg/kg xylazin, Rompun, Bayer, Leverkusen, Germany). After shaving, the skin was opened and laminectomy was performed at T7-T9 level of the thoracic vertebral segments. The spinal cord was compressed by a mouse spinal cord compression device (Curtis et al., 1993). The force and duration of compression were regulated by an electromagnetic device operated through an electrical current flow controller. Compression was performed at the highest force for one second (100% compression according to the operational definition of Curtis et al., 1993; Apostolova et al., 2006). The skin was closed by surgical suture with 6-0 nylon stitches (Ethicon, Norderstedt, Germany). The operated mice were housed individually at 37°C for 12 hours to prevent hypothermia. Afterwards, three mice were housed in one cage and kept in a conditioned room (22°C) with standard water and food provided *ad libitum*.

3.3 EMG recordings

For H-reflex analysis we selected the plantar reflex which, as indicated by pilot experiments, was reproducibly recorded in the mouse. Previous work (Valero-Cabre et al., 2004) has shown that after spinal cord injury in rats the plantar reflex is changed similar to reflexes elicited in other hindlimb muscles such as the gastrocnemius and the anterior tibial muscles. Therefore, we assume that the changes in the plantar H-reflex after spinal cord injury in mice are characteristic for the hindlimb musculature as a whole.

3.3.1 Instrumentation

The experiments were performed in a Faraday cage made of copper metal mesh. All conductible devices used in the cage were grounded. An insulated stimulator (Model 2100 Isolated Pulse Stimulator, A-M systems, Calsborg, WA, USA) was located outside the cage and connected to the stimulating electrode. The signals from the recording electrode were transmitted to a differential amplifier (P55 General Purpose AC Preamplifier, Grass Technologies, West Warwick, RI, USA), magnified (x 1000) and filtered (300 Hz high pass and 1 kHz low pass). The amplified analog signal was delivered to an A/D converter (ADC42, Pico Technology, Cambridgeshire, U.K.) and the digitalized data were displayed and saved on a computer using the Picoscope data acquisition software (PicoScope 5, Pico Technology, Cambridgeshire, U.K.).

3.3.2 Preparation of mice for EMG

Mice were anesthetized using ketamin-xylazin mixture as described above. Ketamin is widely used for H-reflex recordings, because it has been shown to have negligible effects on the electrophysiological recording (Ho and Waite, 2002). The initial dose was strictly controlled according to the body weight for every recording to synchronize the state of anesthesia among animals and prevent voluntary movement during H-reflex recording. Additional anesthesia was given if necessary, with 25% of the initial dose every 30 min, to suppress whisker tremor or voluntary movements. The forelimbs and hindlimbs of the mouse were fixed with tape on a plastic plate (Fig. 1A). The positions of the hindlimbs were controlled to avoid

unnecessary pressure and stretch which could affect the electrophysiological responses of muscles and nerves. To maintain body temperature, the plastic plate was placed on a warm water pad (37°C) driven by a temperature regulating pump system (TP472 T/pump, Gaymar Industries, Orchard Park, NY, USA). The eyes of animals were protected from drying with an eye cream (Bepanthen, Bayer, Leverkusen, Germany).

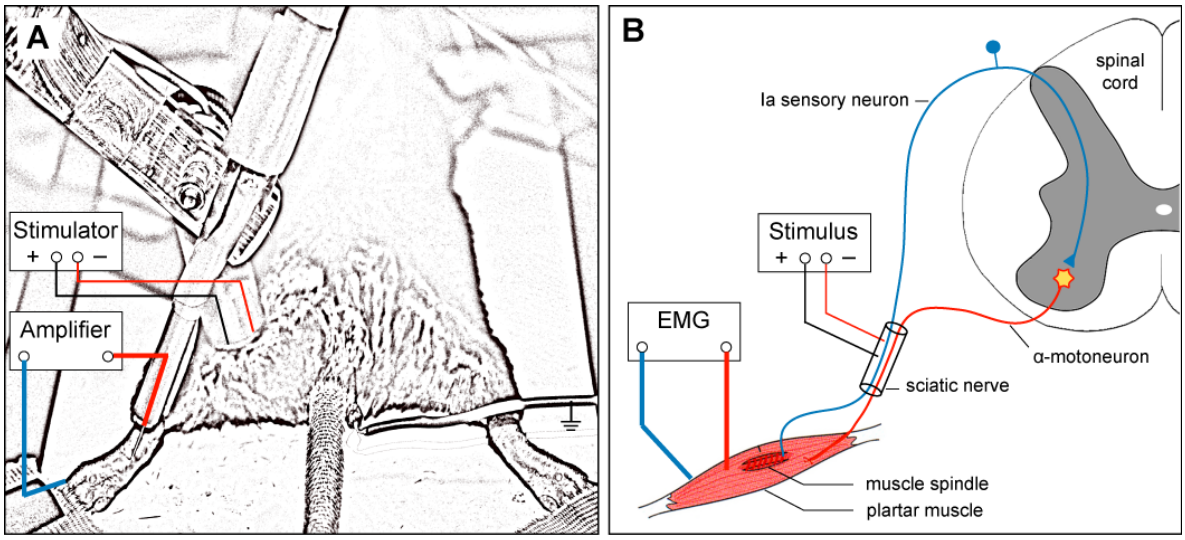

Fig. 1. Preparation of mice for EMG and schematic representation of the H-reflex pathway. (A) For the H-reflex recording, an anesthetized mouse is fixed on a plastic plate. A bipolar stimulating electrode (+ black, - red from Stimulator, A) is inserted into the biceps femoris muscle for sciatic nerve stimulation. For EMG recording, an active recording electrode (red from Amplifier, A) is positioned in the plantar muscles between the second (intermediate) and third (lateral) cuneiform bones and a reference electrode (blue from Amplifier, A) is inserted subcutaneously between the first and second digit. A ground electrode is inserted into the base of tail. (B) Drawing illustrating the neuronal circuit of the H-reflex. Electrical stimulation depolarizes alpha-motoneuron axons in the sciatic nerve (red) leading to a muscle contraction which is recorded using EMG muscle electrodes as a short-latency M-(muscle) wave. Simultaneous depolarization of the Ia afferents from muscle spindles evokes action potentials propagating into the spinal cord and depolarizing Ia synaptic terminals on motoneurons. Transmitter release at the Ia synapse causes eventually motoneuron depolarization and subsequent muscle contraction recorded as a long-latency H-wave.

3.3.3 Electrodes

Sciatic nerve stimulation was performed using two needle electrodes (stainless steel, diameter: 0.4 mm). Small plastic discs were fixed at a distances of 1 cm from the stimulation electrode tips to ensure equal depth of subcutaneous penetration. The stimulating electrodes were inserted between the two heads of the biceps femoris muscle in the thigh so that the cathode was located rostrally to the anode. A ground needle electrode (stainless steel, diameter: 0.4 mm) was applied at the base of the animal's tail. For recording, a reference stainless steel electrode was fixed to the skin between the first and second digit and an active recording electrode (Tungsten, diameter: 0.25 mm) was inserted between the second (intermediate) and third (lateral) cuneiform bones (Fig. 1). Electrode positioning was performed under a stereo microscope.

3.3.4 H-reflex recordings

The H-reflex was elicited by delivering bipolar electrical pulses of 0.2-ms duration to the sciatic nerve. Stimulus intensity was gradually increased until both M- and H-waves with latencies of approximately 2 and 5 ms, respectively, were visible. After the threshold measurement, stimulus intensity was further increased until maximal and stable H-responses were elicited. Thereafter, stimulation continued at the defined suprathreshold level at frequencies of 0.1, 0.3, 0.5, 1, 2, 3, 5 Hz. Six consecutive responses were recorded at each frequency. The amplitudes of M- and H-waves were measured as peak-to-peak values, averaged (excluding the first response at each frequency) and used to calculate H/M ratios.

The latencies of the responses were measured as time elapsed between trigger and peak of each waveform.

3.4 Analysis of motor function

3.4.1 Open-field locomotion scores

The recovery of ground locomotion was evaluated using the Basso, Beattie, Bresnahan (BBB) rating scale (Basso et al., 1995), modified for mice (Joshi and Fehlings, 2002). Hindlimb movements of each animal were observed during free movement in an open-field and evaluated by one investigator using a score between 1 and 21 according to the criteria defined by Joshi and Fehlings (2002). The rating of each animal was repeated during analysis of video recordings for the beam walking test (see below) to confirm the evaluation at slow playback speed. Assessment was performed before and 1, 3, 6 and 12 weeks after injury. Scores for the left and right extremities were averaged.

3.4.2 Single frame motion analysis

In addition to BBB rating, motor abilities of the animals were evaluated using video-based single-frame motion analysis established for the femoral nerve injury paradigm (Irintchev et al., 2005b) and subsequently adapted and expanded for assessment of recovery after spinal cord lesion in mice (Apostolova et al., 2006; Jakovcevski et al., 2007). Here we evaluated three parameters in two different tests: foot-stepping angle and rump-height index during beam walking

(Figs. 2A-C) and extension-flexion ratio measured during voluntary movements without body weight support ("pencil" test, Figs. 2D-E). In every test, the performance of each animal was captured with an A602fc camera (Basler AG, Ahrensburg, Germany) at 100 frames per second and digitized on a computer using a high-speed firewire cable (IEEE 1394) and SIMI Motion 7 software (SIMI Reality Motion Systems, Unterschleissheim, Germany). The digitalized video files were saved in Audio Video Interleaved (AVI) format and subsequently compressed using Xvid Codec (open source MPEG-4 Video Codec, version-1.0.3, http://www.xvid.org/) and VirtualDub software (free video software written by Avery Lee, http://www.virtualdub.org, version-1.5.10) to reduce the file volume without changing the frame rate of 100 per second. The compressed video files were played back at low speed using VirtualDub and single frames in which the animals were seen in defined phases of motion in each motor task (Apostolova et al., 2006) were selected for analyses. The parameters outlined above were estimated by angle and distance measurements using the UTHSCSA ImageTool 3.0 software (free image processing and analysis software from University of Texas Health Science Center at San Antonia, http://ddsdx.uthscsa.edu/dig/).

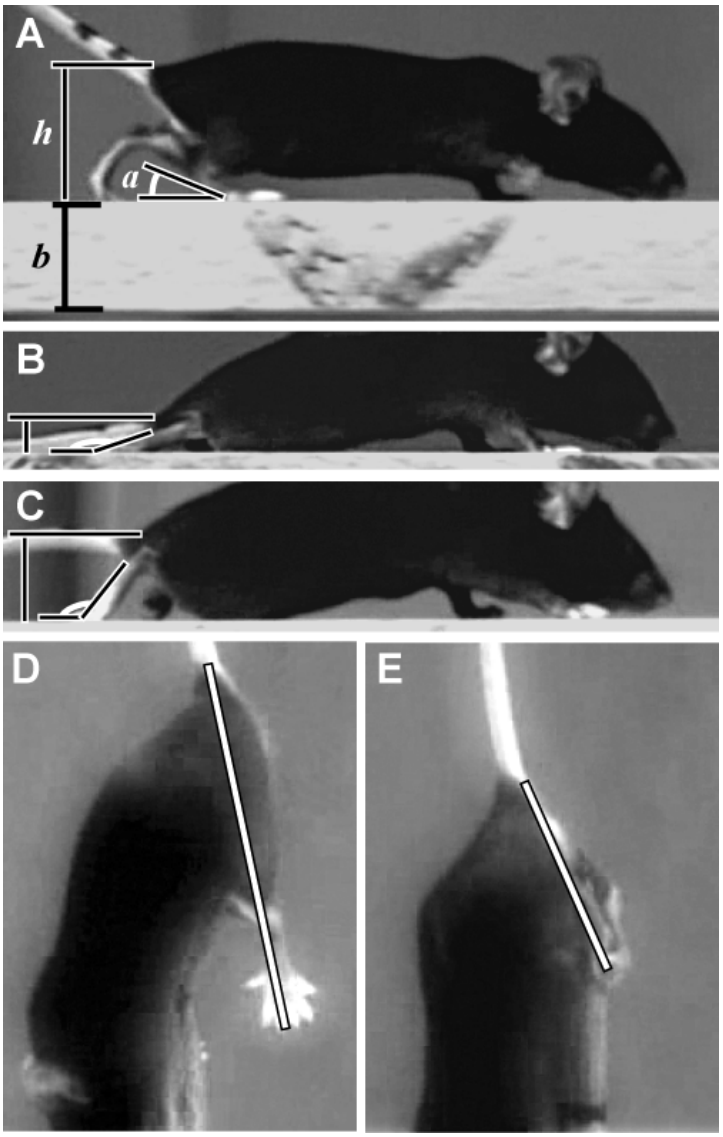

Fig. 2. Single-frame motion analysis parameters. Single frames of video sequences recorded during beam walking (A–C) and voluntary movements (pencil test; D, E) of intact mice (A, D, E) or spinal cord injured mice (B, C, at one and 12 weeks after injury, respectively). The foot-stepping angle, *a*, defined by a line parallel to the dorsal surface of the hind paw and the horizontal line and measured with respect to caudal, is drawn in A–C. Compared with intact mice (A), the angle is largely increased one week after injury (B) and decreases as some recovery occurs in the following weeks (C). The rump height index is calculated as a ratio between the rump height, *h* (distance between the beam and the tail base), and the beam thickness, *b*. The rump height index is smallest one week after injury (B) and increases during the recovery period (C). The extension-flexion ratio is calculated as a ratio between the length of the extremity (white lines in D and E) at maximum flexion (D) and maximum extension (E). This parameter decreases after spinal cord injury due to loss of voluntary motor control.

3.5 Histology

3.5.1 Tissue fixation and sectioning

Twelve weeks after spinal cord injury, after the last EMG and video recording, mice were anesthetized with 16% solution of sodium pentobarbital (Narcoren, 5 µl/g body weight; Merial, Hallbergmoos, Germany). The animals were transcardially perfused with fixative consisting of 4% formaldehyde and 0.1% CaCl2 in 0.1 M cacodylate buffer, pH 7.3, for 15 min at room temperature (RT). After perfusion, the spinal cords were left in situ for 2 h at RT, after which they were dissected out and postfixed overnight (18–22 h) at 4°C in the solution used for perfusion. The lumbar enlargements of the spinal cords were then excised and immersed into 15% sucrose solution in 0.1 M cacodylate buffer, pH 7.3, for 2 days at 4°C, embedded in Tissue Tek (Sakura Finetek, Zoeterwoude, The Netherlands), and frozen for 2 minutes by immersion in 2-methyl-butane (isopentane) precooled to –80°C. Serial transverse sections (25 µm thick) were cut on a cryostat (CM3050; Leica, Nussloch, Germany). Sections were collected on SuperFrost Plus glass slides (Roth, Karlsruhe, Germany) in a standard sequence so that six sections 250 µm apart were present on each slide.

3.5.2 Immunohistochemistry

Immunohistochemistry was performed according to a published protocol (Irintchev et al., 2005a). The following commercially available antibodies were used at optimal dilutions: goat anti-choline acetyltransferase (ChAT, 1:100; Chemicon, Hofheim, Germany), mouse anti-vesicular GABA transporter (VGAT, 1:1000; Synaptic Systems, Göttingen, Germany), mouse anti-vesicular glutamate transporter 1 (VGLUT1, 1:500; Synaptic Systems), and rabbit anti-tyrosine hydroxylase (TH, 1:800; Chemicon, Hofheim, Germany). Water-bath antigen unmasking was performed in 0.01 M sodium citrate solution, pH 9.0, for 30 min at 80°C (Jiao et al., 1999). Nonspecific binding was blocked using 5% normal serum from the species in which the secondary antibody was produced, diluted in phosphate-buffered saline, pH 7.3 (PBS) and supplemented with 0.2% Triton X-100 and 0.02% sodium azide for 1 h at RT. Incubation with the primary antibody, diluted in PBS containing 0.5% lambda-carrageenan (Sigma) and 0.02% sodium azide, was performed for three days at 4°C. After washing in PBS (three times for 15 min at RT), the appropriate Cy3 conjugated secondary antibody, diluted 1:200 in PBS-carrageenan solution, was applied for 2 h at RT. Following a subsequent wash in PBS, cell nuclei were stained for 10 min at RT with bis-benzimide solution (Hoechst dye 33258, 5 µg/ml in PBS; Sigma). Finally, the sections were washed again, mounted in anti-fading medium (Fluoromount G; Southern Biotechnology Associates via Biozol, Eching, Germany) and stored in the dark at 4°C. Photographic documentation was made on an LSM 510 confocal microscope (Zeiss, Oberkochen, Germany).

3.5.3 Quantification of motoneuron soma size and perisomatic terminals

Estimations of soma areas and perisomatic terminals were performed as described previously (Apostolova et al., 2006; Jakovcevski et al., 2007). Transverse spinal cord sections stained for ChAT, VGAT, or VGLUT1 which contained motoneurons caudal to the lesion scar were selected for analyses. Stacks of 1-µm-thick images were obtained on a LSM 510 confocal microscope (Zeiss) using a 63x oil- immersion objective and digital resolution of 1024 x 1024 pixel. Four sections 250 µm apart were analyzed per animal and staining procedure to assure that motoneurons located both close and remote to the lesion scar were sampled. One image per motoneuron at the level of the largest cell body cross-sectional area was used to measure soma area, perimeter, and number of perisomatic terminals. Motoneurons were identified by the ChAT staining for the analyses of cholinergic terminals and by their large, pale nuclei (bis-benzimide staining), large soma size and position in the ventral horn of the spinal cord for analyses of VGAT- and VGLUT1-positive terminals (Fig. 9). The sample size ranged between 20 and 30 motoneurons per animal. Areas and perimeters were measured using the Image Tool 2.0 software program (University of Texas, San Antonio, TX, USA). Linear density was calculated as number of perisomatic terminals per unit length. Mean values of individual animals were used to calculate group mean values.

3.5.4 Estimation of VGLUT1-positive synaptic terminals in the spinal cord

To investigate densities of VGLUT1$^+$ synaptic terminals, transverse spinal cord sections caudal to the lesion scar were used for analysis. Digital images were obtained on a LSM 510 confocal microscope (Zeiss) using a 63x oil- immersion objective and digital resolution of 1024 x 1024 pixel. The grey value was adjusted using threshold to convert the images to grey scale for optimal color intensity with ImageJ software (NIH, USA). Synaptic terminals were detected using particle analysis tool in ImageJ which sorts out objects with defined size (> 2 μm^2) and circularity (0-1). Number of synaptic terminals was sampled in the Clarke's column, lamina VII and lamina IX in each section (Fig. 21). Density was calculated as number of VGLUT1$^+$ synaptic terminals per unit area. Six sections 250 μm apart were analyzed per animal and mean values of individual animals were used to calculate group mean values.

3.5.5 Analysis of monaminergic innervation in lumbar spinal cord

Sagittal spinal cord sections stained for tyrosine hydroxylase (TH) were used to analyze monaminergic axonal innervation in the lumbar spinal cord caudal to the lesion scar. Estimation was performed on an Axioskop microscope (Zeiss) equipped with a motorized stage and Neurolucida software-controlled computer system (MicroBrightField Europe, Magdeburg, Germany). The area of interest, where the TH$^+$ axons were counted, was determined between 250 – 2000 μm from the caudal lesion site in each section (Figs. 22A, B). Four sections 250 μm apart

were analyzed per animal to select samples from diverse sagittal levels and group mean values of individual animals were compared between genotypes.

3.6 Statistical analysis

All numerical data are presented as group mean values with standard error of mean (SEM). Parametric tests were used for comparisons as indicated in the text and figure legends. Analyses were performed using the SYSTAT 9 software package (SPSS, Chicago, IL, USA). The threshold value for acceptance of differences was 5%.

IV Results

4.1 Alteration of the H-reflex and locomotion after spinal cord injury in C57BL/6J mice

4.1.1 Alterations of the H-reflex after spinal cord injury

We first analyzed the reflex responses in C57BL/6J mice prior to compression injury of the spinal cord. Electrical stimulation of the sciatic nerve produced typical EMG responses at baseline stimulation frequency (0.1 Hz) consisting of a short-latency M- (muscle) wave and a long-latency H-wave elicited by stimulation of motor axons and Ia afferents, respectively (Fig. 3A). Increasing the stimulus frequency stepwise from 0.1 Hz to 5 Hz did not alter the M-wave but the amplitudes of the H-wave were strongly reduced reaching values close to zero at 5 Hz (Figs. 3A, C, D). One week after spinal cord injury, the amplitudes of the M-waves were slightly but significantly reduced (-12%) and the H-wave amplitudes were, in contrast, strongly increased (+67%, Figs. 3A-D). No frequency dependence of the M-responses was seen and the H-wave amplitudes were only slightly reduced, even at 5 Hz, compared with the baseline responses (Figs. 3A-D). The latencies of both the M- and the H-waves were significantly shorter than in intact mice (2.0 ± 0.05 ms versus 1.9 ± 0.02 ms and 5.2 ± 0.12 ms versus 4.5 ± 0.05 ms in injured versus intact mice, mean values ± SEM, n = 16 per genotype, $p < 0.05$, two-sided t test). The reduction of the rate depression of the H-wave (decline in amplitude upon increasing stimulation frequency) and increased H/M ratios at baseline stimulation are characteristic findings after spinal cord injury in

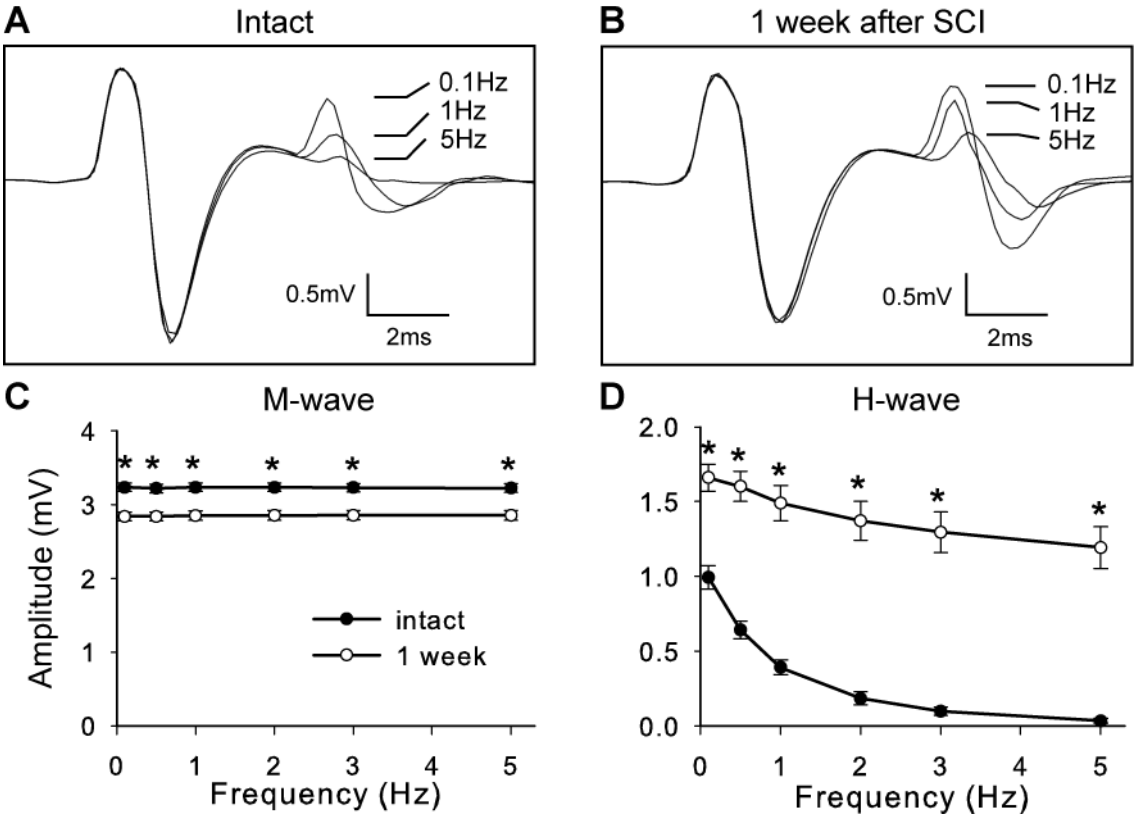

Fig. 3. Alterations of the H-reflex one week after spinal cord injury in C57BL/6J mice. The M- and H-responses were measured in plantar muscles during repetitive stimulation of the sciatic nerve with supramaximal pulses at frequencies ranging between 0.1 and 5 Hz. Panel A and B show representative recordings from a mouse analyzed prior to injury and 1 week after spinal cord injury, respectively. The superimposed traces obtained by stimulation at frequencies of 0.1, 1 and 5 Hz show that the amplitude of the long-latency H-wave in the intact animal (A) is reduced upon increasing stimulation frequency, whereas the M-wave is not affected. The same statements are valid for the recording obtained after injury (B), but compared with intact, the amplitude of the M-wave is decreased and the amplitude of the H-wave at baseline stimulation frequency (0.1 Hz) is increased. These observations were verified by comparisons of the amplitudes of the M- (C) and H-waves (D) before and after injury in 16 animals. Shown are mean values ± SEM. Asterisks indicate significant differences between group mean values for a given stimulation frequency (ANOVA for repeated measurements with Tukey's *post hoc* test, $p < 0.05$).

humans and other mammals (Skinner et al., 1996; Little et al., 1999; Frigon and Rossignol, 2006). The unusual feature in our observations is that increased baseline H/M ratio and reduced rate depression were present already 1 week after injury in the mouse. By contrast, in other mammals these phenomena become apparent only at later time-points after lesion, for example, after 4 weeks in the rat (Thompson et al., 1992; Valero-Cabre et al., 2004; Lee et al., 2005).

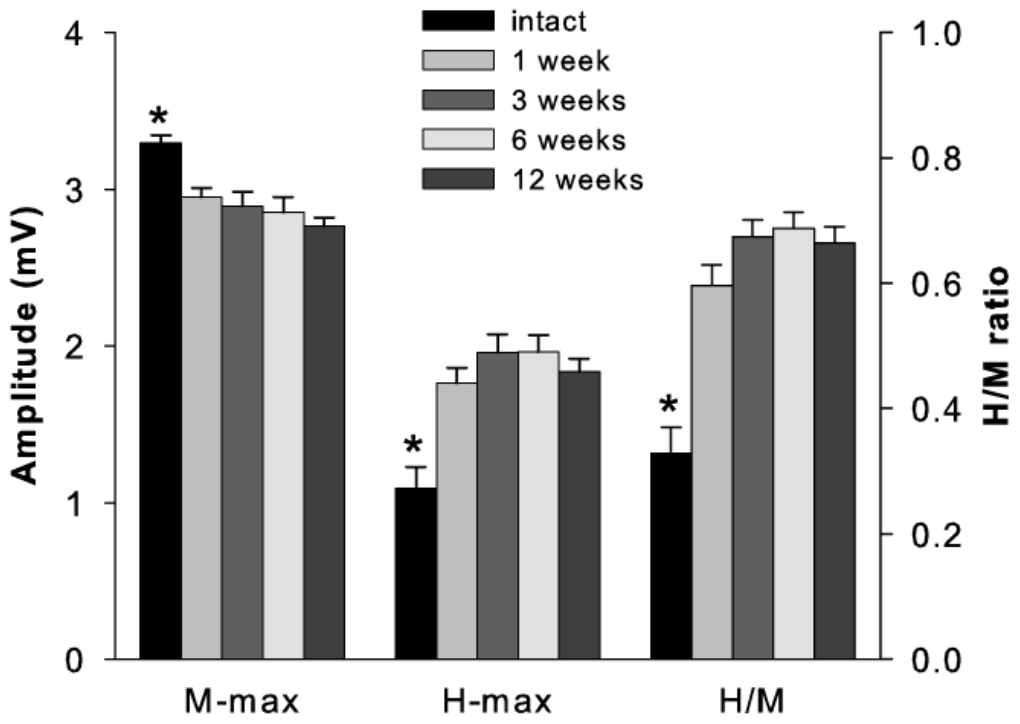

Fig. 4. Alterations of the maximum M- and H-responses at 0.1-Hz stimulation and their ratios (H/M) after spinal cord injury in C57BL6/J mice. Shown are mean values (+ SEM) for 9 mice analyzed prior to injury and 1, 3, 6 and 12 weeks thereafter. Spinal cord injury causes reduction of M-max and increase of both H-max and the H/M ratio, but no alterations occur during the 12-week post-operative observation period. Asterisks indicate significant differences from all other groups (ANOVA for repeated measurements with Tukey's *post hoc* test, $p < 0.05$).

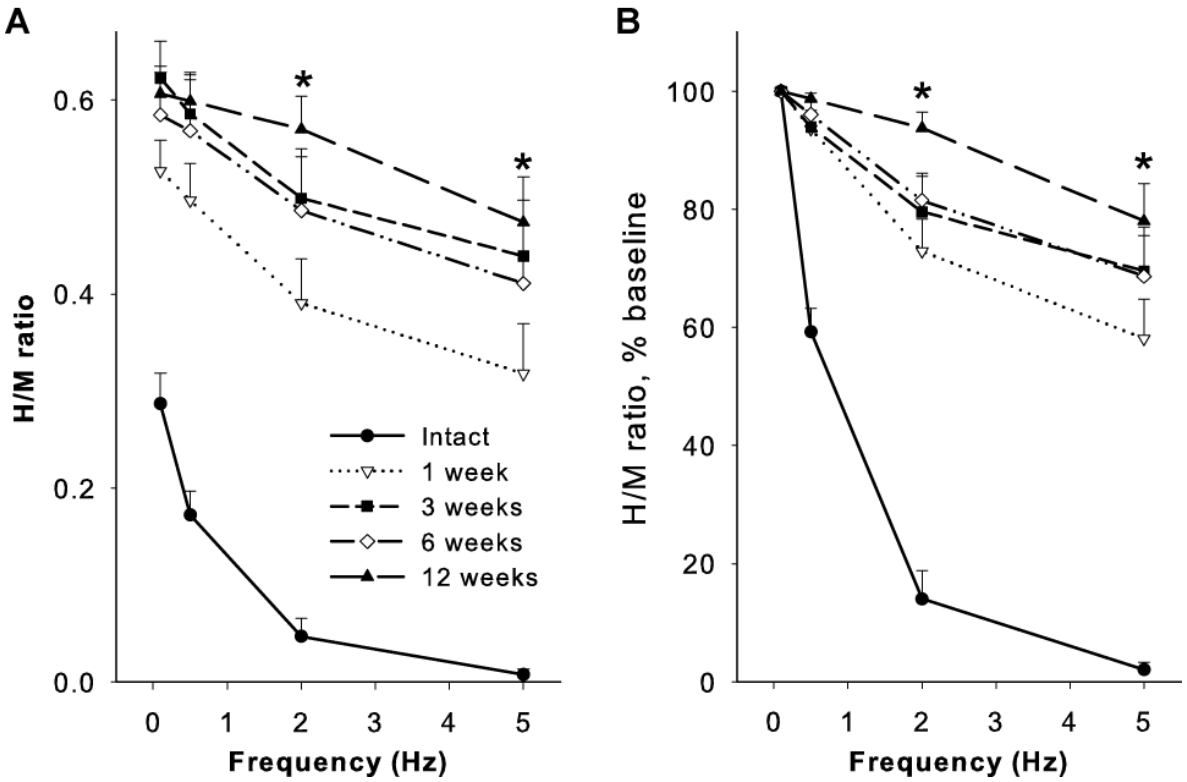

Fig. 5. Alterations in the rate depression of the H-reflex after spinal cord injury in C57BL/6J mice. The H/M ratio was measured at different stimulus frequencies 1, 3, 6 and 12 weeks after spinal cord injury in the animals shown in Fig. 4. Shown are mean values (+ SEM) of absolute ratios (A) and ratios normalized to the individual H/M values at baseline frequency (0.1 Hz, B). For simplicity, values at 1 and 3 Hz (compare Fig. 3) are omitted. Rate depression is significantly reduced between 1 and 12 weeks (asterisks, $p < 0.05$, ANOVA for repeated measurements with Tukey's *post hoc* test).

A considerable degree of spontaneous motor recovery occurs after the first week in spinal cord injured mice (Fig. 7)(Apostolova et al., 2006; Basso et al., 2006; Jakovcevski et al., 2007). We were, therefore, interested whether this functional recovery is accompanied by alterations in the H-reflex. Repeated measurements over a 12-week post-operative period revealed no time-dependent alterations, compared with one week, in the M- and H-wave or the H/M ratio at baseline stimulation frequency (Fig. 4). Compared with 1 week after injury, however, rate depression became increasingly smaller with time after injury and

the H/M ratio reached maximum values at 12 weeks (Figs. 5A, B). Linear regression analysis of H/M ratios normalized to values at baseline frequency (Fig. 5B) confirmed that the slope of linear regression line is significantly decreased at 12 weeks as compared with 1 week after injury (Figs. 6A, B). Therefore, during the time period of functional recovery after compression spinal cord injury in mice there is a progressive decrease in the rate sensitivity of the H-wave. This alteration in time cannot be attributed to unwanted training effects: the mice readily adapt to

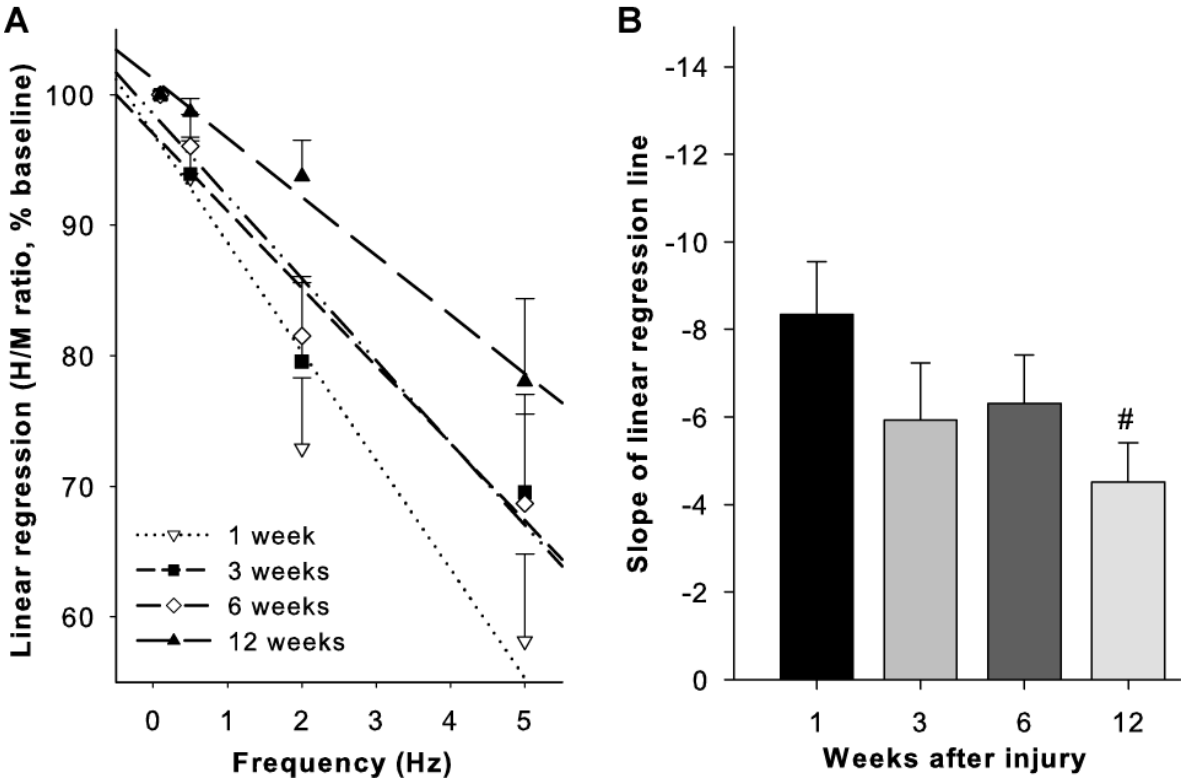

Fig. 6. Linear regression analysis of rate depression and slope of regression line after spinal cord injury in C57BL/6J mice. Rate depression of H/M ratio was analyzed by linear regression to display degree of its reduction as a function of time after injury. Shown are linear regression lines (A) with mean values (+ SEM) of H/M ratio, normalized to values at baseline frequency (0.1 Hz, Fig. 5B), and slopes (+SD) of individual regression lines (B) for 9 mice. The slope of linear regression line confirms that the rate depression of H/M ratio is significantly reduced at 12 weeks compared with that at 1 week after operation (B, crosshatch, $p < 0.05$, ANCOVA for comparing linear regression lines).

walking over the one-meter long beam prior to operation (2-3 trials required) without having to be trained and perform this walking only 4 times over a 12-week time period after injury, at 1, 3, 6 and 12 weeks after injury (typically 4 times 1 meter per recording session). There is no training prior to or in between recording sessions. The “pencil” test, which does not require any initial training, is done only before and at 1, 3, 6 and 12 weeks after injury, and it usually take less than a minute to record the voluntary movements of the mice.

4.1.2 H-reflex alterations and motor recovery after spinal cord injury

In parallel to H-reflex recordings, we performed analyses of motor functions in the C57BL/6J mice described above. Four parameters were used for evaluation: the BBB score, the foot-stepping angle, the rump-height index and the extension-flexion ratio. The first one is a well-known measure of locomotor abilities after spinal cord injury in rodents. The BBB rating estimates different aspects of locomotion, including plantar stepping abilities, limb coordination and trunk stability. In our group of mice, there was a rapid drop of the BBB score after injury, from 21 prior to injury to nearly 1 at one week after injury, indicating a severe degree of locomotor impairment induced by the spinal cord compression (Fig. 7A). In the following weeks, the BBB score increased constantly to reach maximum mean value of 6 at 12 weeks (Fig. 7A). The second parameter used, the foot-stepping angle measured during beam walking (Figs. 2A-C), is a numerical estimate of the plantar stepping ability. Similar to the BBB score, this angle was dramatically changed after spinal cord injury, from 20° prior to injury to 160° at 1 week, and the values were reduced by about 20% in the following 11 weeks (Fig. 7B).

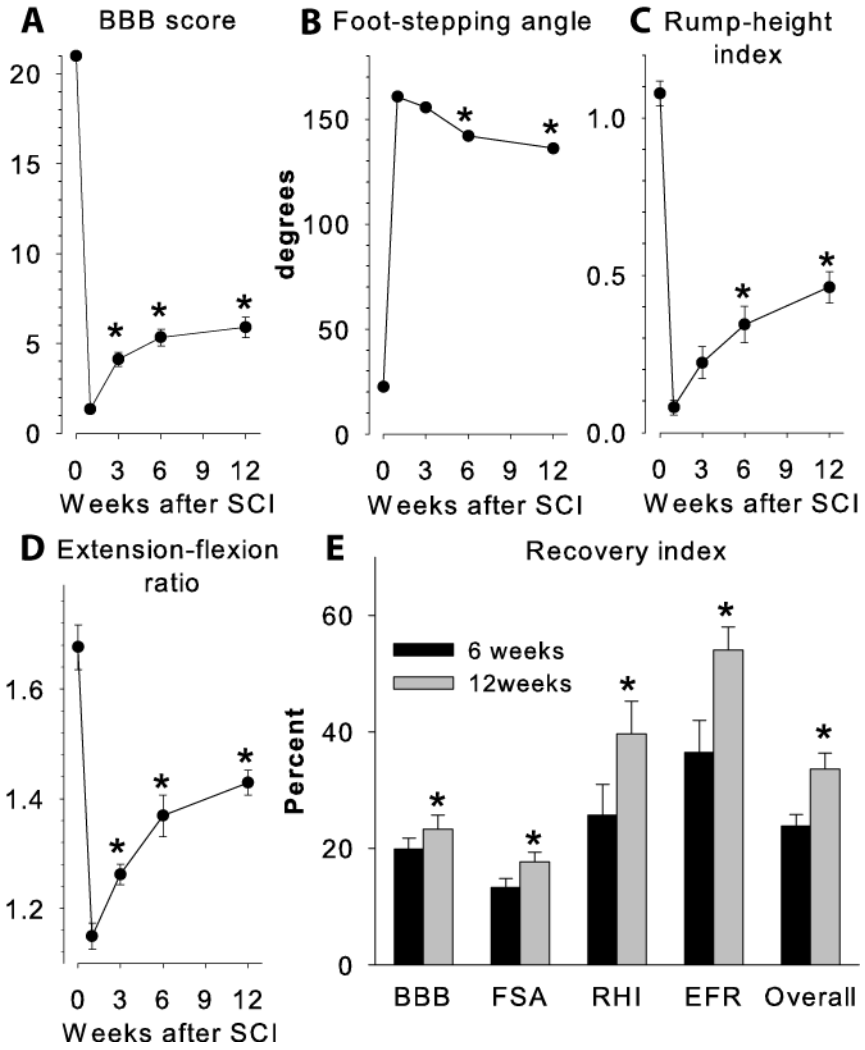

Fig. 7. Functional recovery after compression spinal cord injury in C57BL/6J mice estimated by BBB rating and single-frame motion analysis. Panel A-D show mean values (± SEM) of open-field locomotion scores (BBB, A), foot-stepping angles (FSA, B), rump-height indices (RHI, C), and extension-flexion ratios (EFR, D) before surgery (day 0) and at 1, 3, 6, and 12 weeks after injury in 9 animals used for H-reflex analyses (Fig. 5). Asterisks indicate significant differences between mean values at 1 week and a given time-point thereafter (one-way ANOVA for repeated measurements with Tukey's *post hoc* test, $p < 0.05$). Recovery indices (mean values + SEM, E) calculated from individual animal values for each parameter and overall recovery indices at 6 weeks and 12 weeks after injury are shown in panel E. The recovery index is an individual animal estimate calculated in percent as $[(X_{7+n} - X_7) / (X_0 - X_7)] \times 100$, where X_0, X_7 and X_{7+n} are values prior to operation, 7 days after injury, and a time-point n + 7 days after the spinal cord injury, respectively. In simpler terms, this measure estimates gain of function after the first week ($X_{7+n} - X_7$) as a fraction of the functional loss ($X_0 - X_7$) induced by the operation. Asterisks indicate significant differences between mean recovery indices at 6 and 12 weeks ($p < 0.05$, two-sided paired t test).

Importantly, there was a good correlation between the values of the foot-stepping angle and the BBB score at 12 weeks (Fig. 8C). The third parameter, the rump-height index estimated also during beam walking (Figs. 2A-C), is a measure of the ability to support body weight during ground locomotion. The observations made for this estimate are generally similar to those for the BBB score and the foot-stepping angle with the exception that the final level of recovery was higher than for the other two parameters (Figs. 7C, E). We attribute this phenomenon to recovery of control and coordination of muscles within the hind extremities and, in addition, possibly influences of spasticity (see Apostolova et al., 2006). Finally, the fourth parameter, the extension-flexion ratio measured during the so called “pencil” test (Figs. 2D, C, Apostolova et al., 2006), is an estimate of the ability to initiate and perform voluntary movements without body weight support. As for the rump-height index, we also observed a considerable degree of recovery at 12 weeks (Figs. 7D, E). Since control of voluntary movements requires supraspinal control, the partial recovery of the extension-flexion ratio can be attributed to preservation of descending projections after spinal cord compression and post-traumatic plasticity of these connections. In support of this notion is the finding that a considerable amount of descending projection axons are spared in our compression injury model (Apostolova et al., 2006). The overall conclusion from these observations is that the animals analyzed electrophysiologically have been subjected to a severe injury which is followed by a moderate to low recovery of motor abilities requiring different levels and complexity of spinal and supraspinal control. The functional estimates reported here are in good agreement with previous results using the same experimental paradigm (Apostolova et al., 2006; Chen et al., 2007; Jakovcevski et al., 2007). While in the earlier studies the post-

operative survival period has been limited to 6 weeks, here we extended the observation time period to 12 weeks. As indicated by significant differences in the respective recovery indices (Fig. 7E), functional improvements occur between 6 and 12 weeks.

Using the data sets for motor recovery and H/M ratios, we performed regression analyses in search for covariations between the two types of functional

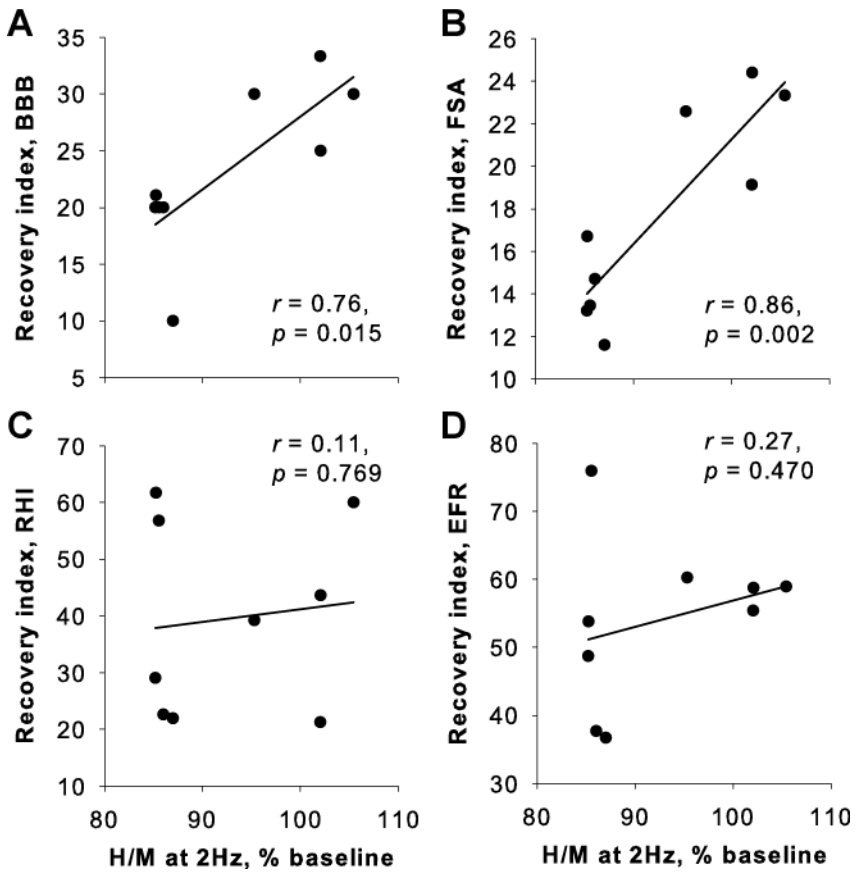

Fig. 8. Correlations between motor recovery indices and H/M rate depression at 2 Hz 12 weeks after spinal cord injury in C57BL/6J mice. H/M rate depression is calculated as ratio of the H/M value at 2 and 0.1 Hz in percent. Significant positive correlations were found between rate depression and recovery indices for BBB score (A) and foot-stepping angle (B), but not rump-height index (D) and extension-flexion ratio (E). A significant positive correlation was also found between BBB scores and foot-stepping angles in individual animals (C). Pearson correlation coefficients and significance levels estimated by ANOVA for regression are shown in the panels.

parameters. We found positive correlations between rate depression, on the one hand, and recovery indices for the BBB score and the foot-stepping angle, on the other (Figs. 8A, B). In contrast, the correlations between rate depression and rump-height index or extension-flexion ratio were poor (Figs. 8C, D). These results suggest that the excitability of the motoneuron pools in the injured spinal cord determines to a significant degree the recovery of plantar stepping abilities but not of coordination or voluntary movements.

4.1.3 Motoneuron synaptic coverage, functional recovery and H-reflex

We next addressed the question whether individual degree of motor recovery and functional properties of the motoneurons after spinal cord injury could be related to alterations in synaptic inputs to motoneurons. We were first interested whether numbers of cholinergic terminals around motoneuron cell bodies, known to form C-type synapses on motoneurons associated with muscarinic type 2 receptors (Davidoff and Irintchev, 1986; Hellstrom et al., 2003), might be important in shaping motoneuron responses. This notion was based on two observations. First, the number of C-terminals in the lumbar spinal cord is greatly reduced, by about one half compared with intact animals, 6 weeks after low-thoracic spinal cord injury and numbers of preserved boutons correlate with plantar stepping abilities of individual mice (Apostolova et al., 2006; Jakovcevski et al., 2007). Second, the cholinergic perisomatic synapses in the intact spinal cord regulate motoneuron excitability during locomotion in uninjured animals (Miles et al., 2007). We analyzed numbers of ChAT-positive terminals around motoneurons located in the lumbar spinal both close and remote to the site of injury in animals

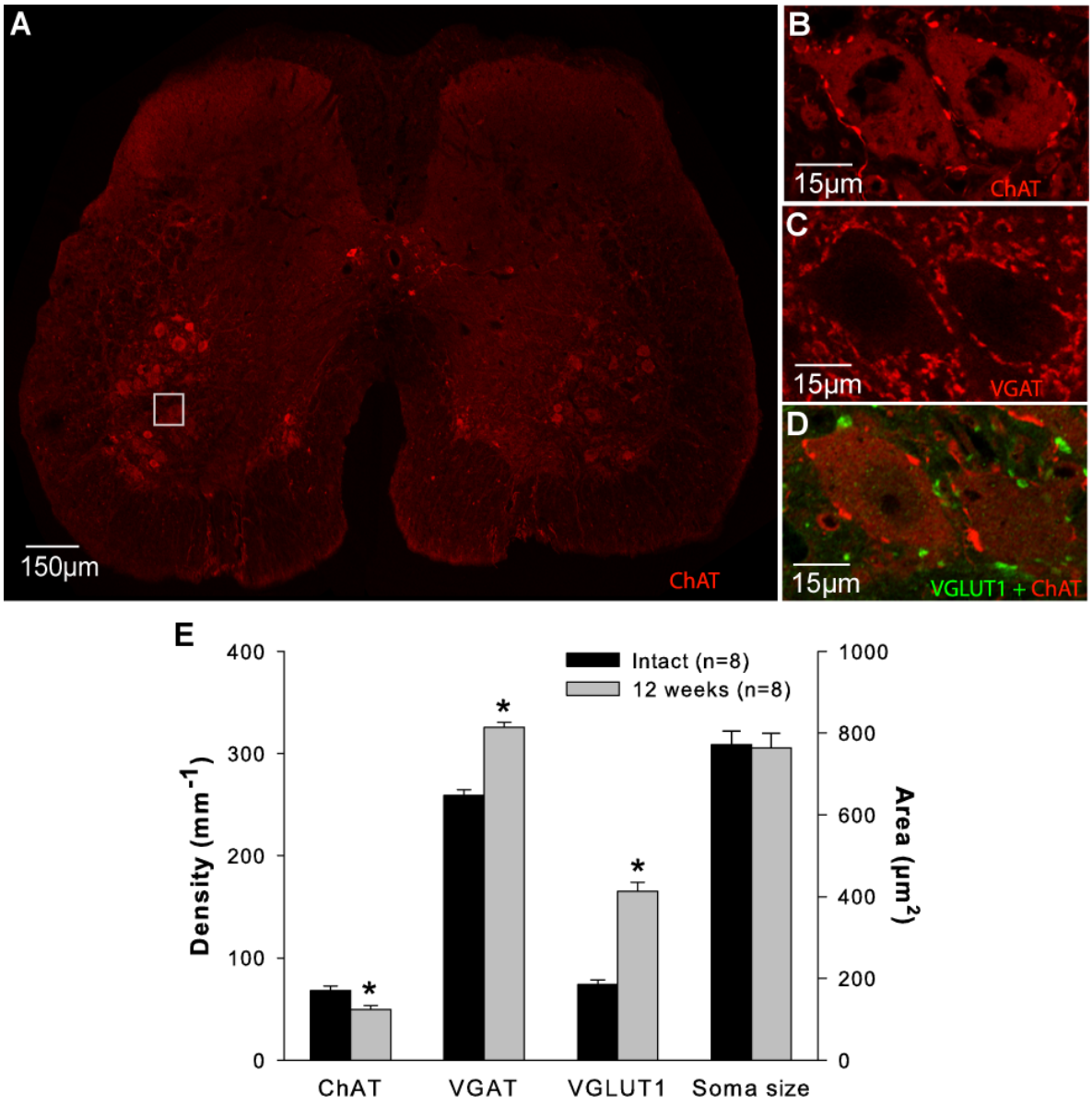

Fig. 9. Analysis of soma areas and perisomatic terminals in C57BL/6J mice. Confocal images (1-µm-thick optical slices) show the distribution of ChAT positive cells in a cross-section from the lumbar spinal cord of a mouse 12 weeks after spinal cord injury (A). (A-D) $ChAT^+$ (B, red), $VGAT^+$ (C, red), and $VGLUT1^+$ (green, double stained with ChAT, red, D) puncta around motoneuron cell bodies in sections from intact C57BL/6J mice. Motoneuron soma size and densities of perisomatic terminals around motoneurons were analyzed in intact mice and mice studied 12 weeks after spinal cord injury (E). Shown are mean values (+ SEM) of soma area of $ChAT^+$ motoneurons ("Soma size") and linear densities of ChAT-, VGAT-, and VGLUT1-positive terminals calculated from mean values of individual animals. Asterisks indicate significant differences between intact and injured animals ($p < 0.05$, two-sided t test for independent samples).

studied physiologically (Figs. 9A, B). We found, in agreement with previous observations (Apostolova et al., 2006; Jakovcevski et al., 2007), a reduction, compared with intact age-matched C57BL/6J mice, in the linear density (number per unit length) of these terminals 12 weeks after injury (Fig. 9E). Scatter plots and regression analysis revealed significant positive correlations between ChAT terminal densities on the one hand, and recovery indices for the BBB score, the foot-stepping angle and the extension flexion ratio (Figs. 10A-C), but not the rump-height index (not shown) or H/M ratios at 2-Hz stimulation (Fig. 10D), on the other.

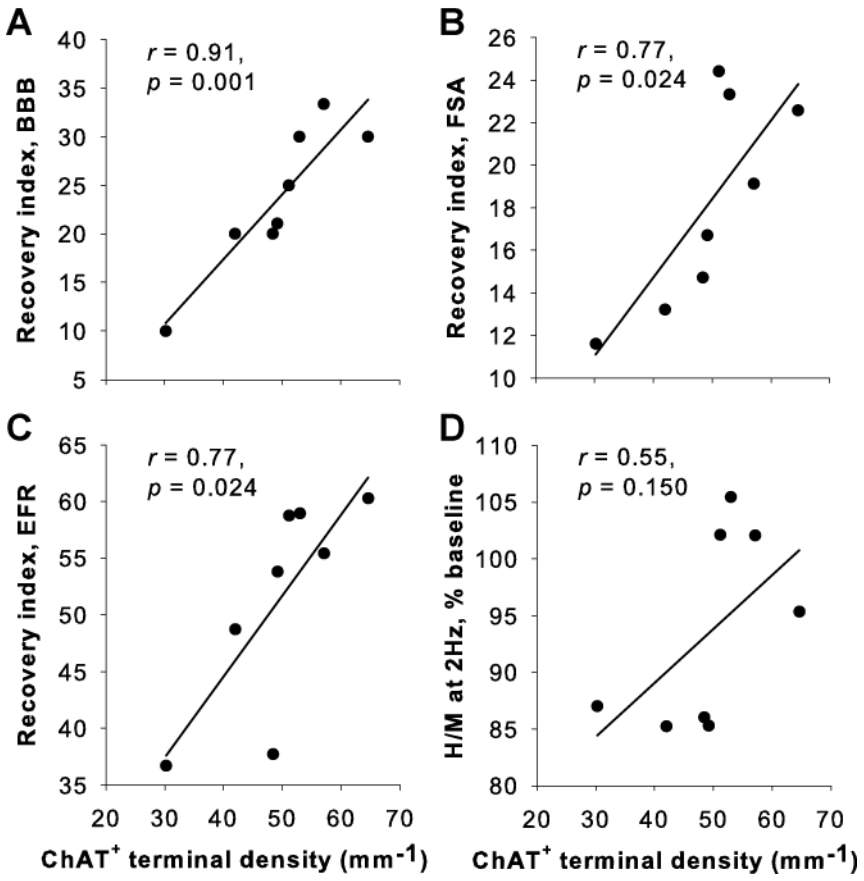

Fig. 10. Correlations between functional parameters and densities of cholinergic terminals around motoneurons at 12 weeks after spinal cord injury in C57BL/6J mice. ChAT positive terminals correlate positively with recovery indices for the BBB score (A), foot-stepping angle (B) and extension-flexion ratio (C) and rate depression of the H-reflex at 5 Hz (D). Pearson correlation coefficients and significance levels estimated by ANOVA for regression are shown in the panels.

These findings confirm our previous observations that higher numbers of ChAT synapses on motoneurons in the distal spinal cord favor specific aspects of motor performance (Jakovcevski et al., 2007). However, inter-individual variations in recruitment of motoneuron pools upon repetitive stimulation might not be strongly dependent on numbers of cholinergic synapses.

In addition to cholinergic synapses, motoneuron cell bodies receive inhibitory (glycinergic and GABAergic) and excitatory (glutamatergic) synapses. We addressed the question whether injury-induced plasticity in these inputs may be related to motor recovery and to alterations in the H-reflex. We estimated, in the animals described above, densities of VGAT-positive ($VGAT^+$, vesicular GABA transporter, Fig. 9C) and VGLUT1-positive ($VGLUT1^+$, vesicular glutamate transporter 1, Fig. 9D) terminals around motoneuron somata, expected to reflect numbers of perisomatic inhibitory and excitatory synapses, respectively (Apostolova et al., 2006; Nikonenko et al., 2006; Jakovcevski et al., 2007). We found remarkably high densities of both types of perisomatic terminals 12 weeks after injury (Fig. 9E). The numbers exceeded the control values for intact animals by 25% and 123% for $VGAT^+$ and $VGLUT1^+$ boutons, respectively, and this increase was not due do reduction of the motoneuron soma areas (Fig. 9E). In contrast, 6 weeks after injury the number of $VGAT^+$ terminals was similar to control values and the number of $VGLUT1^+$ boutons was slightly, by less that 20%, reduced compared with intact mice (Apostolova et al., 2006; Jakovcevski et al., 2007, see their results for wild-type C57BL/6J mice). These observations allow the conclusion that significant alterations in synaptic inputs, both inhibitory and excitatory, to motoneurons occur distal to the site of injury between 6 and 12 weeks after lesion. These alterations must have functional significance although

we did not find correlations between numbers of terminals and H-reflex parameters or motor abilities of individual animals (data not shown). Specifically, the large increase in glutamatergic terminals, which might form axo-somatic, axo-dendritic and axo-axonic synapses in the perisomatic compartment, might be related to H-reflex enhancement in the course of recovery from spinal cord injury.

4.2 H-reflex in mutant mice with enhanced functional recovery after spinal cord injury

Previously studies have shown that functional recovery of mice deficient in the adhesion molecule CHL1 (close homologue of L1, Jakovcevski et al., 2007) and the extracellular matrix protein TNR (tenascin-R, Apostolova et al., 2006) is better than that of wild-type littermates (Table 1). Here we addressed the question whether better recovery of motor functions in these mutant mice is associated with alteration in the H-reflex. We analyzed adult CHL1-deficient (CHL1–/–) mice, TNR-deficient (TNR–/–) mice and wild-type (CHL+/+ and TNR+/+) littermates prior to injury and 1, 3, and 6 weeks after spinal cord injury of similar type and severity as the one used in the previous studies and here for C57BL/6J mice. Comparisons of

Table 1. Summary of differences in functional parameters and H-reflex found between CHL1-deficient mice and wild-type littermates and between TNR-deficient and wild-type littermates without spinal cord injury (0 w) and 1 to 6 weeks (1 – 6 w) after spinal cord compression. Arrows indicate lower (↓) or higher (↑) values in the mutant mice compared with wild-type mice, = indicates no significant difference, / not studied. Data for functional parameters (BBB score, foot-stepping angle, rump-height index and extension-flexion ratio) have been published (Apostolova et al., 2006; Jakovcevski et al., 2007).

Parameter	CHL1-deficient mice				TNR-deficient mice			
	0 w	1 w	3 w	6 w	0 w	1 w	3 w	6 w
BBB score	=	=	↑	↑	=	=	↑	↑
Foot-stepping angle	=	=	↑	↑	=	=	↑	↑
Rump-height index	=	=	=	↑	=	=	=	=
Extension-flexion ratio	=	=	↑	↑	=	=	↑	=
H/M ratio, baseline stimulation (0.1 Hz)	=	=	↑	↑	=	=	↑	↑
H/M ratio, 0.3 – 5 Hz stimulation	=	=	↑	↑	=	=	↑	↑

previously published data on motor recovery of CHL1–/– and TNR–/– mice with the current H-reflex data (Table 1, see 4.2.1 and 4.2.3 for detailed description of the physiological data) shows that the appearance of differences in functional recovery between mutant and wild-type mice, specifically in plantar stepping ability estimated by the BBB score and the foot-stepping angle, goes in parallel with an increase in the H/M ratios. Therefore, we can conclude that better motor recovery in CHL1- and TNR-deficient mice, similar to C57BL/6J mice, is associated with enhanced H-responses. In addition to measurements over a six-week time-period for comparisons with functional recovery, H-reflex in these mice was also analyzed at 12 weeks after injury to investigate long-term alteration of rate depression similar to C57BL/6J mice (Figs. 5, 6).

4.2.1 H-reflex alterations after injury in CHL1-deficient mice

Both prior to and 1 week after injury, we observed similar frequency-dependent responses, as estimated by H/M ratios, in CHL1–/– and wild-type mice (Fig. 11A). Independent of genotype, spinal cord injury caused a marked decrease in the rate depression of the H-reflex at 1 week similar to that in C57BL/6J mice described above. At 3 and 6 weeks, but not at 12 weeks, the absolute H/M ratios in CHL1–/– mice were significantly higher than in wild-type littermates at all frequencies studied (0.1 – 5 Hz, Fig. 11A). Analysis of H/M ratios normalized to baseline values (at 0.1 Hz stimulation), revealed significant differences by ANOVA for repeated measurements between the genotypes only at 6 weeks (Fig. 11B). However, the slope of linear regression lines calculated with the normalized H/M percent values demonstrated differences between the genotypes

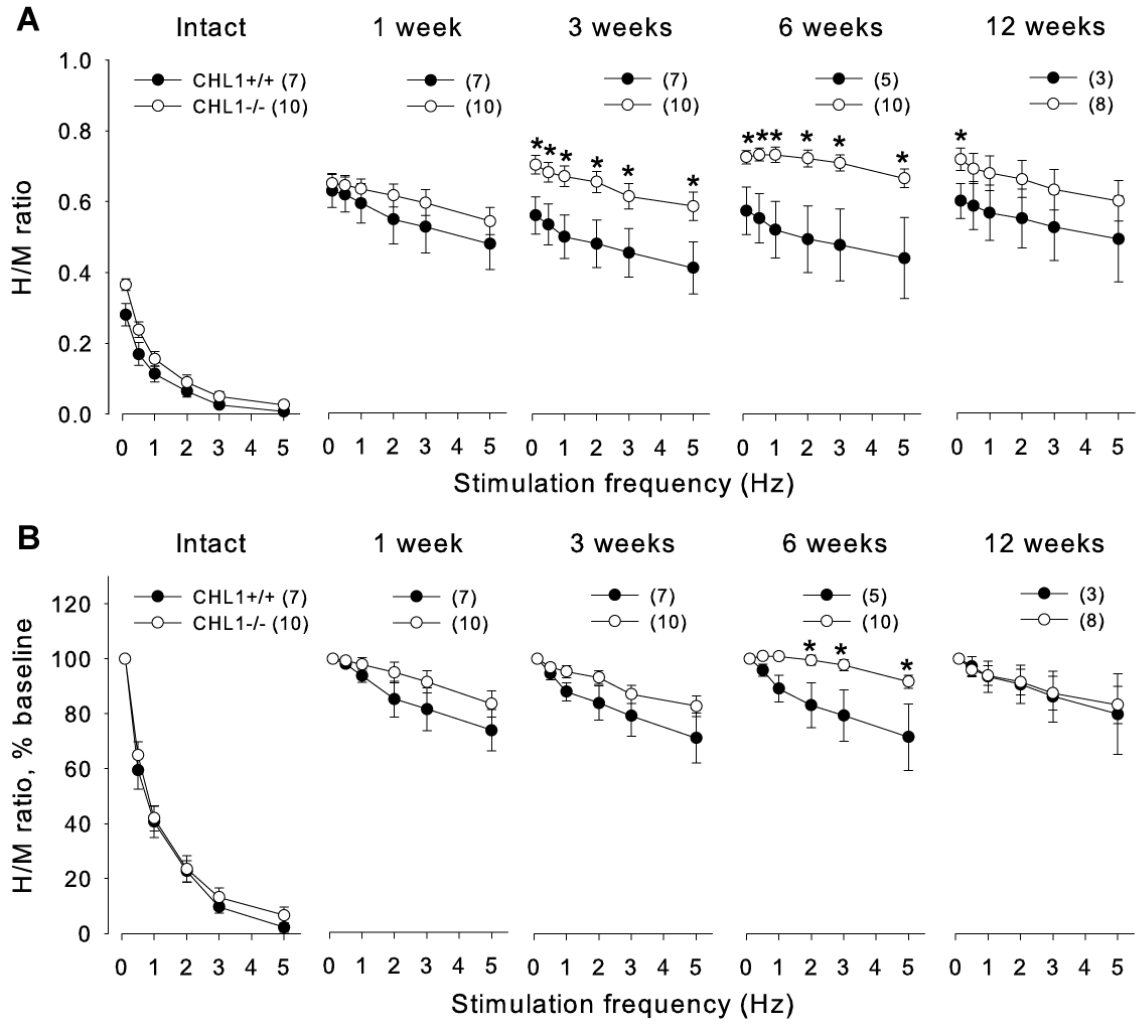

Fig. 11. Rate depression of the H-reflex at different time-points after spinal cord injury in CHL1-/- mice and wild-type littermates. Shown are mean values (± SEM) of absolute H/M ratios (A) and ratios normalized to the individual H/M values at baseline frequency (0.1 Hz, B) for different stimulation frequencies prior to operation and 1, 3, 6, and 12 weeks after spinal cord injury. At 3 and 6 weeks after injury, the H/M ratio in CHL1-/- mice is significantly higher than in wild-type littermates at all stimulation frequencies (A). Rate depression of the normalized H/M ratio is significantly reduced at 6 weeks in CHL1-/- mice (asterisks, ANOVA for repeated measurements with Tukey's *post hoc* test, $p < 0.05$).

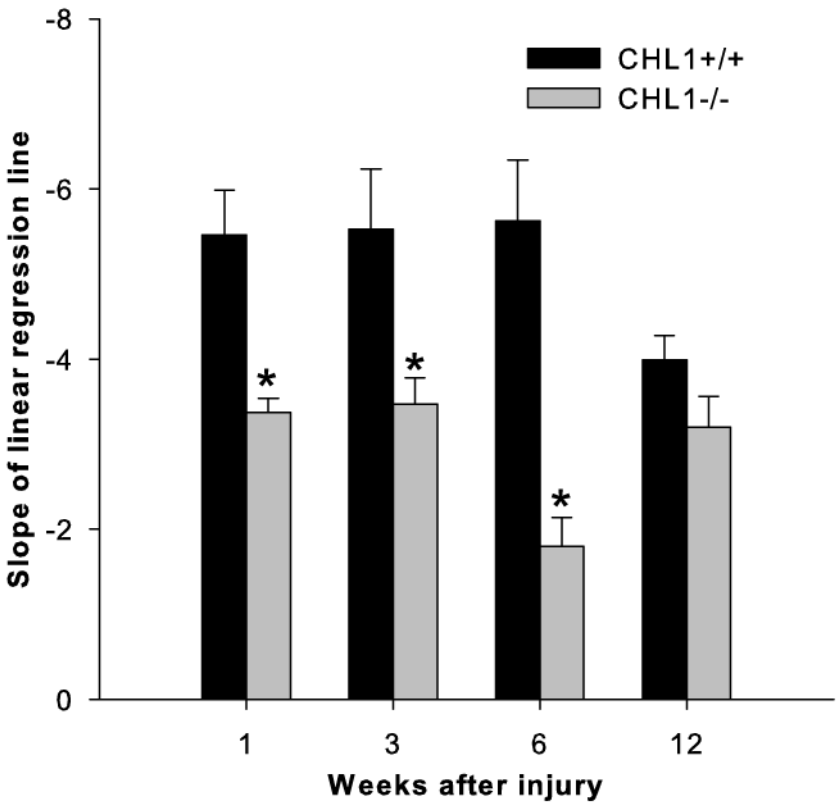

Fig. 12. Slope of linear regression line for H-reflex rate depression after spinal cord injury in CHL1-/- mice and wild-type littermates. Linear regression analysis for rate depression of H/M ratio, normalized to values at baseline frequency (0.1 Hz, Fig. 11B), was performed to display degree of its reduction at a given time point after injury. Shown are slopes (+SD) of individual regression lines in the mice analyzed for H/M ratio (Fig. 11B). The rate depression of H/M ratio is significantly reduced in CHL1-/- mice at 1, 3, and 6 weeks compared with wild-type littermates (asterisks, $p < 0.05$, ANCOVA for comparing linear regression lines).

at 1, 3 and 6 weeks after injury (Fig. 12). These findings allow the conclusion that, similar to C57BL/6J mice, loss of rate sensitivity in H/M ratio and enhancement of H-reflex amplitude are associated with the superior motor recovery observed in CHL1-deficient mice (Jakovcevski et al., 2007). Considering the correlation between rate depression and recovery indices in C57BL/6J mice (Fig. 8), the significant reduction of rate depression at 6 weeks in CHL1–/– mice might be

specifically related to better recovery of the BBB score and the foot-stepping angle in CHL1–/– mice (Table 1).

At 12 weeks after spinal cord injury, the genotype-related differences disappeared, primarily due to increased H-responses in the wild-type mice (Figs. 11A, B, 12). While this increase is expected, regarding the observations on C57BL6/J mice, it is unclear why the H-reflex in CHL1–/– mice does not increase further after the sixth week. It is likely that a physiological maximum of the H-reflex responses exist and this maximum is reached earlier in CHL1–/– than in wild-type mice. Also, an interesting question arising from these observations is whether differences in motor abilities between the genotypes exist at a time-point at which the H-reflex responses are similar, i.e. at 12 weeks after injury. This question cannot be presently answered since no data on motor functions at 12 weeks are present.

4.2.2 Motoneuron synaptic coverage in CHL1-deficient mice at 12 weeks after injury

A previous study has shown that CHL1-deficient mice have less GABAergic ($VGAT^+$) perisomatic terminals and more glutamatergic ($VGLUT1^+$) terminals at 6 weeks after injury compared with CHL1+/+ littermates (Jakovcevski et al., 2007). At 12 weeks after injury, the modulatory cholinergic ($ChAT^+$) terminals showed no difference between both genotypes, similar to the finding of Jakovcevski et al. (2007) at 6 weeks. The $VGLUT1^+$ and the $VGAT^+$ perisomatic terminal densities were also similar in CHL1–/– mice and wild-type littermates at 12 weeks (Fig. 13). From the results of this and the previous study it can be concluded that genotype-related differences in synaptic coverage disappear between the 6^{th} and the 12^{th}

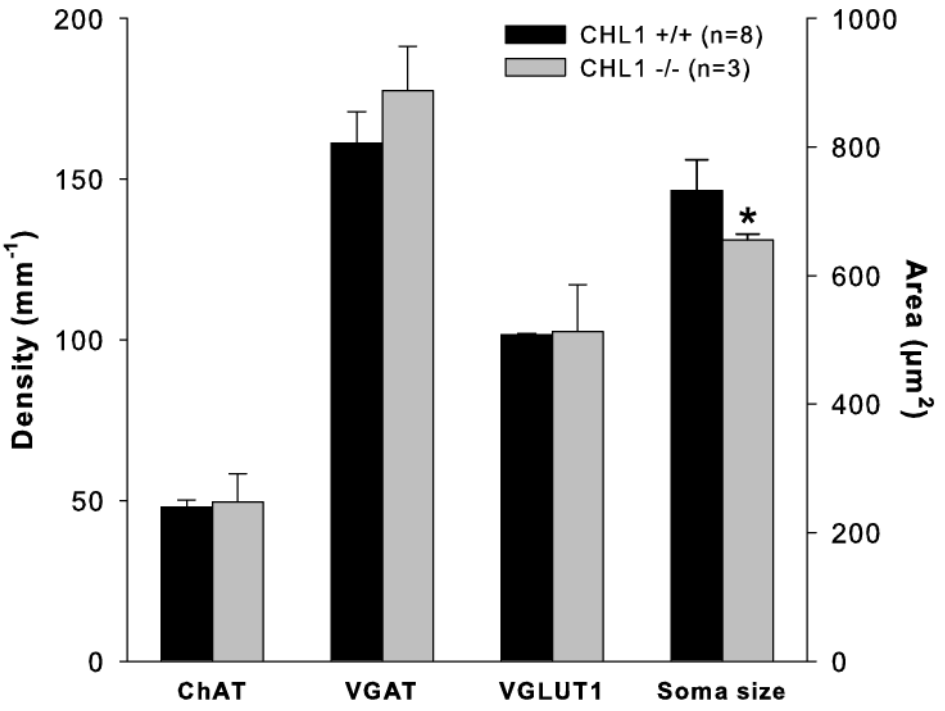

Fig. 13. Analysis of soma areas and perisomatic terminals in CHL1-/- mice and wild-type littermates. Motoneuron soma size and densities of perisomatic terminals around motoneurons were analyzed at 12 weeks after spinal cord injury. Shown are mean values (+ SEM) of soma area of ChAT$^+$ motoneurons ("Soma size") and linear densities of ChAT-, VGAT-, and VGLUT1-positive terminals calculated from mean values of individual animals. Asterisk indicates a significant difference between CHL1-/- mice and wild-type littermates ($p < 0.05$, two-sided t test for independent samples).

week after injury, whereas the soma size in CHL1–/– mice is reduced compared with CHL1+/+ littermates during that time-period (Fig. 13). It is likely that the attenuated differences in the density of excitatory VGLUT1$^+$ boutons and the inhibitory VGAT$^+$ terminals are related to the disappearance of differences between CHL1–/– and wild-type mice at 12 weeks.

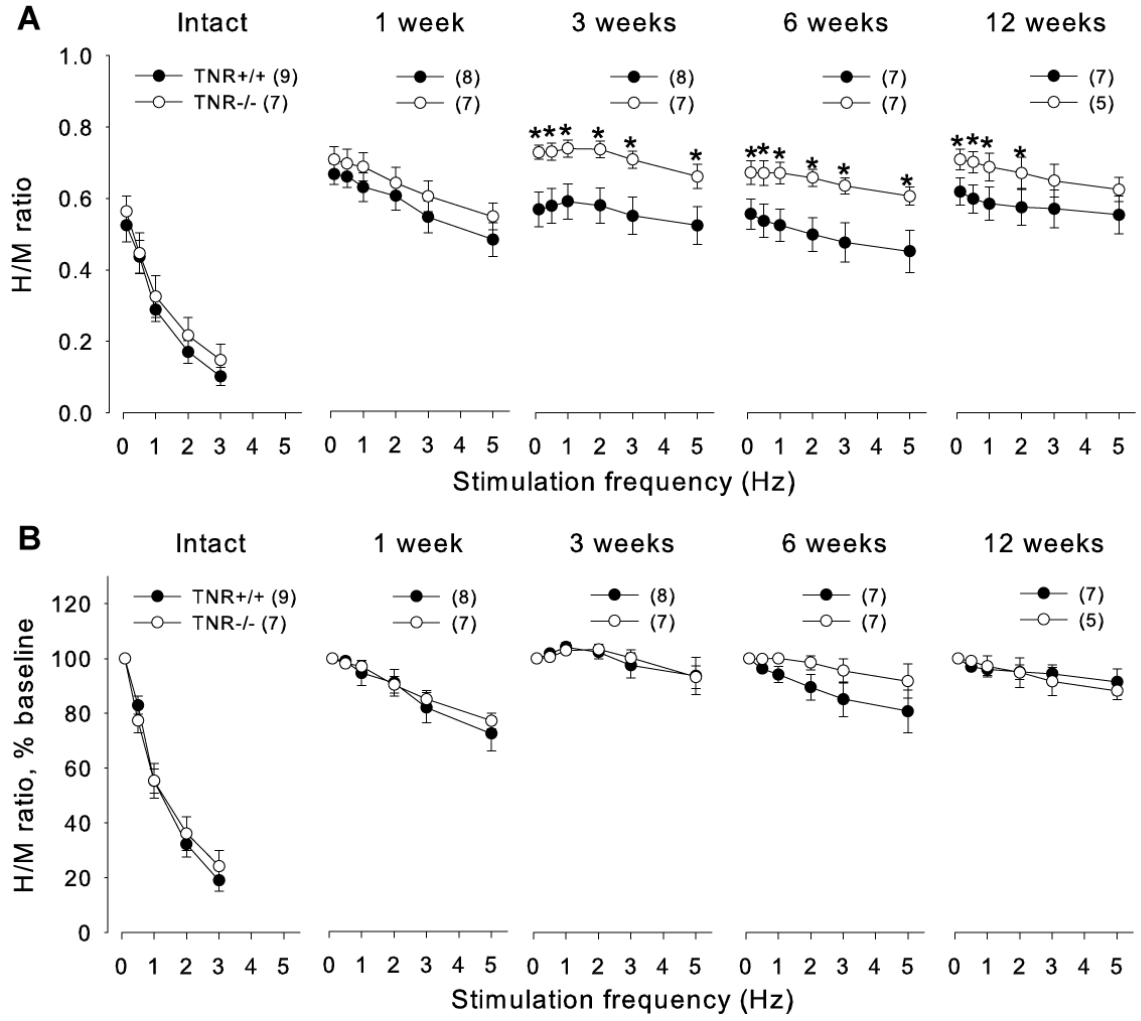

Fig. 14. Rate depression of the H-reflex at different time-points after spinal cord injury in TNR-/- mice and wild-type littermates. Shown are mean values (± SEM) of absolute H/M ratios (A) and ratios normalized to the individual H/M values at baseline frequency (0.1 Hz, B) for different stimulation frequencies prior to operation and 1, 3, 6, and 12 weeks after spinal cord injury. At 3, 6, and 12 weeks after injury, the H/M ratio in TNR-/- mice is significantly higher than in wild-type littermates (A). (asterisks, ANOVA for repeated measurements with Tukey's *post hoc* test, $p < 0.05$).

4.2.3 H-reflex alterations after injury in TNR-deficient mice

H/M ratios measured at different stimulus frequencies showed similar pattern of rate dependency in TNR–/– and TNR+/+ littermate mice before and 1 weeks after injury (Fig. 14A). The absolute values of the H/M ratios at 3 and 6 weeks were significantly elevated in TNR–/–, similar to CHL1–/– mice, compared with wild-type littermate at all frequencies (0.1 – 5 Hz, Fig. 14A). Although normalized ratios did not reveal differences between the genotypes (Fig. 14B), the slope of the linear regression line for normalized ratios was significantly reduced in

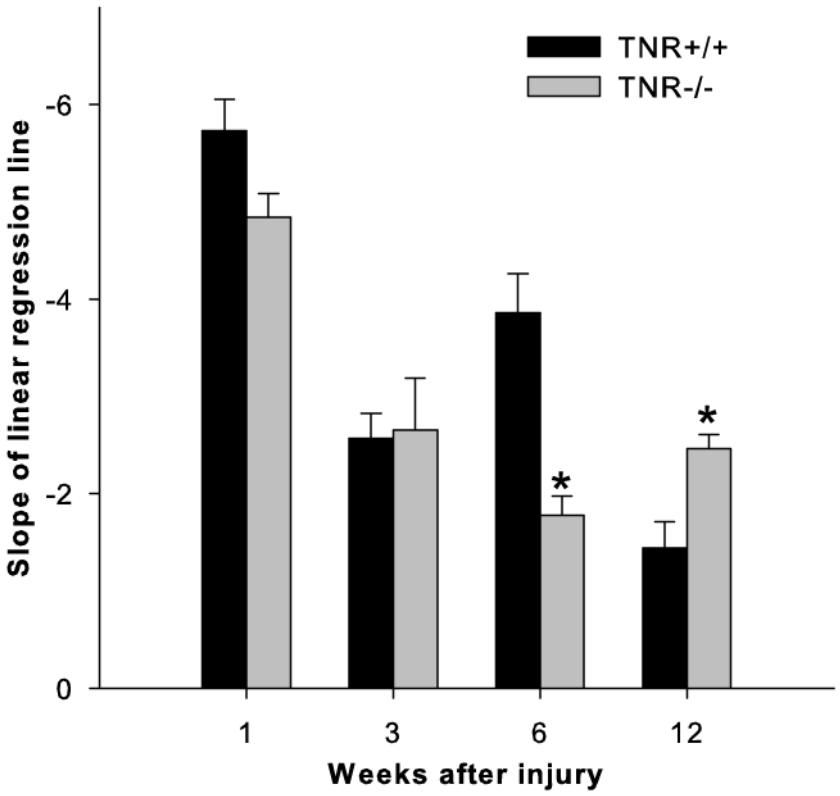

Fig. 15. Slope of linear regression line for H-reflex rate depression after spinal cord injury in TNR-/- mice and wild-type littermates. Linear regression analysis for rate depression of H/M ratio, normalized to values at baseline frequency (0.1 Hz, Fig. 15B), was performed to test for significant reduction at a given time point after injury. Shown are slopes (+ SD) of individual regression lines in the mice analyzed for H/M ratio (Fig. 15B). (asterisks, $p < 0.05$, ANCOVA for comparing linear regression lines)

TNR–/– mice compared with wild-type littermate at 6 weeks (Fig. 15). As observed in CHL1-deficient mice, reduction of rate depression in TNR–/– mice was attenuated (Fig.15B), but in contrast, the absolute H/M ratios were still elevated in TNR–/– mice at 12 weeks after injury (Fig. 14A). While these observations indicate that increased H-responses in general correlate with better functional outcome, it is apparent that specific aspects of the reflex responses are genetically determined.

4.2.4 Motoneuron synaptic coverage in TNR-deficient mice at 12 weeks after spinal cord injury

In addition to the alteration of H-reflex in TNR–/– mice, perisomatic coverage around motoneurons was analyzed at 12 weeks to enable comparisons with previous observations at 6 weeks (Apostolova et al., 2006). At 6 weeks after spinal cord injury, no differences in $ChAT^+$, $VGAT^+$ or $VGLUT^+$ terminals have been observed between TNR–/– mice and wild-type littermates. Here, we found that at 12 weeks after injury the densities of all three types of synapses are significantly higher in TNR–/– that in TNR+/+ mice (Fig. 16). Therefore, as for CHL1–/– mice, synaptic remodeling is still active between the 6^{th} and the 12^{th} week after injury. This type of remodeling differs from that seen in CHL1–/– mice and may in part determine differences, as compared with CHL1–/– mice, in reflex excitability parameters.

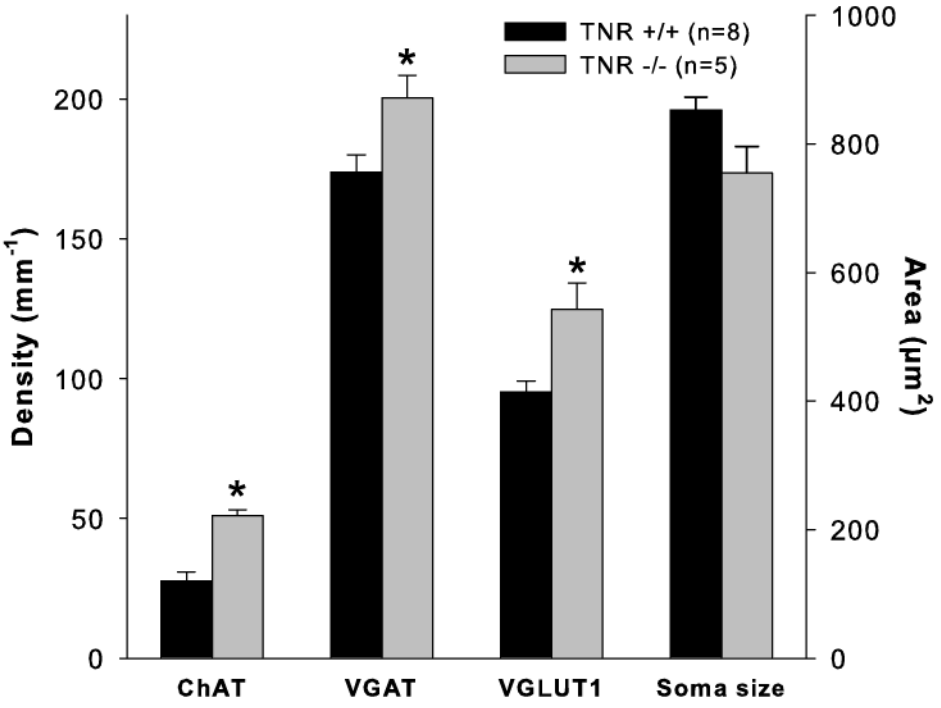

Fig. 16. Analysis of soma areas and perisomatic terminals in TNR-/- mice and wild-type littermates. Motoneuron soma size and densities of perisomatic terminals around motoneurons were analyzed at 12 weeks after spinal cord injury. Shown are mean values (+ SEM) of soma area of $ChAT^+$ motoneurons ("Soma size") and linear densities of ChAT-, VGAT-, and VGLUT1-positive terminals calculated from mean values of individual animals. Asterisks indicate significant differences between TNR-/- mice and wild-type littermates ($p < 0.05$, two-sided t test for independent samples).

4.3 H-reflex alterations and motor recovery in tenascin-C (TNC)-deficient mice

4.3.1 Motor recovery after spinal cord injury in TNC-deficient mice

To estimate the role of TNC in spinal cord regeneration, we first compared motor recovery between TNC–/– and TNC+/+ mice after compression injury using BBB rating (Basso et al., 1995) and objective numerical measures (Apostolova et al., 2006): the foot-stepping angle and the rump-height index during beam walking (Figs. 2A-C) and the extension-flexion ratio measured during voluntary movements without body weight support ("pencil" test, Figs. 2D-E). Motor performance of TNC–/– mice before injury was similar to that of TNC+/+ mice (Fig. 17), in agreement with previously published data that TNC–/– mice have no apparent motor deficits (Morellini and Schachner, 2006). One week after compression injury, both TNC+/+ and TNC–/– mice had severe decline of the BBB score, the rump-height index, and the extension-flexion ratio as well as an increase in the foot-stepping angle (Figs. 17A-D). During the 12 weeks of observation, locomotor abilities recovered to a moderate degree in both genotypes, but this recovery was worse in TNC–/– mice than in TNC+/+ littermates as indicated by the BBB score, the foot-stepping angle, and the extension-flexion ratio (Figs. 17A, B, D). Group mean values statistically differed only for BBB score 12 weeks after injury (Fig. 17A). However, analysis of recovery indices, which estimate gain of function after the first week as a fraction of the functional loss induced by the injury in individual animals (Apostolova et al., 2006), revealed significant differences for the BBB

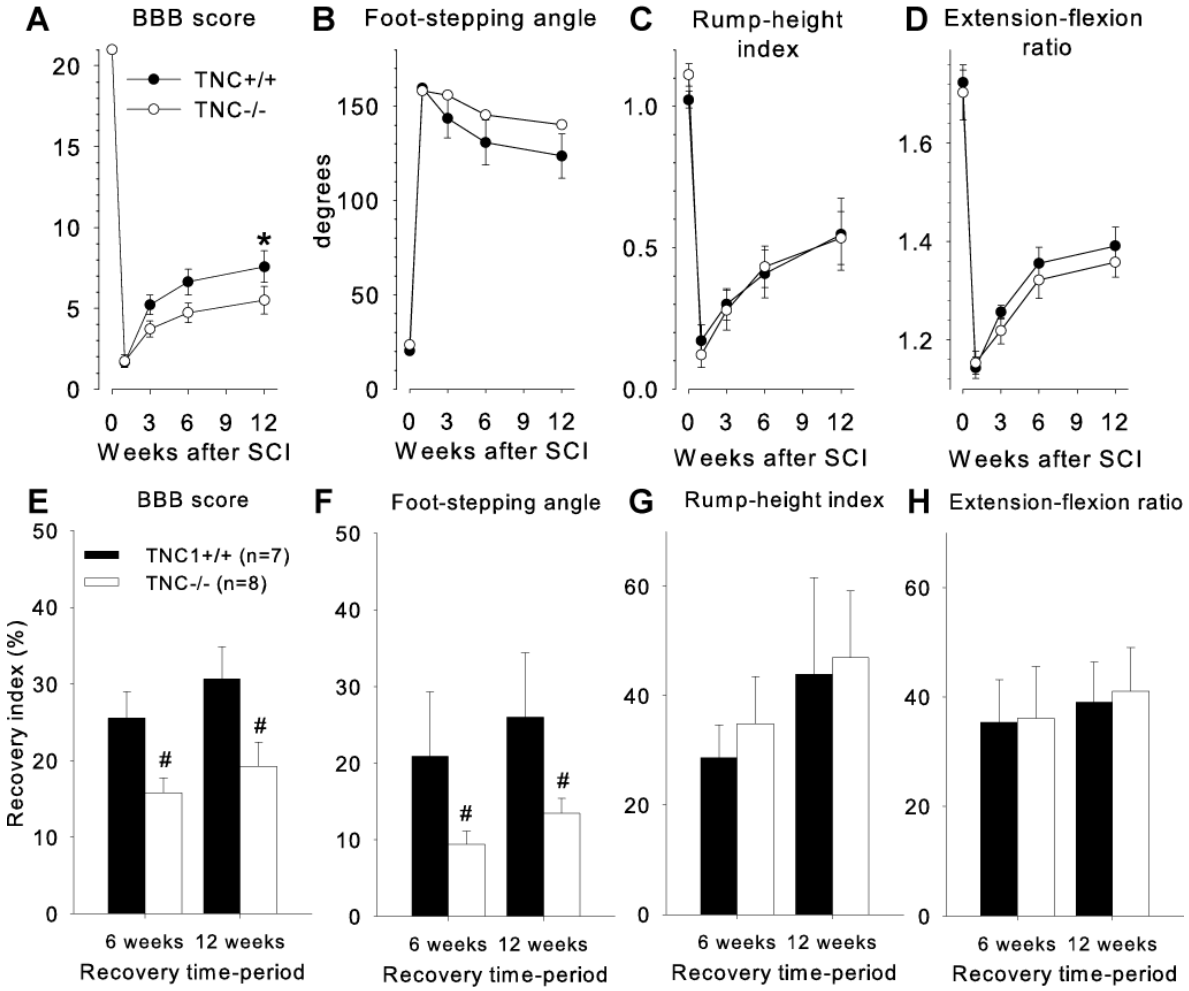

Fig. 17. Functional recovery after compression spinal cord injury in TNC-/- mice and wild-type littermates estimated by BBB rating and single-frame motion analysis. Panels A-D show mean values (± SEM) of open-field locomotion scores (BBB, A), foot-stepping angles (FSA, B), rump-height indices (RHI, C), and extension-flexion ratios (EFR, D) before surgery (day 0) and at 1, 3, 6, and 12 weeks after injury in TNC-/- mice (n=8) and wild-type littermates (n=7). Recovery indices (mean values + SEM) calculated from individual animal values for each parameter at 6 weeks and 12 weeks after injury are shown in panel E-H, respectively. Significant differences between group mean values are indicated by asterisks (A-D), crosshatches indicate differences between mean recovery indices of TNC-/- and TNC+/+ at 6 and 12 weeks (E-H)(ANOVA for repeated measurements with Tukey's *post hoc* test, $p < 0.05$).

score and the foot-stepping angle at 6 and 12 weeks after injury (Figs. 17E, F). These results indicate an adverse effect of TNC ablation on hindlimb locomotion after spinal cord injury.

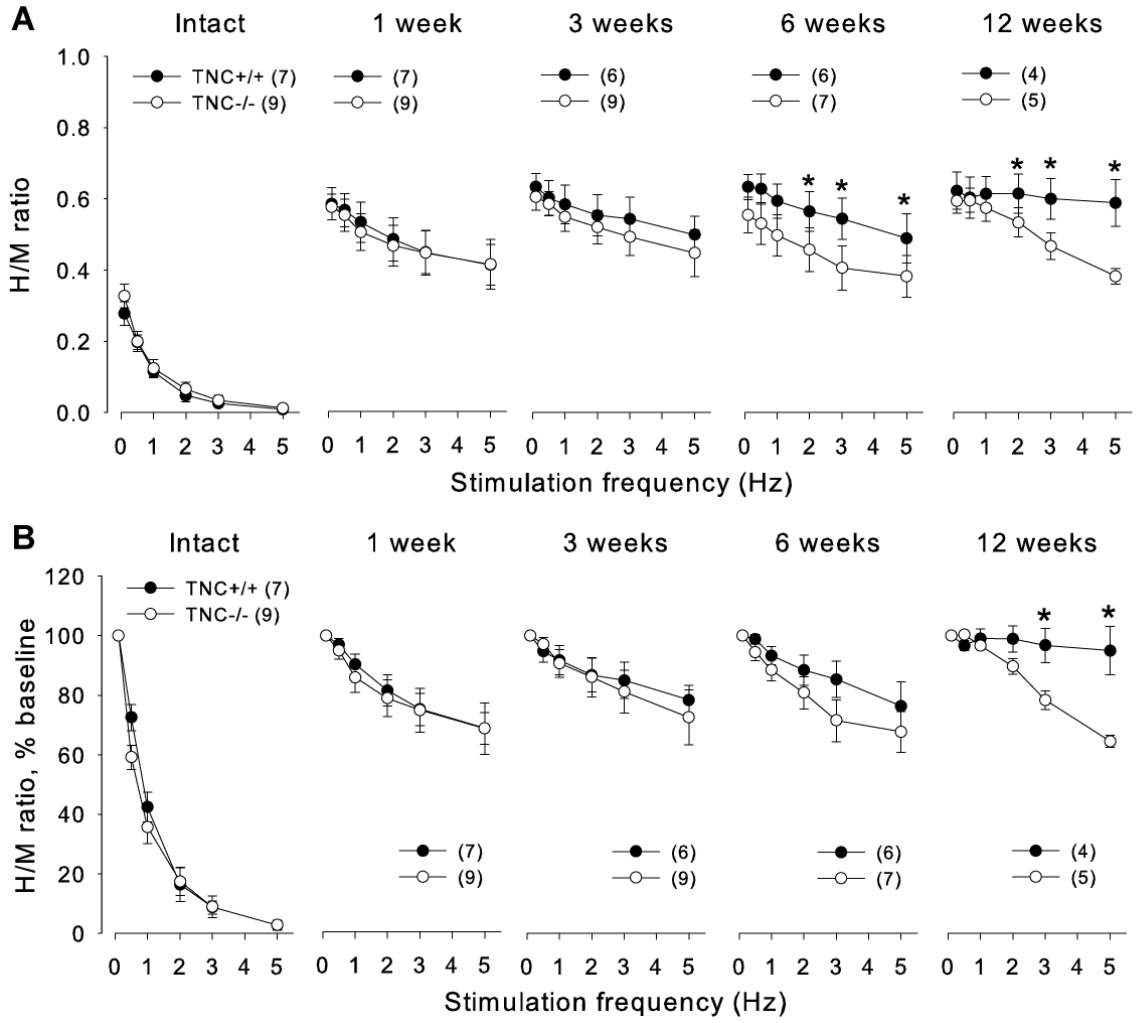

Fig. 18. Rate depression of the H-reflex at different time-points after spinal cord injury in TNC-/- mice and wild-type littermates. Shown are mean values (± SEM) of absolute H/M ratios (A) and ratios normalized to the individual H/M values at baseline frequency (0.1 Hz, B) for different stimulation frequencies prior to operation and 1, 3, 6, and 12 weeks after spinal cord injury. At 6 and 12 weeks after injury, the H/M ratio in TNC-/- mice is significantly lower than in wild-type littermates at high stimulation frequencies (2, 3, and 5 Hz, A). Rate depression of the normalized H/M ratio is significantly increased at 12 weeks in TNC-/- mice (asterisks, ANOVA for repeated measurements with Tukey's *post hoc* test, $p < 0.05$).

4.3.2 H-reflex alteration after spinal cord injury in TNC-deficient mice

In addition to assessment of locomotor recovery, we analyzed the plantar H-reflex in TNC-deficient mice. Prior to injury, the H-reflex responses were strongly reduced in both TNC–/– and TNC+/+ mice when the stimulation frequency was stepwise increased from baseline frequency (0.1 Hz) to 5 Hz (Fig. 18A). One week after injury, the rate depression was severely reduced in both genotypes (Fig. 18A). At later time-points, 3 to 12 weeks, the rate sensitivity declined further, as

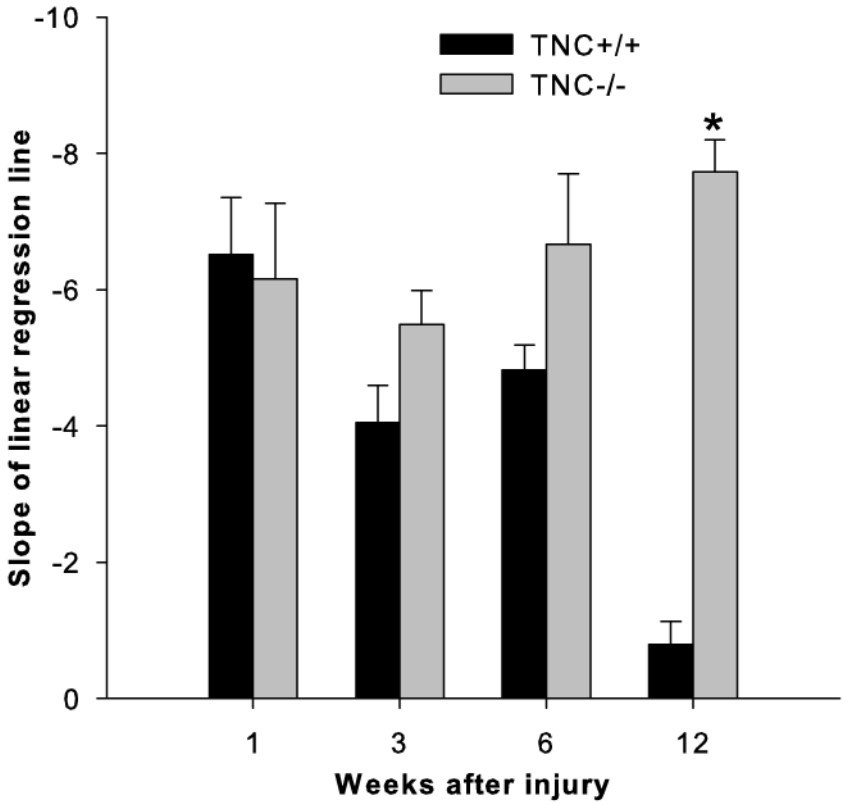

Fig. 19. Slope of linear regression line for H-reflex rate depression after spinal cord injury in TNC-/- mice and wild-type littermates. Linear regression analysis for rate depression of H/M ratio, normalized to values at baseline frequency (0.1 Hz, Fig. 20B), was performed to test for significant reduction at a given time point after injury. Shown are slopes (+ SD) of individual regression lines in the mice analyzed for H/M ratio (Fig. 20B). The rate depression of H/M ratio increased significantly in TNC-/- mice at 12 weeks compared with wild-type littermates (asterisks, $p < 0.05$, ANCOVA for comparing linear regression lines).

compared with one week, in TNC+/+ mice but not in TNC–/– mice (Fig. 18A). Thus, reflex excitability upon repetitive stimulation, as estimated by absolute H/M ratios, was significantly reduced in TNC–/– mice compared with TNC+/+ littermates at 6 and 12 weeks. Analysis of normalized H/M ratios (Fig. 18B) and regression slopes (Fig. 19) revealed higher rate depression in TNC–/– than in TNC+/+ at 12 weeks (Fig. 19, Fig. 6B). Therefore, our combined results suggest that TNC deficiency prevents functionally favorable changes in reflex excitability with time after injury, which in turn leads to worse locomotion.

4.3.3 Morphological changes after spinal cord injury in TNC-deficient mice

To elucidate the possible influence of synaptic circuit remodeling to the worse outcome of spinal cord injury in TNC–/– mice, density of perisomatic terminals around motoneuron was estimated. The linear density of large perisomatic $ChAT^+$ boutons was slightly reduced, as compared with intact mice, after spinal cord injury in both genotypes (Fig. 20A). GABAergic coverage of motoneuron somata, measured by $VGAT^+$ terminals, was significantly higher after injury than in intact animals showing no genotype related differences (Fig. 20B). An extraordinary increase occurred in the excitatory $VGLUT1^+$ synaptic coverage after injury for both genotypes (Fig. 20C). Thus, differences in motor recovery between TNC–/– and TNC+/+ mice cannot be explained by differences in synaptic coverage on motoneurons.

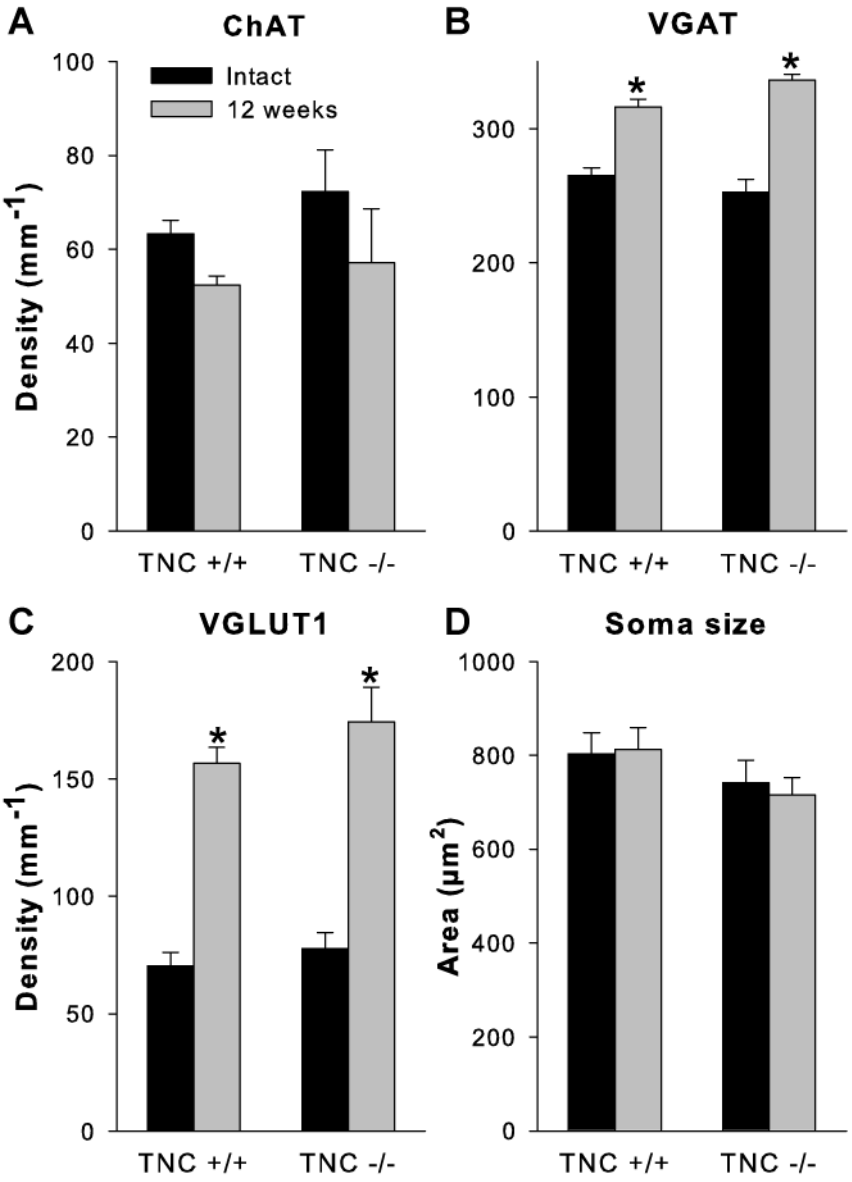

Fig. 20. Analysis of soma areas and density of perisomatic terminals in TNC-/- mice and wild-type littermates. Motoneuron soma size and densities of perisomatic terminals around motoneurons were analyzed in intact mice (n = 4 per genotype) and mice studied 12 weeks after spinal cord injury (n = 5, TNC-/-; n = 4, TNC+/+). Shown are mean values (+ SEM) of soma area of $ChAT^+$ motoneurons (D) and linear densities of ChAT- (A), VGAT- (B), and VGLUT1-positive terminals (C) calculated from mean values of individual animals. Asterisks indicate significant differences between intact and injured animals ($p < 0.05$, two-sided t test for independent samples).

4.3.4 Altered afferent input to the injured spinal cord of TNC-deficient mice

Degree of locomotor recovery after spinal cord injury is largely determined by preservation and functionality of primary afferent inputs (Dietz et al., 2002; Lavrov et al., 2008). We were, therefore, interested whether spinal cord connectivity is altered in TNC–/– mice. We analyzed VGLUT1$^+$ synaptic terminals, which are derived from medium- to large-sized neurons in the dorsal root ganglia and convey mechano- and proprioceptive information to the spinal cord (Brumovsky et al., 2007). We selected three areas for analysis in which VGLUT1$^+$ terminal densities were prominent: the Clarke's column which receives proprioceptive information (Figs. 21A, B), an adjacent part of lamina VII, lamina in the spinal cord containing, among other neurons, last-order interneurons, i.e., innervating motoneurons (Figs. 21A, C), and the motoneuron region, lamina IX, where proprio- and mechanoreceptors form contacts predominantly on motoneuron dendrites (Figs. 21A, D). Analysis of TNC+/+ mice 12 weeks after injury revealed a significant, compared with non-injured mice, decline in the terminal densities in Clarke's column and lamina VII, and, interestingly, a strong increase in lamina IX (Figs. 21E, F, G). Similar injury-related changes were found in TNC–/– mice with one prominent exception: a severe reduction of VGLUT1$^+$ boutons in lamina VII compared with both non-injured TNC–/– mice and injured TNC+/+ mice (Fig. 21F). This genotype-related difference indicates that TNC ablation leads to area-specific deficits in afferent inputs to the injured spinal cord.

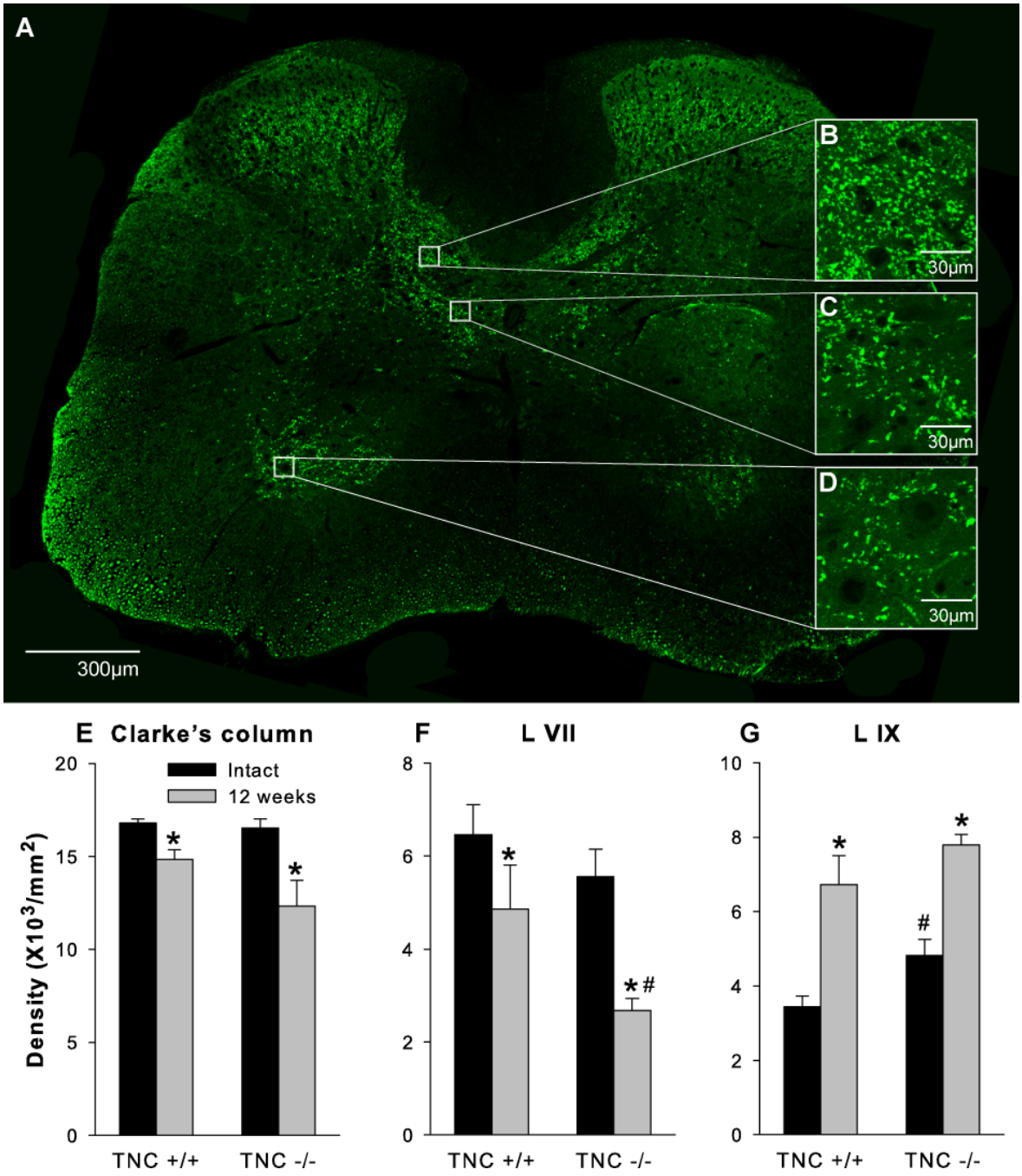

Fig. 21. Analysis of VGLUT1$^+$ terminals in the spinal cord of intact and injured TNC-/- mice and wild-type littermates. A representative section of the spinal cord of a TNC+/+ mouse (A) shows the distribution of VGLUT1$^+$ puncta. Digital images of VGLUT1+ terminals in the Clarke's column (B) and medial lamina VII (LVII, C) and lamina IX (LIX, D) obtained at high magnification were used for estimation of terminal densities (number of puncta per unit area). Mean densities (+ SEM, n = 4) of terminals in the three areas in intact and injured TNC-/- and TNC+/+ mice are shown in panels E-G. Asterisks indicate differences between intact and injured mice of the same genotype, cross-hatches denote differences between similarly treated TNC-/- and TNC+/+ mice ($p < 0.05$, one-way ANOVA with subsequent Tukey's *post hoc* tests).

4.3.5 Adverse effects of TNC deficiency on monaminergic axons

The deficit in the glutamatergic innervation of lamina VII in TNC–/– mice suggested that absence of TNC in the injured spinal cord has a negative impact on axonal survival and/or sprouting. To further analyze this possibility, we examined monaminergic (tyrosine hydroxylase-positive, TH^+) axons in the lumbar spinal cord (Figs. 22A-D). These axons often survive after thoracic compression or contusion injuries and are able to regrow and sprout (Ghirnikar et al., 2001). In support of our notion, we found a dramatic deficit in the TH^+ axonal innervation of the lumbar

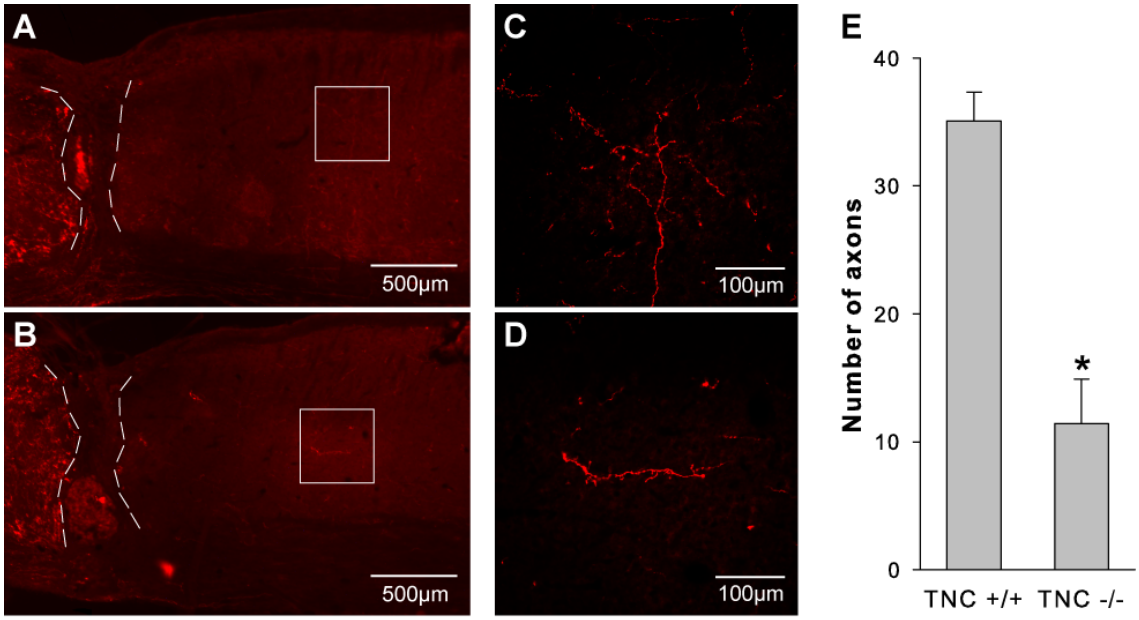

Fig. 22. Analysis of monaminergic axons caudal to the lesion site in the lumbar spinal cord of TNC-/- mice and wild-type littermates 12 weeks after injury. Representative digital images show tyrosine hydroxylase-positive (TH^+) fibers crossing lesion scar area (between reference lines) in the lumbar spinal cord in TNC+/+ (A, C) and TNC-/- (B, D) mice. The identified TH^+ axons, such as those in white squares (A, B) shown at high magnification (C, D), were counted between 250 – 2000 µm from the caudal lesion scar. Shown are mean numbers of TH^+ axons (+ SEM) in TNC+/+ (n = 4) and TNC-/- (n = 3) mice (E). Asterisk indicates significant difference between TNC-/- mice and TNC+/+ littermates ($p < 0.05$, two-sided t test for independent samples).

spinal cord of injured TNC–/– mice compared with TNC+/+ mice (Fig. 22E). Considering that the degree of monaminergic innervation of the lumbar spinal cord is an important determinant of locomotor abilities after spinal cord injury in rats and mice (Edgerton et al., 2004; Fouad and Pearson, 2004; Jakovcevski et al., 2007), we can conclude that the observed axonal deficit accounts, at least in part, for the worse functional outcome in TNC–/– mice.

V Discussion

The results of this study demonstrate that the H-reflex is a useful tool for assessment of motoneuron pool excitability in spinal cord injured mice. In the compression injury paradigm used here, the alterations of the H-reflex had a predictive value for the functional outcome. Enhanced H-reflex responses were associated with better recovery in individual C57BL6/J mice and in two mouse mutants: TNR- and CHL1-deficient mice. In contrast, motor recovery after spinal cord injury in TNC-deficient mice was worse than in wild-type littermates and this was accompanied by reduced H-reflex responses. One possible and probable mechanism underlying the H-reflex alterations after spinal cord injury is structural remodeling of spinal circuitries in the lumbar spinal cord caudal to the lesion site.

5.1 Functional recovery and H-reflex alterations in C57BL6/J mice

Spinal cord injury in mice induced changes in the H-reflex which are well described in humans and other animals: increase in the H-wave amplitude and H/M ratio at baseline stimulation and reduced rate depression upon low-frequency stimulation. The unusual feature with respect to these observations is that the alterations are pronounced, in contrast to other mammals, already one week after injury. This peculiarity is not surprising since even closely related species such as rats and mice react differently to a spinal cord lesion, for example, with respect to neuroinflammatory responses (Donnelly and Popovich, 2008). Still, it will be interesting to investigate which processes leading to hyperexcitability are specifically accelerated in the mouse. This task will be, however, difficult, since

diverse mechanisms are involved in the post-traumatic H-reflex enhancement, for example, increase in motoneuron excitability due to reappearance of large persistent inward ionic currents, decrease of presynaptic inhibition of Ia afferents, enhanced post-activation depression of transmitter release in Ia terminals and reduced reciprocal inhibition by Ia terminals from antagonistic muscles (Hultborn, 2003; Li and Bennett, 2003; Li et al., 2004; Frigon and Rossignol, 2006).

A more intriguing finding than the rapid onset of hyperexcitability in the mouse was that the rate depression in the course of the 12-week observation period progressively decreased, i.e., became more “abnormal” with time. One would expect that, similar to the motor disability, the injury-induced deficits would decline during spontaneous recovery. A similar, unexpected observation in rats has recently been reported (Lee et al., 2005). In rats with mild contusion injury, and thus fairly good functional recovery, the rate depression of the H-reflex decreases, i.e. becomes more abnormal as in our study, between the 1st and 8th post-operative weeks. In contrast, this depression increases, i.e. becomes more “normal”, in rats with complete transection of the spinal cord, an injury leading to poor functional outcome. These observations suggest that reduction in the rate sensitivity of the H-reflex provides functional advantages after incomplete spinal cord injury in both rats and mice. It is possible that a more efficient functionality of reflex pathways at low-frequency stimulation rates allows better, physiologically meaningful control of spinal circuitries by afferent inputs. This notion is in agreement with the idea that enhanced reflex function is beneficial for locomotor recovery after spinal cord injury (Pearson, 2001). The importance of afferent inputs for locomotor abilities has been well documented in cats with complete spinal cord transection in which task-specific alterations in spinal cord circuits occur after they

are trained to stand or walk (Edgerton et al., 2004). Also, stepping in rats with spinal cord transection is improved if motoneuron discharge probability upon Ia afferent stimulation is increased as a result of larger excitatory postsynaptic potentials or decreased after hyperpolarization (Petruska et al., 2007). Finally, H-reflex operant conditioning using an “up-conditioning” protocol leads to increased reflex responses and reduces locomotor deficits after unilateral transection of the lateral spinal cord column (Chen et al., 2006). Taken together, these considerations help to explain the link between rate depression alterations and degree of recovery suggested by our results.

5.2 Synaptic plasticity and H-reflex changes in C57BL6/J mice

Short-term alterations in reflex excitability, occurring within hours and days after spinal cord injury, are explainable by loss of supraspinal and propriospinal inputs, and by alterations in receptors and channels in motoneurons and presynaptic terminals modulating neuronal excitability and transmitter release (Little et al., 1999; Hultborn, 2003; Frigon and Rossignol, 2006). Long-term changes, requiring weeks and even months, are generally associated with structural synaptic plasticity, i.e. formation of new synaptic contacts between primary afferents or spared descending axons, on the one hand, and interneurons and motoneurons in the lumbar spinal cord, on the other. This type of plasticity is achieved by axonal sprouting and its significance for functional recovery after incomplete spinal cord injury is well recognized (Raineteau et al., 2002; Bareyre et al., 2004; Courtine et al., 2008). Twelve weeks after spinal cord compression we observed a remarkable increase, compared with intact animals, of both excitatory

and inhibitory synaptic terminals around motoneuron cell bodies. Although chemically defined, by expression of the vesicular transporters VGAT and VGLUT1, the analyzed synaptic terminals are heterogeneous with respect to cell origins, transmitters (glycine or GABA for VGAT-positive boutons) and post-synaptic targets (mostly motoneuron cell body, but also possibly dendrites and axons in the perisomatic compartment). We thus cannot draw conclusions as to the types of connections which lead to functional recovery and enhanced H-reflex 3 months after injury. It is therefore not surprising that numbers of these heterogeneous terminals did not correlate with H-reflex or functional parameters. Nevertheless, our finding of a large increase in synaptic inputs to motoneurons in the lumbar spinal cord suggest that synaptic remodeling contributes to long-term alterations in reflex responses and functional performance of the animals. A more precise specification of synaptic inputs, for example GABAergic versus glycinergic perisomatic terminals and dendritic versus perisomatic glutamatergic synapses, is required in future studies to understand the relationships between alterations in motoneuron synaptic connections and functional parameters.

5.3 Reflex responses, functional outcome and synaptic alterations in TNR- and CHL1-deficient mice

The conclusion drawn from experiments with C57BL/6J mice, namely, that functional improvements in our experimental paradigm are associated with enhanced reflex excitability, was further substantiated by observations on two mouse mutants. Both TNR- and CHL1-deficient mice developed a more pronounced, as compared with wild-type littermates, hyperreflexia between 3 to 6 weeks after injury and this phenomenon coincided with the appearance of

functional advantages in the time-course of recovery documented in previous studies (Table 1). However, the enhancement of the H-reflex differed qualitatively from that observed in C57BL/6J mice. In the C57BL/6J mice, we observed rate depression attenuation with time after injury. In CHL1-deficient mice, rate depression, calculated as percentage of baseline responses, as well as H/M ratios was dramatically reduced at 1, 3, and 6 weeks after injury compared to that in wild-type mice. In TNR-deficient mice, rate depression was different from that in wild-type mice only at 6 and 12 weeks after injury despite of enhanced H/M ratios similar to CHL1-deficient mice. These observations demonstrate that H-reflex enhancement is associated with better motor recovery in both TNR- and CHL1-deficient mice but differences in rate depression suggest alterations of different synaptic inputs in the two mutants. Increased, as compared to wild-type mice, inhibitory $VGAT^{+}$ terminals on motoneuron cell bodies in TNR –/– mice, but not in CHL1–/– mice, is a plausible explanation for the attenuated H/M ratios in injured TNR–/– mice.

5.4 Reflex responses, functional outcome and synaptic alterations in TNC-deficient mice

In contrast to contributions of enhanced H-reflexes to better recovery in TNR- and CHL1-deficient mice, TNC–/– mice showed decreased H-reflex responses in association with worse recovery of locomotion. These observations lead further support to our hypothesis that alterations of the H-reflex determine the motor outcome.

Upregulation of TNC has previously been observed after lesions of the CNS (Laywell et al., 1992; Nakic et al., 1996; Zhang et al., 1997; Camand et al., 2004)

but its role in tissue repair has not been understood. Our finding that TNC-deficiency leads to worse functional outcome of spinal cord injury in mice provides for the first time evidence that TNC is beneficial for CNS regeneration. The positive effects of TNC on regeneration processes are not restricted to the CNS. Its expression is upregulated in various tissues after injury, for example, peripheral nerve, skin, and skeletal muscle (Mackie et al., 1988; Daniloff et al., 1989; Van Eyken et al., 1992; Irintchev et al., 1993; Mackie et al., 1998) and the absence of TNC has negative impacts on the outcome of facial nerve injury (Guntinas-Lichius et al., 2005) and skeletal muscle damage (Fluck et al., 2003). Therefore, sufficient evidence indicates that TNC promotes tissue healing in various tissues.

A technical novelty in this study was the measurement of primary afferent inputs in the lumbar spinal cord. VGLUT1$^+$ terminal densities were estimated in selected areas including the Clarke's column, lamina VII, and lamina IX, areas in which primary afferents contact interneurons and motoneurons. The observed reduction of spinal afferent inputs to lamina VII, but not in lamina IX or the Clarke's column, in the spinal cords of injured TNC–/– mice can be regarded as a region-specific effect on injury-induced synaptic remodeling underlying functional deficits. This notion is not surprising since TNC is involved in synaptic plasticity (Dityatev and Schachner, 2003). In addition to deficient synaptic remodeling, worse recovery in TNC–/– mice may be related to reduced regrowth or sprouting of monaminergic fibers, as indicated by analysis of TH$^+$ axons. Previous work has shown that degree of motor recovery in mice is positively correlated with numbers of monanminergic fibers in the distal spinal cord (Jakovcevski et al., 2007). Monaminergic transmitters are well known modulators of motoneuron excitability and rhythmic and coordinated movements (Edgerton et al., 2004; Fouad and

Pearson, 2004). For example, descending serotonegic fibers induce a long-lasting depression of lumbar monosynaptic reflexes (Yomono et al., 1992; Honda et al., 2003). After spinal cord injury, various types of serotonergic receptor are upregulated (Fuller et al., 2005; Lee et al., 2007). It is assumed that such receptor upregulation is a compensatory mechanism for the lack or paucity of monaminergic axons and deficient reflex modulation after spinal cord injury. The overall results suggest that worse recovery in TNC–/– mice is associated with restricted synaptic plasticity and reduced axonal sprouting.

5.6 Outlook

The results of this study encourage a more extensive use of H-reflex analysis in mouse models of spinal cord injury. This will provide, in combination with behavioral and structural measures, a better understanding of the mechanisms determining the functional outcome. Of particular interest will be to analyze, for example, the relationships between synaptic inputs, reflex properties and motor abilities in mice subjected to different lesion severities or in mutant mice which display varying degrees of spontaneous recovery compared with wild-type mice. In the latter case, knowledge of specific molecular and cellular deficits could aid in understanding of the pathophysiological mechanisms in the injured spinal cord.

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VIII Appendices

i Abbreviations

%	per cent
°C	degrees Celsius
A/D	analog / digital
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AVI	audio video interleaved
BBB	Basso, Beattie, Bresnahan rating scale
CAMs	cell adhesion molecules
ChAT	choline acetyltransferase
CHL1	close homologue of L1
CHL1–/–	CHL1-deficient mice
CHL1+/+	wild-type littermates of CHL1-deficient mice
CNS	central nervous system
ECM	extracellular matrix
EFR	extension-flexion ratio
EGFL	epidermal growth factor-like repeat
EMG	electromyography
EPSPs	excitatory postsynaptic potentials
FGF-2	fibroblast growth factor
FN-III	fibronectin type III domain
FSA	foot-stepping angle

GABA	gamma-aminobutyric acid
h	hour
HNK-1	human natural killer cell
H-reflex	Hoffmann-reflex
Hz	hertz, unit of frequency (s^{-1})
i.e.	id est (that is)
Ia	type Ia primary afferent fiber
kDa	kilo dalton
kHz	kilo hertz, 10^3 Hz
LIX	lamina IX of grey matter in the spinal cord
LVII	lamina VII of grey matter in the spinal cord
M	molar
min	minute
mRNA	messenger ribonucleic acid
M-wave	muscle-wave
n	number of animals
p	probability
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PNS	peripheral nervous system
r	pearson product-moment correlation coefficient
RHI	rump-height index
RT	room temperature
s	second
SD	standard deviation
SEM	standard error of mean

SFMA	single-frame motion analysis
TA	tenascin assembly domain
TH	tyrosine hydroxylase
TNC	tenascin-C
TNC–/–	TNC-deficient mice
TNC+/+	wild-type littermates of TNC-deficient mice
TNR	tenascin-R
TNR–/–	TNR-deficient mice
TNR+/+	wild-type littermates of TNR-deficient mice
VGAT	vesicular GABA transporter
VGLUT1	vesicular glutamate transporter 1
w	week
µ	micro (10^{-6})

ii Table of figures

iii Curriculum vitae

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1992 - 1995	Daegeon high school in Incheon, Korea
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List of Publications

Lee HJ, Jakovcevski I, Radonjic N, Hoelters L, Schachner M, Irintchev A (2008) Better functional outcome of compression spinal cord injury in mice is associated with enhanced H-reflex responses. Exp Neurol.

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iv Erklärung

Hiermit versichere ich dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe. Ich habe keine anderen als die angegebenen Hilfsmittel und Quellen benutzt und habe die entnommen Stellen als solche kenntlich gemacht. Soweit Fremde Hilfe in Anspruch genommen wurde, ist dies entsprechend im Text vermerkt.

Diese Arbeit ist zuvor keiner Prüfungsbehörde, weder in dieser noch in abgewandelter Form, zum Erwerb des Doktorgrades vorgelegt worden. Auch mit keiner anderen Arbeit habe ich mich zuvor um den Erwerb des Doktorgrades bemüht.

Hamburg, 2009

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