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Molecular determinants of excitability of hindlimb motoneurons after complete spinal cord transection and BDNF overexpression

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Contents

1. Abstract

Spinal cord injury (SCI) causes severe neuronal impairment, resulting in a loss of muscle function and sensation below the injury site. In consequence, the most profound symptom of patients with SCI is the loss of motor ability leading to paraplegia or tetraplegia, depending on the level of injury. Due to the loss of descending supraspinal pathways and to segmental changes in local circuits, the excitability of α-motoneurons (MNs), which is an intrinsic ability of neurons to generate action potentials determined by function and abundance of neurotransmitter receptors and ion channels, is altered. This alteration significantly contributes to the pathology of SCI and the recovery of impaired locomotion. MNs undergo a transition from hypoexcitability during spinal shock at the acute phase to hyperexcitability in the chronic phase of SCI. The hyperexcitability of MNs is considered to be the main reason of a development of muscle spasticity, a common complication of SCI. Understanding of its molecular background might create a possibility of modulation of MN intrinsic excitability which is key to recovery.

Studies using SCI models have investigated the molecular changes in neurotransmission-related molecules in MNs mostly at the late postlesion phase, when hyperexcitability is well established. In experimental SCI in rodents the onset of spasticity can be seen as early as one-week postinjury. However, the pattern and relation of expression level of genes coding for membrane proteins instrumental for excitatory vs inhibitory neurotransmission in the subacute phase of SCI when excitability starts to restore, is not clear.

The first aim of my work was to clarify the direction and extent of transcriptional regulation of receptors mediating excitatory and inhibitory neurotransmission and of functionally associated membrane channels in hindlimb MNs of adult rats, at the second week postinjury, when excitability restores. I hypothesized that after the acute phase, fast molecular changes of lumbar MNs develop in response to the loss of inputs. These responses may disturb the balance of excitatory and inhibitory receptors and related ion channels in MNs. Since the activity of MNs innervating the ankle extensor and flexor muscles is differently affected by SCT, I examined separately gene expression in pools of MNs innervating ankle extensor (Gastrocnemius lateralis; GL) and flexor (Tibialis anterior; TA) muscles.

A promising way to treat SCI is through enrichment of the injured spinal cord with brain derived neurotrophic factor (BDNF). Our previous study showed that BDNF overexpression induced with AAV-BDNF injection caudal to the lesion site improves locomotor abilities, accompanied by upregulation of transcript levels of glutamatergic and GABAergic markers in the interneurons, presynaptic to MNs. While the study demonstrated beneficial role of BDNF in adapting the spinal network to increased activity, undesirable behavioral effects suggesting overexcitability were observed in time. That observation set my second aim, to characterize the effect of spinal AAV-BDNF administration on gene expression of selected proteins studied in the first part of my project, and identify target molecules of pro-excitogenic potential. Limited number of animal studies reported that increased BDNF/TrkB signaling can modulate the excitability of MNs by altering the expression of glutamatergic AMPA and NMDA receptors and of KCC2 chloride ions extruder, responsible for maintaining an inwardly directed driving force for chloride ions, which is a prerequisite for hyperpolarizing GABA-mediated inhibitory neurotransmission. However, a more comprehensive view on the pattern of molecular changes in MNs after BDNF treatment is lacking.

Prior to complete spinal cord transection (SCT) performed at the thoracic Th11 level, fluorescence retrograde tracers were injected to the respective muscles to identify MNs. After SCT, PBS or AAV-BDNF was injected bilaterally to the lumbar L1/2 segment. Non-lesioned rats with injected tracers

served as controls. At two weeks postlesion, locomotor performance of spinal rats was evaluated on a running treadmill. After animal perfusion, GL and TA MNs were isolated from longitudinal spinal sections by laser-assisted microdissection, mRNA was isolated and reverse-transcribed into cDNA. Transcript levels of selected neurotransmitter receptors, ion channels and Cl^- transporters were assayed using quantitative PCR. Immunohistochemical localization and identification of selected receptors was carried out.

BDNF treatment significantly improved locomotor performance of spinal rats on a running treadmill, increasing the mean mBBB score from 0.36 in SCT-PBS rats to 12.19 in SCT-BDNF rats. Quantitative analysis of mRNA levels carried out at 13 days in SCT-PBS group and at peak of locomotor performance in SCT-BDNF group (13-16 days postlesion) revealed that SCT alone increased levels of mRNA coding for AMPAR GluA1 and mGluRs subunits, and down-regulated mRNA coding for GABAAR, GlyR subunits, KCC2, NMDAR, Nav1.6, KCa1.1 and SK2. The level of transcripts of 5-HT_{2B} receptors which become constitutively active after injury and contribute to recovery of MN function and emergence of spasms was markedly up-regulated. BDNF overexpression: (1) tended to rebalance AMPAR and NMDAR subunit mRNA levels, with a trend to decrease AMPAR and increase NMDAR, and (2) further reduced mRNA levels of GABAAR and GlyR subunits. BDNF overexpression also caused a profound increase of mRNA level of 5-HT_{2A} receptors, which augment glutamatergic signaling, acting on timing and amplitude of muscle activity. This set of results suggest that BDNF effects on receptor expression promote pro-excitatory phenotype in MNs, which develops after SCT. Moreover, the degree of down-regulation of KCC2 expression postinjury, which was maintained after BDNF treatment, lets infer on a concomitant accumulation of Cl^- inside MNs, rendering GABA aberrantly “exciting” which would result in decreased GABAergic inhibition.

In conclusion, the experiments led to identification of prominent down-regulation of transcripts coding for molecules controlling inhibition, and of up-regulation of 5HT receptors controlling muscle activity. Correlation analysis of gene expression data revealed profound changes in their patterns, which suggests that in MNs a large set of genes coding for neurotransmission-related membrane proteins undergoes differential regulation postlesion. My results provide new insight into the mechanisms of MN excitability developing in the subacute phase after SCT and demonstrate that both MN groups similarly adapt to a more excitable state, which may increase the occurrence of extensor and flexor spasms already at this stage. The reliable reconstruction of the net effects of BDNF on the excitatory state of MNs requires further studies.

Streszczenie

Uraz rdzenia kręgowego powoduje uszkodzenia dróg nerwowych i dysfunkcję neuronów, prowadząc do osłabienia czynności mięśni i czucia poniżej miejsca urazu. Najpoważniejszym objawem u pacjentów po urazie rdzenia jest utrata zdolności motorycznych, prowadząca do paraliżu kończyn. Ze względu na odcięcie wpływów ze zstępujących szlaków nadrdzeniowych i na segmentalne zmiany w lokalnych obwodach neuronalnych, zmienia się pobudliwość α-motoneuronów (MN). Pobudliwość, która jest zdolnością neuronów do generowania potencjałów czynnościowych, zależy od funkcji i gęstości występowania receptorów neuroprzekaźników oraz kanałów i transporterów jonowych w błonie komórki. Jej zmiana znacząco wpływa na przewodzenie i przywracanie funkcji ruchowych. MN przechodzą w stan obniżonej pobudliwości w ostrej fazie po uszkodzeniu, podczas wstrząsu rdzeniowego, po którym rozwija się nadpobudliwość. Za główną przyczynę rozwoju spastyczności mięśni, która jest częstym powikłaniem SCI, uważa się nadpobudliwość MN w obwodzie neuronalnym odruchu na rozciąganie mięśnia. Zrozumienie podłoża molekularnego spastyczności może pomóc w planowaniu terapii uszkodzeń rdzenia.

W badaniach z wykorzystaniem modeli zwierzęcych opisano zmiany molekularne w MN związane z neuroprzekaźnictwem. Dostępne dane pochodzą z badań późnej fazy po uszkodzeniu, kiedy nadpobudliwość MN jest dobrze ugruntowana. Jednak w doświadczalnym uszkodzeniu rdzenia kręgowego u gryzoni początek spastyczności tylnych kończyn można zaobserwować już po tygodniu od urazu. Zmiany i wzór ekspresji genów kodujących białka błonowe, które odgrywają rolę w neuroprzekaźnictwie w podostrej fazie SCI w której powraca pobudliwość MN, nie są jasne. Pierwszym celem mojej pracy stało się więc wyjaśnienie kierunku i zakresu zmian poziomu transkryptów receptorów pośredniczących w neuroprzekaźnictwie pobudzającym i hamującym oraz funkcjonalnie powiązanych kanałów i transporterów błonowych w MN unerwiających mięśnie stawu skokowego dorosłych szczurów. Zmiany badałem w drugim tygodniu po całkowitym przecięciu rdzenia kręgowego (SCT). Postawiłem hipotezę, że w podostrej fazie w MN rozwijają się szybkie odpowiedzi molekularne na skutek osłabienia dopływu bodźców, związanego z utratą części połączeń nerwowych. Odpowiedzi te mogą zaburzać dynamiczną równowagę pomiędzy receptorami pobudzającymi i hamującymi oraz powiązanymi kanałami jonowymi. Ponieważ aktywność MN unerwiających mięśnie prostowniki i zginacze stawu skokowego zostaje w różnym stopniu zmieniona po SCT, zbadałem ekspresję genów oddzielnie w wyizolowanych grupach MN unerwiających prostownik (Gastrocnemius lateralis; GL; boczna głowa mięśnia brzuchatego łydki) i zginacz (Tibialis Anterior; TA; mięsień piszczelowy przedni) tego stawu.

Drugi cel mojej pracy wynikał z obserwacji, że obiecującym sposobem leczenia uszkodzeń i modulowania pobudliwości MN zmienionej po urazie jest wzbogacenie uszkodzonego rdzenia kręgowego w czynnik neurotroficzny pochodzenia mózgowego (BDNF). Nasze poprzednie badanie wykazało, że nadekspresja BDNF w segmentach położonych doogonowo od miejsca uszkodzenia poprawia zdolności lokomocyjne, czemu towarzyszy wyraźna regulacja w górę (up-regulation) poziomu transkryptów markerów glutaminianergicznych i GABAergicznych w interneuronach rdzenia, presynaptycznych względem MN. Podczas gdy badanie wykazało korzystną rolę BDNF w zwiększeniu aktywności sieci rdzeniowej, z czasem zaobserwowano niepożądane efekty behawioralne, sugerujące nadpobudliwość. Dlatego za cel moich badań obrałem scharakteryzowanie wpływu dordzeniowego podania AAV-BDNF na ekspresję wybranych genów, zbadanych w pierwszej części pracy oraz zidentyfikowanie cząsteczek o potencjale pobudzającym, które ulegają znaczącym zmianom. Wiadomo, że zwiększona sygnalizacja BDNF/TrkB może modulować pobudliwość MN poprzez zmianę ekspresji

receptorów glutaminianu typu NMDA i AMPA oraz transportera jonów chlorkowych KCC2. Ten transporter odpowiada za utrzymywanie skierowanej do wewnątrz „siły napędowej" dla jonów chlorkowych, co jest warunkiem hiperpolaryzacji i hamowania aktywności komórek za pośrednictwem GABA. Brakuje jednak bardziej kompleksowego obrazu wzorca zmian molekularnych w MN po uszkodzeniu rdzenia kręgowego i wywołaniu nadekspresji BDNF.

Przed wykonaniem SCT na poziomie segmentów piersiowych Th11/12, które prowadzi do porażenia kończyn dolnych, wstrzykiwano do odpowiednich mięśni znaczniki fluorescencyjne, umożliwiające identyfikację MN GL i TA. Po SCT podawano do odcinka lędźwiowego L1/2 obustronnie PBS (operowana grupa kontrolna SCT-PBS) lub AAV-BDNF (grupa SCT-BDNF). Szczury z wstrzykniętymi znacznikami ale bez uszkodzeń służyły jako kontrole. Po dwóch tygodniach od uszkodzenia oceniano sprawność lokomotoryczną zwierząt na ruchomej bieżni. Po sperfundowaniu zwierząt i wypreparowaniu MN GL i TA metodą mikrodysekcji laserowej, izolowano mRNA i przeprowadzano odwrotną transkrypcję do cDNA. Z użyciem ilościowej metody qPCR mierzono poziomy transkryptów wybranych receptorów neuroprzekaźników, kanałów jonowych oraz transporterów jonów chlorkowych. Przeprowadzono lokalizację immunohistochemiczną i identyfikację wybranych receptorów.

Wzbogacenie rdzenia kręgowego w BDNF znacząco poprawiło sprawność ruchową szczurów, zwiększając średni wynik oceniany w skali mBBB z 0,36 w grupie SCT-PBS do 12,19 u szczurów SCT-BDNF. Ilościowa analiza poziomu transkryptów, przeprowadzona grupie SCT-PBS po 13 dniach od uszkodzenia, wykazała wielokrotny wzrost ekspresji mRNA podjednostek AMPAR GluA1 i mGluRs oraz spadek poziomu mRNA GABAAR, GlyR, KCC2, ale też NMDAR, Nav1.6, KCa1.1 i SK2. Poziom transkryptów receptorów 5-HT_{2B}, których konstytutywną aktywność po urazie wiązaną z rozwojem spastyczności opisano w literaturze, uległ znacznemu podwyższeniu po SCT. Nadekspresja BDNF, badana w punkcie czasowym, w którym sprawność szczurów SCT-BDNF osiągnęła maksimum (13-16 dni po uszkodzeniu), spowodowała (1) tendencję do przywracania równowagi poziomów mRNA podjednostek AMPAR i NMDAR oraz (2) dalsze zmniejszenie poziomu ekspresji mRNA podjednostek GABAAR i GlyR. Wykryto również znaczny wzrost poziomu mRNA receptora 5-HT_{2A}, który wzmacnia sygnalizację pobudzającą MN, wpływając na czas i amplitudę aktywności mięśni. Ten zbiór wyników sugeruje, że wpływ BDNF na ekspresję receptorów promuje fenotyp pro-pobudzeniowy w MN, który rozwija się po SCT. Co więcej, stopień osłabienia ekspresji KCC2 po urazie, który utrzymywał się po podaniu AAV-BDNF, pozwala wnioskować o współzachodzącej akumulacji jonów chlorkowych w MN. Ten stan może odwrócić skutek lokalnego działania GABA na receptory z hamującego na pobudzający, co prowadzi do zmniejszenia hamowania neuronów.

Wyniki doświadczeń przedstawione w niniejszej dysertacji doprowadziły do zidentyfikowania: (1) spadku poziomu transkryptów kodujących białka kontrolujące hamowanie MN oraz (2) zwiększenia ekspresji receptorów 5HT kontrolujących aktywność mięśni. Analiza korelacyjna danych ujawniła głębokie zmiany we wzorcach ekspresji genów, co sugeruje, że w MN duży zestaw genów kodujących białka błonowe związane z neuroprzekaźnictwem podlega zróżnicowanej regulacji już w drugim tygodniu po uszkodzeniu. Uzyskane wyniki dostarczają wglądu w mechanizmy pobudliwości MN rozwijające się w fazie podostrej poniżej miejsca uszkodzenia. Wskazują, że w obu grupach MN dochodzi do zbliżonych zmian w kierunku stanu bardziej pobudliwego, co może zwiększać występowanie skurczów prostowników i zginaczy już we wczesnej fazie po urazie rdzenia. Pełne zrekonstruowanie wpływu BDNF na stan pobudzenia MN po urazach rdzenia kręgowego wymaga dalszych badań.

2. List of abbreviations

5-HT	5-hydroxytryptamine
5-HTRs	5-HT receptors
AADC	aromatic L-amino acid decarboxylase
AAV	adeno-associated virus
ACh	acetylcholine
AChE	acetylcholinesterase
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APs	action potentials
ARs	adrenergic receptors
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
C boutons	large cholinergic synaptic terminals apposing α-motoneurons
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
ChAT	choline acetyltransferase
Chrm2	muscarinic receptor gene
CNS	central nervous system
CPGs	central pattern generators
DA	dopamine
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ELISA	enzyme linked-immunosorbent assay
EMG	electromyography
EPSPs	excitatory postsynaptic potentials
ES	electrical stimulation
FB	fast blue retrograde tracer
GABA	γ-aminobutyric acid
$GABA_ARs$	γ-aminobutyric acid type A receptors
GAPDH	glyceraldehyde 3–phosphate dehydrogenase
GL	gastrocnemius lateralis muscle
GLT-1	glutamate transporter 1
Glu	glutamic acid; here used to abbreviate glutamate
GlyRs	glycine receptors
IF	immunofluorescence
iGluRs	ionotropic glutamate receptors
IPSPs	inhibitory postsynaptic potentials
KCC2	K-Cl co-transporter
LMD	laser microdissection
LTD	long-term depression
LTP	long-term potentiation
M2	muscarinic receptor subtype 2
mAChR	muscarinic acetylcholine receptor
mAHP	medium afterhyperpolarization
mBBB	modified Basso-Beattie-Bresnahan scale
mGluR	metabotropic glutamate receptor
MN	motoneuron
mRNA	messenger ribonucleic acid
NA	noradrenaline
nAChR	nicotinic acetylcholine receptor
NMDAR	N-methyl-D-aspartate receptor
PBS	phosphate-buffered saline

PBST	phosphate-buffered saline + 0.1% Triton
PCR	polymerase chain reaction
PICs	persistent inward currents
RT	room temperature
RT-qPCR	real-time quantitative PCR
SCI	spinal cord injury
SCT	spinal cord transection*
SDS	sodium dodecyl sulfate
SD	standard deviation
SEM	standard error of the mean
Sol	soleus muscle
SP	spinal rats
TA	tibialis anterior muscle
Th	thoracic
TH	tyrosine hydroxylase
TrkB	tropomyosin-related kinase B receptor
VAChT	vesicular acetylcholine transporter
VGLUT1	vesicular glutamate transporter 1
VGLUT2	vesicular glutamate transporter 2

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*here used to abbreviate “complete spinal cord transection” term

3. Introduction

3.1. Spinal cord injury

According to WHO International Perspectives on spinal cord injury, between 250 000 and 500 000 people suffer a spinal cord injury (SCI) every year around the world. Among the SCI cases, up to 90% of SCI are traumatic, caused by a sudden, traumatic impact on the spine that dislocates, fractures, crushes or compresses one or more vertebrae (Ahuja et al., 2017; Alizadeh et al., 2019). As shown in **Figure 3.1**, traumatic SCI primarily results from motor vehicle accidents (38%), slips or falls (30.5%), violence (13.5%), sports related injuries (9%) and medical or surgical injuries (5%) (National Spinal Cord Injury Statistical Center, 2016). Pathologically, traumatic SCI causes a severe temporary or permanent neuronal dysfunction including sensory loss and motor paralysis below the level of the injury, dysfunctions of autonomic nervous system, chronic pain and spasticity (Hubli et al., 2011; Binder, 2013). People with spinal cord injury are two to five times more likely to die prematurely.

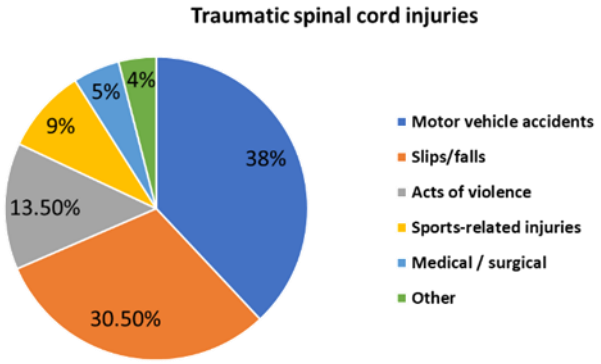

Figure 3.1. Causes of Traumatic Spinal Cord Injury. Image has been modified from (National Spinal Cord Injury Statistical Center, 2016) (http://dx.doi.org/10.1080/10790268.2016.1210925).

Based on the pathophysiological outcomes, traumatic SCI can be divided into primary and secondary injuries. The primary injury is caused by the initial mechanical fracture and dislocation of vertebrae, and is followed by the secondary injury inducing continuous cellular and molecular damage to the spinal cord, which includes vascular damage, cavitation, ionic imbalance, excitotoxicity, calcium influx, inflammation, and may lead to cell death (Ahuja et al., 2017; Alizadeh et al., 2019). Necrosis is the prominent cell death mechanism in the cavity of the lesion and is almost immediately initiated following injury. Apoptosis, the most extensively studied mechanism of cell death after SCI, is a time-limited mechanism which expands from the epicenter of the injury site to distal regions as far as 8 mm

along the spinal cord in rats (Hassannejad et al., 2018) (**Figure 3.2**). It initiates at about one hour postinjury up to two weeks, with apoptotic cell deaths of neurons and oligodendroglial cells reported in decreasing numbers as late as 3 weeks after SCI in animals (Hassannejad et al., 2018) and, similarly, in humans (Emery et al., 1998).

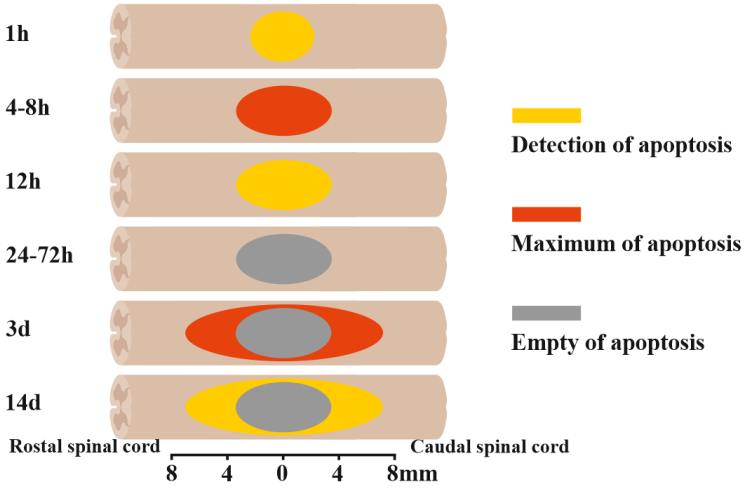

Figure 3.2. Spatio-temporal course of apoptosis after SCI in rats. Apoptosis expands from the epicenter of the injury site to distal regions as far as 8 mm along spinal cord initiating about one hour postinjury and occurring up to two weeks. There are two peaks of apoptosis during this process: the first one is at 4-8 hours postinjury at the lesion epicenter; the second one is at 3 days postinjury about 7-8 mm caudal and rostral to the lesion epicenter (Hassannejad et al., 2018). Schematic is modified from Hassannejad et al., 2018.

To study SCI, a series of experimental models using distinct species have been developed (reviewed in (Ahuja et al., 2017; Sharif-Alhoseini et al., 2017; Minakov et al., 2018)). Depending on the severity of SCI, they can be clearly classified as models of complete and incomplete injury (Kirshblum et al., 2011) (**Figure 3.3**). Obviously, the major difference between the two types of SCI is the degree of the disconnection of spinal cord neuronal network in segments located below the injury site from the supraspinal centers. In physiological conditions, the cerebral command systems, consisting of forebrain motor cortex, basal ganglia, midbrain and brainstem, control the muscle contraction and body movements through the descending tracts to the spinal cord and receive the sensory feedback through ascending tracts from the spinal cord to the brain (Jordan and Sławińska, 2014; Grillner and El Manira, 2020). After incomplete SCI, only part of the descending and ascending tracts in the region of injury is destroyed. Therefore, some neuronal signals can be conveyed by the preserved pathways to the areas caudal to the injury site enabling impaired regulation of the sensory and motor processing, and providing

the possibility of faster and more remarkable degree of recovery compared to complete SCI (Raineteau and Schwab, 2001). In contrast, complete SCI causes a total loss of sensation and muscle function

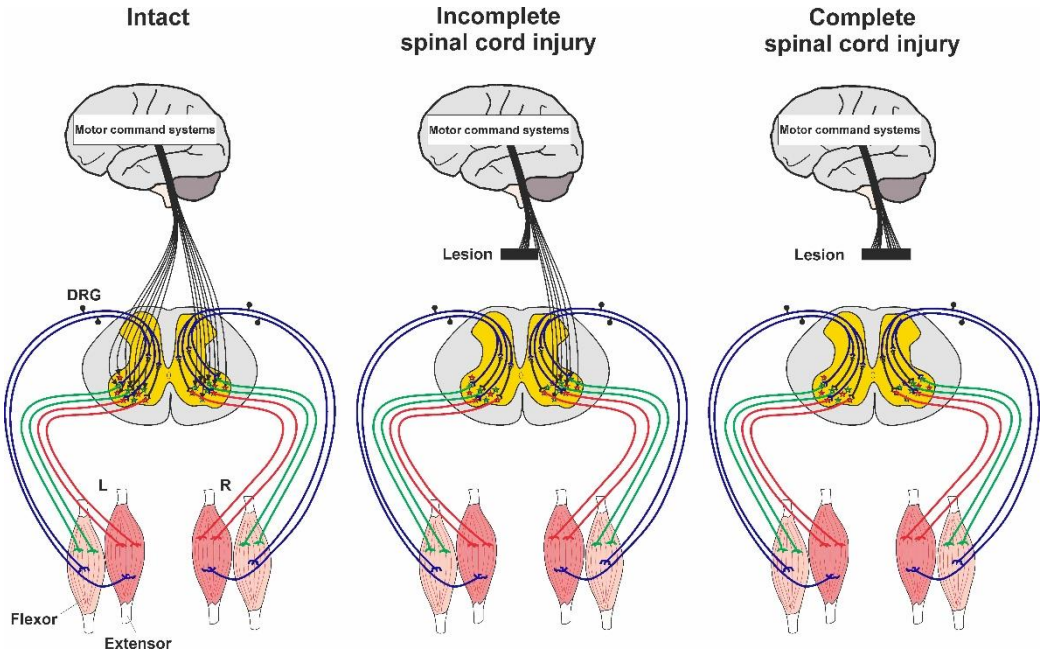

Figure 3.3. Incomplete and complete SCI. Under physiological conditions, the supraspinal systems (motor cortex, basal ganglia, midbrain and brain stem) control body movements including locomotion through the descending tracts to the spinal cord, and ascending tracts from the spinal cord to the brain. Intrinsic neural circuits of the spinal cord can function independently without influences from higher motor centers and allow for the alternating and rhythmic locomotor movements. Activated motoneurons (MNs) (green: flexor MNs; red: extensor MNs) are responsible for contraction of the effector muscles in the periphery; they transmit signals to neuromuscular junctions where stimulation of respective, functionally different muscles to contract occurs. Sensory signals from the muscle are transmitted to the spinal cord by sensory nerve fibers (in violet). The signals transmitted to MNs reach them directly or through interneurons. MNs execute locomotion by contracting flexor (in light red) and extensor (in dark red) muscles. Incomplete SCI destroys part of these tracts; some signals are still able to reach areas caudal to the injury. Complete SCI causes a total loss of sensation and muscle function because no transmission from the higher locomotor centers occurs. Despite that, the intraspinal neural circuits remain intact and maintain some potential to activate MNs.

below the lesion site due to the aforementioned loss of signaling from descending tracts, including loss of a feedback from ascending tracts (Waters et al., 1991; Dietz et al., 2009). Based on the mechanism leading to primary injury, transection models (imitating laceration), contusion and compression (with their combinations: impact plus persistent compression; impact alone with transient compression) and distraction models are in use (Cheriyan et al., 2014; Kjell and Olson, 2016; Ahuja et al., 2017; Sharif-Alhoseini et al., 2017; Alizadeh et al., 2019). Although the compression and contusion models are

considered to be the most relevant to human SCI and suitable for the study of pathophysiological changes in the injured spinal cord, transection models have been particularly used to investigate, whether and to which extent neuronal regeneration and compensation such as axonal sprouting can occur, and whether its support by bioengineered scaffolds or bioactive molecules can elicit functional recovery (Gao et al., 2013; Cheriyan et al., 2014; Medalha et al., 2014; Ziemlińska et al., 2014; Han et al., 2015; Lukovic et al., 2015; Alizadeh et al., 2019; Płatek et al., 2020). Transection models can be further divided into complete and partial transection, including dorsal or lateral hemisection and unilateral transection (Cheriyan et al., 2014). Among these models, complete transection (SCT) is the only type of SCI with separation of connections and loss of communication with the motor centers above the lesion site. Therefore, that model is suitable for the investigation of a potential and properties of the intrinsic spinal neural circuit in locomotion recovery in conditions excluding any direct influence from higher motor centers (Cheriyan et al., 2014; Alizadeh et al., 2019).

3.2. Alterations of Motoneuron Excitability by Spinal Cord Injury

3.2.1. Functions of MNs during motor activity

Motor activity such as walking and swimming is a recurrent and well patterned rhythmic behavior, which requires the reciprocal actions of the antagonist groups of flexor and extensor muscles at different limb joints (Sherrington, 1906; Sherrington, 1908; Kiehn and Dougherty, 2013; Britz et al., 2015). The rhythm and pattern of locomotion is generated by the Central Pattern Generators (CPGs), consisting of spinal interneurons located within the cervical and lumbar regions of the spinal cord (Cazalets et al., 1995; Marcoux and Rossignol, 2000; Ballion et al., 2001; Barthélemy et al., 2007; Kiehn and Dougherty, 2013; Ramirez-Jarquin and Tapia, 2018), and acting on motoneuronal populations that innervate axial and limb muscles, according to specific gait pattern requirements. These CPG circuits are under direct control of supraspinal centers (from the cortex to brainstem) that are involved in the decision making and planning for movement initiation, motivational aspect of locomotor behavior, direction change and speed of the underlying motor output programs (Armstrong, 1986; Rossignol, 1996; Jordan et al., 2008; Heckman and Enoka, 2012).

Spinal cord motoneurons (MNs) are classified as lower MNs; they receive inputs from supraspinal locomotor centers, spinal interneurons and peripheral sensory pathways (**Figure 3.3**). During locomotion, the extensor and flexor MNs, whose perikarya are located in the ventral horn of the spinal

cord, are activated in a precise rhythmic alternating pattern coordinated by signals from different groups of excitatory and inhibitory interneurons in the CPGs; these MNs transmit the signals along their axons to the muscle, acting by releasing the neurotransmitter acetylcholine at the neuromuscular junctions, where the proper rhythm and alternation of muscle contraction is generated (**Figure 3.4**) (Britz et al., 2015; Kiehn, 2016; Arber, 2017; Ramirez-Jarquin and Tapia, 2018). MNs not only serve as final motor executor after the convergence of the signals from CPG networks, but also are able to shape the final motor output with its intrinsic and conditional membrane properties, including excitability (Kiehn et al., 2000). MNs act as the key players that integrate signals from the brain and peripheral sensory pathways and shape the output to the muscles, organs and glands which execute motor function.

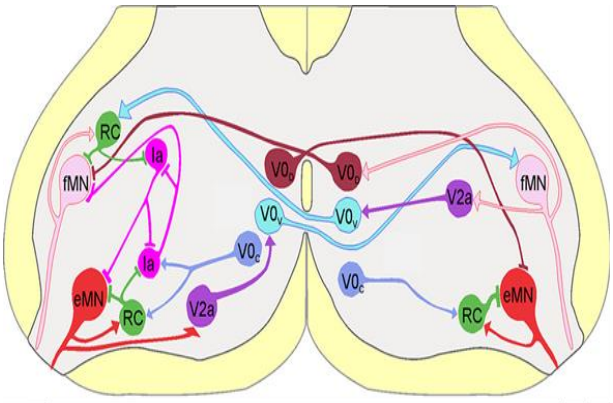

Figure 3.4. Schematic representation of the spinal neuronal network in which flexor (fMN) and extensor (eMN) MNs converge the signals from several types of interneurons (INs) in CPGs. These INs include excitatory V2a, $V0_V$ and $V0_C$, inhibitory $V0_D$, Renshaw cell (RC; inhibitory) and Ia inhibitory neurons. Dorsal horn upper laminae are cut off for simplicity. The schematic is modified from (Ramirez-Jarquin and Tapia, 2018).

3.2.2 Modulation of the excitability of MNs after SCI

As a result of SCT, impairment or loss of motor ability below the injury site occurs (**Figure 3.5**) due to the pathological perturbations in spinal motor circuits (Hubli et al., 2011; Binder, 2013). Among factors which contribute to impairment of motor network, a key contributor is an altered excitability. That intrinsic property of each neuron is an ability to generate action potentials (APs) in response to the stimulus, that determines appropriate function. APs generated by MNs are responsible for the contraction and relaxation of limb muscles that produce movements and complex behaviors. There are data indicating that SCI affects the excitability of MNs, which in turn contributes to development of pathological symptoms. By using the Hoffman reflex (H-reflex), which is an electrical analogue of spinal stretch reflex allowing to estimate alpha-MN excitability, MN excitability was shown to be changed.

Figure 3.5. The rat with a complete spinal cord transection at low thoracic level displays a total loss of motor ability of hindlimbs. **A.** The animal is assisted by the experimenter, who secures proper position of the trunk on the treadmill platform, by holding the animal's body. **B.** Unassisted animal moves in an open field. In both cases the hindlimbs are dragged on the ground with the feet on their dorsum. Photos taken by Benjun Ji; graphic processing done by Anna Głowacka.

What is a sequence of changes in electrical properties of MNs postlesion? Immediately after SCI, depression of spinal reflexes and hyperexcitability of MNs was described caudal to the injury site due to a sudden loss of supraspinal excitatory inputs; it contributes to a clinically defined spinal shock (Leis et al., 1996; Hiersemenzel et al., 2000; Singhal and Aggarwal, 2016). Subsequently, spontaneous recovery of locomotion can occur owing to the restoration of the excitability of MNs and spinal sensorimotor circuits (Onifer et al., 2011; D'Amico et al., 2014a). However, at the chronic phase of SCI, the progressive development of exaggerated reflexes (hyperreflexia) and hyperexcitability of MNs located below the injury site at weeks or months after injury tends to result in maladaptive changes, such as spasticity. Spasticity is a common SCI complication, which exacerbates locomotion deficits and worsens the life quality of individuals (Adams and Hicks, 2005; Brashear, 2005; Edgerton and Roy, 2010). Recent studies have shown that reduction of chronic, SCI-induced hyperexcitability of MNs, not only alleviates spasticity but also improves the locomotion recovery below the level of injury (Chen et al., 2018; Bilchak et al., 2021; Gong et al., 2021). Therefore, manipulating excitability of MNs to achieve its functionally relevant level is a promising approach to treat SCI and improve motor functions.

In normal subjects, the excitatory [mainly glutamatergic], inhibitory [GABAergic and glycinergic] and modulatory (serotoninergic [5-HT], noradrenergic [NA], dopaminergic [DA] and cholinergic [ACh]) signaling to MNs were shown to contribute to MN excitability, which is determined by a pre- and postsynaptic abundance, distribution and functional properties of the neurotransmitter receptors, ion channels and transporters (reviewed by (Rekling et al., 2000)). In subsequent years several studies

demonstrated that SCI results in specific changes in density of synaptic inputs to MNs (Kitzman, 2006; Ichiyama et al., 2008; Macias et al., 2009; Skup et al., 2012), and in gene expression, protein translation and biophysical properties of some neurotransmitter receptors in MNs (Li et al., 2004; Boulenguez et al., 2010; Ryge et al., 2010; Garcia et al., 2018); these alter the threshold and the probability of generation of APs, and subsequently change the excitability of MNs. Based on these data, one of the research goals of my study was to characterize motoneuronal expression of the neurotransmitter receptor and ion channel molecules, which contribute to excitability of lumbar MNs, in the early postlesion phase when excitability recovers.

Although severe SCI eliminates majority or all the descending supraspinal inputs to MNs located below (in the quadrupeds caudal to) the injury site, MNs remain under control of peripheral sensory inputs and intersegmental interneurons within the intact spinal sensorimotor circuitry (**Figure 3.6**). The principal inputs from these circuits to MNs consist of excitatory, inhibitory and cholinergic modulatory afferents (**Figure 3.6**).

The signaling to MNs is mediated by excitatory glutamatergic ionotropic receptors (AMPAR and NMDAR) and inhibitory GABAergic and glycinergic ionotropic receptors (GABAR and GlyR), generating excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs), respectively (Hendry, 2016). Important to their proper function is the aforementioned 5-HT, NA, and DA modulatory signaling, which is cut-off after severe SCI, and cholinergic signaling, which is largely maintained (Skup et al., 2012). These inputs contribute to MN activation by amplifying the excitatory and inhibitory effects on them, and by modulating ion channels which conduct persistent inward currents (PICs), which are particularly strong in spinal MNs (Heckman et al., 2005; Heckman et al., 2009). Similar to the descending 5-HT system, DA system may serve as a potential target to promote recovery of locomotor function following SCI (Sharples et al., 2014). These neuromodulators contribute to network flexibility. Altered density and strength of synaptic inputs on MNs after SCI result in changes of threshold potential, resting membrane potential and afterhyperpolarization which determine the excitability of MNs (Lamas et al., 2009; Sanchez et al., 2011; Power et al., 2012). At the molecular level, the corresponding adaptive or maladaptive changes of postsynaptic receptors, ion channels and transporters in MN plasmalemma contribute to abnormal excitability of MNs (Rekling et al., 2000; Li et al., 2004; Boulenguez et al., 2010; Ryge et al., 2010; Garcia et al., 2018).

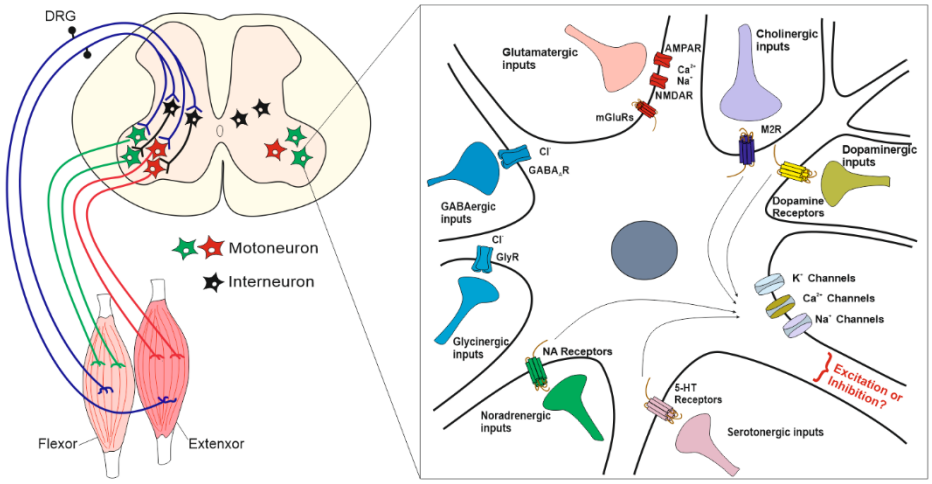

Figure 3.6. Stimuli from the periphery are transmitted to spinal cord MNs by sensory nerve fibers, directly or through interneurons. The left panel shows that intrinsic spinal sensorimotor circuitry remains morphologically preserved after SCI. In these conditions MNs maintain the potential to transmit signals contracting different groups muscles (e.g., the flexor and extensor muscles). The right schematic shows that the MNs are equipped with synaptic neurotransmitter receptors which consist of three categories: excitatory, inhibitory and modulatory.

In the following chapters, I describe the current knowledge on the molecular basis and contribution of excitatory, inhibitory and modulatory signaling to MNs properties, which underly changes of their excitability after SCI.

3.2.2.1. Excitatory glutamatergic signaling

In the spinal cord, glutamate (Glu) neurotransmission is critical for setting the speed and rhythm of locomotor movements, which is controlled by neurons located in the midbrain and hindbrain nuclei (Hagglund et al., 2010; Talpalar and Kiehn, 2010; Ryczko et al., 2017; Caggiano et al., 2018). MNs receive excitatory inputs from Ia primary afferents (M-type boutons) and from afferents of supraspinal origin, either directly or via interneurons (S-type boutons) (Carp and Wolpaw, 2010). Intrinsic properties of MNs determine how these inputs are transformed into a sequence of action potentials that elicit muscle contraction. As already mentioned, Glu neurotransmission is initiated by activation of ionotropic glutamate receptors (iGluRs) and modulated by metabotropic glutamate receptors (mGluRs) after Glu binding; the process is dependent on Glu concentration in the synapse and in peri-synaptic regions, which is controlled by neurons and astrocytes (Reiner and Levitz, 2018).

So far there is evidence that the density of glutamatergic inputs on lumbar MNs after thoracic SCT is decreased, which in rats transected as neonates concerns mostly S-type boutons (Ichiyama et al., 2011),

while in rats transected as adults includes also M-type boutons (Kitzman, 2006; Ichiyama et al., 2008; Macias et al., 2009; Skup et al., 2012). A postsynaptic response to spinal injury includes transcriptional regulation of AMPA, NMDA and mGlu receptor subtypes as well as Glu transporters expression, which has been described so far for other than lumbar MNs (Grossman et al., 1999, 2000; Ryge et al., 2010; Wienecke et al., 2010; Kim et al., 2011; Mantilla et al., 2012; Gransee et al., 2017). The net outcome of these effects is an abnormal excitation state of MNs (Cregg et al., 2017; Mantilla et al., 2017). Although the effects of spinal injury on MNs and their inputs have been extensively studied, the view of identified alterations remains inconsistent.

After SCT a development of neuronal dysfunction below the lesion site is progressive. While the peripheral input to spinal cord remains, it is affected and reorganized. This and other aforementioned types of excitatory inputs to lumbar MNs, which use different vesicular glutamatergic transporters: VGLUT1 (M-type boutons) and VGLUT2 (S-type boutons), decrease significantly also after less severe injuries. A loss of Glu boutons was reported at one day after a thoracic compression injury in neonatal mice, recovering quickly by 6 days postinjury (Chawla et al., 2017), and in the subacute phase (1 week), being restored to control level late postlesion (9 weeks) in adult rats with a staggered thoracic hemisection (Beauparlant et al., 2013). In contrast, after SCT of adult rats or mice, the number of VGLUT1- and VGLUT2-positive synaptic boutons which abut lumbar MNs decreases markedly in the subacute phase (Grycz et al., 2019) and remains decreased between 11 weeks and 3 months postinjury (Khalki et al., 2018; Yokota et al., 2019). These data reflect different potential for recovery, dependent on the severity of spinal cord damage.

Of note is that, although the density or number of Glu boutons is decreased after SCT, their efficiency may increase. That finds explanation in the increased proportion of primary sensory Ia afferent terminals (VGLUT1-positive, M-type) devoid of GABAergic P boutons responsible for presynaptic inhibition on GL and TA MNs, indicating that a decrease of Ia afferent inhibition occurs, which has been shown to contribute to enhance spinal reflexes and the development of spasticity (Calancie et al., 1993; Khalki et al., 2018; Caron et al., 2020).

In target neurons, NMDA and non-NMDA (AMPA and kainate) receptor subtypes mediating Glu neurotransmission respond differentially to the lesion (Hartmann et al., 2004; Anggono and Huganir, 2012).

3.2.2.1.1. AMPAR and NMDAR Properties and Function

Glutamatergic receptors are primarily expressed on postsynaptic membranes, where they modulate synaptic transmission and plasticity (Choi, 1994b; Hollmann and Heinemann, 1994; Petralia et al., 1994; Dingledine et al., 1999). As mentioned earlier, these receptors consist of two main families: ionotropic and metabotropic receptors. For the purpose of this thesis, I describe briefly the function of ionotropic receptors (iGluRs) only.

IGluRs are ligand-gated ion channels, allowing the Na^+ and K^+, and Ca^{2+} in and out the neurons after Glu binding. Changes in their activation and abundance contribute to basal excitatory synaptic transmission and, importantly, several forms of synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD) (Tocco et al., 1992; Maren et al., 1993; Asztely and Gustafsson, 1996). AMPAR and NMDAR are two most important members of iGluR family.

AMPAR are mainly tetramers of four distinct subunits: GluA1, GluA2, GluA3, and GluA4 (**Figure 3.7 A,B**) (Keinanen et al., 1990; Hollmann and Heinemann, 1994), mediating fast excitatory neurotransmission and synaptic plasticity in the brain and spinal cord [(Hartmann et al., 2004), for reviews, see (Collingridge et al., 2004; Anggono and Huganir, 2012)]. A large portion of AMPARs in the CNS comprises of heteromers containing GluA1 and GluA2 subunits (Wenthold et al., 1996; Sans et al., 2003; Lu et al., 2009). One of the most important features of AMPAR is controllable Ca^{2+}-permeability which depends on whether the GluA2 subunit is present within the tetramer. A genetic manipulation at the transcript level, RNA editing, alters a codon encoding glutamine (Q) to a codon encoding arginine (R) in the GluR2 mRNA (Sommer et al., 1991; Puchalski et al., 1994). The additional positive charge of arginine (R) residue in the pore of AMPAR tetramer prevents the influx of Ca^{2+} into the cells [(Burnashev et al., 1992), see review in (Jonas and Burnashev, 1995; Isaac et al., 2007)]. Therefore, Q-R edited GluA2 renders the GluA2-containing AMPAR impermeable to Ca^+ (Sommer et al., 1991; Puchalski et al., 1994; Greger et al., 2003). Consequently, the differential subunit composition between GluA2-containing AMPAR, and GluA2-lacking AMPAR at synapses, confer distinct biophysical and molecular properties to AMPARs, and mediate the dynamics of this fast-excitatory signaling (Gan et al., 2015; Compans et al., 2016). A change in AMPAR composition is considered crucial in diseases, such as amyotrophic lateral sclerosis (ALS), postulated to be caused by overactivation of AMPAR (Vandenberghe et al., 2000; Liu and Zukin, 2007; Buckingham et al., 2008;

Wright and Vissel, 2012). Therefore, in my study, I decided to search for changes in expression level and abundance of GluA1 and GluA2 subunits in MNs after SCT and BDNF treatment.

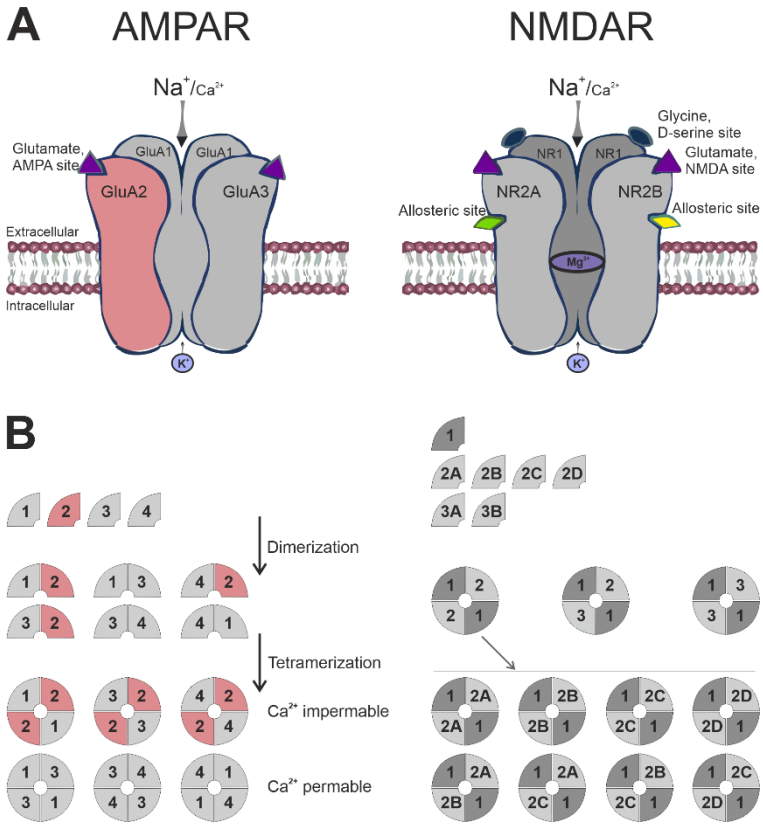

Figure 3.7. Subunit compositions of AMPAR and NMDAR. A: AMPAR and NMDAR structure in plasma membrane. B. Possible combination of subunits and the resulting changes in the receptor properties. Schematics adapted from (Greger et al., 2017) (AMPAR) and (Pham and Gardier, 2019) (NMDAR).

NMDARs are also tetrameric, consisting of three subunit families: GluN1, GluN2 and GluN3 (**Figure 3.7**) (Moriyoshi et al., 1991; Ikeda et al., 1992; Monyer et al., 1992; Ishii et al., 1993; Ciabarra et al., 1995; Mori and Mishina, 1995; Laube et al., 1998). GluN1 subunit is obligatory in the NMDAR tetramers to regulate the trafficking of GluN2 or GluN3 subunits to assemble functional NMDAR complexes (Hollmann and Heinemann, 1994; Garcia-Gallo et al., 2001; Fukaya et al., 2003). GluN2 subunits are encoded by four different genes GluN2A, GluN2B, GluN2C and GluN2D (Ikeda et al., 1992; Monyer et al., 1992), in which GluN2A and GluN2B have been the most extensively studied and believed to play important roles in synaptic plasticity; different GluN2A-D subunits endow NMDAR with distinct electrophysiological and pharmacological properties (Traynelis et al., 2010; Paoletti, 2011; Wyllie et al., 2013). The switched expression profile of GluN2A/ GluN2B during development modifies synaptic strength and controls the LTD/LTP threshold by controlling Ca^{2+} entry and intracellular

signaling cascades [reviewed in (Yashiro and Philpot, 2008)]. Since great majority of functional NMDARs in the CNS are GluN1/ GluN2 assemblies (Traynelis et al., 2010; Hansen et al., 2014), our research focused on the regulation of GluN1 and GluN2 subunit expression in MNs.

3.2.2.1.2. The roles of AMPAR and NMDAR in SCI

Altered function of the two kinds of receptors was reported to be involved in many neurological disorders and cell death (Van Damme et al., 2003; Liu and Zukin, 2007; Bezprozvanny and Mattson, 2008; Mota et al., 2014; Zhang et al., 2016b). In the spinal cord, the normal expression pattern of AMPAR and NMDAR has been evaluated in the number of studies (Furuyama et al., 1993; Tolle et al., 1993; Jakowec et al., 1995b; Jakowec et al., 1995a; Petralia et al., 1997; Rigby et al., 2002; Polgar et al., 2008). The well documented roles of AMPAR and NMDAR in spinal LTP and LTD concern their contribution to pain transmission and chronic pain in the dorsal horn neurons [reviewed in (Larsson and Broman, 2011; Zhuo, 2017)]. However, how the AMPAR and NMDAR are regulated in lumbar MNs, needs to be explored more.

In incomplete SCI models, including spinal cord contusion (Grossman et al., 1999, 2000; Brown et al., 2004), in MN disorders (Mennini et al., 2002; Bigini et al., 2006) and after peripheral nerve injury (Virgo et al., 2000; Doolen et al., 2012; Chen et al., 2013), AMPAR and NMDAR expression patterns are altered. In the early, acute phase after SCI, at the injury site, where extracellular Glu is increased dramatically albeit transiently (Panter et al., 1990; Liu et al., 1991; Farooque et al., 1996) the level of expression and activation time of NMDAR and AMPAR subtypes change. These events result in neuronal injury through an excitotoxic cascade which is initiated by excessive Na^+ and Ca^{2+} influx through AMPAR and NMDAR (Choi, 1994b, a; Liu et al., 1997; Grossman et al., 1999). Using spinal cord microdialysis in the rat, Corona and Tapia revealed that activation of GluA2-lacking, Ca^{2+}-permeable AMPAR, and not the accumulation of extracellular Glu in the lumbar segments *per se* is associated with permanent hindlimb paralysis and a remarkable loss of MNs (Corona and Tapia, 2004, 2007). Accordingly, administration of antagonists of NMDAR and AMPAR at the injury site reduced neuronal damage and loss (Liu et al., 1997; Li and Tator, 2000; Corona and Tapia, 2007, 2008). Thus overactivation of AMPAR and NMDAR largely determines MN vulnerability in the spinal cord at the injury site and raises the question on the degree and time of their activation in more distant segments of the spinal cord after injury.

Using partial SCI models, it was shown that postsynaptic localization of GluA2-lacking, Ca^{2+} permeable AMPAR on MNs is increased (Ferguson et al., 2008). Furthermore, Huie and coworkers revealed changes of composition and phosphorylation state of GluA1 and GluA2 subunits on MNs located below site of SCT and showed their relation to the maladaptive plasticity (Huie et al., 2015). Blocking the Ca^{2+} permeable AMPARs restored adaptive motor responses in a sensorimotor spinal training task. These data and results demonstrating that AMPAR activity impacts motor training, including instrumental learning, suggest potential for dynamic modulation of AMPAR composition and function in spinal cord motor systems (Joynes et al., 2004; Hoy et al., 2013). Changes in NMDAR expression were also associated with functional recovery after spinal cord injury (Joynes et al., 2004; Ferguson et al., 2012; Huie et al., 2015). In a contusive model of thoracic SCI in rats, gene expression of NMDAR subunits was upregulated in distant lumbar MNs at 24 hours and 1 month postinjury (the latter correlating with the recovery of hindlimb motor function) (Grossman et al., 2000), while AMPAR GluA2 subunit transcript level was downregulated (Grossman et al., 1999). Similar effects were also observed in cervical (C), phrenic (PMNs) and sacral MNs at 2-3 weeks postinjury: hemisection at C2 led to upregulation of NMDAR transcript level but a decrease of AMPAR level in PMNs below the lesion site, at C3-5 (Mantilla et al., 2012; Gransee et al., 2017). Importantly, blockade of NMDAR was shown to reduce diaphragm EMG amplitude and suppress spontaneous PMN bursting (Cregg et al., 2017; Mantilla et al., 2017), indicating that NMDAR-mediated neurotransmission was crucial for rhythmic diaphragm activity (Cregg et al., 2017; Mantilla et al., 2017). In rats with SCT at the sacral S2 level, MNs innervating tail muscles located in S3-S4 also showed increased expression of genes encoding NMDAR subunits at 3 weeks and 2 months postinjury; that response was interpreted as contributor to the hyperexcitability of MNs and tail spasticity (Ryge et al., 2010; Wienecke et al., 2010). So far, only one study on NMDAR and AMPAR subunit expression in lumbar MNs after thoracic SCT, done in mice, showed no difference from control levels when measured at 3 months (Yokota et al., 2019). Thus most of the data available speak for a change of MN phenotype after SCI towards enrichment in NMDAR and impoverishment in AMPAR, with not clear demonstration of the changes in balance between Ca^{2+} permeable and impermeable receptor forms, at certain stage after SCI. The shift in receptor abundance from AMPAR to NMDAR was suggested to increase MN excitability through prolonging excitatory postsynaptic currents and slowing receptor desensitization (Rekling et al., 2000; Mantilla et al., 2012). With this respect, the studies aimed to characterize abundance and responses of AMPARs and

NMDARs to the SCT in functionally different MN pools, which control extensor and flexor muscles are especially important to understand the postlesion motor dysfunction. Such data are lacking.

The last major aspect of Glu neurotransmission is its availability in the synaptic cleft. In the acute phase after SCI, at the injury site and in its proximity, extracellular Glu concentration increases dramatically (although transiently), being sufficient to evoke excitotoxicity. It results in primary and secondary neuronal damage and death through a cascade of molecular events initiated by excessive Ca^{2+} and Na^{+} influx through prolonged action of Glu receptors (Panter et al., 1990; Liu et al., 1991; Choi, 1992, 1994b; Farooque et al., 1996; Liu et al., 1997; Grossman et al., 1999). That significant elevation of extracellular Glu is detected during the first 30 mins postinjury and returns to basal levels after 1-2 hours (Panter et al., 1990; Liu et al., 1991). Glu concentration is the highest at the injury zone and declines steeply with a distance from the injury epicenter, becoming undetectable several millimeters away (McAdoo et al., 1999). The "safety valve" is a Glu transporter 1 (GLT-1), which is responsible for the vast majority of Glu uptake, preventing excitotoxicity in CNS (Rimmele and Rosenberg, 2016). In the spinal cord, GLT1 deletion causes MNs loss and motor deficits, indicating that GLT1 is a crucial contributor to the mechanisms removing Glu from the synaptic cleft and supporting survival of spinal MNs postlesion (Sugiyama and Tanaka, 2018). In the rat, after contusive SCI, GLT-1 was reported to be increased in the acute phase (within 24 hours) at the lesion epicenter and several millimeters rostral and caudal to it (Vera-Portocarrero et al., 2002; Kim et al., 2011). Later on, the expression of GLT-1 significantly decreases below control level in the subacute phase (7 days and 14 days) and intermediate phase (4, 6 and 8 weeks), as shown in a crush SCI, leading to reduced Glu removal and to excitotoxic neuronal damage (Olsen et al., 2010; Kim et al., 2011; Li et al., 2014b). Consistently, transplantation of glial progenitors that overexpress GLT1 into the injury site preserves diaphragm function after cervical contusion SCI ((Li et al., 2015), but see (Li et al., 2014b)).

At the lumbar region of my interest, after low thoracic contusion SCI, GLT-1 protein level was reported to slightly decrease for the first 3 days and then return to control levels up to 8 weeks (Kim et al., 2011), indicating that GLT1 is less affected in regions distant from injury epicenter,. Earlier study and that by Kim and coworkers showed that other Glu transporters, glial GLAST and neuronal EAAC1, are also increased in the acute phase (within 24 hours) at the injury epicenter but subsequently decreased below control level up to 8 weeks (Vera-Portocarrero et al., 2002; Kim et al., 2011). Interestingly, in

contrast to GLT1, GLAST was significantly increased far from the lesion site, in L4-5 region, from the first days to 8 weeks after SCI (Kim et al., 2011). These data indicate that SCI temporally and spatially affects the expression of the Glu transporters, which modulate Glu level in the synaptic cleft and further Glu neurotransmission.

3.2.2.2. Inhibitory glycinergic and GABAergic signaling

In the spinal cord, inhibitory neurotransmission is mediated by postsynaptic glycinergic receptors (GlyRs) and GABA receptors (GABARs), when activated by binding glycine or GABA, respectively, released from presynaptic terminal. On the lumbar MNs of adult intact rats, glycinergic axon terminals are four times more frequent than GABAergic axon terminals, suggesting that glycinergic system is the major contributor to MNs inhibition in adulthood (Khalki et al., 2018; Sadlaoud et al., 2020b; Bras and Liabeuf, 2021; Mazzone et al., 2021). Despite that, both glycinergic and GABAergic inhibitory mechanisms were indicated as regulatory for the excitability of MNs and spinal network.

Ionotropic GlyR and $GABA_AR$ are ligand-gated chloride (Cl^-) channels whose activation leads to the influx of chloride ions, membrane hyperpolarization and decreased neuronal firing. The ability of MNs to transmit inhibitory signals is impaired after SCI. The group of Kjehn, exploring tail spasticity model after SCT, reported that at 3 weeks and 2 months after SCT in adult rats, transcript levels of GlyR subunits were changed, with Glra1 upregulated, but Glra2 downregulated; the study from Vinay group showed that the protein abundance of GlyR and $GABA_AR$ at the membranes of lumbar MNs was changing reversibly in time; GlyR was decreased at 3 weeks, restored to control level at 8 weeks, but decreased below control level when analyzed at 16 weeks after SCT (Ryge et al., 2010; Wienecke et al., 2010; Sadlaoud et al., 2020b).

Importantly, the degree of spasticity, determined by rate-dependent depression (RDD) of the H reflex, was shown to be correlated with the time course of changes in GlyR protein expression in lumbar MNs following SCT, indicating that spasticity development was significantly influenced by glycinergic signaling in MNs (Sadlaoud et al., 2020b). In line with these observations, administration of glycine aimed to activate GlyRs results in reduced spasticity; pharmacological blockade of GlyRs enhanced spasticity in animals with SCI (Simpson et al., 1995).

In addition to the abundance of GlyR and $GABA_AR$/$GABA_BR$ on MNs, another crucial factor

which determines a processing of inhibition is intraneuronal Cl^- homeostasis. That is primarily regulated by K^+-Cl^- co-transporter KCC2 and Na^+-K^+-Cl^- cotransporter NKCC1 (Côté, 2020). In mature neurons these transporters, the major Cl^- extruder (KCC2) and importer (NKCC1), significantly affect the polarity and efficacy of the $GABA_AR$ and GlyR- mediated synaptic transmission (Chamma et al., 2012; Côté, 2020). In normal adult animals, KCC2 and NKKC1 maintain a low-level of intracellular Cl^-, and in these conditions the activated $GABA_AR$ and GlyR allow influx of Cl^-, generating a hyperpolarization effect. The group of Vinay and ours showed that spinal cord transection at thoracic segments in adult rats downregulates the KCC2 protein expression in plasma membranes of lumbar MNs (Boulenguez et al., 2010; Ziemlińska et al., 2014; Khalki et al., 2018). The data on response of NKCC1 are inconsistent; although increased NKCC1 protein level, identified by western blot, was observed in the lumbar enlargement after SCT ((Cote et al., 2014; Mekhael et al., 2019), but see (Boulenguez et al., 2010)), immunostaining for NKCC1 in the ventral horn failed to show any changes (Cote et al., 2014). Further studies are needed to further characterize the response and identify cell populations which display changes of NKCC1 after SCI. What is relatively clear at a current stage of the studies, is that a decrease of KCC2 in MNs leads to accumulation of high concentrations of Cl^- in MNs, evoking in consequence a depolarizing effect instead of inhibitory response after $GABA_AR$ and GlyR activation, to contribute to hyperexcitability of MNs and spasticity (Boulenguez et al., 2010; Cote et al., 2014; Côté, 2020). In line with this description are the data showing that physical exercise, which restored the levels of KCC2 and NKCC1 towards control levels in the lumbar spinal cord, also led to reduced spasticity and promoted functional recovery (Cote et al., 2014; Khalki et al., 2018). Consistently, pharmacologically enhancing KCC2 activity or blocking NKCC1 mirrored the effects of exercise, decreasing spasticity and improving functional recovery of hindlimbs after chronic SCI (Cote et al., 2014; Chen et al., 2018; Mekhael et al., 2019; Bilchak et al., 2021). These data indicate that KCC2 and NKCC1 are potential pharmacological targets to treat spasticity and improve locomotor network following SCI. To sum up, SCI downregulating KCC2 and GlyR/$GABA_AR$ neurotransmission and upregulating NKCC1 may lead to disinhibition of MNs and cause an increase the excitability of MNs.

3.2.2.3. Neuromodulatory signaling

Different from ionotropic signaling, neuromodulatory signaling is mediated by metabotropic receptors, acting through a signaling cascade triggering G-proteins and changing the concentration of

intracellular second messengers (Heckman et al., 2009). The major neuromodulators acting on MNs are 5-HT, NA, DA and ACh. Among them, 5-HT, NA and DA derive mostly from the brain and are released in spinal cord from descending tracts, while ACh is released by cholinergic $V0_C$ interneurons located in the spinal cord. All they were shown to profoundly modulate MNs excitability through modulating the actions of ion channels and pumps on MNs (Miles and Sillar, 2011).

3.2.2.3.1. Serotonergic signaling

In the mammalian spinal cord, 5-HT is mainly released by the descending projections which originate in the raphe nuclei of the brainstem (Dahlstroem and Fuxe, 1964; Fuxe, 1965; Steinbusch, 1981). Via binding to 5-HT receptors (5-HTRs), 5-HT elicits signaling which affect sensory and motor function and neuronal plasticity within the spinal cord. 5-HTR family consist of seven types (5-HTR1 - 5-HTR7) and fourteen subtypes, majority of which have been detected in the spinal cord neurons (Perrier et al., 2013; Zhang, 2016). In MNs, 5-HTR1 (A, B, D), 5-HTR2 (A, B, C), 5-HTR3, 5-HTR5A and 5-HTR7 are expressed and play roles in modulating excitability of MNs through a range of ion channels mediating K^+, Ca^{2+} , and Na^+ conductance [reviewed in (Perrier et al., 2013; Zhang, 2016).

SCI results in a 5-HT fiber loss around MNs caudal to the injury site (Anden et al., 1964; Magnusson, 1973; Zhang, 2016; Yokota et al., 2019). In consequence, an increased expression and activity of 5-HTRs was reported using different animal models, and interpreted as a compensatory response (Fuller et al., 2005; Hayashi et al., 2010; Kong et al., 2010; Kong et al., 2011; Mantilla et al., 2012; Perrier et al., 2013; Ren et al., 2013; Zhang, 2016; Mantilla et al., 2017; Garcia et al., 2018). In addition, after SCI, some subtypes of 5-HTRs turn into a constitutively active state; because their activation does not depend on 5-HT availability, signaling through them may contribute to hyperexcitability of MNs and hyperreflexia (Murray et al., 2010; Murray et al., 2011a; D'Amico et al., 2013b).

There remains 2-15% of the normal levels of 5-HT in spinal cord caudal to the lesion site (Magnusson, 1973; Newton and Hamill, 1988; Schmidt and Jordan, 2000), proposed to be released from intraspinal 5-HT neurons (Newton et al., 1986; Newton and Hamill, 1988) and cells containing aromatic L-amino acid decarboxylase (AADC) (Li et al., 2014c; Wienecke et al., 2014). Since the number of intraspinal 5-HT neurons is negligible (3-9 cells/spinal cord was reported) (Newton et al., 1986; Newton and Hamill, 1988), AADC cells may play major role in 5-HT residual supply. Because SCI increases the

ability of AADC cells to synthesize 5-HT from 5-HTP (Li et al., 2014c; Wienecke et al., 2014) the upregulated 5-HTRs postinjury could be activated by 5-HT from these remaining sources (Zhang, 2016; Huang et al., 2021a).

Activation of 5-HTR2 subtype on MNs facilitates generation of voltage dependent Ca^{2+} and Na^{+} persistent inward currents (PICs) through modifying the gating behavior of the Ca^{2+} channels (low-threshold L-type calcium channels CaV1.3) and Na^{+} channels (Heckman et al., 2003; Perrier and Delgado-Lezama, 2005; Harvey et al., 2006b; Li et al., 2007; Heckman et al., 2009). PICs increase MN excitability through amplifying subsequent synaptic inputs and generating plateau potentials on MNs (Heckman et al., 2003; Heckman et al., 2008). After acute SCI, the immediate loss of the descending inputs from supraspinal pathways results in disappearance of PICs, which further leads to hyperpolarization of MNs and clinically generates hyporeflexia of spinal shock (Ditunno et al., 2004). At the chronic phase of SCI, upregulated 5-HTR2 was reported to facilitate the reappearance of PICs which produce a sustained depolarization of MNs to develop MN hyperexcitability and muscle spasticity which can last for weeks to months after SCI (Ditunno et al., 2004; Heckman et al., 2009; D'Amico et al., 2014b). Consistently, application of antagonists of 5-HTR2 decreases the occurrence of PICs and impairs the firing of MNs (Harvey et al., 2006b, c; Li et al., 2007).

Recently, the ability of 5-HTRs to form complexes with NMDAR to affect NMDA-induced MN depolarization has been discovered; probably through forming a macromolecular complex containing 5-HTR2B and NMDAR GluN1 subunit (Benhadda et al., 2021). 5-HTR2B activation potentiates NMDAR-induced depolarization of rat spinal cord dorsal horn neurons in a neuropathic pain model and also frog spinal cord MNs (Holohean and Hackman, 2004; Aira et al., 2013). Similarly, 5HTR2C was shown to form a protein complex with NMDAR GluN2A subunit and to enhance NMDAR MN depolarization through phosphorylation of Src tyrosine kinase in the frog spinal cord (Bigford et al., 2012). Therefore, it is reasonable to speculate that altered activity of 5-HTRs after SCI may modulate MN excitability through affecting NMDAR-induced depolarization.

However, activation of 5-HTRs does not always display excitatory effect on the MN output. For instance, in addition to 5-HTR2A-mediated direct excitatory effect on MNs, activation of 5-HT2A receptors also increases cell membrane expression and function of KCC2 on MNs and reduces SCI-induced spasticity (Bos et al., 2013; Sanchez-Brualla et al., 2018). Another dimension of serotonergic

modulation of MN function is activation of presynaptic 5-HTR1 (1B, 1D or 1F) located on sensory afferent terminals and excitatory interneurons, which can be evoked by receptor agonists. Such treatment inhibits polysynaptic EPSPs on MNs, therefore reducing CaPIC activation and alleviating muscle spasms after SCI (Yoshimura and Furue, 2006; Murray et al., 2011b; D'Amico et al., 2013a).

In the injured spinal cord, administration of 5-HT or increasing endogenous 5-HT levels by inhibiting its reuptake result in the net effect of increasing MN excitability (Slawinska et al., 2013; Leech et al., 2014; Nardone et al., 2015; Perrier, 2016; Perrin and Noristani, 2019).

3.2.2.3.2. Noradrenergic signaling

Descending noradrenergic (NA) projections to the spinal cord are mainly derived from brainstem Locus Coeruleus (A6 cell group), the region around the superior olivary nuclei (A5 cell group) and subcoeruleus/medial parabrachial systems (A7 cell group) (Pickel et al., 1974; Hancock and Fougerousse, 1976; Amaral and Sinnamon, 1977; Nygren and Olson, 1977; Westlund et al., 1982, 1983). NA binds to three G-protein coupled noradrenergic receptors: α1-, α2- and β-adrenergic receptors (ARs), which are divided into 9 subclasses: α1A, α1B, and α1D; α2A, α2B, and α2C; β1, β2, and β3 subtypes (Bylund, 1992; Graham et al., 1996). In the spinal cord high levels of α1-ARs were found in the MN region of the ventral horn lamina IX (Day et al., 1997; Giroux et al., 1999) with α2-ARs relatively sparsely distributed in that region (Young and Kuhar, 1980; Roudet et al., 1994; Stone et al., 1998; Giroux et al., 1999). Noteworthy, immunochemical labeling has shown that lumbar MNs express α1A, α2A- and β1-ARs at early postnatal periods (Tartas et al., 2010). β-ARs were also detected in the ventral horn by histochemical fluorescence (Melamed et al., 1976; Mizukami, 2004; Arora et al., 2021) and ligand binding autoradiography (Patterson and Hanley, 1987).

In the neonatal rat-isolated spinal cord preparation, bath-applied NA enhanced the excitability of spinal lumbar MNs by hyperpolarizing the threshold for action potential production and increasing the input membrane resistance (Elliott and Wallis, 1992; Sqalli-Houssaini and Cazalets, 2000; Fedirchuk and Dai, 2004). Consistently, activation of postsynaptic α1- and β-ARs by agonists enhances the excitability of ventral MNs by inducing PICs and increasing the firing frequency of APs in MNs of not only neonatal rats (Tartas et al., 2010; Shoji et al., 2019) but also adult animals (Lee and Heckman, 1998, 1999; Harvey et al., 2006c). These results indicate that descending noradrenergic projections also modulate the excitability of MNs.

Spinal cord transection dramatically but not completely decrease NA concentration in the spinal below the lesion site (Anden et al., 1964; Magnusson, 1973; Commissiong et al., 1978; McNicholas et al., 1980). After SCT, about 2-5% of the normal level of NA is still detected (Magnusson, 1973; Roudet et al., 1993; Roudet et al., 1994), which is suggested to be released from the intraspinal neurons or sympathetic fibers (McNicholas et al., 1980; Nozaki et al., 1980; Cassam et al., 1997; Takeoka et al., 2010). This residual release of NA was proposed to facilitate activation of CaPIC in MNs, contributing to increased MN excitability and muscle spasms after SCI, owing to denervation supersensitivity developing after SCI (Rank et al., 2007). Accordingly, SCT at low thoracic level was reported to upregulate densities of α1- and α2-ARs in the lumbar segments at 2 weeks and one month postinjury, but they were normalized in longer survival times (>2 months) (Roudet et al., 1993; Roudet et al., 1994; Giroux et al., 1999). Importantly for the functional outcome, the time course of AR upregulation corresponds to recovery period of hindlimb locomotion after spinal lesion (Barbeau and Rossignol, 1987; Harnie et al., 2019). Administration of ARs agonist to SCI subjects modulates spinal neuronal excitability and improves locomotor recovery (Forssberg and Grillner, 1973; Maynard, 1986; Barbeau et al., 1987; Chau et al., 1998; Gao et al., 2019). Sustained activation of specific ARs during SCI contributes to the development of muscle spasticity. Similarly to 5-HT receptors, α1-ARs, in particular α1A-ARs subtype, became constitutively active after SCI; in consequence AR signaling contributes to hyperexcitability of MNs and facilitates muscle spasms by activating PICs on MNs both in animal models (Harvey et al., 2006c; Rank et al., 2011) and human patients (D'Amico et al., 2013b). On the contrary, α2-ARs which do not respond with constitutive activity to SCI, mediate an inhibitory effect of residual NA on MN excitability and muscle spasms by reducing long polysynaptic EPSP that triggers PICs (Tremblay and Bedard, 1986; Rank et al., 2011).

3.2.2.3.3. Dopaminergic signaling

In mammals, DA descending tracts to spinal cord originate from hypothalamic A11 neurons (Björklund and Skagerberg, 1979; Skagerberg and Lindvall, 1985; Barreiro-Iglesias et al., 2008; Tay et al., 2011; Koblinger et al., 2014; Ozawa et al., 2017), acting as the primary supplier of DA in the spinal cord to modulate spinal motor circuits. In line with that, lesions of A11 nuclei or spinal cord transection in rodents result in a significant decrease of DA concentrations in the caudal spinal cord (Magnusson, 1973; Commissiong et al., 1978; Zhao et al., 2007). DA exerts its actions via five functionally distinct

G-protein-coupled receptors that are classified as two subgroups: excitatory D1-like (D1 and D5) and inhibitory D2-like (D2, D3, and D4) (reviewed in (Beaulieu and Gainetdinov, 2011; Beaulieu et al., 2015)), all of which are identified in spinal cord MNs (Zhu et al., 2007).

DA inputs on spinal MNs diversely modulate their excitability across species, developmental stage and DA concentration (Picton and Sillar, 2016; Jha and Thirumalai, 2020). In rodents DA displays the concentration-dependent excitatory (through D1-like receptors) and inhibitory (through D2-like receptors) effects on the output of spinal network (Barriere et al., 2004; Sharples et al., 2015; Sharples et al., 2020). DA can directly trigger skeletal muscle tone (Schwarz and Peever, 2011) and enhance excitability of MNs by decreasing both the first spike latency and mAHP amplitude and by potentiating AMPAR mediated neurotransmission, shown in mice (Han et al., 2007). That process is mediated via activating D1-like receptors but not D2-like receptors on MNs (Han and Whelan, 2009). Another line of evidence in mice demonstrated that D1 receptor can form heteromers with adenosine A1 receptor on MNs to control their excitability; through their interaction adenosine tonically inhibits D1R-mediated excitatory signaling (Rivera-Oliver et al., 2019).

SCI results in a marked reduction of DA concentrations in the spinal cord below the lesion site, due to the interruption of descending tracts from A11 neurons (Magnusson, 1973; Commissiong et al., 1978). However, similarly to studies on 5-HT and NA intrinsic sources in the spinal cord, recent studies have shown that a population of ADDC positive and/or TH positive neurons residing in the rat spinal cord might be the potential source of DA (Hou et al., 2016; Ren et al., 2016; Ren et al., 2017; Qiao et al., 2021). It has been suggested that the ability of these cells to produce DA is repressed by DA descending tracts in physiological conditions (Ren et al., 2016; Hou et al., 2021). SCI would cause removal of suppression and allow these cells to regain the ability to synthesize DA (Hou et al., 2016; Ren et al., 2016; Hou et al., 2021; Qiao et al., 2021).

Data on the effects of SCI on the expression or distribution of DARs are relatively sparse. After a SCT at T10 level of adult rats, mRNA levels of TH and D2R were reported to be downregulated in the caudal spinal cord L1 segment at both the subacute (3 weeks) and chronic stages (8 weeks), while the level of D1R mRNA was unaltered (Zhao et al., 2021). Since available analysis of gene expression concerned segmental characteristics only, precise patterns of expression and distribution of the DARs on MNs needs to be disclosed in further studies.

3.2.2.3.4. Cholinergic signaling

Unlike the aforementioned monoaminergic pathways, the cholinergic signaling in the spinal cord is intrinsic; all cholinergic neurons that connect to spinal sensorimotor networks are located within the spinal cord (Mille et al., 2021). MNs receive cholinergic inputs from premotor INs that release ACh to modulate the excitability of these MNs and motor output (Nascimento et al., 2020).

The excitability of spinal MNs is modulated by ACh via the activation of mAChRs muscarinic receptors, particularly M2 type mAChRs which is the predominant subtype expressed in the spinal cord neurons including MN (Wieckowska et al., 2018). M2 receptor cluster on the postsynaptic membrane in and around the C boutons synapses of large cholinergic inputs abutting on somata and proximal dendrites of MNs. They derive from Pitx2- and Dbx1-expressing V0c cholinergic INs located lateral to the central canal (lamina X and medial lamina VII) and play crucial roles in MN firing, recruitment and motor outputs during locomotion ((Miles et al., 2007; Zagoraiou et al., 2009; Nascimento et al., 2020), for review (Witts et al., 2014)). Except M2 type mAChRs, SK type channels, Kv2.1 channels, TMEM16F, sigma 1 (S1R) and neuroligin 1 receptors have also been characterized in C bouton complex, providing a molecular basis of modulation of MN excitability by cholinergic IN (Deardorff et al., 2014; Witts et al., 2014; Mille et al., 2021). In rodents, turtles and salamanders, activation of M2 mAChRs was shown to increase the excitability of spinal MNs by reducing spike afterhyperpolarization (AHP) mediated by SK channels or reducing spike half-width though Kv2.1 channels ((Chevallier et al., 2006; Miles et al., 2007; Nascimento et al., 2020), for review (Mille et al., 2021)).

A loss of cholinergic inputs has been observed on lumbar MNs at 6 weeks after thoracic SCT (Apostolova et al., 2006; Jakovcevski et al., 2007; Mehanna et al., 2010; Skup et al., 2012), and also on sacral MNs at 1-12 weeks after sacral SCT in rats, the latter accompanied by a coinstantaneous shrinkage of the cholinergic INs perikarya located around the central canal (Kitzman, 2006; Kapitza et al., 2012). The increased number of inputs on lumbar MNs in mice at 3 months after thoracic SCT has been also reported (Yokota et al., 2019). The loss of cholinergic inputs correlates with impaired locomotor performance (Jakovcevski et al., 2007), confirming that cholinergic inputs are crucial during locomotion.

Postsynaptically, at the second week after SCT, the transcript level of M2 receptor was downregulated in the L3-6 segments (Wieckowska et al., 2018). At the chronic phase (6 weeks postinjury) SCT selectively increased synaptic distribution of M2R protein as compared to extra-

synaptic M2R density on plasma membrane of TA MNs (Wieckowska et al., 2018). The enhanced synaptic accumulation of M2R after SCT was proposed to be a compensatory mechanism in response to reduced cholinergic inputs and contribute to generation of clonic movements observed in chronic spinal rats (Wieckowska et al., 2018). Such response of mAChR may support a development of hyper-cholinergic state that enhances MN output in paraplegic animals (Jordan et al., 2014).

3.3. Treatment of spinal cord injury

In research and clinical studies, the attempts to evoke and improve the motor recovery focus on two main directions to: (1) regain the neural connectivity across the lesion; (2) modulate the spinal cord network preserved below the injury site. After incomplete SCI, the sensation and muscle contractions are partially retained owing to spared descending and ascending tracts (Raineteau and Schwab, 2001). Over time, spontaneous sprouting and regeneration of corticospinal and rubrospinal neurons can lead to formation of synaptic connections (Ghosh et al., 2009; O'Shea et al., 2017). By manipulating these two processes of sprouting and regeneration of groups of fibers which may bypass the site of incomplete injury, improvement of motor abilities can be achieved (Raineteau and Schwab, 2001). In rare cases of SCT in humans, modelled in animal studies (also by our Group), sprouting of axons across the lesioned area is extremely limited because glial scar and lesion cavity, as well as non-permissive environment of inhibitory molecules obstructs axonal growth from neurons surviving above the lesion (Jakeman et al., 1998; Boyce et al., 2007; Ollivier-Lanvin et al., 2015; Krupka et al., 2017; Gaudet and Fonken, 2018; Sofroniew, 2018; Marchionne et al., 2020) (**Figure 3.8**). Although many attempts have been made to reconnect the two parts after experimental SCT, the effectiveness of these approaches is not very satisfying. Regardless, some breakthroughs are achieved in recent years. Lu and coworkers, taking advantage of combinatorial therapy consisting of cAMP injection into the brainstem, cell grafts in the lesion site, and brain derived neurotrophic factor (BDNF) expression within and below the lesion site, successfully provoked axonal regeneration across the lesion site in rats with SCT (Lu et al., 2012). Anderson and coworkers also utilizing combined treatment addressing three essential growth-conditioning factors (neuron intrinsic growth capacity, growth-supportive substrate, chemoattraction), successfully stimulated propriospinal axon regeneration across astrocyte scar borders and lesion core in spinalized mice and rats (Anderson et al., 2018). Although the improvement of locomotor function was

not obvious in those studies, a proof was obtained that neural connectivity recuperation across anatomically complete SCI lesions is achievable.

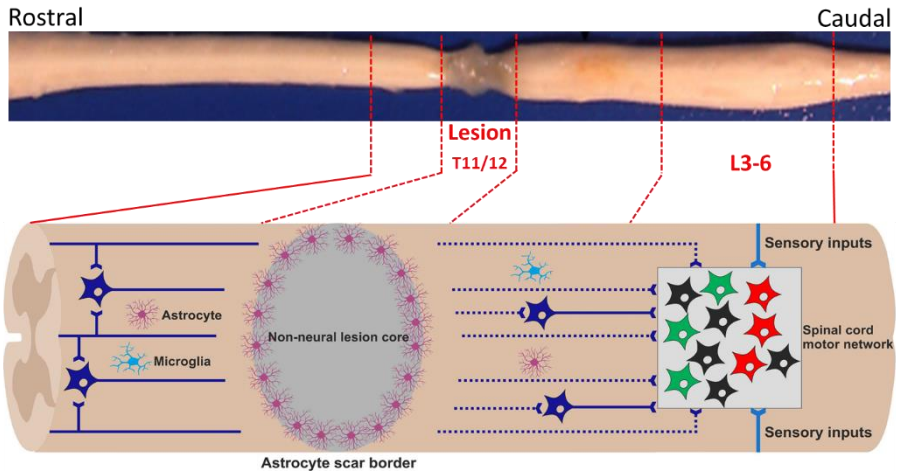

Figure 3.8. Complete SCI at low thoracic 11/12 level. Formation of astrocyte scar blocks regeneration of the transected fibers, leading to permanently disrupted connections between supraspinal motor centers and spinal cord motor network located below the injury site. In effect complete loss of motor functions occurs. Black neurons designate interneurons; red and green MNs designate neurons which innervate Gastrocnemius lateralis (GL) and Tibialis Anterior (TA) muscles, respectively. GL and TA were selected for the current study.

Caudal to the injury site (**Figure 3.8**), the intrinsic spinal locomotor circuitries controlling locomotion remain intact. Although spared circuitries are usually functionally dormant at the pathological state postinjury, its preservation provides a promising possibility that by reactivating these circuits an improvement of motor function is achievable (Gao et al., 2021). In the circuitry, sensory proprioceptive Ia or cutaneous feedbacks from the muscles, joints and skin are integrated in the Central Pattern Generator (CPG) networks and transmit signals to MNs that innervate skeletal muscles (Rossignol et al., 2006; Rossignol and Frigon, 2011; Grillner and El Manira, 2020). Assuming that they receive adequate excitatory and inhibitory drives, the circuitries can be recruited into operation mode. Therefore, to regain locomotor abilities after SCI, the main task is to alter electrical and synaptic properties of spinal locomotor circuitries to achieve a functional state. That was proposed either by manipulating the peripheral sensory afferent feedback or by directly activating local locomotor pattern generators within the spinal cord (Rossignol and Frigon, 2011). Treadmill locomotor training, electrical stimulation, pharmacological or cell based therapy of subjects with the injured spinal cord are the main approaches which has been shown to be capable of transforming nonfunctional spinal circuits into functional and adaptive states to regain hindlimb locomotion (Courtine et al., 2009; Rossignol and

Frigon, 2011). The aim of these approaches is to elicit especially long-lasting improvement in locomotor function through engaging the learning ability of spinal cord.

3.3.1. Locomotor training

Locomotor training is a classic sensorimotor rehabilitation approach to improve locomotor performance of individuals with SCI. Early studies in cats showed that subjects with complete transection of the spinal cord at thoracic level recovered full weight-bearing locomotion of hindlimbs after a few weeks' daily training on a treadmill (Lovely et al., 1986; Barbeau and Rossignol, 1987; de Leon et al., 1999). Less advanced recovery applies also to spinal rodents. In our and others' studies, daily locomotor training was shown to gradually improve hindlimb movements in spinalized rats and mice (Macias et al., 2009; Skup et al., 2012; Hubli and Dietz, 2013; Smith and Knikou, 2016). After appropriate training and treatment, the animals with SCI can regain hindlimb stepping with alternation, plantar foot placement and body weight support, indicating that intrinsic locomotor network is reactivated. Compared to subjects with incomplete SCI, SCT subjects need to be assisted manually or by a robotic device during the whole treadmill training period due to total loss of voluntary motor control (Hubli and Dietz, 2013). Even so, motor learning during training improves the coordination of bilateral muscle activation, which is essential for locomotion (Hubli and Dietz, 2013). The major drive to reactivate and reorganize the intraspinal locomotor network after training is a reinforcement of activity-dependent sensory feedback from muscular and cutaneous receptors, and modulation of neuronal excitability (Smith and Knikou, 2016). Electrophysiological experiments revealed that treadmill stepping-evoked recovery in SCT rats transected as neonates or young adults, was associated with significant changes in cellular properties of spinal hindlimb MNs, such as a reduction in action potential afterhyperpolarization (AHP) and change in synaptic inputs to MNs comparing with spinalized rats without training (Petruska et al., 2007). AHP is an important parameter determining neuron excitability; it tends to limit high-frequency burst firing of neurons, and its amplitude is decreased in spinal MNs during fictive locomotion in the cat (Brownstone et al., 1992; Brumberg et al., 2000). At the molecular level, locomotor training elevates levels of both glutamate and glycine in the spinal cord lumbar region of rats with neonatal, thoracic spinal cord transection (Cantoria et al., 2011), and normalizes a balance of excitatory and inhibitory signaling on MNs (Tillakaratne et al., 2002; Ichiyama et al., 2011; Wieckowska et al., 2018), which is essential for proper activity of MNs. Taken together, the repetitive

activation of the preserved spinal network by locomotor training is effective in modulation of sensorimotor circuitries to achieve appropriate excitability level, and leads to some recovery of a weight-bearing locomotor capacity of animals with SCI (Edgerton et al., 2008). Outcomes of locomotor training also support the concept that learning is not exclusive to the brain, since spinal cord also can learn from training without commands from supraspinal motor centers (Grau et al., 2020).

3.3.2. Electrical stimulation

Electrical stimulation (ES) is another efficient approach to treat SCI. Basic and clinical research have applied epidural or transcutaneous ES on injured spinal cord to restore rhythmic motor ability after incomplete or complete SCI (Lu et al., 2016; James et al., 2018; Bonizzato et al., 2021; Malone et al., 2021; Sharif et al., 2021; Wang et al., 2021). ES is capable of reactivating the dormant network and restore its excitability allowing motor-pattern generation based on proprioceptive input processing from muscles, bones and skin to MNs (Angeli et al., 2014; Hofstoetter et al., 2015; Formento et al., 2018; Audet and Lecomte, 2021). This is consistent with the fundamental roles of proprioceptive inputs in reorganization of sensorimotor circuits during recovery (Takeoka and Arber, 2019; Takeoka, 2020). In rats with SCT, ES enhances excitability of spinal circuitry and improves gait quality and weight-bearing stepping (Wenger et al., 2016). However, in human SCI patients, the requirements of ES frequencies and amplitudes are more restricted and the enhancing effects can be exploited just in a narrow range of ES parameters; that is due to antidromic collisions during ES causing that large amount of proprioceptive input is blocked (Formento et al., 2018). The loss of proprioceptive afferents reduces the effectiveness of ES in boosting recovery after SCI, confirming that preserved proprioception within sensorimotor circuits is highly necessary to promote motor recovery with ES.

3.3.3. Pharmacological, cell- and biomolecule-based therapies

Distinct from locomotor training and electrical stimulation that stimulate the entirety of the spinal cord or segmental networks, pharmacological or cell-based manipulation can be more targeted to modulate defined motor circuitries. The pharmacological agents include a variety of receptor agonists/antagonists (Teng et al., 2003; Baastrup and Finnerup, 2008; Hama and Sagen, 2012; Chen et al., 2017; Gotoh et al., 2020; Simmons et al., 2021), ion channels blockers (Liu et al., 2011; O'Hare Doig et al., 2017; O'Hare Doig et al., 2020), antioxidants and anti-inflammatory drugs (Bains and Hall, 2012; Fakhri et al., 2018; Hayta and Elden, 2018; Vilchis-Villa et al., 2019; Lambrechts and Cook, 2021) and

neurotrophic factors (e.g. BDNF, NT3, NGF, FGF) (Hollis and Tuszynski, 2011; Awad et al., 2015), which trigger a series of signaling pathways facilitating neuroprotection, structural plasticity and functional recovery. Cell-based therapies use transplantation of specific types of cells into the SCI microenvironment to provide trophic support for neuroprotection, axonal remyelination and regeneration, angiogenesis, immunomodulation and inter-neuron communication ((Hesp et al., 2018; Kobashi et al., 2020; Gong et al., 2021; Olmsted et al., 2021), for review (Ashammakhi et al., 2019; Ahuja et al., 2020)). Cell types are multitudinous, including neural stem cells, oligodendrocyte progenitor cells, mesenchymal stem cells, Schwann cells, olfactory ensheathing cells or genetically modified cells that can express specific proteins (e.g., growth factors) (for review (Ahuja et al., 2020)). Biomaterial scaffolds have also been used to create bridges across the irreversible lesions, improve efficiency of delivery of therapeutic agents or drugs and enhance neuronal survival (Tran et al., 2020; Shultz and Zhong, 2021; Xu et al., 2021), for review (Shen et al., 2021)).

The ultimate goal of all these approaches is to reactivate impaired spinal neuronal network and restore motor ability after SCI. Administration of agonists/antagonists or channel blockers of neurotransmitter receptors and ion channels exerts modulatory effects on the excitability of MNs. For instance, administration of monoaminergic agonists that activate serotoninergic and adrenergic receptors in spinalized animals can restore MN excitability (Heckman et al., 2009). On the other hand, CLP257, a KCC2 enhancer, increases KCC2 membrane expression and function in lumbar MNs, ameliorating symptoms of spasticity in a rat model of SCT (Bilchak et al., 2021). Nimodipine, an L-type calcium channel blocker, also prevents spasticity after SCI (Marcantoni et al., 2020). Recent study on transplantation of human spinal GABAergic neurons into injured rat spinal cord revealed a reduced MN hyperexcitability, alleviated spasticity and improvement of locomotion after impacted SCI, by reorganizing inputs to MNs after the grafted GABA neurons integrate into the local neural circuit (Gong et al., 2021). These examples show that MNs, a final motor output of the spinal network, is a promising target for treatment of SCI.

3.4. Does BDNF Treatment Modulate Excitability of Spinal Cord Motoneuron?

3.4.1. BDNF properties

BDNF is a member of the neurotrophin family widely expressed in numerous groups of neurons in the central and peripheral nervous system; BDNF signaling is crucial for neuronal survival, growth, differentiation and regeneration (Hofer and Barde, 1988; Lipsky and Marini, 2007; Macias et al., 2007; Macias et al., 2009; Colucci-D'Amato et al., 2020). Three isoforms are translated from the *bdnf* gene, including pro-domain of BDNF, pro-BDNF and mature BDNF (Foltran and Diaz, 2016; Kowianski et al., 2018) (**Figure 3.9**). Except that the mature form of BDNF protein elicits cellular responses via binding to tropomyosin receptor kinase B (TrkB) receptor, both the pro-domain of BDNF and pro-BDNF have also been identified as independent signaling molecules, which regulate distinct cellular processes through binding to specific receptors.

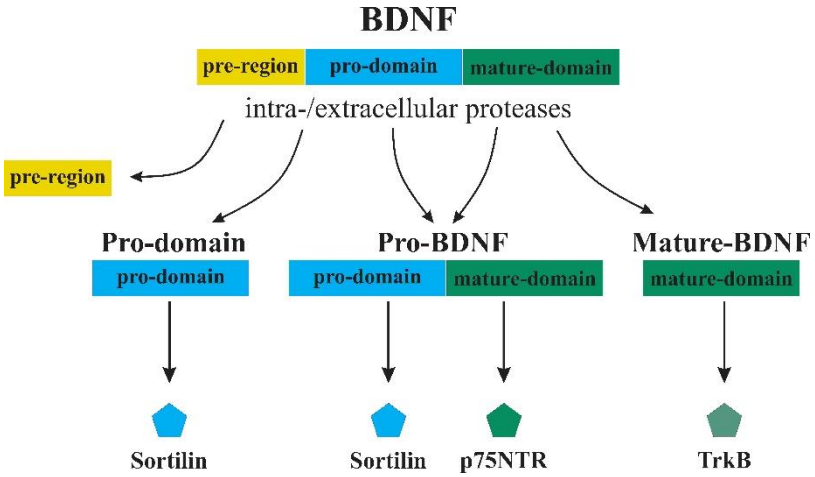

Figure 3.9. BDNF isoforms and their binding receptors. Three isoforms including pro-domain of BDNF, pro-BDNF and mature BDNF are produced after intra- or extracellular cleavage. The pro-domain of BDNF binds preferentially to the sortilin receptor, while pro-BDNF exhibits a high affinity to p75 receptor. The mature BDNF displays highest affinity to the TrkB receptor. A schematic is adapted from (Kowianski et al., 2018).

The pro-domain of BDNF binds to a Vps10p domain protein, sortilin, while pro-BDNF exhibits a high affinity to p75 receptor (containing the death domain which mediates pro-apoptotic signaling) and lower affinity to TrkB receptor (Chen et al., 2005; Anastasia et al., 2013; Zanin et al., 2017). In contrast to pro-BDNF, mature BDNF binds to TrkB with high affinity, and with lower affinity to the p75 receptor (Reichardt, 2006). The different receptor binding properties endows the three BDNF isoforms distinct roles. Although the exact functions of the pro-domain of BDNF are unclear, it was shown to function as an independent ligand to negatively modulate neuronal architecture and morphology, causing growth cone retraction (Dieni et al., 2012; Anastasia et al., 2013) and reduction of dendritic spine density (Guo

et al., 2016). Pro-domain of BDNF was shown to facilitate hippocampal long-term depression (LTD) (Mizui et al., 2015) and its activity was also linked to Alzheimer's disease (Lim et al., 2015). Compared with pro-domain of BDNF, pro-BDNF displays distinct functions. Pro-BDNF binding to p75 receptor can regulate neuronal survival and give rise to retraction of neurite growth cones and reduction of dendritic spine density via triggering signaling cascades including JNK, caspase 3, Ras and NF-kB (Teng et al., 2005; Sun et al., 2012; Arango-Lievano et al., 2015). Application of pro-BDNF also facilitated NMDAR- dependent LTD in hippocampal slices following low frequency stimulation (Pang et al., 2004).

The mature BDNF is the most extensively studied isoform, displaying opposite functions to pro-BDNF. The properties of mature BDNF, such as promoting dendritic growth, axonal branching, formation and maturation of synapses (Cohen-Cory and Fraser, 1995; Martínez et al., 1998; McAllister et al., 1999), underlines the crucial roles of mature BDNF in the development of both central and peripheral nervous system (McAllister et al., 1999; Bibel and Barde, 2000). Signaling of mature BDNF via TrkB receptor is also a major regulator of synaptic plasticity in the mammalian CNS, triggering long term potentiation (LTP) and mediating activity-dependent changes in structure and function of synapses through mitogen activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), phosphoinositide 3-kinase (PI3K) and phospholipase C γ (PLC γ) pathways (Numakawa et al., 2010; Leal et al., 2015; Kowianski et al., 2018). My dissertation is with emphasis on regulatory roles of the mature BDNF. Therefore the “BDNF” in the remainder of the text refers to mature BDNF.

3.4.2. BDNF in the treatment of SCI

Our and other studies have shown that in the spinal cord, BDNF is expressed in numerous groups of neurons including MNs, astrocytes, oligodendrocytes, microglia and macrophages, regulating the survival and function of these cells (Dougherty et al., 2000; Gómez-Pinilla et al., 2001; Ikeda et al., 2001; Skup et al., 2002; Macias et al., 2007; Macias et al., 2009). Although both increased and decreased levels of BDNF and TrkB have been detected after SCI (Dougherty et al., 2000; Hutchinson et al., 2004; Macias et al., 2009; Garraway and Huie, 2016) raising the discussions on its deficit or lack of deficit and availability in postlesion conditions, BDNF is regarded as a potential promoter of motor functions after SCI, owing to its crucial regulatory role in axonal regeneration, neuronal excitability and synaptic transmission (Seebach et al., 1999; Arvanian and Mendell, 2001). Early studies showed that BDNF rescues spinal MNs from injury-induced cell death (Yan et al., 1992; Clatterbuck et al., 1994; Novikov

et al., 1997). Locomotor training improved the recovery of locomotor function of hindlimbs in spinal animals, which is coinstantaneous with an upregulated BDNF expression in the lumbar spinal cord (Gómez-Pinilla et al., 2002; Gomez-Pinilla et al., 2007; Macias et al., 2009; Cote et al., 2011; Joseph et al., 2012; Wang et al., 2015). In agreement with these data, the locomotion recovery after treadmill training in rats with SCI can be inhibited by blocking of BDNF-TrkB signaling, indicating that the training-triggered upregulation of BDNF is essential for functional recovery after SCI (Li et al., 2019). Similarly, electrical stimulation is also able to increase BDNF levels in the spinal cord, documented in the rat contusion SCI model (Ghorbani et al., 2020) and peripheral nerve injury model (Al-Majed et al., 2000; Huang et al., 2013). Electrical stimulation was shown to act on BDNF levels through activating a Ca^{2+}- and Erk-dependent signaling pathways (Wenjin et al., 2011). Given that locomotor training and electrical stimulation also lead to alteration of MN excitability, while preparing our project we speculated that the upregulated BDNF contributes in part to this changed MN excitability. The stimulation of BDNF content by these two approaches underlines the rationale for exogenous application of BDNF protein or its gene, which can be used as an independent intervention to promote recovery of locomotion of animals with SCI.

So far, approaches for exogenous application of BDNF to the injured spinal cord include grafts of tissues or cells expressing BDNF, injection of viral vectors to deliver BDNF gene, intrathecal infusion by minipump, biomaterial-based drug delivery system or 3D- printed scaffolds integrated with BDNF (Ziemlińska et al., 2014; Harvey et al., 2015; Hernandez-Torres et al., 2017; Ghosh et al., 2018; Charsar et al., 2019; Marchionne et al., 2020; Liu et al., 2021; Sieck et al., 2021). Purified or recombinant BDNF protein, mRNA of BDNF or tissues/cells secreting BDNF have been used for the application, which is rostral or caudal to the injury or targets the injury site (Harvey et al., 2015; Crowley et al., 2019). For instance, a graft with BDNF or NT3 neurotrophic factors into T12 spinal transection site promoted and enhanced locomotor recovery in untrained, spinalized cats, which was comparable with that in cats receiving a training instead (Boyce et al., 2007; Ollivier-Lanvin et al., 2015). By using a programmable and implantable mini-pump, BDNF was administered to the lumbar spinal cord in cats with thoracic SCT, which significantly improved the recovery of weight-bearing plantar stepping (Marchionne et al., 2020). Directly injected BDNF mRNA nano-micelles into the injured tissue was also shown to improve motor function recovery in mice with contusion SCI (Crowley et al., 2019). Importantly, our previous

study has shown that overexpression of BDNF in lumbar spinal segments caudal to the lesion site, achieved from the adeno-associated (AAV) viral vector carrying BDNF transgene, may lead to robust early locomotor recovery even in rats with complete spinal transection; rats did not require tactile stimulation of the tail to trigger treadmill walking and performed steps with plantar feet placement (Ziemlińska et al., 2014), what disclosed the potential of lumbar spinal circuits completely devoid of descending inputs to reorganize and trigger motor activity.

3.4.3. BDNF regulates MN excitability in the locomotion recovery after SCI

Despite that variety of studies demonstrate that BDNF can promote locomotor recovery of animals with SCI, the cellular and molecular mechanisms underlying functional improvement are not fully elucidated. In our previous work, in spinal rats which received AAV-BDNF and were planned to receive 5 weeks of locomotor training, extensive myoclonus with multiple hindlimb jerks and tremor triggered by the training did not allow to continue the experiments in that schedule (E. Ziemlińska, unpublished observations). Animal behavior suggested generation of hyperexcitability within the spinal network, but no analysis of molecular changes could be done to identify response of MNs to that treatment.

In the spinal animals with partial lesion, BDNF was shown to promote sprouting and formation of connections between corticospinal neurons and the spared descending propriospinal interneurons; that allowed the signals from corticospinal tract to bypass the injury site and reach the distal network (Vavrek, 2006). However, in SCT animals treated with BDNF, the growth of axons is barely beyond the lesion cavity (Jakeman et al., 1998; Boyce et al., 2007; Ollivier-Lanvin et al., 2015; Krupka et al., 2017; Marchionne et al., 2020). Therefore, in these conditions BDNF- induced recovery of hindlimb locomotion was deemed to be unlikely associated with axonal regeneration across the lesion site into caudal spinal cord. Rather, BDNF transported anterogradely or retrogradely from the administration site to the lumbar spinal cord and subsequent BDNF/TrkB signaling was proposed to trigger activation of the local locomotor circuitry within the CPG to facilitate locomotion (Jakeman et al., 1998).

Of note, BDNF appears to impact the intrinsic excitability of the spinal MNs. Application of BDNF to the medial gastrocnemius muscle which led to retrograde transport of BDNF to MNs innervating the muscle, significantly increased an electrical excitability of the targeted MNs (Gonzalez and Collins, 1997). Administration of AAV-BDNF into injured spinal cord also increased excitability of ankle extensor MNs in the completely transected rat spinal cord, which was indicated by a reduced rheobase

(Boyce et al., 2012). These effects are most probably owing to the enhancement of excitatory synaptic transmission at Glu synapses (Carvalho et al., 2008; Shinoda et al., 2014; Rauti et al., 2020). So far, increased BDNF availability in the spinal cord was shown to modulate presynaptic components of inhibitory signaling and the potassium chloride co-transporter KCC2 expression in MNs (Rivera et al., 2004; Ziemlińska et al., 2014), as well as gating of ion channels including Na^+, Ca^{2+} and K^+ channels (Blum et al., 2002; Rose et al., 2004). Expanding the scope of investigation on changes affecting neurotransmitter receptors and channels in MNs to identify (1) potential molecular players in generating hyperexcitability in MNs and (2) BDNF role in this phenomenon, is ahead of us.

4. Assumptions and Aims of the thesis

In the spinal cord appropriate excitability of MNs is indispensable for the running of motor network. MN excitability shapes motor output and motor activity through directly innervated muscles (Rossi et al., 2012; Taylor et al., 2020). Injury to the spinal cord leads to dysfunction of motor network, in which MNs with altered excitability fail to properly drive skeletal muscle contractions. Hyperexcitability of MNs has been implicated in SCI-caused involuntary muscle contractions and generation of spasticity impeding the recovery of locomotion at late postlesion period (D'Amico et al., 2014b). Development of its molecular background requires identification, but in animal models changes in neurotransmission-related molecules in MNs were studied mostly at the late postlesion phase, when hyperexcitability is well established. Because in rodents the onset of spasticity can be seen as early as one week postinjury, I hypothesized that after the acute phase, fast molecular changes of lumbar MNs develop in response to the loss of inputs, which may disturb the balance of excitatory and inhibitory receptors and related ion channels and transporters in MNs.

It became the first aim of my work to clarify the direction and extent of transcriptional regulation of receptors mediating excitatory and inhibitory neurotransmission and of functionally associated membrane channels and transporters in hindlimb MNs at the second week after SCT, when the excitability restores. Since the activity of MNs innervating the ankle extensor and flexor muscles is differently affected by SCT, I aimed to examine separately gene expression in pools of MNs innervating ankle extensor (Gastrocnemius lateralis; GL) and flexor (Tibialis anterior; TA) muscles.

There is a premise that brain derived neurotrophic factor (BDNF) spinal overexpression promotes locomotion recovery in part through modulating the excitability of the spinal cord MNs, but may promote also undesirable hyperexcitability. I hypothesized that BDNF treatment may alter molecular changes in MNs caused by SCT.

Therefore, the second aim of my work was to characterize the effect of intraspinal AAV-BDNF administration on gene expression of the proteins studied in the first part of the study, and identify target molecules of pro-excitogenic potential at two weeks after treatment, when significant locomotion recovery is observed, but episodes of myoclonic jerks are also reported.

5. Materials and Methods

5.1. Basic Materials, Experimental Apparatus, Animals

Table 5.1 Chemical compounds and producers

Compound	Company
Acids: HCl, H_3PO_4, CH_3COOH	POCH, Gliwice, Poland
Acrylamide, bis-acrylamide	Sigma-Aldrich, St. Louis, MO, US
Ammonium persulphate (APS)	Sigma-Aldrich, St. Louis, MO, US
Benzamidine hydrochloride	SERVA Electrophoresis GmbH, Heidelberg, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, St. Louis, MO, US
Coomasie® Brillant Blue R-250 (Blue G)	SERVA Electrophoresis GmbH, Heidelberg, Germany
DEPC (diethylpyrocarbonate)	Carl Roth, Karlsruhe, Germany
Glycerol	Carl Roth, Karlsruhe, Germany
Inorganic salts: NaH_2PO_4 x H_2O, Na_2HPO_4, NaCl, KCl, $MgCl_2$, $NaHCO_3$	POCH, Gliwice, Poland
Jung tissue-freezing medium	Leica, Nussloch, Germany
Polyvinyl alcohol - Mowiol (4-88)	Sigma-Aldrich, St. Louis, MO, US
NaOH	POCH, Gliwice, Poland
NP40	Sigma-Aldrich, St. Louis, MO, US
Organic solvents: acetone, ethanol, heptane, methanol	POCH, Gliwice, Poland
Paraformaldehyde	Sigma-Aldrich, St. Louis, MO, US
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, St. Louis, MO, US
Sodium Dodecyl Sulphate (SDS)	SERVA Electrophoresis GmbH, Heidelberg, Germany
TEMED (N,N,N',N' -tetrametyloetylenodiamina)	Sigma-Aldrich, St. Louis, MO, US
Thimerosal	Sigma-Aldrich, St. Louis, MO, US
Tris-base	SERVA Electrophoresis GmbH, Heidelberg, Germany
Tris-EDTA (TE) buffer; pH 8.0	Promega Corporation, Madison, WI, US
Triton X-100	Sigma-Aldrich, St. Louis, MO, US
Tween 20	MP Biomedicals, LLC, Santa Ana, CA, US
β-mercaptoethanol	Sigma-Aldrich, St. Louis, MO, US

Table 5.2 Experimental Apparatus

Name	Company
Analytical balance AB204-S	Mettler-Toledo GmbH, Columbus, US
Autoclave 2100	Prestige Medical Ltd, Blackburn, United Kingdom
Centrifuge 5430 R	Eppendorf AG, Hamburg, Germany
Centrifuge MPW-60	MPW Med. Instruments, Warsaw, Poland
Eppendorf BioSpectrometer® basic	Eppendorf AG, Hamburg, Germany
Eppendorf Cuvette (50-2000µl)	Eppendorf AG, Hamburg, Germany
GeneAmp PCR System 2400	Perkin Elmer, Waltham, MA, US
Heidolph Rotamax 120 platform shaker	Heidolph Instruments GmbH, Schwabach, Germany
IKA Color squid red	IKA Werke GmbH & Co. KG, Staufen, Germany
IKA VXR Vibrax	IKA Werke GmbH & Co. KG, Staufen, Germany
Leica cryostat (CM1850)	Leica Biosystems GmbH, Wetzlar, Germany
Leica Laser Microdissection system LMD7000	Leica Microsystems GmbH, Wetzlar, Germany
LightCycler® 96 Instrument	Roche Diagnostics GmbH, Mannheim, Germany
Mini Protean III vertical electrophoresis apparatus	BioRad, Munich, Germany
Multi-Spin	Biosan, Riga, Latvia
Nikon Eclipse 80i fluorescence microscope, CCD Evolution VF digital camera	Nikon Corporation, Tokyo, Japan Media Cybernetics, Inc., Silver Spring, US
NV-GS400 video camera	Panasonic Corporation, Kadoma, Osaka, Japan
pH meter 430	Corning Incorporated, New York, US
Polytron homogenizer PT 1200E	Kinematica AG, Luzern, Switzerland
Thermo Shaker TS-100	Biosan, Riga, Latvia
Treadmill	Panlab S.L. Barcelona, Spain
Ultrasonic-0.5 cleaner	Polsonic Sp.zo.o. Warsaw, Poland
ULTRA-TURRAX mechanical homogenizer T8	IKA-Werke GmbH & Co. KG, Staufen, Germany
Vortex-Genie 2	Scientific Industries Inc, New York, US
Zeiss LSM780 confocal microscope	Carl Zeiss AG, Jena, Germany

Table 5.3. Experimental Kits

Name	Company
ZR RNA MiniPrep™ isolation kit, No. R1065	Zymo research, Irvine, US
Arcturus PicoPure™ RNA Isolation Kit No. KIT0204	Thermo Fisher Scientific, Waltham, US
QuantiTect® Whole Transcriptome kit, No.207043, 207045	Qiagen, Hilden, Germany
Quant-iT™ PicoGreen™ dsDNA Assay Kit, No. P7589	Thermo Fisher Scientific, Waltham, US
Transcriptor First Strand cDNA Synthesis Kit, No. 04897030001	Roche Molecular Systems Inc, Pleasanton, US
LightCycler 480 probe master No. 04887301001	Roche Molecular Systems Inc, Pleasanton, US
ChemiKine™ BDNF Sandwich ELISA Kit, No. CYT306	Merck Millipore, Burlington, MA, US

Animals

The study has been performed on 72 adult male Wistar rats weighing on arrival 230–350 g. Animals were outbred colony (International Laboratory Code Registry: Cmdb:Wi) of the Wistar rats (RRID_RGD_13508588) supplied by Medical University of Białystok, Poland. The animals were bred in the animal house of the Nencki Institute of Experimental Biology in Warsaw, with free access to water and pellet food, under standard humidity and temperature conditions, at a 12 h light-dark cycle. Rats were initially housed in groups of 4–6 and following surgery they were housed individually. Experimental protocols involving animals, their surgery and care were approved by the First Local Ethics Committee in Warsaw (no 535/2014 and 782/2015) and were in compliance with the guidelines of the European Community Council Directive 2010/63/UE of 22 September 2010 on protection of animals used for scientific purposes.

Table 5.4. Group of Rats and Experimental Procedures. Survival time: 13-16 days*

Analytical procedure	Animal group	Tracer IM injection	SCT	intraspinal injection		Locomotor performance	WB	IF	LCM/ qPCR
				PBS	AAV -BDNF				
LCM	Control	+							9
	SCT	+	+						9
	SCT-PBS	+	+	+		+			7
	SCT-BDNF	+	+		+	+			7
Biochemistry	Control						6		
	SCT-PBS		+	+			5		
	SCT-BDNF		+		+		5		
Immuno-histochemistry	Control	+						8	
	SCT-PBS	+	+	+		+		8	
	SCT-BDNF	+	+		+	+		8	

*Rats survived 13-16 days depending on their progress in locomotor performance

5.2. Methods

5.2.1. Retrograde tracing of motoneurons

The animals were given subcutaneous injection of Butomidor (butorfanolum, 1.5 mg/300 g b.w.; Richter Pharma, Wels, Austria) (Table 5.5) as an analgesic premedication and then were anesthetized with isoflurane (1–2.5% in oxygen; Baxter, Lessines, Belgium) via a facemask. This procedure is advised and generally accepted in this type of surgery by scientific and veterinary community. The skin overlaying selected muscles was shaved and disinfected with 3% hydrogen peroxide at the incision sites over the bellies of the GL, TA and Sol muscles. Cholera toxin conjugated with Alexa Fluor 555 (0.01% solution in phosphate buffered saline, Molecular Probes, US), cholera toxin conjugated with Alexa Fluor 488 (0.01% solution in phosphate buffered saline, Molecular Probes, US) and Fast Blue (2% aqueous solution, Dr. Illing Plastics GmbH, Germany) were injected bilaterally to GL, TA and Sol muscles respectively, to label MNs innervating GL, TA and Sol muscles 2 weeks before spinal cord transection. The position of the needle in the muscle belly was carefully inspected throughout with the use of magnifying glasses. Injection lasted around 10 mins; the needle was left in the muscle for at least 5 min after the injection was completed in order to avoid leakage of the tracer. The injection site was cleaned and the skin sutured. The analgesic Tolfedine (tolfenamic acid 4%, 4 mg/kg, s.c.; Vetoquinol, Lure Cedex, France) was given during five postoperative days; Baytril (Enrofloxacinum 5 mg/kg; Bayer GmbH, Leverkusen, Germany) was administered over five consecutive days to prevent infection.

Thereafter plastic collars (Harvard Apparatus, Holliston, Massachusetts, US) were put on each animal to protect their wounds from licking for two days, and rats were returned to individual cages with access to food and water ad libitum.

Table 5.5. Medicines and drugs for animal surgery, post-surgery care and perfusion

Name	Producer	Action	Dose	Administration
AErrane (Izofluran)	Baxter, Lessines, Belgium	General anesthesia	3-4% - introduction 1.5-3.5% maintaining	Inhalation, mixture with oxygen
Butomidor	Richter Pharma AG, Wels, Austria	Butorphanol, 10mg/ml – premedication	3.3 mg/kg	intraperitoneal
Heparin	WZF, Polfa S. A. Poland	5000 j.m./ml - anticoagulant	0.1 ml	intracardiac, during perfusion
Xylocaine	Astra Zeneca AB, Sweden	Lidocaini hydrochloridum 2%	0.4 ml	intracardiac, during perfusion
Vetbutal	Biowet Puławy, Poland	Sodium pentobarbital 50 mg / ml - terminal anesthesia	80 mg/kg	intraperitoneal
Tolfedine	Vetoquino, Lure Cedex, France	Tolfenamic acid 4% - non-steroidal antiinflammatory drug, analgesic	4 mg/kg	subcutaneous
Baytril	Bayer Animal Health GmbH, Leverkusen, Germany	Enrofloxacin 2.5% - antibiotic	5 mg/kg	subcutaneous
Sul-Tridin 24%	ScanVet Sp. z o.o., Gniezno, Poland	Sulfadiazine 200 mg / ml trimethoprim 40 mg / ml; prevents infections	30 mg/kg	subcutaneous
Vetaflunix	VET-AGRO Sp. z o. o., Lublin, Poland	Flunixin 50 mg / ml (as flunixin meglumine) anti-inflammatory, analgesic drug	2.5 mg/kg	subcutaneous

5.2.2. Spinal cord transection

Rats were spinalized at low thoracic segments under aseptic conditions (**Figure 5.1**). The caudal thoracic vertebrae were exposed by incision of the skin and muscles with a fine scalpel. The vertebrae

were stabilized by insertion of hooks into the connective tissue and muscles around the incision. A laminectomy was performed at the level of T9/10 vertebrae. The dura was opened and lignocainum hydrochloricum (2%; Astra Zeneca AB, Sodertalje, Sweden) was applied to the surface of the spinal cord. The spinal cord was then completely transected using surgical scissors and the gap between the rostral and caudal ends was enlarged by aspiration to approximately 1 mm, washed with warm 0.9% NaCl (approximately 36°C) and dried with absorbable cellulose. The tissue surrounding the lesion area was closed with surgical sutures and the skin over the wound was closed with stainless steel staples. The antibiotic Sultridin (30 mg/kg, Norbrook, Ireland) was administered during five consecutive days and an analgesic Vetaflunix (2.5 mg/kg, subcut., VET AGRO, Poland) for five postoperative days. Immediately after the surgery, the rats were placed individually in warm cages, and inspected until fully awaken. The animals were attended for general inspection, cleaning of their body and for manual bladder expression twice daily. The animals exhibited no significant health problems for weeks after spinalization except for occasional bladder bleeding during the initial post-surgery days. Spontaneous micturition returned in the second post-surgery week.

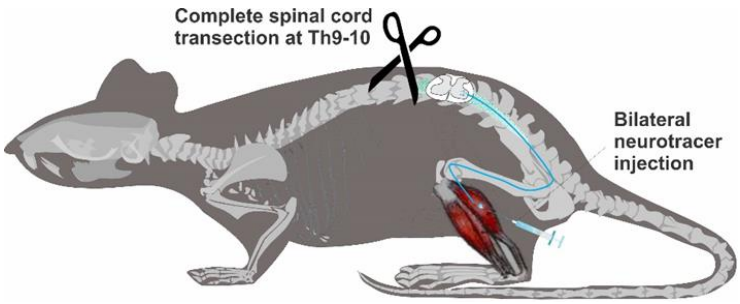

Figure 5.1. Experimental model 1: Complete spinal cord transection. Neurotracers were injected bilaterally into the ankle extensor (GL and Sol) and flexor (TA) muscles to label the respective MNs. Two weeks later, complete transection of the spinal cord was done at the spinal cord Th9-10 segments.

5.2.3. AAV-BDNF and PBS intraspinal injections

Surgical procedures (**Figure 5.2**) were performed as described (Macias et al., 2009) except that the animals were given a subcutaneous injection of butorphanol analgesic (Butomidor, Richter Pharma, Wels, Austria; 3.3 mg/kg) as a premedication and then anesthetized with Isoflurane (Baxter, Lessines, Belgium, 1–2.5% in oxygen) via facemask. The first laminectomy was performed at the thoracic (Th) 9–10 vertebrae, and the cord was completely transected at the Th9–10. The second laminectomy was

performed at the Th 11–12 vertebrae to expose the spinal cord for injections. After opening the dura and pia maters AAV-BDNF or PBS were injected via a fine glass capillary, inserted into the spinal cord bilaterally, about 0.7 mm from the midline, at 1 mm in depth. Surgical stereomicroscope Nikon SMZ 1000 was used to control positioning and movement of the capillary and avoid bending of the spinal cord. One μL of either a viral particle solution (3.6×10^8 AAV particles) or PBS were injected at a speed of 0.1 mL per minute (sp 101i syringe pump, WPI, Sarasota, FL, US). Injections were given bilaterally within half an hour after spinal cord transection. Five minutes after injection, the capillary was removed, the cut tissues were sutured, the skin over the wound was closed with sterile stainless-steel staples, and 5 mL of 0.9% NaCl was injected SC. The antibiotic Sultridin (30 mg/kg, Norbrook, Ireland) and analgesic Vetaflunix (2.5 mg/kg, SC, VET AGRO, Poland) was administered during five consecutive days. The rats were inspected daily, and bladders were voided manually.

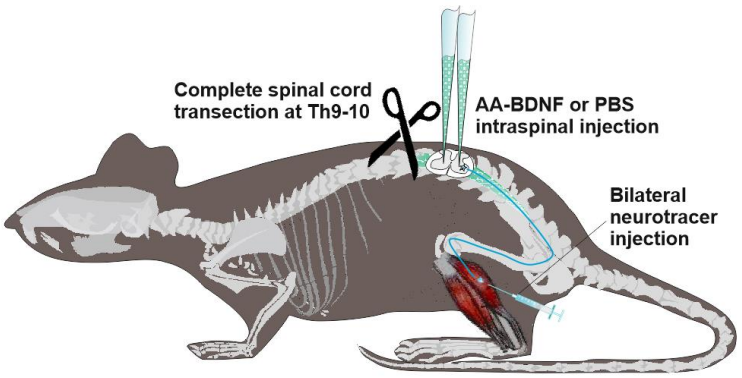

Figure 5.2. Experimental model 2: AAV-BDNF overexpression in spinal neurons. First, neurotracers were injected bilaterally into the extensor and flexor muscles. Two weeks later, complete transection of the spinal cord was done at Th9-10 segments, and AAV-BDNF or PBS was injected into L1-2 segment.

5.2.4. Evaluation of treadmill locomotion by the use of the modified BBB scale

Basso-Beattie-Bresnahan (BBB) scale for evaluation of quadrupedal locomotion, modified by Antri and co-workers (mBBB) (Antri et al., 2002) was used, as this allows for evaluation of hindlimb movements on a moving treadmill when assisted by the experimenter. Thirty-eight rats were subjected to intramuscular injections of tracers as described above. Among them, thirty animals were subjected to subsequent complete spinal cord transection together with intraspinal injections either of PBS (N=15, SP-PBS group) or the AAV-BDNF vector (N=15, SP-BDNF group) below the lesion site (see details in

Table 1 and 2). The control group consisted of eight non-lesioned rats. The locomotion of the SP-PBS and SP-BDNF rats was examined on a running treadmill.

Five days before surgery, rats were accustomed to the treadmill walking twice daily, as described previously (Macias et al., 2009; Ziemlińska et al., 2014). Examination of locomotion of spinal animals started five days after surgery. Both forelimbs and rostral trunk were placed on a platform located 1 cm above the treadmill belt, while the hindlimbs were placed on the running treadmill. An experimenter limited lateral and vertical movements of the trunk and secured the proper position of the trunk and forelimbs during testing (**Figure 3.4**). All rats were examined to walk on the treadmill running with a speed of 0.05 m/s during two 2 min sessions with 1 min break in home cages. All treadmill walking sessions were recorded with a digital camera (Panasonic NV- GS400) at 25 frames/s. The camera was placed perpendicularly to the longitudinal axis of the rat's body, so that the side views of the left and right hindlimb (1 min each) were recorded during session. To monitor the timeline of motor recovery after BDNF or PBS treatments, locomotion of the rats was examined at the 5^{th}, 8^{th}, 11^{th}, 13^{th} days post-lesion, and in the SP-BDNF group this examination was additionally performed at 14^{th}, 15^{th}, 16^{th}, 17^{th} days (**Table 6.2**). The locomotion sessions were followed by 1 min observation in the open-field. This timetable has been chosen as in our previous study it was shown that BDNF overexpression in the lumbar segments improved locomotor performance of spinalized rats on a running treadmill at early time point (~2 weeks) with slower but unstable improvement observed till 6–7 weeks. At this late time point, BDNF treated rats displayed increased frequency of clonic movements which attenuated the quality of locomotor movements (Ziemlińska et al., 2014). Testing days of each rat are specified in Table 2.

5.2.5. Tissue preparation and Laser Microdissection (LMD)

Rats were deeply anesthetized with a lethal dose of Morbital (pentobarbital 120 mg/kg, Biowet Puławy Ltd., Poland) and perfused transcardially with 250 ml ice-cold 0.01 M PBS ([in mM] 154 NaCl, 1.3 Na_2HPO_4, 2.5 NaH_2PO_4, pH 7.4). The vertebral column was excised, placed on ice. The spinal cord lumbar (L) 3-6 segments were rapidly dissected in a cold room, put immediately in dry ice-precooled tubes and stored in -80 ˚C until sectioning.

The frozen L3-6 segments were surrounded by Jung tissue-freezing medium (Leica, Nussloch, Germany, cat no 14020108926) and cut into 25 µm thick longitudinal sections on the cryostat (Leica

CM1850) at -20˚C. The sections were mounted onto RNase-free PET-Membrane Frame Slides (Leica, No. 11505190), 4 sections/slide. Slides were put immediately to the dry ice precooled box and stored at -80 ◦C until processing for LMD. Prior to LMD, slides were dehydrated with ethanol in the following order: 70%, 80%, 90% and 100% ethanol, 30s each, followed by 100% ethanol (fresh) for 30s. After dehydration sections were cleared in 100% xylene washes (1x for 30s, 1x for 180s) and air dried for 3-5 mins. Leica Laser Microdissection system LMD7000 was used to isolate the MNs (**Figures 5.3, 5.4**).

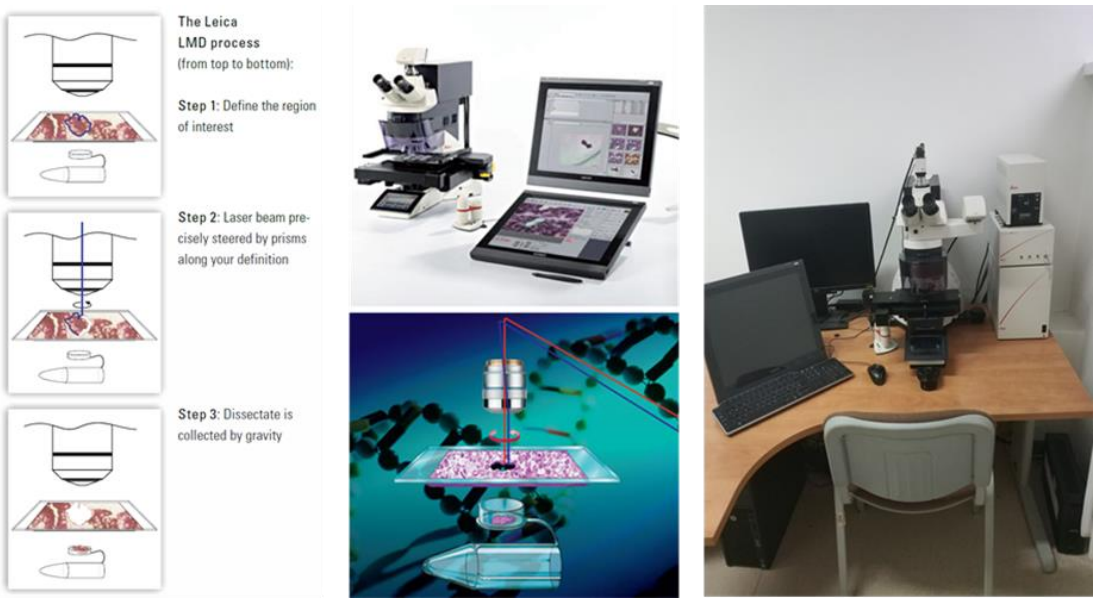

Figure 5.3. Laser Microdissection (LMD) set-up. Stages of: selection of the area of interest, cell isolation and transfer are shown on the left.

For LMD the RNase-free caps of 0.2 ml tubes were put on the tube holder for sample collection and filled with 20 µl extraction buffer (from Arcturus™ PicoPure™ RNA Isolation Kit, KIT0204, Applied Biosystems). The air-dried slide was placed on the slide holder of the LMD 7000 system. Under LMD system microscope (objectives x10, 0.32 NA and x63, 0.7 NA) attached to the software unit, MNs were identified, selected and then cut with UV laser. After the collection was complete for one slide, the microfuge tube was removed from the holder. Collected MNs were lysed by incubating the sample in the buffer for 30 mins at 42 ◦C. The lysates were spinned down for 2 mins at 800 x g to collect them at the bottom of the tubes. The sample tubes were immediately frozen by placing them on dry ice and then stored at -80˚C. To limit RNA degradation, samples were collected for up to 60 mins per slide.

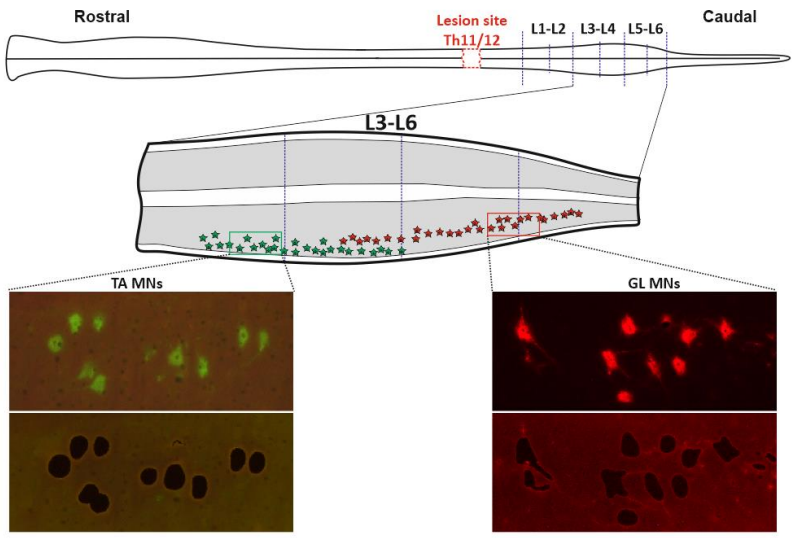

Figure 5.4. **Laser microdissection procedures.** Upper panel: scheme of the spinal cord with the lesion site (marked in red) at the Th 11/12 segments, and lumbar segments under study; the distribution of GL MNs (red) and TA MNs (green) in L3-6 is shown on the diagram enlargement (middle panel). Lower panel: representative photomicrographs showing GL and TA MNs before LMD and the tissue section remaining after microdissection. The figure is modified from our study (Grycz et al., 2019).

5.2.6. RNA isolation from microdissected MNs and qPCR

The total RNA from the GL and TA MNs was extracted using Arcturus PicoPure™ RNA Isolation Kit (Thermo Fisher Scientific, No. KIT0204) according to the manufacturer's instructions. RNA was then pre-amplified and reverse-transcribed using a QuantiTect® Whole Transcriptome kit (Qiagen, No.207043, 207045). The final concentration of the cDNA was determined by utilizing Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, No. P7589). RT-qPCR was used to analyze gene expression level with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control gene. The RT-qPCR reactions were performed in a 20 µl reaction mixture containing 7.4 µl PCR grade H_2O, 10 µl LightCycler 480 Probes Master solution (Roche, cat no. 04887301001) master, 0.4 µl forward primer (20 µM) of target gene, 0.4 µl reverse primer (20 µM) of target gene, 0.4 µl probe (10 µM) of target gene, 0.1 µl forward primer (20 µM) of GAPDH gene, 0.1 µl reverse primer (20 µM) of GAPDH gene, 0.2 µl probe (10 µM) of GAPDH gene, 1 µl diluted cDNA sample. Probes and primers are listed in **Table 5.6**. Roche LightCycler 96 was used to run the reactions with the following thermal cycling profile: preincubation at 95°C for 10 minutes, 65 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 10 seconds. Data were analyzed by using

the LightCycler software. The $2^{-\Delta\Delta Ct}$ method was used for relative quantification of gene expression level based on the target and reference genes' Ct (cycle threshold) value, the cycle number at which the amplification curve reaches the threshold line.

Table 5.6. List of probes and primers

Gene symbol	Name of coded protein	Accession number	Roche UPL No.	Forward primer (5'–3')	Reverse Primer (5'–3')	Amplicon length/nt
Gria1	AMPAR GluA1	NM_031608.1	69	tcccttgaccataaccttgg	ctcatgaatggcttggagaa	61
Gria2	AMPAR GluA2	NM_017261.2; NM_001083811.1	67	ggaggtgattccaaggaaaag	cccccgacaaggatgtaga	77
Gria3	AMPAR GluA3	NM_032990.2; NM_001112742.1	94	ccaagaatgcaccactgaag	ctgcgggagacatccact	109
Gria4	AMPAR GluA4	NM_017263.2; NM_001113184.1; NM_001113185.1	25	ggctaccattatatcatcgcaaa	ggtgtattaaaatctaccaactggaat	110
Grin1	NMDAR GluN1	NM_001270602.1	95	cctacacagctggcttctacag	cgaaggaaactcaggtggat	94
Grin2a	NMDAR GluN2A	NM_012573.3	94	cgtcatggtctccaggagtaa	cactgaagggttcgaggaaa	62
Grin2b	NMDAR GluN2B	NM_012574.1	106	tcctgcagctgtttggagat	gctgctcatcacctcattctt	95
Grin2c	NMDAR GluN2c	NM_012575.3	94	ggcactcctgcaacttctg	gttctggcagatccctgaga	76
Grin2d	NMDAR GluN2d	NM_022797.1	25	acatggtgcgatacaaccag	tgaaggcgtccagtttcc	75
Gabra1	$GABA_AR$ α1	NM_183326.2	129	tgacagtcattctctcccaagtc	tcagaacggtcgtcactcc	87
Gabra2	$GABA_AR$ α2	NM_001135779.2	94	gacagacttctggatggttaca	ggtcacatagatgttggtggag	87
Gabra3	$GABA_AR$ α3	NM_017069.3	129	cctacttgccatgtatcatgact	ggtcatggtgagaacagtggtg	113
Gabrb3	$GABA_AR$ β3	NM_017065.1	80	atcgagctcccacagttctc	tcaatgagagtcgagggtagg	91
Gabrg2	$GABA_AR$ γ2	NM_183327.1	75	ttgaagtgggagacacaaggt	cggacataaccacatagtcacc	111
Glra1	GlyR α1	NM_013133.1	69	ccggcaacacaaggaact	tctccaccctcatcatcctt	69
Glrb	GlyR β	NM_053296.1	29	ggatctattcaagagacaacaatgg	gcttgagtctggggtcgtt	79
Slc12a4	KCC1	NM_019229.2	101	acagagcgggatcgagag	ctctggtccatgtcatctgg	130
Slc12a5	KCC2	NM_134363.1	75	ttctggacaaccaccctca	ggcagaagcccttcacct	84
Slc12a6	KCC3	NM_001109630.1	124	gcgtcttacctgggtagtgg	tggagatggcagttaacattgta	95
Scn1a	Nav1.1	NM_030875.2	67	agaaccaggccacattggag	gttctgatgctgttgctgcc	120
Scn3a	Nav1.3	NM_013119.2	80	acagcgagagcaggagaga	cgtttcagtggtggtaccgt	83

Scn8a	Nav1.6	NM_019266.3	67	agagcaaaaagaggccgagt	tcttcaatggcgtcttccga	120
Scn9a	Nav1.7	NM_133289.2	129	ggtcatggtgattgggaacc	ttgtttgcatcggtgtcttcc	111
Cacna1b	Cav2.2	NM_001195199.1 NM_147141.1	73	cggcactggagatcaagc	gcagattagcccacacagaag	96
Cacna1d	Cav1.3	NM_001389225.2	69	ccctttatccaccctgcgatg	agattcttcgaggacgggga	98
Kcnn1	SK1 (K_{Ca}2.1)	NM_019313.1	124	tcggaaacaccagcgtaagt	cttcacagtccggagcttct	64
Kcnn2	SK2 (K_{Ca}2.2)	NM_001309404.1; NM_019314.2	113	gcgttttgttatgaagactttaatga	gtgacatcctgttgatcatggta	132
Kcnn3	SK3 (K_{Ca}2.3)	NM_019315.2	69	ttcagggctcccaattca	atttagctggctgccttgc	72
Kcnma1	KCa1.1	NM_031828.1	56	cctcacacagcccttttgc	gggtgaggatattgtcattgaag	95
Grm1a	mGluR1A	NM_017011.1	101	caccgaagaagatgaattgga	gaatcctcaggggtcagctt	69
Grm5	mGluR5	NM_017012.1	82	tccagcagcctagtcaacct	gattttccgttggagcttagg	71
Chrm2	M2R	NM_031016.2	64	ccaccttcagactgtcaacaatta	catggagaaaacacctatgatgag	76
Htr1a	5-HTR1A	NM_012585.1	113	gggcaactccaaagagcac	cgggggcataggagttagat	62
Htr2a	5-HTR2A	NM_017254.1	69	agcactcgagccaaactagc	tggaagagcttttctgatgaca	71
Htr2b	5-HTR2B	NM_017250.2	129	cgcggtaataatccccacca	ccaccgccaaggacattaga	110
Htr2c	5-HTR2C	NM_012765.3	69	aagaaggtgttcgttggcct	ccgtttctcgtctagctgcc	106
Htr3a	5-HTR3A	NM_024394.2	65	ggctaactacaagaagggagtgc	accatatgtaggtggtcagaacc	131
Adra1a	NAα1A	NM_017191.2	50	gcgtctgggtgctttctttg	ggctcctcattgatctggca	100
Adra1b	NAα1B	NM_016991.2	129	ttggctccccttcttcatcg	gagcacgggtagatgatggg	135
Adra1d	NAα1D	NM_024483.2	129	tgtgctggttccccttcttc	agcgggttcacacagctatt	124
GAPDH	GAPDH	NM_017008.4	*	ctgcaccaccaactgcttag	tgatggcatggactgtgg	92

*sequence: tttggcatcgtg. Synthesized by Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

5.2.7. Immunofluorescence

Obtaining the tissue material for immunofluorescence analysis

Rats were deeply anesthetized with a lethal dose of sodium pentobarbital administered intraperitoneally. After confirming that the rat was unresponsive to pain stimuli, the abdominal wall was cut, the chest opened and 0.5 ml of a mixture of heparin (anticoagulant) and xylocaine (4: 1) was injected intracardially. One minute later, the aorta was cannulated, the right atrium was incised and the animal was perfused with an IKA peristaltic pump (the protocol and solutions used are shown in **Table 5.7, 5.8**).

Table 5.7. List of solutions used for perfusion and perfusion parameters

Type of solution	volume	Pumping speed	Perfusion time
Perfusion buffer	250ml	100 ml/min	2.5 min
4% paraformaldehyde (PFA) solution in 0.2M PB	150ml	100 ml/min	1.5 min
4% PFA in 0.2M PB	300ml	22 ml/min	15 min

Table 5.8. Chemical composition of solutions used for animal perfusion and tissue preparation

Name	Composition
0.2 M PB pH 7.4 phosphate buffer (A+B)	(A) 0.2 M NaH_2PO_4 x 2H2O (B) 0.2 M $NaHPO_4$
0.01 M Phosphate Buffered Saline (PBS) pH 7.4	10 mM PB pH 7.4 150 mM NaCl 3.7 mM KCl
0.01 M Phosphate Buffered Saline with 0.2% Triton X-100 (PBST) pH 7.4	PBS, pH 7.4 0.2% Triton X-100
Saline solution in phosphate buffer for animal perfusion	10 mM PB pH 7.4 140 mM NaCl 4.8 mM KCl 6 mM $NaHCO_3$
Tissue cryoprotection solution	10, 20 and 30% sucrose in 0.1M PB 0.1% Thimerosal
Antifreeze solution (for storing tissue sections in the freezer)	50 g sucrose 100 ml glycerol 42 ml 0.2M PB 125 ml H2O
Mowiol solution (medium for sealing slides)	48 ml 0.2M Tris-HCl pH 8.5 24 g glycerol 9.6 g Mowiol (4-88) 24 ml dH2O

Table 5.9. List of antibodies and reagents for immunofluorescence

Primary antibodies (1°Ab)			
Antigen	**Host**	**Dilution**	**Company**
Anti-GluA1	Rabbit	1:100	Abcam, Cambridge, UK (ab15303)
Anti-GluA2	Goat	1:200	Santa Cruz Inc., Dallas, Texas, US (sc-7610)
Anti-GluN1	Goat	1:100	Santa Cruz Inc., Dallas, Texas, US (sc-1467)
Anti-GluN2A	Rabbit	1:500	Alomone Labs, JerUSlem, Israel (AGC-002)
Anti-VGluT1	Mouse	1:1000	Synaptic Systems GmbH, Göttingen, Germany (135 011)
Anti-Synaptophysin	Guinea pig	1:1000	Synaptic Systems GmbH, Göttingen, Germany (101 004)
Secondary antibodies (2°Ab) and fluorochromes			
Antigen	**Host**	**Dilution**	**Company**
Anti-Goat (AF488)	Donkey	1:500	Jackson IR, West Grove, PA, United States (705-546-147)
Anti-Goat (AF647)	Donkey	1:500	Invitrogen, Inc., Waltham, US (A32849)
Anti-Rabbit (AF555)	Donkey	1:500	Invitrogen, Inc., Waltham, US (A32794)
Anti-Rabbit (Cy3)	Donkey	1:800	Jackson IR, West Grove, PA, US (711-167-003)
Anti-Guinea pig (AF647)	Donkey	1:500	Jackson IR, West Grove, PA, United States (706-605-148)
Anti-Guinea pig (CF405S)	Donkey	1:500	Biotium, Inc., Fremont, CA, US (20356)
Serum			
NDS (Normal Donkey Serum)			Jackson IR, West Grove, PA, United States (017-000-121)

The isolated blocks of spinal cord segments, dorsal ganglia, tibial nerve, and the soleus and gastrocnemius muscles were further fixed in a 4% paraformaldehyde solution in 0.1 M PB for approximately one hour at 4° C. For cryoprotection, the material was placed for several days in buffered sucrose solutions of increasing concentrations (**Table 5.8**) at 4° C, until they were saturated. The tissue was stored in a 30% sucrose solution at 4° C.

Preparation of spinal cord sections

Lumbar segments of the spinal cord were placed in plastic tubs filled with Tissue-freezing Medium (Leica Microsystems GmbH, Wetzlar, Germany) and immersed in cooled heptane (-80° C) for quick freezing. The plastic tubs were then stabilized for 1 hour at -18° C. The blocks were transferred to the cryostat chamber and fixed on the specimen disc. Transversely cut sections, 25 µm thick, were stored as free-floating in the anti-freeze solution (**Table 5.8**) at -18ºC.

Immunofluorescence detection of proteins

Transverse sections of the spinal cord were used to analyze the localization and measure the level of immunofluorescence-labeled proteins. Protein localization was identified by means of an indirect reaction, using two antibodies: primary (1° Ab), detecting the tested antigens, and secondary (2° Ab), coupled with a specific fluorochrome, recognizing the primary antibody.

A specificity control of the reaction was performed omitting the primary antibodies in the incubation medium, to test if there is a nonspecific binding of the secondary antibodies to the tissue. A single, double or triple labeling with primary antibodies was used to detect antigens that identify different proteins and to assess their co-localization. The actual reactions were preceded by test reactions with single and triple labeling. Comparison of the results of these reactions showed that the simultaneous triple labeling did not impair the intensity of the reaction products and did not result in nonspecific binding of the antibodies. The protocol for the IF reactions performed is shown in Table 5.10.

Table 5.10. Immunohistochemical procedure

Action	Buffer	Time/ Temperature
1. Washing	PBS-T	3 x 5 min
2. Blocking	5% NGS w PBS-T	60 min / RT
3. 1°Ab	3% NGS w PBS-T	overnight / 4°C
4. Washing	PBS-T	3 x 5 min
5. 2°Ab	PBS-T	60 min / RT
6. Washing	PBS-T	2 x 5 min
7. Washing	PBS	2 x 5 min
8. Applying sections to the slides		
9. Drying	–	~ 5-10 min, RT
10. Mounting	Mowiol	

Confocal microscopy: image acquisition and selection of optical parameters

The Zeiss LSM780 confocal microscope was used to collect images of IF reactions. The PL APO 63x / 1.4 Oil DIC lens was used. Stacks of digital images with dimensions of 2048 × 2048 pixels, with a pixel size of 0.05 μm, sampled in the Z axis every 0.15 μm were collected. Since the perikarya of α-MNs are large, there is usually part of MN in tissue sections of the core 25 μm thick. Typically, the

collected images consisted of a stack of about 64 scans, which corresponds to MN fragment of about 13 μm in the Z axis. An argon and UV laser was used to induce fluorescence. Constant exposure parameters were used for each of the four channels in which the labeling was recorded.

5.2.8. Subcellular fractionation and Western Blot

Subcellular fractionation

The fractionation procedures referred to prior work with mouse and rat spinal cord tissue. Spinal cord samples were homogenized using an electric homogenizer (Polytron homogenizer PT 1200E, Table 5.2) in 20 volumes of lysis buffer. Homogenates were centrifuged at 5,000 x g for 5min at 4° C to generate a supernatant (S1) and a nuclear pellet (P1). The supernatant (S1) was collected into a new tube and centrifuged again at 13000 x g for 30 min at 4°C to produce a supernatant (S2) and a modestly synaptoneurosomal-enriched pellet fraction (P2). The S2 was collected into a new tube and the P2 was resuspended in 50μL of PBS containing protease and phosphatase inhibitor with vortexing and sonicating. All sample fractions were divided into two portions. One portion was used for measuring total protein concentration by Bradford method which will be described below; the other portion was stored at -80° C until used for Western Blot.

Measurements of total protein concentration with Bradford method

The total protein concentrations of the spinal cord subcellular fractions were measured by a modified Bradford method. The assay was carried out in 10mm Eppendorf Cuvettes using Eppendorf BioSpectrometer® basic (**Table 5.2**) according to the protocol from the Bradford Protein Assay kit (Bio-Rad, Munich, Germany). First, 100μL aqueous BSA solutions (in concentrations: 0; 20; 40; 60; 80 and 100μg/ml; 0 was used as blank) were prepared in duplicate to prepare the standard curve. In the meantime, two dilutions of experimental samples in 100 were prepared in ddH_2O. Then, 1mL Bradford working solution (**Table 5.11**) was added into each tube and mixed well with gentle pipetting.

Table 5.11. Solutions used for subcellular fractionation, Western Blot and protein assay

Type of solution	**Formulation**
Lysis buffer	10mM Tris-HCl (pH 7.5) 320 mM Sucrose in H_2O (containing Roche Protease and Phosphatase Inhibitor Inhibitor Cocktail, Roche)
Loading Buffer 5x - for loading samples	62 mM Tris-HCl pH 6.8 10% (w/v) SDS 5% (w/v) β-Mercaptoethanol 50% (v/v) glicerol 0.13% (w/v) bromophenol blue
Bradford's reagent A (Determination of protein concentration)	100 ml of 95% ethanol 200 ml of 88% orthophosphoric acid 350 mg of Serva Blue G
Bradford working solution B (Determination of protein concentration)	425 ml of dH2O 15 ml of 95% ethanol 30 ml of 88% orthophosphoric acid 30 ml Bradford Reagent A
Solution A - component of both polyacrylamide gels - denaturing electrophoresis (SDS-PAGE)	30% acrylamide 0.8% bis-acrylamide
Solution B - component of the resolving polyacrylamide gel - denaturing electrophoresis (SDS-PAGE)	1.5 M Tris-Cl pH 8.8 0.4% SDS
Solution C - component of the stacking polyacrylamide gel - denaturing electrophoresis (SDS-PAGE)	0,5 M Tris-Cl pH 6.8 0.4% SDS
10% ammonium persulfate (APS)	10% APS w dH_20
7.5% (1.5 mm thick) resolving gel - SDS-PAGE	2.5 ml solution A 2.5 ml solution B 5 ml dH_20 50 µl 10% APS 10 µl TEMED
5% (1.5 mm thick) stacking gel - SDS-PAGE	0.67 ml solution A 1ml solution C 2.3 ml dH_20 30 µl 10% APS 5 µl TEMED
Electrophoresis buffer - SDS-PAGE (1x)	25 mM Tris-base 192 mM glycine 0.1% SDS
Buffer for electrotransfer (1x)	25 mM Tris-base 192 mM glycine 0.01% SDS
Solution 0.02M TBST	20 mM Tris-HCl pH 7.4 150 mM NaCl 0.01% Tween 20
5% BSA solution - to block the PVDF membrane to prevent non-specific antibody binding	5% BSA in TBST pH 7.4
5% BSA solution - for 1° Ab dilution	5% BSA in TBST pH 7.4

After incubation for 5 minutes at RT, the absorbance of the samples was spectrophotometrically measured at wavelength 595nm using Eppendorf BioSpectrometer® basic (**Table 5.2**). Concentrations of the experimental samples were calculated based on the standard curve. To keep the reliability of the results, BSA standard curve was prepared for each measurement.

Table 5.12. Reagents used for subcellular fractionation and Western Blot techniques

Reagent	Application	Company
Spectra™ Multicolor Protein Ladder	Molecular weight standard (10–260 kDa)	Thermo Scientific, Inc., Waltham, US
ECL™ Western Blotting Detection Reagents	Detection of proteins by WB method	GE Healthcare UK Ltd, Little Chalfont, UK
Bovine Serum Albumin (BSA)	Component of buffers	Sigma-Aldrich, St. Louis, MO, US
Complete ULTRA	A set of protease inhibitors	Roche Applied Science, Penzberg, Germany
PhosSTOP	Set of phosphatase inhibitors	Roche Applied Science, Penzberg, Germany

Table 5.13. Antibodies used to identify proteins by Western Blot technique

Antigen	Host	Dilution	Company
Anti-GluA2	Goat	1:1000	Santa Cruz Inc., Dallas, Texas, US (sc-7610)
Anti-GluN1	Goat	1:1000	Santa Cruz Inc., Dallas, Texas, US (sc-1467)
Anti-β-actin	Mouse	1:1000	Santa Cruz Inc., Dallas, Texas, US (sc-47778)
Anti-Goat (HRP)	Rabbit	1:2500	Invitrogen, Inc., Waltham, US (31402)
Anti-Mouse (HRP)	Goat	1:3000	Bio-Rad Inc., Hercules, California, US (170-5047)

SDS-PAGE

The polymerized gels (1.5 mm thick) consisting of 7.5% Separation gel and 5% stacking gel were prepared according to the protocol in **Table 5.11**. The border between separation gel and stacking gel was kept around 1 cm away from the lower edge of the comb. The Mini-Protean III apparatus (**Table 5.2**) was assembled by placing the prepared polymerized gels in the electrophoresis apparatus and filling the apparatus with ice-cold electrophoresis running buffer (**Table 5.11**). Subcellular fractions prepared and stored at -80° C in fractionation procedure were boiled for 5 minutes at 100° C in 1× concentrated

sample loading solution containing SDS (**Table 5.11**). For SDS-PAGE, around 10-30μg total protein in each sample was loaded into the gel well. The spectra multicolor broad range protein ladder (**Table 5.12**) was used as the molecular weight standard for proteins. One sample was repeated on all gels and used as an internal standard. After the electrophoresis instrument was set to a stable voltage state, the electrophoresis was started at voltage 90 V. After the sample passed through the stacking gel, the voltage was adjusted to 120 V. The electrophoresis was ended when the bromophenol blue reached the bottom of the gel.

Protein transfer to PVDF membranes

(1) Cut a suitable sized PVDF membrane (cut a corner to make a mark) which can fully cover the gel. In order to increase the hydrophilicity of the PVDF membrane, the membrane was soaked in 100% methanol for 1 min and then stabilized in the pre-cooled transfer buffer (min. 30 min). The Mini Gel Holder Cassette containing filter paper and sponge was also soaked in the pre-cooled transfer buffer in a square tray.

(2) After electrophoresis, the upper (stacking) gel was removed. One corner of the separation gel was cut to mark it, then gel was carefully peeled off from the glass plate, and placed in the pre-cooled transfer buffer.

(3) Mini Gel Holder Cassette was opened in the pre-cooled transfer buffer and the cassette was assembled in the order from bottom to top: the sponge on the black side (cathode) → 3 layers of filter paper → gel → PVDF membrane → 3 layers of filter paper → sponge → the white side of the cassette (anode). When place each layer, a glass coated rod was used to drive off the air bubbles. Then the cassette was closed and inserted into the electrode holder in the transfer electrophoresis tank filled with pre-cooled transfer membrane buffer. The transfer electrophoresis tank was surrounded in ice to keep low temperature. The transfer was carried out at a constant voltage of 60 V for 2 h.

Antigen detection

After transfer, the PVDF membrane was stabilized for 5-10 minutes in TBST buffer (**Table 5.11**). In order to detect proteins with different molecular weights in the same membrane, the PVDF membrane was cut into strips based on the molecular weights of the target proteins using as a reference the bands of molecular weight standards.

1. Blocking: in order to block non-specific binding sites on the membrane, the membrane was incubated in a 5% BSA solution in TBST for 1 hour at RT, with the side which was adjacent to the gel facing up.

2. Primary antibody (Ab) incubation: the antibodies that specifically recognized the epitopes of the target protein, as tested in the preliminary assays, were used (**Table 5.13**). The membrane was incubated in a Ab solution suspended in 2.5% BSA in TBST at 4° C overnight.

3. Secondary Ab incubation: after incubation with primary Ab, the membrane was washed 3 times with 1×TBST for 10 mins each time. Next, the membrane was incubated at RT for 1 hour with a secondary Ab solution (HRP conjugated) (**Table 5.13**) suspended in TBST buffer.

4. HRP-ECL autoradiography: after incubation with secondary Ab, the membrane was washed 3 times with TBST, 10 mins each. ECL solution (GE Healthcare, **Table 5.12**) was used to detect labeling according to its instructions. The chemiluminescent signal was recorded on an X-ray film or Fluor-S® MAX MultiImager (Bio-Rad). The membranes were exposed several times to optimize the signal.

5.2.9. Statistical analysis

The mean and standard deviation (SD) or standard error of the mean (SEM) were calculated for each data set. The Grubbs test of outliers was carried out for all data and outliers were discarded (https://www.graphpad.com/quickcalcs/Grubbs1.cfm). Statistical significance was accepted at $P \lesssim 0.05$. The STATISTICA 13.1 software (StatSoft Inc, Tulsa, OK, US) was used to analyze data. The following tests were used: the Shapiro-Wilk test to verify normality of distribution of all data; the Levene's test to verify homogeneity of variance in the experimental groups. Mann-Whitney U-test was used for comparisons of two independent groups of samples throughout the study, because normality of data distribution was not met in case of some data. Wilcoxon test for dependent samples were used for comparisons of data from TA and GL MNs within groups. The scatter plot and bar graphs were drawn by using GraphPad Prism 9.2.0 software (trial version) and CorelDraw software.

6. Results

6.1. Molecular identification of pro-excitogenic receptor and channel phenotypes in the lumbar MNs in after SCT

6.1.1. SCT at low thoracic level does not cause degeneration of MNs at lumbar 3-6 segments

GL and TA MNs were visualized under LMD microscope in fluorescence mode, as shown in **Figure 6.1A** (enlargement from **Figure 5.4)**. GL MNs were located within the gray matter of the lumbar L4-6 segment, whereas majority of TA MNs were located in L3-4 segment, consistent with other studies (Nicolopoulos-Stournaras and Iles, 1983; Skup et al., 2012; Chopek et al., 2015; Mohan et al., 2015).

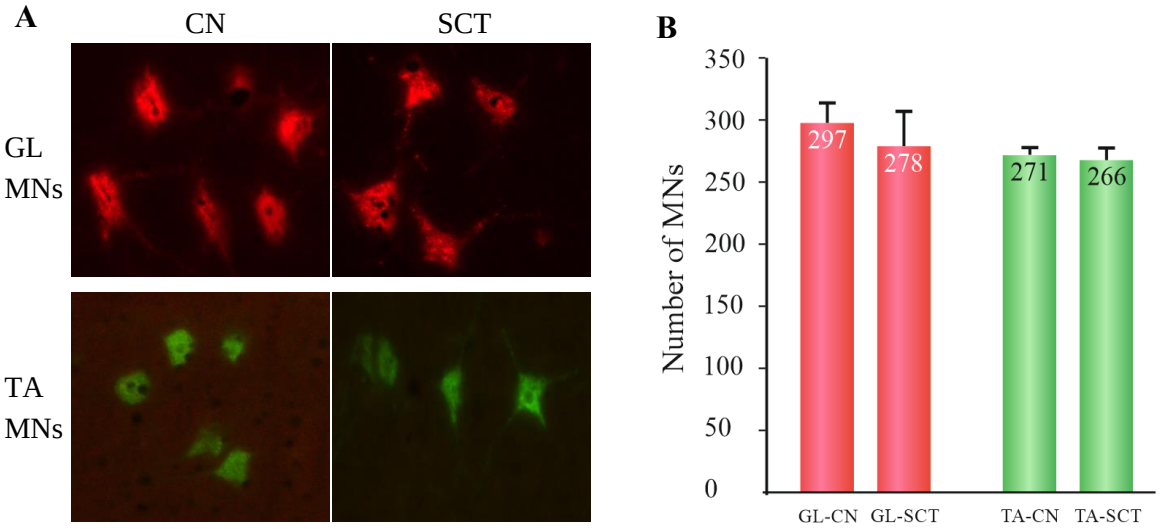

Figure 6.1. The morphology (A) and number (B) of GL and TA MNs isolated from the intact (CN) and spinalized (SCT) rats. A: representative photomicrographs of lumbar sections with traced GL and TA MNs show that the morphology and size of MNs is comparable between groups. B: the number of GL and TA MNs isolated from the CN and SCT groups of rats. Data are means +/- SEM. Differences between groups are non-significant (Mann-Whitney U Test). Nine rats per group were examined.

One profound feature of SCI is neuronal loss at and around the injury site. At the injury epicenter of severe SCI, cells die by necrosis or apoptosis (Li et al., 1996; Byrnes et al., 2007; Ling et al., 2013; Hassannejad et al., 2018). Significant loss of MNs has been observed by 48 h postinjury at 4.0 mm from the epicenter (Ling et al., 2013). Since in the present study the spinal MNs located at 13-15 mm from Th11 injury epicenter were the subject of gene expression analysis, it raised the necessity to identify whether neuron loss or shrinkage which may affect the collection of the material and transcript levels occurs. Therefore, during microdissection, the numbers of traced GL and TA MNs were counted on each

horizontal L3-6 section (**Figure 6.1A**). The results showed that the number of MNs of a given type was comparable between intact and spinalized rats (GL: SCT = 94% CN, p = 0.53; TA: SCT = 98% CN, p = 1.00; Mann-Whitney U Test).

In the spinal group the tracer deposits filled the cell bodies and the dendritic processes of MNs indicating that tracer transport to MNs and its maintenance were not impaired as compared to the control group. In addition, the morphology of the MNs perikarya in the lumbar segment was similar between CN and SCT rats, and no labeled apoptotic profiles or shrunken neurons were found in sections from SCT rats (**Figure 6.1B**). Thus, these results strongly suggest that complete SCT at thoracic level did not cause degeneration of MNs located at distal lumbar L3-6 segment.

6.1.2. Transcription rate of receptor and ion channel subunits in GL and TA MNs of the intact rats

In the present study, thirty-four genes (**Table 5.6**) were analyzed. The selected genes encode the major subunits of the excitatory (AMPA, NMDA), inhibitory ($GABA_A$, Gly), modulatory (mGlu, mACh, 5-HT, and NA) receptors, ion channels (Na^+, Ca^{2+} and K^+) and K^+ - Cl^- cotransporters (KCCs).

In intact rats, all groups of genes showed similar expression pattern in the GL and TA MNs (**Figure 6.2**). Among them, genes encoding for majority of subunits of inhibitory receptors: *Gabrb3* ($GABA_A$ β3), *Gabrg2* ($GABA_A$ γ2), *Glra1* (Gly α1), *Glrb* (Gly β), and *Kcnma1* (KCa1.1) channel demonstrated the highest transcript level, followed by *Scn8a* (Nav1.6), *Scn1a* (Nav1.1), *Grin2a* (GluN2A), *Grin2b* (GluN2B), *Kcnn2* (SK2), and *Gabra2* ($GABA_A$ α2) and *Gabra3* ($GABA_A$ α3) receptor subunits.

The remaining sets of genes displayed relatively low transcript levels in both pools of examined MNs. Among receptors, the subunits which combine to form subclasses of inhibitory $GABA_AR$ and GlyR were more highly expressed than subunits of other neurotransmitter receptors and ion channels, both in GL and TA MNs.

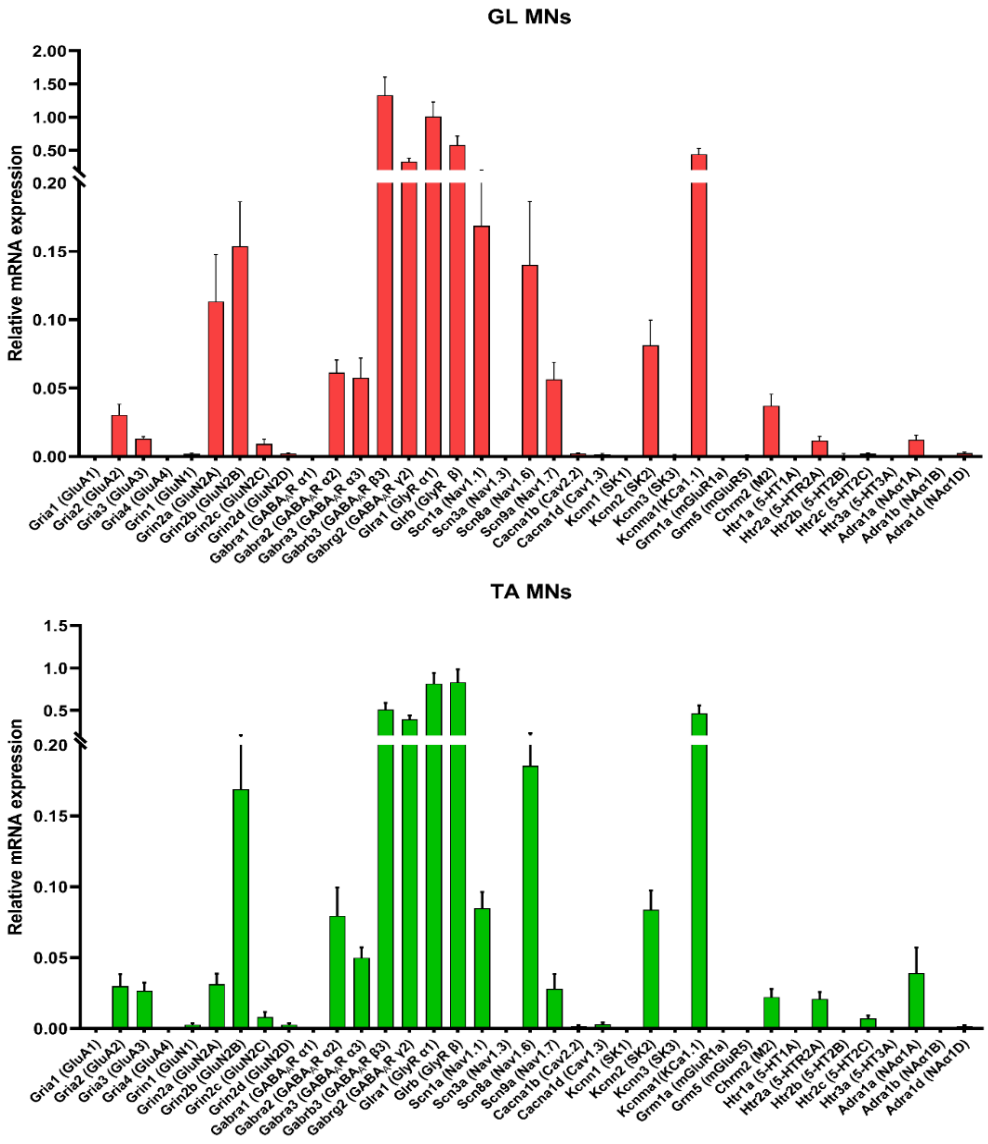

Figure 6.2. Comparison of transcript levels of genes encoding major subunits of ionotropic excitatory (AMPA and NMDA), inhibitory ($GABA_A$ and Gly) and neuromodulatory (mGlu, 5-HT, and NA) receptors, ion channels (Na^+, Ca^{2+} and K^+) and K^+-Cl^- cotransporters KCCs in pools of MNs innervating Gastrocnemius lateralis (GL) and Tibialis Anterior (TA) muscles in intact rats. All groups of genes showed similar expression pattern in GL and TA MNs. N = 9 rats.

6.1.3. SCT differentially alters transcript level of subunits of glutamatergic AMPA, NMDA and mGluRs receptors

AMPARs are mainly tetramers consisting of GluA1, GluA2, GluA3, and GluA4 subunits, encoded by *Gria1*, *Gria2*, *Gria3* and *Gria4* genes, respectively (Hollmann and Heinemann, 1994; Diering and Huganir, 2018). As described in the Introduction, GluA2 undergoes a Q/R editing which alters a codon encoding glutamine (Q) to a codon encoding arginine (R) in GluA2 mRNA, rendering GluA2 virtually Ca^{2+} impermeable (Puchalski et al., 1994). Consequently, GluA2-containing AMPAR is impermeable to

Ca^{2+}, while GluA2-lacking AMPAR is Ca^{2+} permeable. In the brain, in the basal physiological state the vast majority of AMPARs consist of GluA2/GluA3, GluA1/GluA2 subunit combinations, with a small population of GluA1/GluA1 homomers (Diering and Huganir, 2018). Based on the quantitative comparisons our findings suggest that the same relationship takes place in the lumbar spinal cord motoneurons: *Gria2* (GluA2) and *Gria3* (GluA3) transcript levels were much higher than *Gria1* (GluA1) and *Gria4* (GluA4) transcript levels (**Figure 6.3**), suggesting that GluA2/GluA3 is the major subunit combination of AMPAR in GL and TA MNs. The expression level of *Gria4,* much lower than of the other subunits of AMPAR, is consistent with observation that *Gria4* is primarily expressed in early postnatal development, while *Gria1-3* are expressed in most neurons in the brain in the adulthood (Diering and Huganir, 2018).

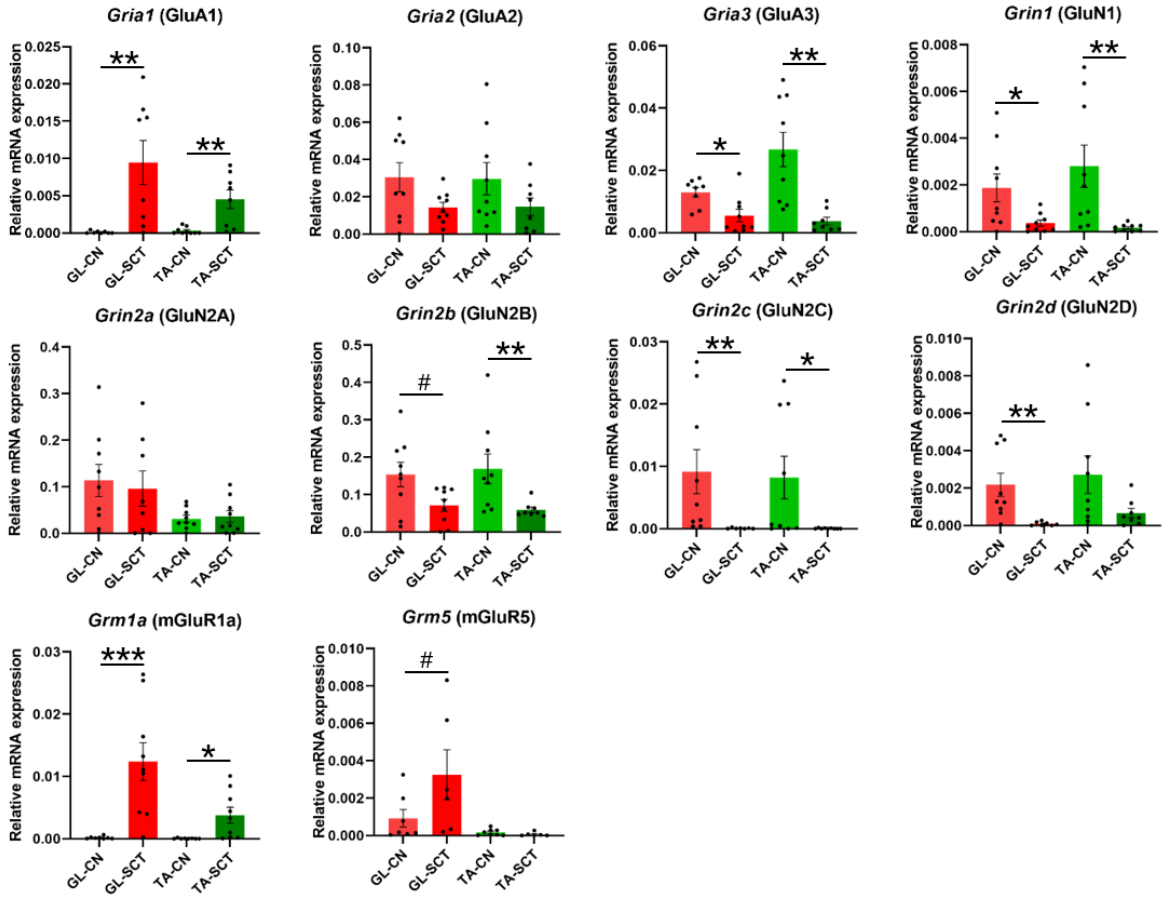

Figure 6.3. SCT differentially affects the transcript levels of AMPAR and NMDAR subunits, with the same impact in GL and TA MNs. SCT led to a significant increase of *Gria1* (GluA1) subunit, and a decrease of *Gria3* (GluA3) subunit. A significant decrease of transcripts of all subunits of NMDAR except for *Grin2a* (GluN2A) was found. Upregulation of the transcript level of *Grm1a* (mGluR1A) in GL and TA MNs was accompanied by an increase of transcript level of *Grm5* (mGluR5) in GL MNs but not in TA MNs. Data are means +/- SEM. Mann-Whitney U-test, # $p<0.08$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

SCT led to a significant increase of transcript level of *Gria1* (GluA1) subunit ($p = 0.01$ both in GL and TA MNs), a decrease of *Gria3* (GluA3) subunit ($p = 0.004$ in GL MNs, $p = 0.002$ in TA MNs), while *Gria2* (GluA2) subunit was unchanged in either of the two MN groups (**Figure 6.3**). The result suggests that the proportion of GluA1/GluA2 subunit combination in AMPARs may be increased, while that of GluA2/GluA3 subunit combinations is decreased. Further speculation of the consequences of that direction of changes is that more GluA1/GluA1 homomers may be formed after injury. Because AMPARs containing GluA1/GluA1 subunit combination are Ca^{2+} permeable, the switch from GluA2-containing AMPARs to GluA2-lacking AMPARs increases the possibility of MN enrichment in synaptic Ca^{2+}-permeable AMPARs (CP-AMPAR) resulting in membrane depolarization of the MNs and sustained AP firing. Existence and dynamic regulation of Cp-AMPARs enable these special receptors to serve as signaling molecules presumably via calcium influx (Man, 2011).

In the brain, the most common structure of NMDAR, which is also a tetrameric complex, contains two GluN1 and two GluN2 (typically one GluN2A and one GluN2B) subunits (Kohr, 2006). We show that SCT led to decreased transcript levels of *Grin1* (GluN1)*, Grin2b* (GluN2B), *Grin2c* (GluN2C) and *Grin2d* (GluN2D) subunits, while it did not alter *Grin2a* (GluN2A) transcript level neither in GL nor TA MNs (**Figure 6.3**). As a consequence, the general decrease of NMDAR subunit gene expression after SCT may lead to decreased NMDAR signaling in GL and TA MNs.

Metabotropic glutamatergic receptors mGluRs play roles in modulation of neurons' excitability after ligand binding. In MNs of intact rats, we demonstrate that *Grm1a* (mGluR1A) showed higher mRNA expression than *Grm5* (mGluR5), which is in line with a result of early *in situ* hybridization study by Tölle's group (Berthele et al., 1999). We found that SCT results in an increased transcript level of group I mGluRs. Namely, the transcript level of *Grm1a* (mGluR1A) was increased profoundly both in GL and TA MNs after SCT, with *Grm5* (mGluR5) elevated in GL MNs but not in TA MNs.

6.1.4. SCT downregulates transcript level of GABAergic and Gly receptor subunits and of KCC transporters similarly in GL and TA MNs

Inhibition of MNs is primarily mediated by GABAergic and glycinergic neurotransmission which is regulated by intraneuronal chloride (Cl^-) homeostasis. We report that SCT leads to profound decreases of the transcript levels of all examined subunits of inhibitory receptors $GABA_AR$ and GlyR both on GL and TA MNs (**Figure 6.4**).

Next, we examined expression level of genes encoding K^+ - Cl^- co-transporters: *Slc12a4* (KCC1), *Slc12a5* (KCC2) and *Slc12a6* (KCC3), which primarily extrude chloride ions out of the neurons regulating the intraneuronal Cl^- homeostasis. Among them, *Slc12a5* (KCC2) displayed the highest transcript level in intact animals (**Figure 6.4**), confirming that this neuron-specific KCC2 is the major Cl^- extruder of comparable abundance in GL and TA MNs.

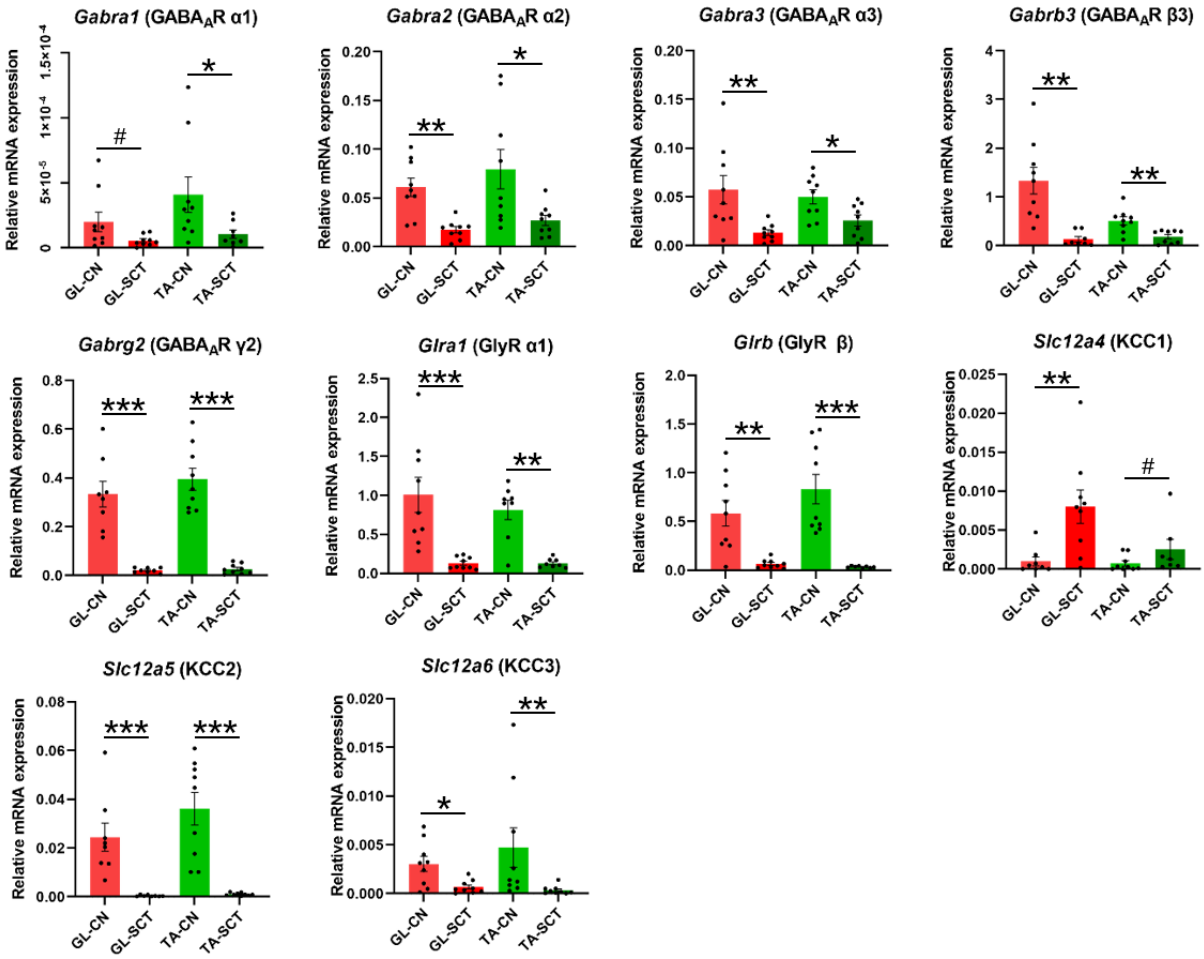

Figure 6.4. **SCT downregulates the transcript level of all subunits of $GABA_A$ and Gly receptors as well as KCC2 and KCC3 transporters, with the same impact in GL and TA MNs**. The opposite response was found for KCC1 transporter, both in GL and TA MNs. Data are means +/- SEM. Mann-Whitney U-test, # p<0.08; * p<0.05; ** p<0.01; *** p<0.001.

SCT caused a significant downregulation of *Slc12a5* (KCC2) and *Slc12a6* (KCC3) genes, while *Slc12a4* (KCC1) gene expression was upregulated (**Figure 6.4**). The predominant decrease of transcript level of KCC2 and KCC3, if followed by changes in respective proteins, may be indicative of decreased abundance and function resulting in accumulation of high concentrations of Cl^- in the cytoplasm of GL and TA MNs. If so, a depolarizing effect instead of inhibitory response may be expected after ligand

binding to $GABA_A$ and Gly receptors (illustrated in **Figure 7.1**).

6.1.5. SCT downregulates transcript level of Nav1.6, KCa1.1(BK), and SK2 channels similarly in GL and TA MNs

Action potentials (AP) are directly initiated by the opening of voltage-gated (VG) Na^+ and Ca^{2+} channels and are negatively regulated by the activity of Ca^{2+}-activated K^+ (SK and BK) channels. SK channels mediate the outward K^+ currents, generate the medium afterhyperpolarization (mAHP), which reduces the firing frequency of APs, leading to decreased excitability of neurons (Stocker, 2004). Thus increased persistent Na^+ and Ca^{2+} inward current (PIC) and decreased K^+ outward current increase the probability of APs generation and subsequently the excitability of neurons. Therefore, we examined whether SCT affects the transcript levels of major Na^+, Ca^{2+} and K^+ channels related to the generation of APs.

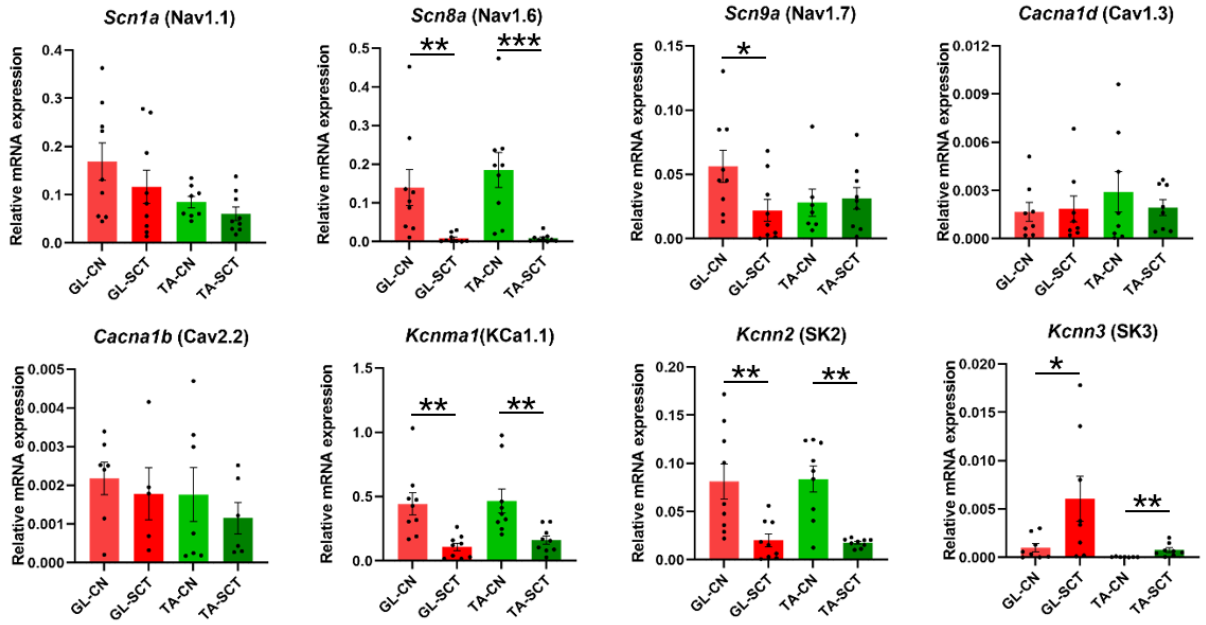

Figure 6.5. SCT downregulates the transcript level of the Nav1.6, KCa1.1 (BK), and SK2 channels similarly in GL and TA MNs. Expression of voltage-dependent Ca^{2+} channels: *Cacna1b* (Cav2.2) and *Cacna1d* (Cav1.3) was unaffected by SCT. Data are means +/- SEM. Mann-Whitney U-test, # p<0.08; * p<0.05; ** p<0.01; *** p<0.001).

Among the four examined VG Na^+ channels, *Scn1a* (Nav1.1) and *Scn8a* (Nav1.6) displayed the highest and comparable transcript levels in GL and TA MNs, followed by the moderate *Scn9a* (Nav1.7) expression (**Figure 6.5**). *Scn3a* (Nav1.3) was expressed in an extremely low level in both MN groups (not shown), in line with a study showing similar differences in expression signal in the lumbar spinal cord using *in situ* hybridization (Fukuoka et al., 2010). After SCT, *Scn8a* (Nav1.6) was significantly

downregulated both in GL and TA MNs. *Scn9a* (Nav1.7) was selectively decreased in GL MNs while *Scn1a* (Nav1.1) and *Scn3a* (Nav1.3) were kept unaltered.

The expression of two VG Ca^{2+} channels: *Cacna1b* (Cav2.2) and *Cacna1d* (Cav1.3) in the intact rats was two orders of magnitude lower than that of VG Na^{+} channels, and was unaffected by SCT. On the opposite, the expression of small-conductance Ca^{2+}-activated K^{+} (SK) channels was markedly changed. In GL and TA MNs of intact animals, *Kccn2* (SK2) displayed the highest transcript level, followed by the *Kccn3* (SK3), while the transcript level of *Kccn1* (SK1) was extremely low, suggesting that SK2 undergoes extensive turnover and plays the major roles in these MNs. These results are in line with the study showing that SK2 protein is expressed in all α-MNs of the lumbar segment in the rat, whereas SK3 is expressed preferentially in small-diameter α-MNs (Deardorff et al., 2013). Regulation of gene expression of SK2 and SK3 in response to the lesion was in opposite direction: a significant decrease of transcript level of *Kccn2* (SK2), parallel in both groups of MNs, was accompanied by increased *Kcnn3* (SK3) level.

Highly abundant *Kcnma1* transcripts of large-conductance (KCa1.1, BK) channel, which holds the ability to integrate changes in intracellular calcium and membrane potential contributing to regulation of outward K+ currents, were significantly downregulated both in GL and TA MNs (**Figure 6.5).**

6.1.6. SCT alters transcript levels of 5-HT receptors but not NA receptors

Finally, we examined expression of serotoninergic 5-HT receptors and noradrenergic NA receptors (**Figure 6.6**), which have been demonstrated to play crucial roles in modulating the firing of MNs in spinal cord (Heckmann et al., 2005; Heckman et al., 2009). Examination of transcript levels of the 5-HTR subunits after the lesion revealed that the regulation of gene expression of Htr1a (5-HTR1A) and Htr2b (5-HTR2B) subunits, abundant in normal rats, markedly increased, and a degree of their up-regulation was similar in both groups of MNs. These changes were accompanied by selective increase of Htr2a (5-HTR2A) subunit in GL MNs only, and a selective decrease of Htr2c (5-HTR2C) subunit in TA MNs.

Among the examined NA receptor subunits, *Adra1a* (NAα1A) and *Adra1d* (NAα1D) expression was comparable, whereas the *Adra1b* (NAα1B) displayed very low expression (data not shown),

suggesting that NAα1A) and NAα1D predominate in GL and TA MNs. Expression of none of these subunits was changed.

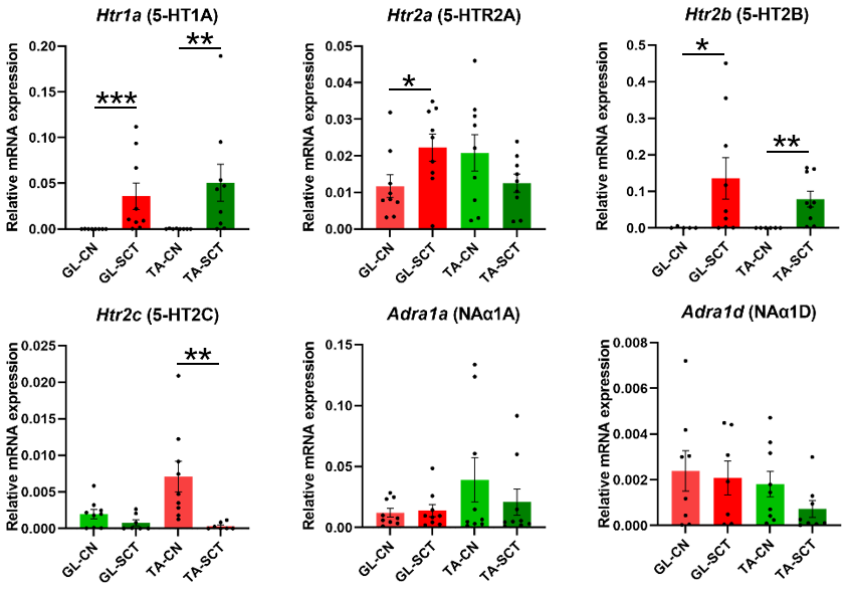

Figure 6.6. SCT upregulates the transcript level of 5-HT 1A and 2B receptors similarly in LG and TA MNs. Increased gene expression of *Htr1a* (5-HT1A), *Htr2a* (5-HT2A) and *Htr2b* (5-HTR2B) subunits of 5-HT receptors in GL and TA MNs is accompanied by a decrease in *Htr2c* (5-HT2C) subunit in TA MNs but not GL MNs. SCT does not alter gene expression of NA receptor subunits. Data are means +/- SEM. Mann-Whitney U-test, # $p<0.08$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

6.1.7. Correlation analyses of gene expression in GL and TA MNs after SCT

Correlation analyses were carried out by using classical Pearson correlation tests for every pairwise combination of all genes (R package). The heat-mapped correlograms were produced by using corrplot package under R, with a scale from -1 to 1. The correlograms were generated for GL and TA MNs from both CN and SCT animals to search for the differences in the co-expression pattern of all gene pairs. First, the correlation analysis indicated that the correlated patterns of gene expression differ between GL MNs and TA MNs from CN animals (**Figure 6.7**). Second, the patterns of correlated gene expression are changed after SCT both in GL and TA MNs (**Figure 6.7**). For instance, in GL MNs of CN rats there was a clear negative or positive correlation between the expression of NMDAR GluN2A subunit and ion channels subtypes (**Figure 6.7, box A**), but these correlations were lost after SCT (**Figure 6.7, box A'**). Similarly, there are also relationships among Na^+ and Ca^{2+} channel subtypes in GL MNs of CN rats (**Figure 6.7, box B**), which are substantially weakened or disappear after SCT (**Figure 6.7, box B'**). The same observation concerns TA MNs (**Figure 6.7, box C and C'**).

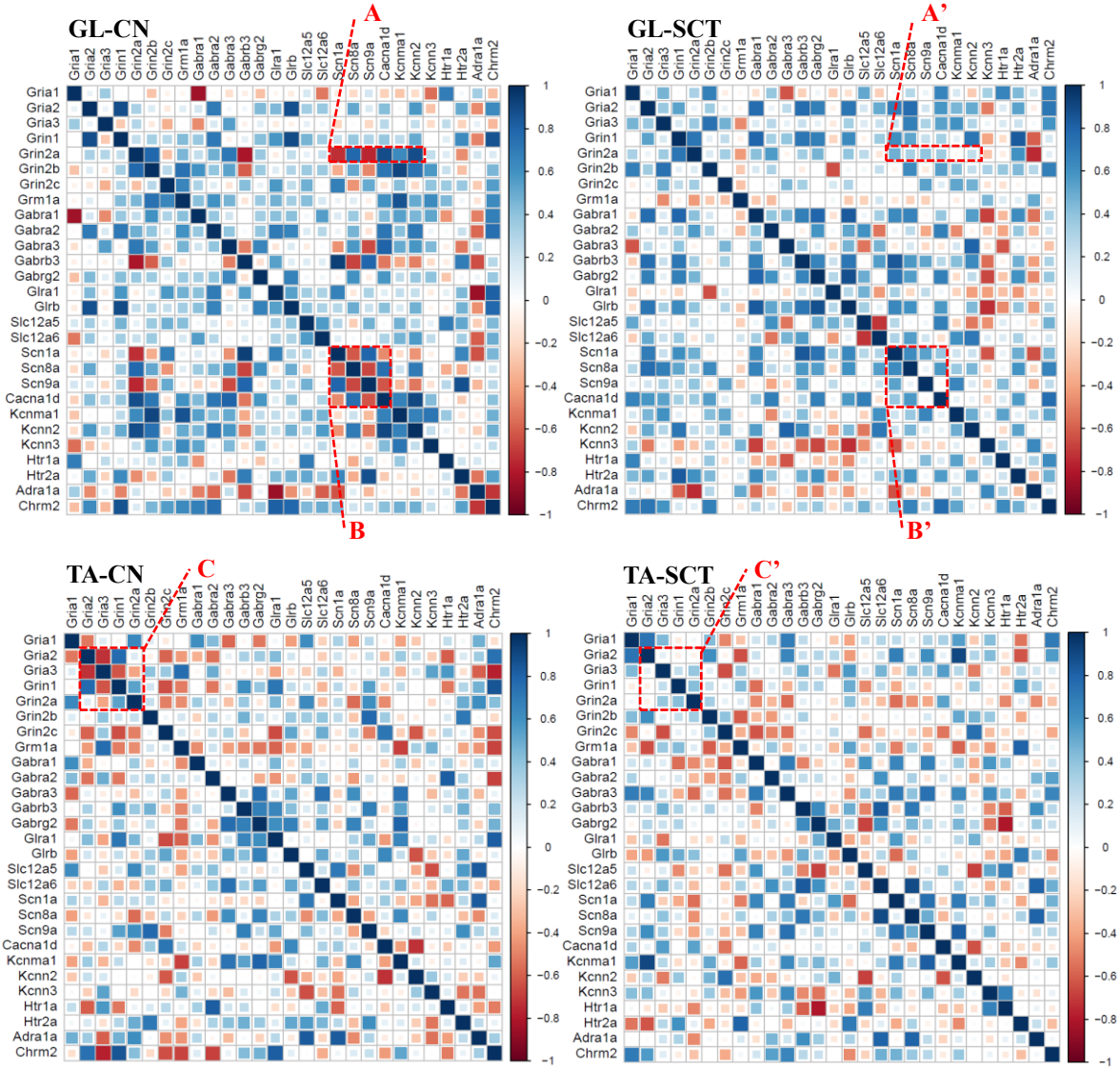

Figure 6.7. Changes in correlated gene expression as a result of SCT in GL and TA MNs. The order of the genes along the X- and Y-axis of each correlogram are the same for comparison of the changes between groups.

The correlograms with hierarchical clustering were also analyzed (**Figure 6.8**). With the function of hierarchical clustering, the genes with similar expression patterns group together as a cluster, indicating these genes are co-regulated in either CN or SCT groups of rats. The results showed there are clearly different clusters containing different genes between GL and TA MNs from CN animals. Furthermore, SCT substantially altered the distribution and density of these clusters (**Figure 6.8**). For instance, in GL MNs of CN rats, gene *Gria2*, *Grin1*, *Glrb* and *Gabra2* are positively correlated in one cluster (**Figure 6.8, box A**). However, SCT dispersed the four genes into four different clusters (**Figure 6.8, box A'**). Similar effects of SCT also occurs in TA MNs.

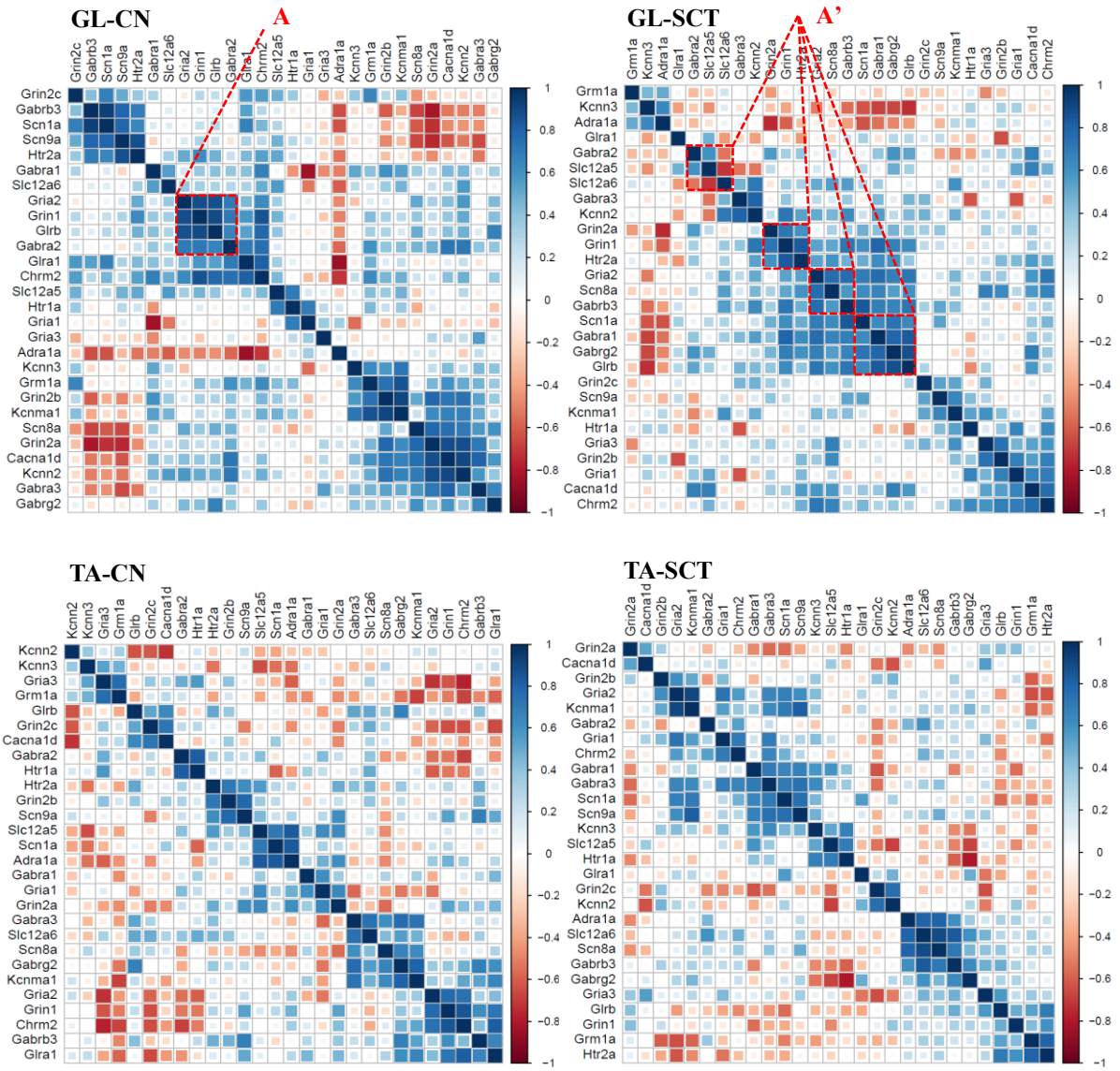

Figure 6.8. Changes in clustered correlated gene expression as a result of SCI in GL and TA MNs. The order of the genes along the X- and Y-axis of each correlogram are automatically clustered depending on the R-values of the genes.

These results suggest the set of genes coding for neurotransmission-related membrane proteins undergoes differential regulation in different functional types of MNs under the influence of spinal cord injury already in the second week postinjury. The direction of changes in GL and TA MNs is summarized **in Table 6.1.**

Table 6.1. Direction changes in gene expression in GL and TA MNs at 2 weeks after SCT

Gene symbol	GL	TA	Gene symbol	GL	TA
Gria1	↑	↑	*Slc12a4*	↑	↑
Gria2	–	–	*Slc12a5*	↓	↓
Gria3	↓	↓	*Slc12a6*	↓	↓
Grin1	↓	↓	*Scn1a*	–	–
Grin2a	–	–	*Scn8a*	↓	↓
Grin2b	↓	↓	*Scn9a*	↓	–
Grin2c	↓	↓	*Cacna1b*	–	–
Grin2d	↓	–	*Cacna1d*	–	–
Grm1a	↑	↑	*Kcnn2*	↓	↓
Grm5	↑	–	*Kcnn3*	↑	↑
Gabra1	↓	↓	*Kcnma1*	↓	↓
Gabra2	↓	↓	*Htr1a*	↑	↑
Gabra3	↓	↓	*Htr2a*	↑	–
Gabrb3	↓	↓	*Htr2b*	↑	↑
Gabrg2	↓	↓	*Htr2c*	↓	↓
Glra1	↓	↓	*Adra1a*	–	–
Glrb	↓	↓	*Adra1d*	–	–

6.2. Overexpression of BDNF leads to locomotor recovery and pro-excitogenic phenotype in the lumbar MNs after SCT

6.2.1 Evaluation of treadmill locomotion of PBS-treated and BDNF-treated rats with mBBB scale

I first evaluated motor abilities of the SCT rats on a running treadmill (**Figure 6.9**) using modified BBB (mBBB) scale (Antri et al., 2002; Antri et al., 2003) and then in the open-field. No pressure stimulation of the tail was applied while rats were walking on the moving treadmill or when were tested in the open-field. Evaluation of treadmill locomotion was carried out by three experienced observers independently evaluating motor behavior of every animal according to the common protocol. Disputable scores were verified and finally accepted by all observers.

The rats walking on the running treadmill differed markedly between groups. All animals from the SCT with PBS intraspinal injection (SCT-PBS) group, tested on the 13th postsurgery day, kept their paws on the dorsal side and were unable to support their body weight when placed on the running treadmill

(**Figure 6.9 A**). They performed several spontaneous hindlimb movements, but no alternative movements were detected. In contrast, at the same time point, SCT rats with AAV-BDNF treatment (SCT-BDNF) were able to display step-like hindlimbs movements, with right-left alternation, plantar foot placement and body weight support (**Figure 6.9 B and C**). The quantitative analysis of the bipedal treadmill locomotion in the two groups of rats was done using mBBB scale (Antri et al., 2002; Antri et al., 2003) with four major levels and 22 scores [level 1 (scores 0–1), level 2 (scores 2–9), level 3 (score 10), level 4 (scores 11–22)] which are assigned to the recovery degree of motor capabilities. Among them, level 1 and score 0 amounts to no movements, while level 4 and score 22 is equal to locomotion of intact animal.

In my experiments, the motor capabilities of twelve of fourteen SCT-PBS rats on the treadmill on the 13th day were ranked at the level 1, and the remaining two rats were ranked at the level 2. In contrast, in SCT-BDNF group at the same time point, twelve out of thirteen rats reached level 4. The remaining SCT-BDNF rat was classified at level 2 with score 4.5 which is still higher than in all of SCT-PBS rats (**Table 6.2**). The mean scores for the locomotor performance on the treadmill in 13th day after treatment were 0.36 +/- 0.74 in SCT-PBS versus 12.19 +/- 3.31 in SCT-BDNF groups (Mann-Whitney U test, p=0.006) (**Table 6.2** and **Figure 6.10**), showing that BDNF overexpression significantly improved the locomotion of the spinalized rats on the running treadmill and that this process was progressive. Discrepancies between the scores among SCT-BDNF rats on the 13th day of testing suggested that some animals already reached their potentially highest level of motor abilities (e.g., *Zd7, BJ2, BJ3, BJ6 in Table 6.2) w*hile the others did not. To verify the possibility of further improvement the observation of all SCT-BDNF was prolonged by 2 consecutive days, revealing that indeed in majority of SCT-BDNF subjects the improvement of performance reached the peak between 13th and 16th day after BDNF treatment, when all rats were ranked at level 4 with a mean score 14.62 +/- 2.32 (**Table 6.2**). Compared with the performance on 13th day, further improvement was also significant (Wilcoxon test, P = 0.008) (**Figure 6.10**). I conclude that my results not only confirm a beneficial effect of AAV-BDNF treatment on motor abilities of the spinalized rats, but also reveal the timeline of the recovery process detectable as early as one week following the treatment.

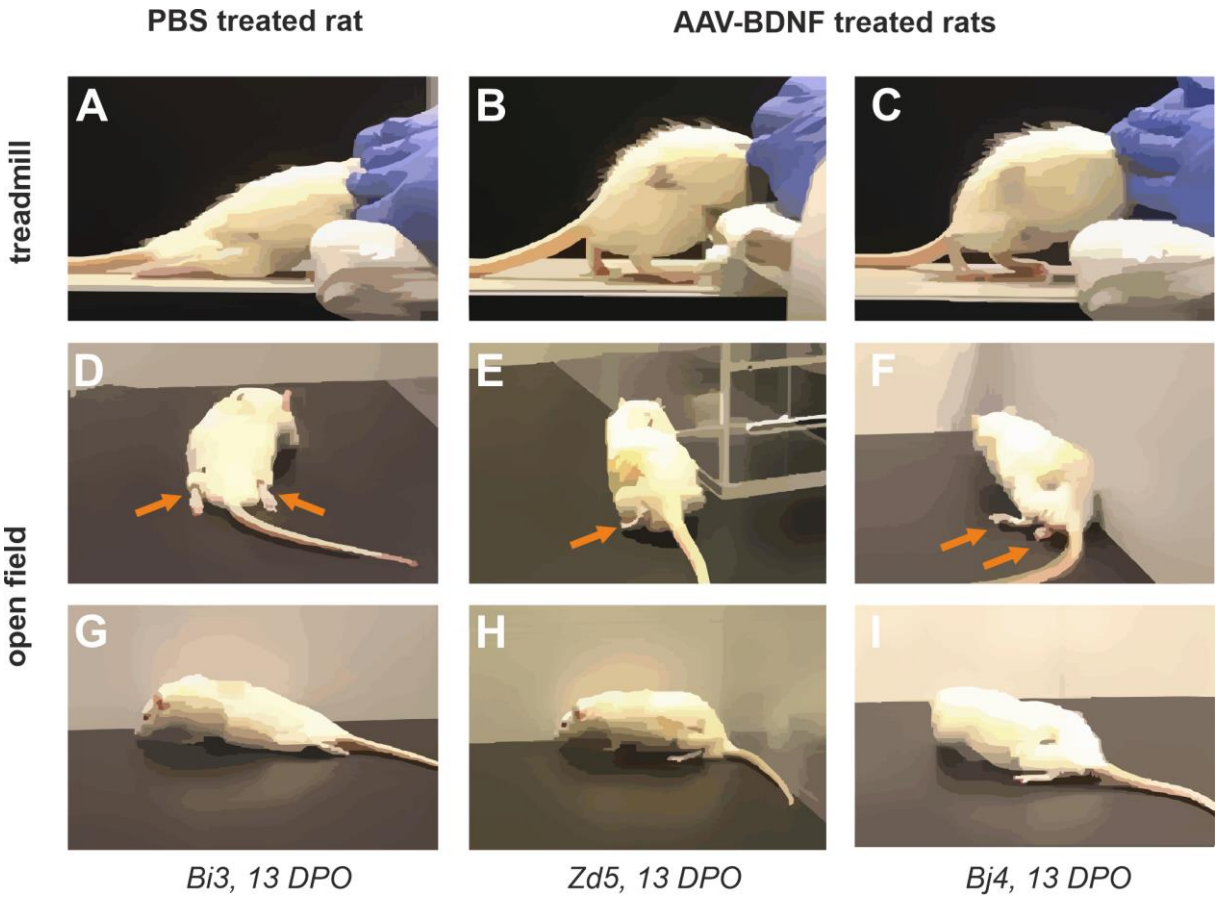

Figure 6.9. **Locomotor performance of PBS (Bi3, 13 DPO) and AAV-BDNF (Zd5 and Bj4, 13 DPO) treated, spinal rats on a running treadmill (A-C) and in the open field (D-I).** D-I The typical position of the feet during movement in the open field is shown: the spinal rat not treated with AAV-BDNF drags the feet lying on the dorsum of the feet, while the AAV-BDNF- treated rats are able to move their feet and put them on the plantar aspect of the feet enabling walking on the running treadmill.

Table 6.2. Locomotor capabilities of spinal rats in PBS-treated (SCT-PBS) and BDNF-treated (SCT-BDNF) groups evaluated during hindlimb walking on a running treadmill using mBBB scale

SCT-PBS					SCT-BDNF						
Group rat code	Testing day	Modified BBB Scale		Sacrifice day	Group rat code	Testing day	Modified BBB Scale		Testing day (best locomotor performance)	Modified BBB Scale		Sacrifice day
		Level	Score				Level	Score		Level	Score	
Zc4		1	0	14	**Zd1**		2	4.5	**16**	4	15.5	17
Zc5		1	0	14	**Zd2**		4	11	**14**	4	16.5	16
Zc6		1	1	14	**Zd3**		4	11	**16**	4	11.5	16
Zc7		1	0	14	**Zd4**		4	11	**16**	4	11	16
Zc8		1	0	15	**Zd5**		4	11.5	**15**	4	15.5	16
Zc9		1	0	15	**Zd7**		4	13	**13**	4	13	17
Zc10	**13**	2	2	14	**Bj1**	**13**	4	16	**14**	4	17	15
Bi1		1	0	14	**Bj2**		4	16	**13**	4	16	16
Bi2		1	0	14	**Bj3**		4	13	**13**	4	13	16
Bi3		1	0	14	**Bj4**		4	11.5	**14**	4	15.5	16
Bi4		2	2	14	**Bj5**		4	11	**15**	4	11.5	16
Bi5		1	0	14	**Bj6**		4	18	**13**	4	18	16
Bi6		1	0	14	**Bj7**		4	11	**16**	4	16	16
Bi7		1	0	15								
Mean			**0.36**					**12.19**			**14.62**	
SD			**0.74**					**3.31**			**2.32**	

The table shows the level and scores for the motor capabilities of SCT-PBS and SCT-BDNF rats on the running treadmill (based on the mBBB scale modified by Antri et al., 2002). The mean scores for SCT-PBS (13th day), SCT-BDNF (13th day) and SCT-BDNF (day with best performance) were 0.36, 12.19 and 14.62, respectively. The differences between the scores in groups are significant, as shown in **Figure 6.10.**

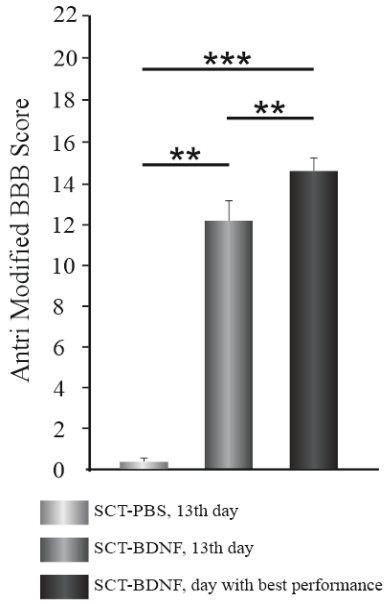

Figure 6.10. **Locomotor performance of SCT-PBS and SCT-BDNF rats during hindlimb walking on a running treadmill, evaluated by using mBBB scale.** There is a significant difference between SCT-PBS (N=14) and SCT-BDNF (N=14) rats at 13th day after treatment (Mann-Whitney U test; $p = 0.006$), indicating that AAV-BDNF treatment brings the marked improvement of locomotor performance of the spinal rats on the running treadmill. In majority of rats from SCT-BDNF group, the motor abilities were further improved in the following testing days, comparing with that of 13th days (Wilcoxon test, $p = 0.008$). Data are means +/- SEM. ** $p<0.01$; *** $p<0.001$).

In an open field, the SCT-PBS rats moved with their forelimbs, dragging the body which symmetrically or asymmetrically lied on the surface of the ground. Their hindlimbs were dragged on the dorsal aspect of the feet and displayed sporadic movements much less frequent than the steps of forelimbs. (**Figure 6.9 D, G**). The AAV-BDNF treatment enabled spinalized rats to generate some step-like movements of their hindlimbs, with plantar foot placement, occasional right-left alternation and sporadic, weak body weight support in the open field (**Figure 6.9 E-I**). Sporadically, the hindlimb movements performed a few step-like movements in-phase with the forelimbs. According to our observation, five out of thirteen rats could keep the body symmetric during most of the time in the open field. They displayed occasional alternative step-like hindlimb movements with plantar feet placement and weak or no weight support. The other eight rats kept their body either asymmetrically or symmetrically with occasional bilateral plantar foot placement (**Figure 6.9 E-H**). Referring to these results, we may conclude that BDNF overexpression also improves the motor abilities of these rats in the open field.

To sum up, although our previous work has already shown that BDNF overexpression improved hindlimb locomotion of spinalized rats, my results revealed the timeline of the recovery process following the treatment, through improving the experimental procedures and increasing the number of experimental animals. My results further confirmed and strengthened the conclusion that BDNF treatment markedly improves the locomotor performance of the spinalized rats on the running treadmill already at the second week after surgery. In the next step, I verify whether the membrane receptor,

channels and transporters expression, examined by using biochemical and immunohistochemistry methods, would correlate with the behavioral results.

6.2.2 Molecular identification of changes in MN neurotransmitter receptor and channel phenotypes after AAV-BDNF treatment

6.2.2.1. PBS or BDNF intraspinal injection to SCT rats did not alter morphology of MNs in lumbar 3-6 segments

Our analysis of animal behavior has shown that AAV-BDNF administration significantly improves the locomotor performance of spinalized rats on the running treadmill. Based on these observations, we hypothesized that the evoked BDNF overexpression promotes locomotion recovery in part through increasing the excitability of both ankle extensor and flexor MNs. Therefore, we examined the expression levels of a range of genes in GL (extensor) and TA (flexor) MNs, expecting to emerge from among the candidate genes those revealing a profound change towards higher excitation state.

In previously described experiment I have shown that SCT at thoracic level did not cause degeneration of MNs located at lumbar L3-6 segments (**Figure 6.1**). Here, I examined whether PBS or BDNF intraspinal injection to spinalized rats affect MNs number and morphology at lumbar 3-6 segments. GL and TA MNs were clearly visualized under LMD microscope in fluorescence mode, and during microdissection the numbers of traced GL and TA MNs were counted on each horizontal L3-6 section. **Figure 6.11 A** presents representative photomicrographs showing GL and TA MNs of CN, SCT-PBS and SCT-BDNF groups before LMD and the tissue section remaining after microdissection. The morphology of MNs of a given type was similar across CN, SCT-PBS and SCT-BDNF groups of rats. The number of MNs of a given type was comparable among intact (CN), SCT-PBS and SCT-BDNF rats (GL: SCT-PBS = 89% CN, SCT-BDNF = 88% CN, $p = 0.40$; TA: SCT-PBS = 97% CN, SCT-BDNF = 102% CN, $p = 0.83$; One-way ANOVA with Tukey's post-hoc test, **Figure 6.11 B**). Thus, these results suggest that PBS or BDNF intraspinal injection to spinalized rats did not cause alterations in morphology and number of MNs at lumbar 3-6 segments.

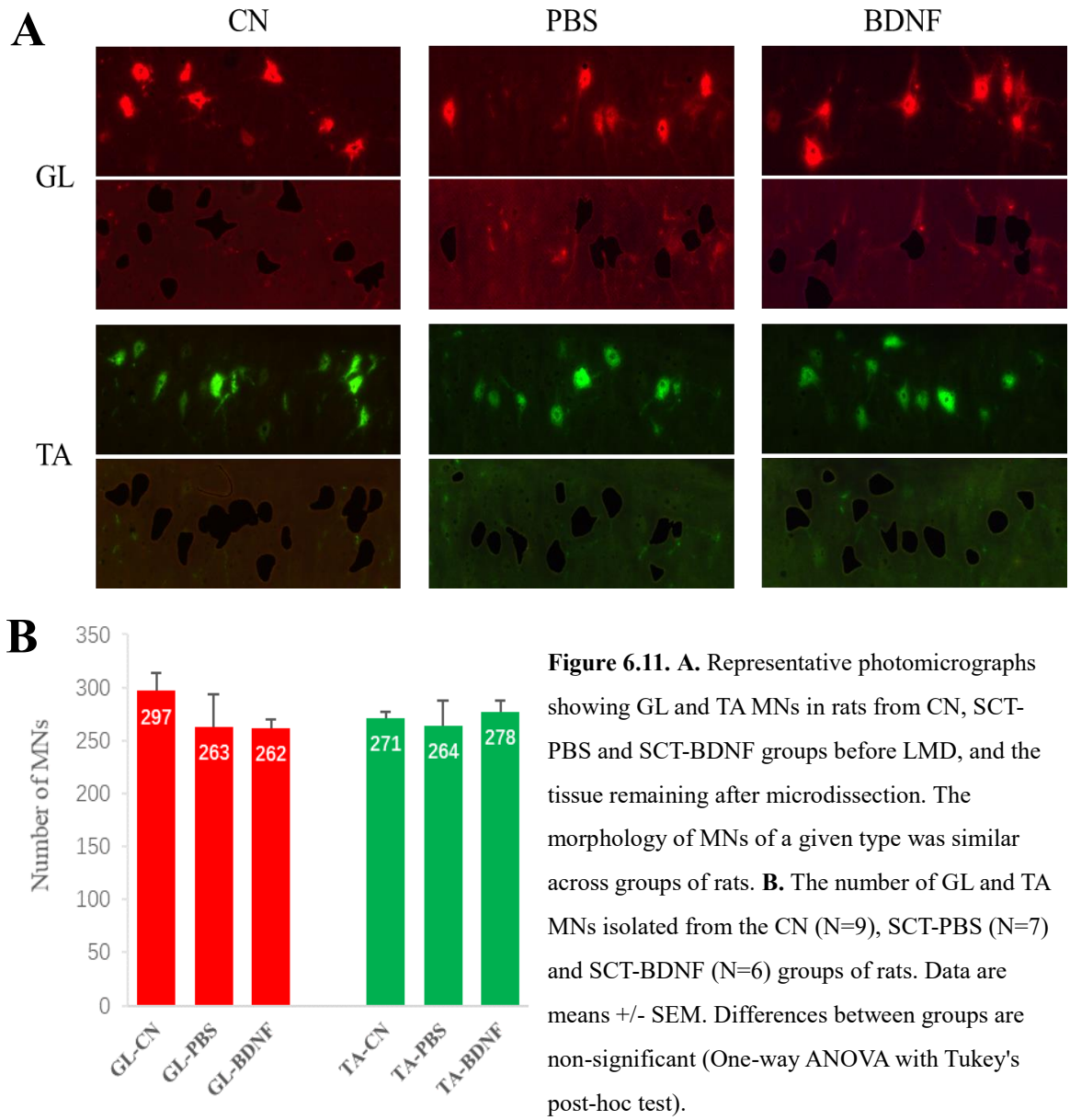

Figure 6.11. A. Representative photomicrographs showing GL and TA MNs in rats from CN, SCT-PBS and SCT-BDNF groups before LMD, and the tissue remaining after microdissection. The morphology of MNs of a given type was similar across groups of rats. **B.** The number of GL and TA MNs isolated from the CN (N=9), SCT-PBS (N=7) and SCT-BDNF (N=6) groups of rats. Data are means +/- SEM. Differences between groups are non-significant (One-way ANOVA with Tukey's post-hoc test).

6.2.2.2. AAV-BDNF intraspinal injection to SCT rats causes BDNF overexpression in GL and TA MNs

The expression of BDNF in pools of GL and TA MNs, which are located in these segments, was much lower comparing with that in homogenates from whole L3-6 segments ((Głowacka et al., 2022), submitted). Out of 9 rats BDNF expression was above the limit of detection in 4 rats only, in which it was in a range of 1E-09 – 5E-08. Two weeks after SCT, the level of spinal BDNF expression in the SCT-PBS group showed a tendency to decrease, and in GL and TA MNs it was undetectable in majority of rats (**Figure 6.12**). AAV-BDNF administration resulted in high BDNF mRNA levels in GL MNs and TA MNs of 4 rats; in the remaining rats no BDNF overexpression was found (**Figure 6.12**).

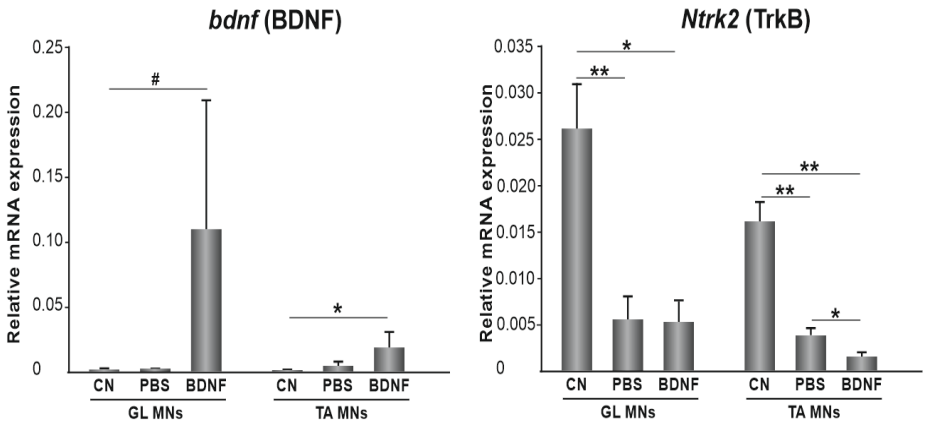

Figure 6.12. The effect of AAV-BDNF spinal injection on BDNF mRNA and TrkB mRNA level in GL and TA MNs 2 weeks after SCT. Transcript levels were measured with a qPCR assay in samples from 9 CN, 7 SCT-PBS and 6 SCT-BDNF rats. Data are means +/- SEM. # p<0.08; * p<0.05; ** p<0.01; Mann-Whitney U-test.

Spinalization caused a profound decrease of TrkB in pools of GL and TA MNs, suggesting a deficit in BDNF/TrkB signaling. However, in TA MNs TrkB mRNA level was reduced further by AAV-BDNF administration, while in GL MNs it was not affected by the treatment (**Figure 6.12**). These results suggest that majority of lumbar MNs maintains some degree of responsiveness to BDNF signaling at two weeks after the lesion. That signaling may be further reduced in TA MNs by spinally overexpressed BDNF, differentiating the degree of functional impairment between GL and TA.

6.2.2.3. BDNF overexpression modulates gene expression of molecules of pro-excitogenic potential in GL and TA MNs

Glutamatergic AMPAR and NMDAR. In SCT-PBS group, the mRNA level of *Gria2* tended to increase in GL MNs without changing its level in TA MNs. On the contrary, the level of *Gria3* was down-regulated both in GL and TA MNs (GL: CN vs SCT-PBS, p=0.003; TA: CN vs SCT-PBS, p=0.003; Mann-Whitney U Test) (**Figure 6.13**).

BDNF overexpression led to a tendency to decrease the mRNA level of *Gria2* level in both groups of MNs with its profound down-regulation in TA MNs. On the contrary, BDNF overexpression had no effect on decreased *Gria3* expression in either MN group (**Figure 6.13**).

Among NMDAR subunits, neither the PBS nor AAV-BDNF treatment alter the transcript level of *Grin1* in GL MNs. However, *Grin1* was significantly downregulated in TA MNs of SCT-PBS group (TA: CN vs SCT-PBS, p=0.03; Mann-Whitney U Test) and restored by BDNF overexpression to control level

(TA: SCT-PBS vs SCT-BDNF, p=0.02; Mann-Whitney U Test) (**Figure 6.13**). The mRNA level of *Grin2a* was significantly decreased in SCT-PBS group in GL MNs, showed a tendency to decrease in TA MNs (GL: CN vs SCT-PBS, p=0.006; TA: CN vs SCT-PBS, p=0.10; Mann-Whitney U Test), which was alleviated by BDNF overexpression (**Figure 6.13**).

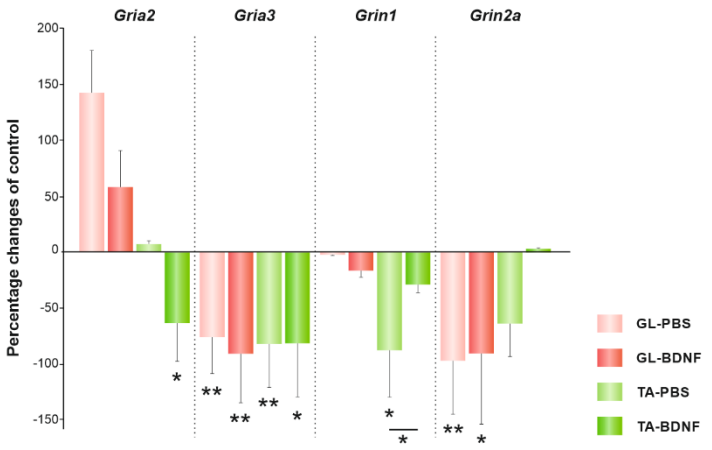

Figure 6.13. The effect of AAV-BDNF spinal injection on glutamatergic AMPAR and NMDAR mRNA levels in GL and TA MNs 2 weeks after spinal cord transection. Data are from 9 Control, 7 SCT-PBS and 6 SCT-BDNF rats. Data are means +/- SEM. * p<0.05; ** p<0.01; Mann-Whitney U-test.

Inhibitory receptors GABAAR and GlyR and KCC2. In SCT-PBS group the transcript levels of *Gabra2* were downregulated both in GL and TA MNs (**Figure 6.14**). BDNF caused further decrease of *Gabra2* in TA MNs but not GL MNs. *Gabra3* was regulated contrary to *Gabra2:* it was not changed after SCT and BDNF tended to increase its expression in both MN groups (n.s.).

Almost all remaining transcripts of inhibitory receptors' subunits were significantly down-regulated in SCT-PBS group. Also, *Slc12a5* mRNA level was found to be dramatically reduced both in GL and TA MNs comparing with intact rats. BDNF overexpression moderately deepened suppression of these genes (*Gabrg2, Glra1, Glrb* in GL MNs), evoked suppression of upregulated *Glra1* gene in TA MNs, or did not change reduced transcript levels (*Slc12a5*) (**Figure 6.14).**

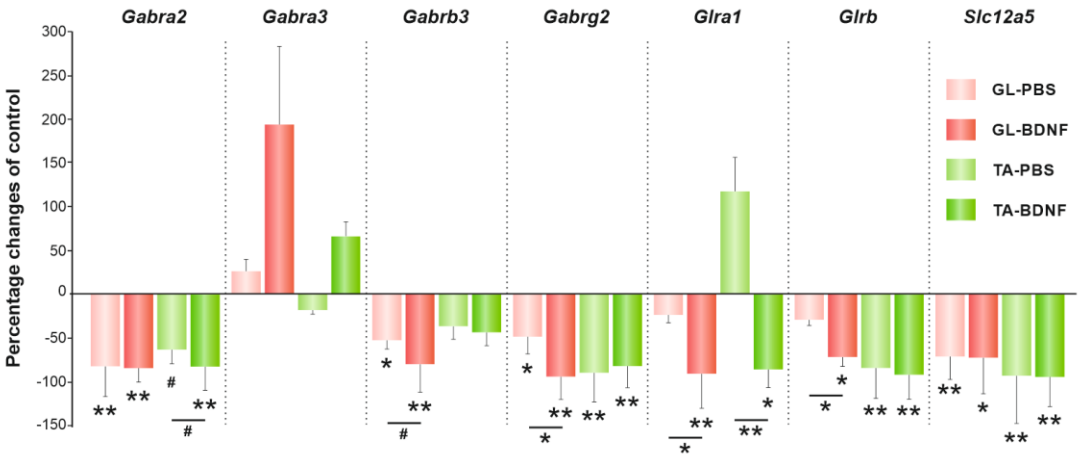

Figure 6.14. The effect of AAV-BDNF spinal injection on the mRNA levels of inhibitory GABAAR, GlyR and KCC2 in GL and TA MNs 2 weeks after spinal cord transection. Data are from 9 Control, 7 SCT-PBS and 6 SCT-BDNF rats. Data are means +/- SEM. # p<0.09; * p<0.05; ** p<0.01; Mann-Whitney U-test.

Ion channels: SCT-PBS profoundly elevated the transcript level of *Scn1a* in both GL and TA MNs (GL: CN vs SCT-PBS, p=0.004; TA: CN vs SCT-PBS, p=0.001; Mann-Whitney U Test), and less profoundly elevated *Cacna1d* transcript levels in GL (**Figure 6.15**). BDNF overexpression counteracted the effects of spinalization and restored expression of *Scn1a* to control level (GL: SCT-PBS vs SCT-BDNF, p=0.01; TA: SCT-PBS vs SCT-BDNF, p=0.02; Mann-Whitney U Test).

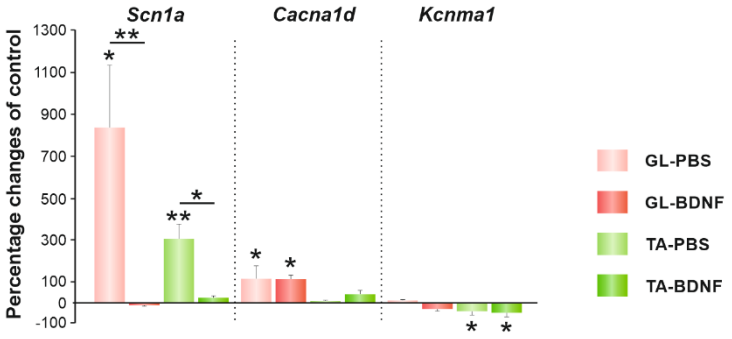

Figure 6.15. The effect of AAV-BDNF spinal injection on the mRNA levels of Na^+, Ca^{2+} and K^+ channels in GL and TA MNs 2 weeks after spinal cord transection. Data are from 9 Control, 7 SCT-PBS and 6 SCT-BDNF rats. Data are means +/- SEM. * p<0.05; ** p<0.01; Mann-Whitney U-test.

Changes in *Kcnma1* expression were small, but in TA MNs of SCT-PBS group, its level was downregulated (TA: CN vs SCT-PBS, p=0.05; Mann-Whitney U Test). BDNF overexpression resulted in a slight decrease of *Kcnma1* in both GL and TA MNs comparing with SCT-PBS, but the effect was not statistically significant (**Figure 6.15**).

Neuromodulatory receptors. In the SCT-PBS group, the expression of *Htr2a* was increased in GL MNs (GL: CN vs SCT-PBS, p=0.02; Mann-Whitney U Test), but not in TA MNs comparing with intact rats (**Figure 6.16**). BDNF overexpression further elevated the transcript level of *Htr2a* in GL and moderately increased the level of *Htr2a* in TA MNs (GL: SCT-PBS vs SCT-BDNF, p=0.02; TA: SCT-PBS vs SCT-BDNF, p=0.07; Mann-Whitney U Test). SCT-PBS downregulated the mRNA level of *Adra1a* in TA MNs, but not in GL MNs (TA: CN vs SCT-PBS, p=0.01; Mann-Whitney U Test). AAV-BDNF treatment led to a slight decrease of *Adra1a* in GL MNs and increase in TA MNs, although the effects were not statistically significant (**Figure 6.16**).

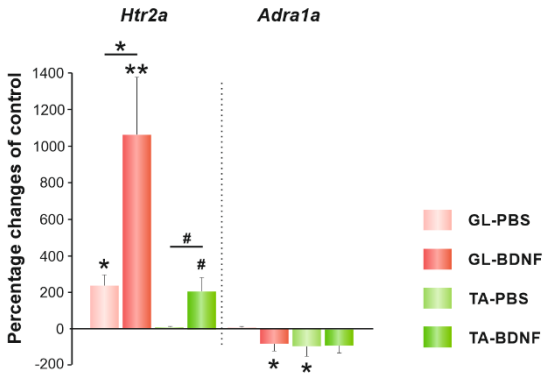

Figure 6.16. The effect of AAV-BDNF spinal injection on the mRNA levels of serotoninergic Htr2a and noradrenergic Adra1a in GL and TA MNs 2 weeks after spinal cord transection. Data are from 9 Control, 7 SCT-PBS and 6 SCT-BDNF rats. Data are means +/- SEM. # p<0.08; * p<0.05; ** p<0.01; Mann-Whitney U-test.

6.2.2.4. Correlation analyses of gene expression in GL and TA MNs after SCT and AAV-BDNF treatment

Correlation analyses of gene expression in GL and TA MNs after SCT (SCT-PBS) and AAV-BDNF treatment (SCT-BDNF) were also performed. It showed that the patterns of correlated gene expression were altered in GL and TA MNs in response to SCT-PBS and SCT-BDNF (**Figure 6.17 and 6.18**). In GL MNs of CN rats, there are several significant correlations (marked by *) which disappeared in SCT-PBS group. SCT-PBS resulted in more positive correlations that formed several clusters (**Figure 6.17,** boxes in red dotted line), comparing with the more dispersed and even correlations in CN group. AAV-BDNF treatment disrupted the clustered pattern showing in SCT-PBS group and inclined to more dispersed pattern like that in CN group, although it was still different from CN group (**Figure 6.17**). In TA MNs of CN rats, the correlations among the genes were generally weaker comparing with that in

SCT-PBS and SCT-BDNF groups (**Figure 6.18**). Both the SCT-PBS and SCT-BDNF led to a redistribution of the correlation patterns, which was differ from that in CN group (**Figure 6.18**).

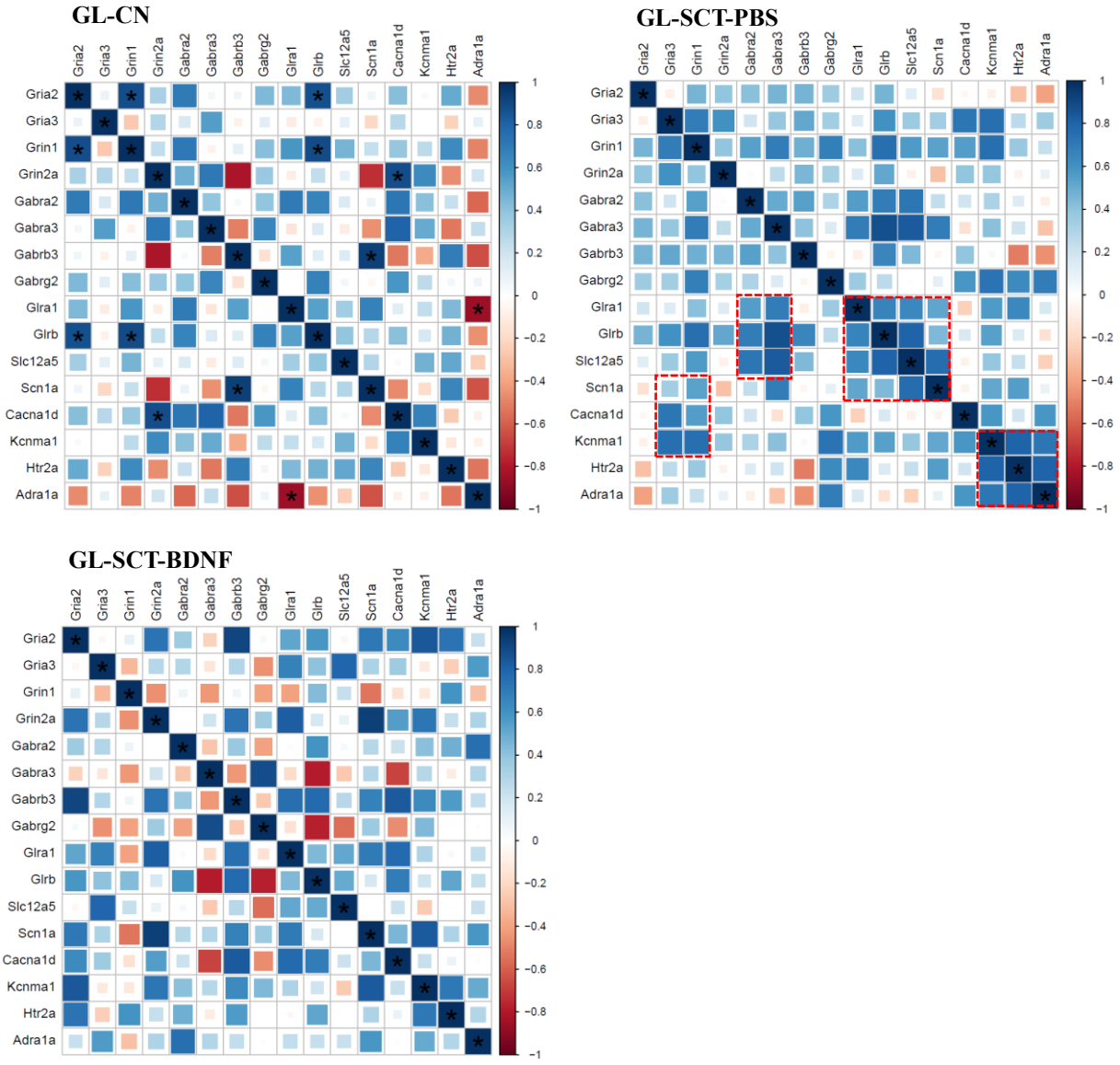

Figure 6.17. Changes in correlated gene expression as a result of SCT and AAV-BDNF treatment in GL MNs. The order of the genes along the X- and Y-axis of each correlogram are the same for comparison of the changes between groups. * significant FDR corrected p-value in R.

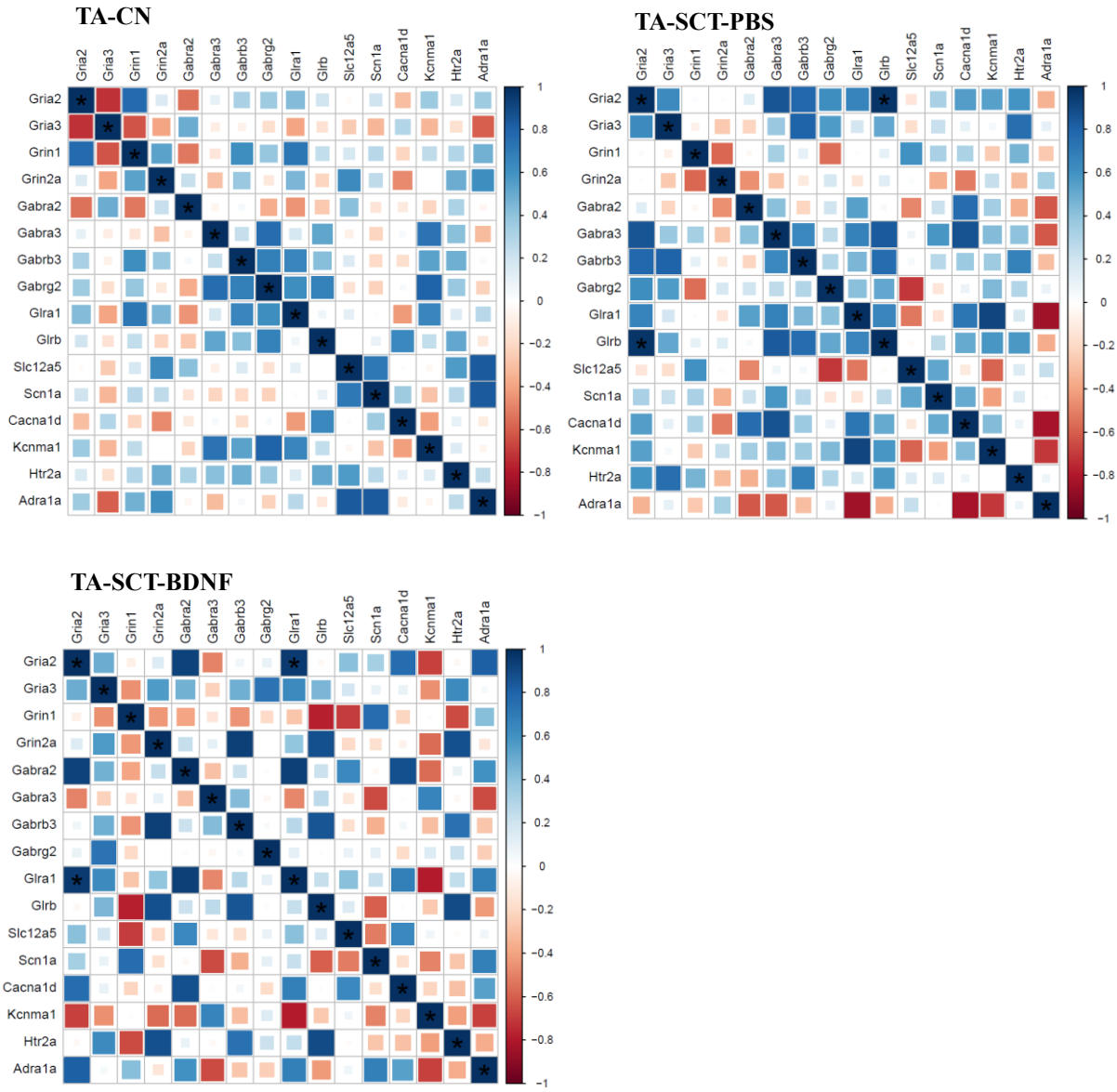

Figure 6.18. Changes in correlated gene expression as a result of SCT and AAV-BDNF treatment in TA MNs. The order of the genes along the X- and Y-axis of each correlogram are the same for comparison of the changes between groups. * significant FDR corrected p-value in R.

6.2.2.5. BDNF overexpression effects on the molecular changes in the whole lumbar segments after SCT

There is no doubt that GL and TA MNs play crucial roles in processing locomotion. However, the function of the spinal neuronal network cannot be fulfilled without premotor neurons. The net is an entirety containing cells whose intricate communications endow the precise function. BDNF overexpression affects not only MNs, but also other types of cells.

Our previous study showed that BDNF overexpression altered the expression levels of presynaptic interneuronal molecules involved in glutamatergic (VGluT2) and GABAergic (GABA, GAD65, GAD67) neurotransmission in spinal cord lumbar segments at six weeks postinjury (Ziemlińska et al., 2014). As the neurotransmitter receptors, ion channels and transporters are widely expressed in the spinal cord on different types of cells, our additional task was to examine whether effects of AAV-BDNF administration would be seen at the transcript level of selected receptors in samples from whole lumbar 3-6 (L3-6) segments. The result could roughly indicate the general tendency of regulation of neuronal excitability in response to SCT and BDNF overexpression.

In intact rats, transcript levels of *Gria2*, *Grin2a*, *Glra1* and *Gabrg2* subunits were the highest followed by *Gria1* and *Grin2b*, while *Grin1* are the lowest (**Figure 6.19**). Similarity of expression level between *Gria2*, *Grin2a*, *Glra1* and *Gabrg2* subunits suggests that there is a similar abundance of Glutamatergic, Glycinergic and GABAergic receptors in the L3-6 network. That relation did not concern characteristics of receptor expression GL and TA MN pools, which revealed significantly higher expression of GABAAR and GlyR than iGluR subunits (**Figure 6.2**).

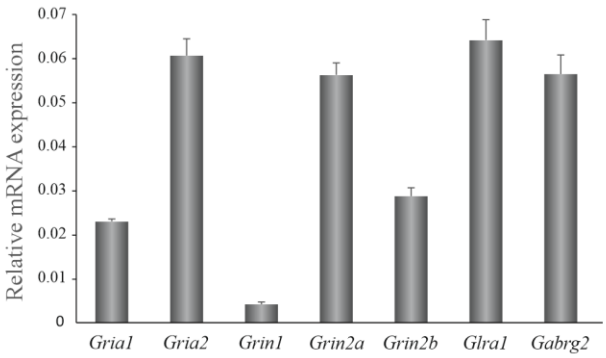

Figure 6.19. The transcript levels of AMPAR *Gria1* (GluA1) and *Gria 2* (GluA2), NMDAR *Grin1 (GluN1)*, *Grin 2a*, (GluN2A) and *Grin2b* (GluN2B), GlyR *Glra1* (GlyR α1) and $GABA_AR$ G*abrg2* ($GABA_AR$ α2) subunits in the spinal L3-6 segment of intact rats.

SCT resulted in a moderate increase in *Grin1* transcript level which was accompanied by a marked decrease of *Grin2a*, and *Glra1* and *Gabrg2* transcripts of inhibitory receptor subunits in L3-6 segment at 2 weeks postinjury (**Figure 6.20**). The results suggest primarily that inhibitory signaling is decreased and a balance between inhibitory and excitatory neurotransmission is disturbed. BDNF overexpression further increased the transcript level of *Grin1* and decreased the level of *Gabrg2* (**Figure 6.20**). In

addition, BDNF overexpression tends to increase the mRNA levels of *Gria1* and *Gria2* with respect to their levels in spinalized rats only, suggesting that up-regulation of AMPAR signaling is promoted (**Figure 6.20**).

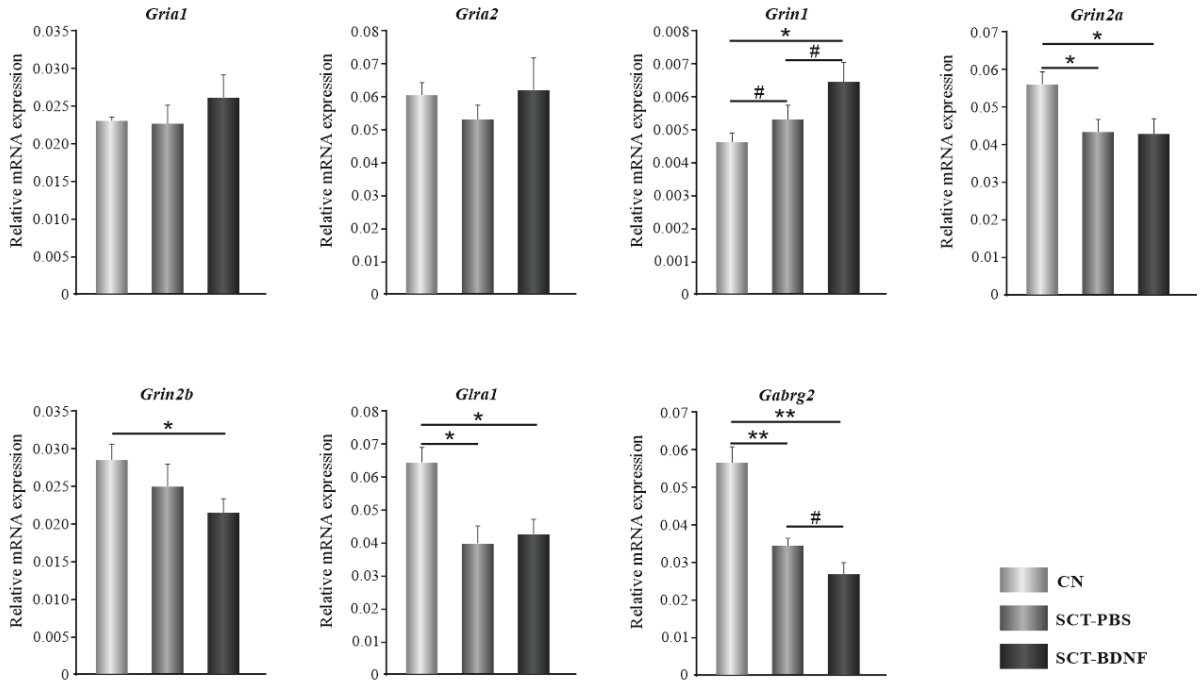

Figure 6.20. The effect of BDNF overexpression on the AMPAR, NMDAR, GlyR and GABAAR subunit levels in the spinal cord L3-6 segment at 2 weeks postinjury. Data are means +/- SEM. # $p<0.08$; * $p<0.05$; ** $p<0.01$; Mann-Whitney U-test.

We identified some clear similarities between responses of MNs and of the whole network to BDNF. First, BDNF overexpression increased the transcript level of *Grin1* both in TA MNs and L3-6 segments. The decreased levels of inhibitory receptor subunits in GL and TA MNs are in line with the downregulated transcript levels of inhibitory *Glra1* and *Gabrg2* after SCT in L3-6 segments. The significantly decreased *Gabrg2* level in GL MNs after BDNF overexpression is also parallel with downregulation of *Gabrg2* in L3-6 segments. These results suggest that the disinhibition is a universal effect of SCT in the spinal cord and that BDNF treatment might further promote the excitation state of the spinal cord neuronal network.

7. Discussion

7.1. SCT tends to increase the excitability of MNs innervating ankle extensor GL and flexor TA muscles

I demonstrate that complete SCT at low thoracic level leads to early, significant changes in expression of the genes coding for major receptors and ion channels related to the control of excitability in MNs. The results provide new insight into the mechanisms of MN excitability developing in the subacute postlesion phase below the site of SCT, involving a multiple increase in transcripts coding for AMPAR GluA1 and 5-HTR 1A, 2A and 2B subunits, and a profound decrease in all $GABA_AR$ and GlyR subunits, KCC2, KCC3 chloride extruders and VG SK2 and BK potassium channels. We report that lumbar GL and TA MN groups, despite differential electrical properties (Cotel et al., 2009; Kim et al., 2020) similarly adapt to a more excitable state, which may increase the occurrence of extensor and flexor spasms.

7.1.1. Functional implications of early changes in gene expression of glutamatergic receptors after SCT

Among glutamatergic receptors, ionotropic AMPAR and NMDAR directly affect membrane potentials through controlling the flux of Na^+ and Ca^{2+} ions. The reported here changes in the level of transcripts of AMPAR GluA1 and GluA3 subunits, with maintenance of the most abundant transcripts of GluA2 subunit, suggest that AMPARs containing GluA1 homomers may become more abundant than other subunit combinations in AMPARs produced and undergoing trafficking to synapses in MNs. That such combination is possible, show early experiments which revealed that endogenous GluA1 in brain neurons is mostly tetrameric (Greger et al., 2003). Because emerging evidence supports that the regulated expression and trafficking of GluA1-homomeric subtype of AMPARs mediates synaptic plasticity (Ge and Wang, 2021), the observed GluA1 stimulation in MNs may be a manifestation of postlesion plasticity.

AMPAR subunit composition determines both trafficking modes and transmission properties (Hollmann and Heinemann, 1994; Shi et al., 2001; Malinow, 2003). The trafficking of AMPARs to and from synapses is a key mechanism for regulating the strength of synaptic transmission (Barry and Ziff, 2002; Malinow, 2003). Early studies have shown that the majority of AMPARs in the telencephalon contain the GluA2 subunit (Ozawa et al., 1998; Dingledine et al., 1999) which dominates major AMPAR

transmission properties (single-channel conductance, rectification, and Ca^{2+} permeability) through Arg607, a residue introduced into the GluA2 pore loop by RNA editing at the Q/R site (Sommer et al., 1991). Editing at this site is specific for GluA2, as GluA1, GluA3, and GluA4 carry a Gln (Q) at this pore-lining position (Seeburg, 1996). The vast majority of GluA2 (>99%) in adult brain is edited to Arg (R). Editing at this site was found to be efficient and crucial for brain function, turning AMPAR from Ca^{2+} permeable (CP) to Ca^{2+} impermeable (CI-) (Seeburg, 1996). Our reasoning is that the central role of GluA2 subunit in AMPAR is reflected by its highest transcript level among other AMPAR subunits in MNs in normal rats, found in this study, and by relative stability of its expression after SCT.

Importantly, apart from determining conductance properties Arg607 also controls AMPAR traffic by restricting channel exit from the endoplasmic reticulum (ER) (Greger et al., 2002). Exit from the ER poses the first rate-limiting step for membrane proteins destined for the cell surface, and is subject to tight control (Ellgaard and Helenius, 2003). ER-retained GluA2 is very stable and forms an intracellular pool. That contrasts with GluA1, which is mostly post-ER and concentrated at synapses. Concentration of GluA2 in the ER facilitates its incorporation during channel assembly, and thus explains its presence in the majority of AMPARs in the brain (Greger et al., 2002; Seeburg, 2002).

Two transitions can be postulated based on the results: (1) the aforementioned transition from Ca^{2+} impermeable (CI-) AMPAR to Ca^{2+} permeable (CP-) AMPAR, and (2) transition in contribution of NMDAR and AMPAR in mediating of excitatory events. First, CP-AMPARs which lack GluA2 subunit have a higher single-channel conductance than the CI-AMPARs which contain GluA2 (Cull-Candy and Farrant, 2021). Therefore, there is a larger influx of Na^{+} and Ca^{2+} through CP-AMPARs. For instance, switch to CP-AMPAR (lacking GluA2 or containing unedited GluA2) increases neuronal excitability in hypothalamus of hypertensive rats (Li et al., 2012), in the hippocampus of mice with seizures (Konen et al., 2020), in spinal dorsal horn neurons of rats with inflammatory pain (Vikman et al., 2008; Li et al., 2021), and also in differentiated human MNs in amyotrophic lateral sclerosis model (Huang et al., 2021b).

Our speculation on the "NMDAR to AMPAR functional transition" is based on the reasoning taking into account that although single receptor conductance of NMDAR is higher comparing to that of AMPAR (Traynelis et al., 2010), NMDARs are blocked by Mg^{2+} at resting conditions and activate slower than AMPARs. Besides, AMPARs represent the majority Glu receptors on postsynaptic sites and

produce a faster response (Goncalves et al., 2020). n certain circumstances, such as epileptiform activity, CP-AMPARs can be a richer source of intracellular Ca^{2+} than NMDARs, as concluded from comparison of the effect of blockade of NMDARs vs CP-AMPARs, the former reducing the additional conductance to a lesser extent (Amakhin et al., 2021). Therefore, despite NMDAR subunit transcript level is reduced after SCT, increased abundance of CP-AMPARs, if occurs, might be able to depolarize the postsynaptic membrane by providing a sufficient influx of Ca^{2+}. There is experimental evidence to support that reasoning: (1) after NMDAR ablation excitability of hippocampal CA3 neurons is enhanced (Fukushima et al., 2009), and (2) a significant increase in AMPAR/NMDAR ratio was found in spinal dorsal horn neurons of rats with inflammatory pain which reflects, at least in part, an increase in neuronal excitability (Gold and Flake, 2005). That was associated with reduction in the quantal amplitude of NMDAR-mediated synaptic currents (Vikman et al., 2008). Our data suggest that the quantitative switch in Glu receptor expression levels from NMDAR to AMPAR may contribute to an increase or at least maintenance of MN excitability. How long the increased levels of CP-AMPARs may be maintained remains uncertain since following a depolarization a block of CP-AMPARs by endogenous intracellular polyamines was reported (Twomey et al., 2018).

7.1.2 Functional implications of downregulation of gene expression of GABAergic and glycinergic receptors, KCC2 and ion channels after SCT

In the present study we showed that SCT significantly downregulated the transcript levels of all examined subunits of $GABA_AR$ and GlyR, as well as the Cl^- extruder KCC2 and KCC3, suggesting a decreased synthesis of these molecules and subsequent development of reduced inhibition on MNs. Development of hindlimbs spasticity at 3 weeks postinjury was found associated with significantly decreased protein level of $GABA_A$ receptors and GlyR receptors on lumbar MNs in rats with SCT (Sadlaoud et al., 2020a). Similarly, decreased gene expression of these receptors also contributes to the hyperexcitability of sacral MNs and tail spasticity in rats with SCT at the sacral segments (Wienecke et al., 2010). KCC2 chloride ions extruder has been well shown to strongly influence the efficacy and polarity of the $GABA_AR$- and GlyR- mediated synaptic transmission (Chamma et al., 2012). In basal conditions, KCC2 maintains a low-level of intracellular Cl^-; activated $GABA_AR$ and GlyR allow influx of Cl^- into neurons to generate an inhibition of neurons by hyperpolarizing the postsynaptic membrane.

Downregulation of KCC2 in MNs was found to decrease the strength of postsynaptic inhibition and therefore contributed to spasticity formation after spinal cord injury (Boulenguez et al., 2010) (**Figure 7.1**). Herein, my results suggest that reduced expression of inhibitory receptors and chloride ions KCC2 and KCC3 extruders in MNs occurs at the early postinjury time point. Surprisingly, the transcript level of KCC1 was increased after SCT. KCC1 is ubiquitously expressed in brain, kidney, colon, heart, red blood cells and dorsal root ganglion (Gillen et al., 1996; Hebert et al., 2004; Crable et al., 2005; Mao et al., 2012; Côté, 2020), assumed to act as a housekeeping gene but also to mediate the symport of K^+ and Cl^- ions, but their function in MNs was not described. The increase of KCC1 may serve a compensation for the profound decrease of KCC2 and KCC3 to protect these MNs.

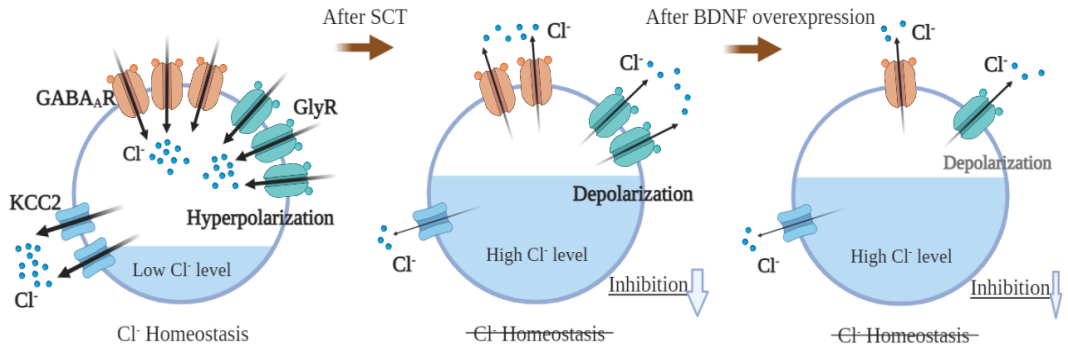

Figure 7.1 Proposed effects of SCT and BDNF overexpression on the Cl^- homeostasis in MNs. KCC2 is the major Cl^- extruder in mature neurons. By regulating intraneuronal chloride ions homeostasis, KCC2 strongly influences the efficacy and polarity of the $GABA_AR$ and GlyR mediated synaptic transmission. In basal conditions, KCC2 maintains a low-level of intracellular Cl^-; activated $GABA_AR$ and GlyR allow influx of Cl^-, generating a hyperpolarization. After SCT, a decrease of the KCC2 may lead to accumulation of high concentrations of Cl^- in MNs, evoke a depolarizing instead of inhibitory response, suggesting the possibility of a decrease of inhibition. The unaltered, low KCC2 level after BDNF overexpression shown in my study suggest that high concentrations of Cl^- are maintained in MNs. Therefore, activation of $GABA_AR$ and GlyR signaling may turn to excitatory instead of inhibitory. Deepened decrease of $GABA_AR$ and GlyR in MNs after BDNF overexpression may in these conditions be interpreted as promoting partial recovery of inhibition.

7.1.3. SCT affects gene expression of ion channels and modulatory receptors

The process of generation of action potential (AP) can be divided into two zones: input zone (synaptic potentials) and output zones (AP) (Giuliodori and Zuccolilli, 2004). Temporal and spatial summation of the postsynaptic potentials (EPSP and IPSP) propagates to the axon hillock and decides to fire an AP or not. When a net depolarized membrane potential reaches to the threshold, voltage-gated

ion channels, such as Na^+, Ca^{2+} channels, are responsible to generate APs. Therefore, the expression and abundance of ion channels are crucial for the initiation of APs and excitability of neurons. We have demonstrated that SCT increases the probability of generating EPSPs by upregulating the CP-AMPAR transcript levels, but reduces the IPSPs probability through downregulating the transcript levels of GABAR and GlyR, possibly decreasing the inhibitory signaling. My reasoning is that after the summation of EPSP and IPSP, an increased net depolarized membrane potential may be expected; in consequence, it may increase the probability to control the action of ion channels and generate of APs.

However, my results show that the changes of gene expression of ion channels after SCT are diverse. The transcript level of two voltage-dependent Ca^{2+} channels: Cav2.2 and Cav1.3 were unaltered, while the major voltage-dependent Na^+ channel Nav1.6 in rat spinal MNs was significantly downregulated. In addition, SCT also significantly decreased Ca^{2+}-activated K^+ channels SK2 and KCa1.1 in GL and TA MNs. Downregulated Nav1.6 gene seems to lead to a decrease of the probability of AP generation, but on the other side, the decreased SK2 and KCa1.1 might lead to reduced mAHP and therefore enhance the firing of AP and excitability of neurons. Furthermore, in addition to the revealed changes at the level of gene expression, their activity and biophysical properties may be altered and modulated by neuromodulatory receptors.

Group I mGlu1 and mGlu5 receptors are both expressed in the spinal cord lumbar MNs and they are predominantly expressed on postsynaptic membranes (Vidnyánszky et al., 1994; Anneser et al., 2004; Ma et al., 2006), modulating MN firing and the intensity of locomotor-related output (Nistri et al., 2006; Iwagaki and Miles, 2011). At the molecular level, their activation often results in cell depolarization and an increase in neuronal excitability by elevating intracellular Ca^{2+} level either via releasing of calcium ions from internal stores or via Ca^{2+} channels (Fagni et al., 2000; Ireland and Abraham, 2002; Niswender and Conn, 2010; Li et al., 2014a; Yu et al., 2018). Following SCI in rats, upregulation of Group I mGluRs was observed in spinal cord neurons (Gwak and Hulsebosch, 2005). In rat superficial spinal dorsal horn neurons, Group I mGluRs has been shown to mediate Ca^{2+} influx via L-type of voltage-gated Ca^{2+} channels (Heinke and Sandkuhler, 2007). Therefore, we may propose that the increased level of transcripts coding for group I mGluRs, found in the current study, if translated into changed mGluR protein level may contribute to the increase of intracellular Ca^{2+} level by modulating Ca^{2+} channels and triggering the release of Ca^{2+} from internal stores in GL and TA MNs.

After SCI, persistent sodium inward currents (Na^+ PICs) and persistent calcium currents (Ca^{2+} PICs) are spontaneously developed in MNs, contributing to MN firing (Li et al., 2004). The two inward currents are directly generated by voltage-gated Na^+ channels and Ca^{2+} channels, respectively (Harvey et al., 2006a; Heckman et al., 2008; Rank et al., 2011). Ca^{2+} channel subtype Cav1.3 was demonstrated to be responsible for generating Ca^{2+} PICs and contribute to the hyperexcitability of MNs after SCI (Carlin et al., 2000; Li and Bennett, 2003; Jiang et al., 2021). However, it is not clear which subtypes of voltage-gated Na^+ channels mediate the Na^+ PICs. It is reasonable to propose that Nav1.1 and Nav1.6 may play the roles, since they are the most abundant at the transcript level in the two groups of MNs, as revealed in the current study. Furthermore, the generation of Na^+ PICs and Ca^{2+} PICs is facilitated after activation of 5-HT_2 and NAα1 receptors which modulate function of the relative Na^+ and Ca^{2+} channels (Harvey et al., 2006a; Heckman et al., 2008; Rank et al., 2011), therefore leading to a general increase of the excitability of MNs.

In the current study, in both MN groups, spinalization led to increased transcript level of 5-HT_{1A} and 5-HT_{2B} receptors, suggesting a positive effect of these receptors' activation on MN excitability. The increased MN firing frequency may contribute to change in properties as well, as a result of 5-HT_{1A} inhibition of SK channels and reduced mAHP (Wikström et al., 1995; Grunnet et al., 2004). I conclude that this common response, accompanied by a MN-type specific change (an increase of 5-HT_{2A} in GL MNs and a decrease of 5-HT_{2C} in TA MNs) might be modulated differently in GL and TA MNs.

7.2. Overexpression of BDNF leads to locomotor recovery and promotes pro-excitogenic phenotype of lumbar MNs after SCT

One promising way to treat SCI and modulate SCI-altered excitability of MNs is through administration of brain derived neurotrophic factor (BDNF) to the injured spinal cord. BDNF is a classic member of neurotrophin family that supports the survival, differentiation and maturation of neurons and regulates glutamatergic signaling, synaptic function and spine morphology during development of the nervous system, promoting protection and regeneration of neurons (McAllister et al., 1999; Bibel and Barde, 2000; Numakawa et al., 2010; Kowianski et al., 2018). After being secreted into extracellular cleft, BDNF binds to TrkB receptor and acts on both presynaptic terminals and postsynaptic neurons to regulate neurotransmitter release (Li et al., 1998; Tyler et al., 2002), and modulate membrane potentials

and cell functions through receptor-mediated signaling pathways (Numakawa et al., 2010; Kowianski et al., 2018). Based on these features, number of studies were undertaken which identified that administration of BDNF to the animals with different SCI improve their motor abilities (Ziemlińska et al., 2014; Hernandez-Torres et al., 2017; Krupka et al., 2017; Ghosh et al., 2018; Charsar et al., 2019; Crowley et al., 2019; Li et al., 2019; Marchionne et al., 2020; Sieck et al., 2021). Moreover, the recovery of hindlimb locomotion after BDNF treatment was accompanied with enhanced excitability of lumbar MNs innervating hindlimb muscle (Boyce et al., 2012). These data were the starting point of my experiments, to consider BDNF treatment improving motor function after SCI in part through modulating the MN excitability.

My results showed that BDNF overexpression by AAV-BDNF intraspinal injection into spinal cord L1 segment profoundly improved locomotor performance of spinalized rats on a running treadmill, which is consistent with our previous study on long-term effects of BDNF overexpression (Ziemlinska et al., 2014). Moreover, through improving the experimental procedures and increasing the number of experimental animals, my results further strengthened the above conclusion. My results also showed that the AAV-BDNF administration increased the transcript level of BDNF in GL and TA MNs, that is in line with our study that shows the transcript level is significantly increased in the whole L3-6 spinal segment where the GL and TA MNs are located ((Głowacka et al., 2022), under review).

7.2.1. BDNF and glutamatergic signaling

BDNF increases Glu release by modulating the distribution and size of synaptic vesicles within presynaptic terminals (Takei et al., 1998; Tyler and Pozzo-Miller, 2001; Tyler et al., 2006). Postsynaptically, BDNF/TrkB signaling regulates the expression, traffic and phosphorylation of AMPAR and NMDAR (Narisawa-Saito et al., 2002; Xu et al., 2006; Caldeira et al., 2007; Carvalho et al., 2008; Carreño et al., 2011; Zhang et al., 2016a). In the spinal cord, the regulation of Glu signaling via BDNF/TrkB has been extensively studied in the neuropathic pain pathways in the dorsal horn neurons. Binding of BDNF to TrkB receptors on dorsal horn neurons activates downstream signaling that triggers phosphorylation of GluN2B and GluN1 subunits of NMDAR and further enhances influx of Ca^{2+} through NMDAR (Slack and Thompson, 2002; Slack et al., 2004; Gao et al., 2005; Obata and Noguchi, 2006; Geng et al., 2010; Chen et al., 2014; Ding et al., 2015; Liu et al., 2015; Li et al., 2017).

As the result, the excitability of dorsal horn neurons is increased, which leads to enhancement of neuropathic pain (Zhou et al., 2021). Current evidence indicates that BDNF exerts similar modulatory functions in spinal cervical MNs; it has been suggested that BDNF/TrkB signaling may enhance the excitability of phrenic MNs via NMDAR signaling (Gill et al., 2016). Localized acute or chronic intrathecal delivery of BDNF to phrenic MNs pool promotes functional recovery of rhythmic EMG activity of diaphragm muscle after cervical SCI (Mantilla et al., 2013; Hernandez-Torres et al., 2017; Sieck et al., 2021), prevented by knockdown of TrkB expression or inhibition of TrkB kinase activity in phrenic MNs (Mantilla et al., 2013; Mantilla et al., 2014). Intrapleural delivery of AAV-TrkB also enhanced the recovery of ipsilateral rhythmic diaphragm muscle activity postinjury, and simultaneously increased mRNA expression of NMDAR in phrenic MNs (Gransee et al., 2017), suggesting an association between BDNF/TrkB signaling and Glu neurotransmission, which may reflect increased excitability of phrenic MNs during functional recovery of diaphragm. BDNF is capable of promoting survival of axotomized MNs in spinal cord lumbar segments during development (Yan et al., 1992; Kishino et al., 1997). However, how BDNF/TrkB signaling influence Glu signaling in lumbar MNs is not clear.

In my study, BDNF overexpression resulted in a general decrease of the transcripts of AMPAR subunits but an increase of the transcripts of NMDAR subunits, suggesting a rebalance of abundance of AMPAR and NMDAR, comparing to changes provoked by SCT. Although I have discussed above that the switch in expression levels from NMDARs to AMPARs after SCT may contribute to an increase or at least maintenance of MN excitability, based on the aforementioned evidence which I gathered, I propose a speculation that enhanced BDNF/TrkB signaling after AAV-BDNF administration might increase the excitability of MNs in a NMDAR-dependent way. Whether it is the case needs to be confirmed by further studies.

7.2.2. Multitarget impact of BDNF on inhibitory signaling: GABAergic/glycinergic receptors

BDNF/TrkB signaling can either downregulate or upregulate the expression of KCC2 in neurons via distinct signaling pathways (Lee-Hotta et al., 2019). So far, the data regarding how BDNF treatment modulates KCC2 expression after SCI are not conclusive. After chronic SCI, a decreased expression of

KCC2 was shown to contribute to the development of hyperreflexia and spasticity (Boulenguez et al., 2010). These symptoms can be alleviated by physical exercise. Treadmill training upregulated BDNF expression which increased expression of KCC2 on the plasma membranes of lumbar MNs after SCI (Tashiro et al., 2015; Beverungen et al., 2020; Li et al., 2022); elevated levels of KCC2 restored Cl^- homeostasis and reduced hyperexcitability of MNs, ameliorating spasticity. Consistently, in these two studies, blocking BDNF signaling during the training session prevented the increase of KCC2 expression and failed to ameliorate allodynia and spasticity. These results appear to illustrate a promising role of activity-dependent endogenous BDNF in modulation of KCC2 expression, MN excitability and recovery after SCI. Interestingly, intrathecal injection of exogenous BDNF into spinal cord lumbar enlargement at 15 d after SCI increased the relative amount of plasmalemmal versus cytoplasmic KCC2, indicating the exogenous BDNF contributes to attenuation of spasticity when the hyperreflexia and hyperexcitability had already developed (Boulenguez et al., 2010).

In my study, comparing with intact rats, the SCT with PBS injection resulted in decreased mRNA levels of the majority of examined subunits of $GABA_AR$, GlyR and KCC2, which is in line with the effects of SCT described in the first chapter. My results showed that BDNF overexpression resulted in a further downregulation of *Gabrb3*, *Gabrg2*, *Glra1*, *Glrb* in GL MNs and *Gabra2*, *Glra1* in TA MNs; besides, the deficits of KCC2 transcripts caused by SCI were not counteracted by enhanced BDNF signaling in either MN type. Therefore, assuming that the transcript level is positively correlated with its protein level, I conclude that after SCT both lumbar extensor and flexor MNs are impoverished in KCC2. At a first glance, it seems that the results are in contrast to our expectations, because we considered that the enhanced BDNF/TrkB signaling might partly recover the impaired inhibitory neurotransmission in MNs to attenuate the development of the hyperexcitability of MNs and improve locomotor performance. Spinal disinhibition has been recognized as one mechanism to underlie hyperexcitability of MNs during SCI. Indeed, as I have illustrated on the **Figure 7.1,** a decrease of the KCC2 after SCT may lead to accumulation of high concentrations of Cl^- inside GL and TA MNs, which causes a shift in $GABA_AR$ and GlyR function from inhibition to depolarization state, leading to a decrease of inhibition. However, low KCC2 level, found unaltered after BDNF overexpression in my study, together with concomitant decrease of $GABA_AR$ and GlyR transcripts in MNs suggest, that high concentrations of Cl^- are maintained in the MNs but less excitatory drive resulting from GABAR and

GlyR contribution occurs, which also can be interpreted as a partial recovery of inhibition on MNs.

In this reasoning, the data obtained by our group previously, are important. Namely, chronic administration of exogenous BDNF up to 6-7 weeks resulted in a reduced level of KCC2 caudal to lesion site, which combined with elevated levels of GABA and GABA-synthetic enzymes (GAD65 and GAD67) (Ziemlińska et al., 2014), might serve as the conditions to develop hyperreflexia, observed in that time postlesion. The high level of intracellular Cl^-, flowing out the neurons through GABA or/and glycine receptors, increases plasma membrane potential and results in the depolarization of the neuron; depolarization raises Ca^{2+} influx through voltage-dependent Ca^{2+} channels and NMDAR, further leading to increased excitability (Lee-Hotta et al., 2019).

Taken together, these data, including those obtained by me, raised the question: which determinants or conditions trigger BDNF to elicit either the upregulation or downregulation of the expression of KCC2 during SCI? The most probable factor is the concentration of BDNF available rather than its source. Physical training may induce the proper synthesis and release of BDNF as a result of adaptive plasticity of the animals to adapt to environmental change, which tends to alleviate the symptoms of SCI. On the other hand, BDNF tissue levels achieved after exogenous administration of BDNF protein or trangene are hard to control, what may lead to the amount of BDNF surpassing the requirement of the body, as described by our group, when overwhelming BDNF expression at 6-7 weeks after AAV-BDNF administration lead to spasticity-liked symptoms attenuating the quality of locomotor recovery accompanied with a decrease of KCC2 expression (Ziemlińska et al., 2014).

Interestingly, a study showing that application of exogenous BDNF significantly decreased the KCC2 mRNA levels in non-axotomized corticospinal neurons, while it increased KCC2 mRNA in axotomized corticospinal neurons (Shulga et al., 2008), suggest that axotomy may intervene the regulatory effects of BDNF on KCC2 expression. However, in my study spinal lumbar MNs are not axotomized after a thoracic SCT. Moreover, compared to the severe cell death of axotomized neurons at the lesion site of the spinal cord, neurons are not affected far from the lesion site(Bjugn et al., 1997; Crowe et al., 1997; Grossman et al., 2000; Hassannejad et al., 2018), as I showed also in my study in spinal and spinal, BDNF-treated rats.

7.2.3. Selectivity of BDNF- upregulating effects on monoaminergic receptors: 5-HT2ARs

BDNF can exert excitatory actions and raise excitability of MNs also via mediating the gating of ion channels including Na^+, Ca^{2+}, K^+ at the plasma membrane of neurons (Blum et al., 2002; Rose et al., 2004). My results showed that BDNF overexpression did not cause significant effects on the transcript level of Cav1.3 and Kca1.1 channels, but counteracted the Nav1.1 (one of the major Na^+ channels expressed in GL and TA MNs) elevation caused by SCT-PBS and restored it to control level.

The descending serotonergic (5-HT) or noradrenergic (NA) signaling from brain stem to spinal cord play crucial modulatory role in regulating the excitability of the spinal neuronal network. Given that, they have also been regarded as potential target to treat SCI. Administration of agonists of 5-HTRs and ARs, or graft of brainstem embryonic tissue were shown to trigger the restoration of coordinated hindlimb locomotion after SCI in adult animals (Chau et al., 1998; Ribotta et al., 2000; Sławińska et al., 2000; Slawinska et al., 2013; Ganzer et al., 2018; Kwaśniewska et al., 2020). At the cellular level, 5-HTRs and ARs are well known to modulate MN excitability in intact and SCI rats, especially 5-HTR_2 and $AR_{\alpha 1}$ in facilitating MN PICs (Heckman et al., 2009; Rank et al., 2011).

A substantive number of studies demonstrated that treatment of SCI with BDNF administration promoted the sprouting of 5-HT fibers and enhanced the serotonergic innervation of MNs (Sasaki et al., 2009; Han et al., 2015; Liu et al., 2017; Ghosh et al., 2018; Charsar et al., 2019; Kandalam et al., 2020). However, the knowledge of the connection between BDNF and t receptors in SCI is limited. The results obtained in this study showed that BDNF overexpression significantly elevates the transcript level of 5-HTR_{2A} beyond control level in GL and TA MNs, without affecting the $AR_{\alpha 1A}$, suggesting an increased serotonergic signaling dependent modulation which may enhance the excitability of MNs. There are also studies showing that activation of 5-HT_{2A} receptors by agonists upregulates the function and membrane expression of KCC2 in lumbar MNs and in dorsal horn neurons, thereby restoring the described earlier chloride ions homeostasis and endogenous inhibition, and reducing spasticity and neuropathic pain after SCI in rats (Bos et al., 2013; Sanchez-Brualla et al., 2018). In my study, KCC2 transcript level was not altered by BDNF overexpression, but we cannot exclude the possibility that the KCC2 protein was redistributed. Further studies are needed to disclose whether the protein level of the above molecules was adapted by BDNF overexpression.

7.2.4. How does BDNF overexpression change the protein level of the receptors?

The molecules examined in my study are clearly expressed on MNs displaying specific roles. For instance, I showed that AMPAR and NMDAR are detected in the cytoplasm and in the plasma membrane compartments (**Figure 7.2 A, B**, **Figure 7.3**). Subcellular fractionation of the tissue from L3-6 segments and western blot results showed that AMPAR GluA2 subunit and NMDAR GluN1 are mainly associated with membrane fraction (**Figure 7.2 C**). These observations indicate that these molecules are synthesized in the GL and TA MNs and transported to plasma membrane structures.

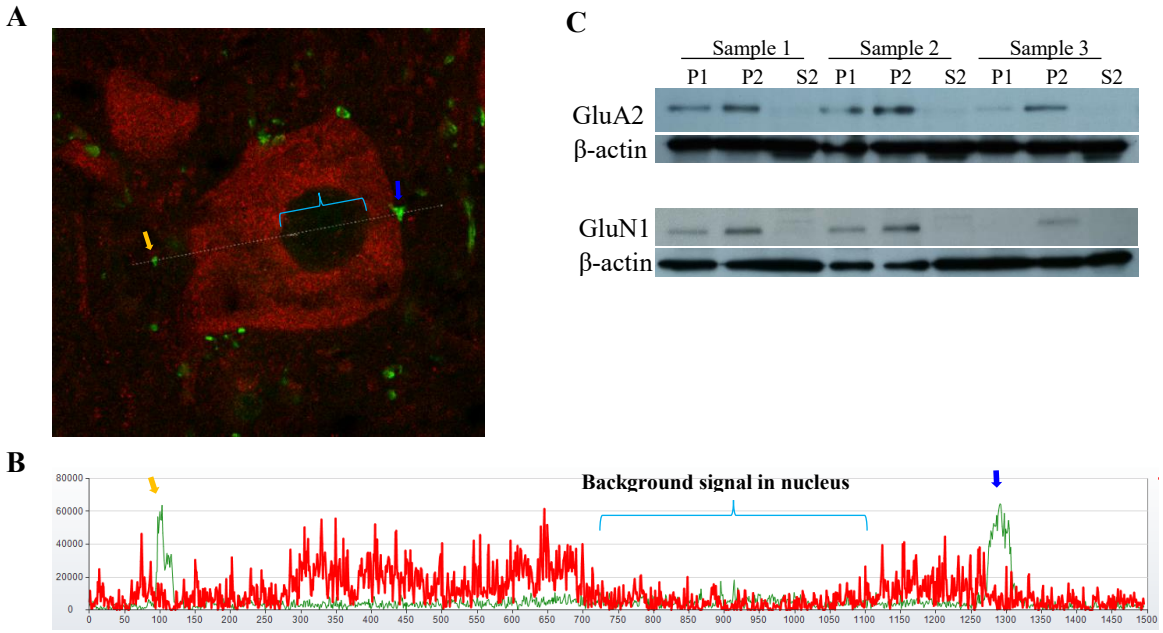

Figure 7.2. NMDAR GluN1 and VGLUT1 IF signal distribution on TA MNs. A. The IF intensity of GluN1 observed in the cytoplasm and membranes of MNs. VGLUT1 maker of Glu inputs is located in terminal boutons (green, arrows). B. The histogram presents intensity of line profile (white dotted line on A) of the staining. Arrows point peaks of VGLUT1 signal. Green: VGLUT1; Red: NMDAR GluN1. C. Subcellular fractionation and western blot results show that AMPAR GluA2 and NMDAR GluN1 are dominating in membrane fractions. P1: nuclear; P2: membrane; S2: cytoplasm fraction.

In the current study, I have shown the transcriptional regulation of the genes responsible for MN excitability in response to SCT and BDNF overexpression. Whether this transcriptional regulation is translated into protein alterations needs to be disclosed in further studies.

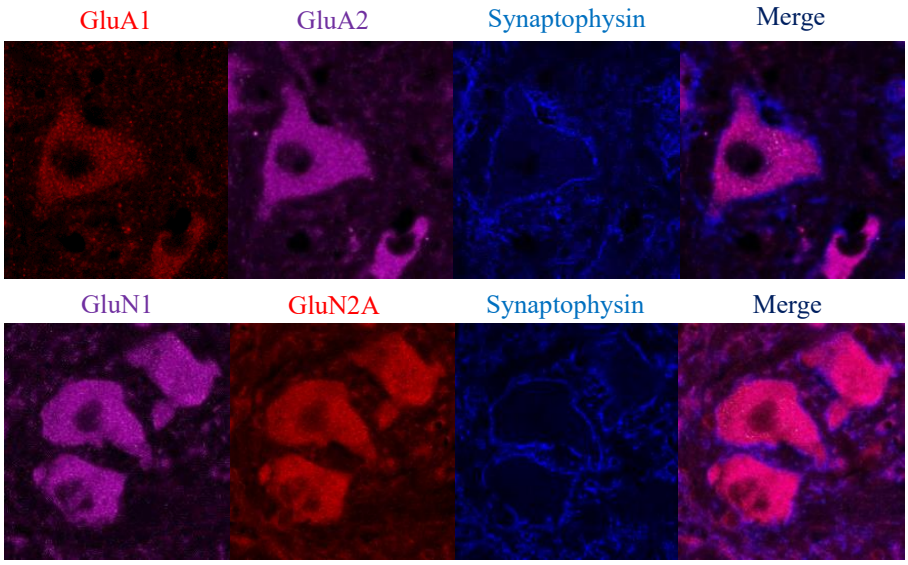

Figure 7.3. Representative example of AMPAR (GluA1 and GluA2 subunits), NMDAR (GluN1 and GluN2A subunits) and presynaptic maker synaptophysin expression and distribution on large MNs in the ventral horn of spinal cord.

To sum up, increasing the availability of BDNF for the impaired neurons to improve their function is a useful approach to treat SCI, as confirmed by this study. The treatment significantly improves the recovery of locomotion of SCT animals. At the molecular level, BDNF increases the MN excitability and activity of the locomotor network within the CPG. Despite beneficial effects, BDNF administration time period and dose are crucial for the outcomes, because high doses markedly exceeding the physiological ones and long term BDNF treatment cause undesirable effects. Our previous study showed that BDNF local overexpression improved the locomotor performance of spinalized rats on a running treadmill at early time point (~2 weeks) and at late time points (6–7 weeks). However, at the late time point, the BDNF treated rats displayed increased frequency of clonic movements which attenuated the quality of locomotor movements (Ziemlińska et al., 2014). These spasticity-like symptoms were also observed in a study exploring a cervical hemisection (Fouad et al., 2013). In my study a recovery of locomotion in the spinalized rats treated with AAV-BDNF was progressive. First step-like hindlimb movements in SP-BDNF rats was observed as early as on the 8th day after treatment and marked improvement of their performance reached the peak between 13th and 16th days. The results of my work indicate that controlling the administration time and dose of BDNF with concomitant treatment aimed to modulate selected receptors and channels is a promising strategy to improve function and network activity.

8. Summary and Conclusions

1. SCT results in disinhibition and increased modulation of transcription of the majority of genes coding for major receptors, transporters and channels associated with neurotransmission, similarly in GL and TA MNs. This result suggests that both the ankle extensor and flexor MNs were adapted to be more excitable at the subacute phase.

2. Correlation analysis indicated that the patterns of gene expression differ between GL MNs and TA MNs from Control animals, and are changed after SCT. The revealed patterns suggest that the set of genes coding for neurotransmission-related membrane proteins undergoes differential regulation in extensor and flexor MNs in physiological conditions and after injury.

3. BDNF overexpression caused a profound improvement of the locomotor performance of spinalized rats accompanied by marked changes in receptor expression suggesting promotion of the pro-excitatory phenotype in MNs, which develops after SCT. The reconstruction of the net effects of BDNF on the excitatory state of MNs requires further studies.

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