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Gene expresion in rodent spinal neuronal populations and their response to injury

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It is not what you look at that matters, it's what you see ...

H. D. Thoreau

Abstract

Motor neurons are the centre of convergence for all neural activity relating to movements. The activity integrated in the motor neurons is transmitted to appropriate muscles generating coordinated muscle contractions. Motor neurons, long considered passive integrators of the motor signal, have been shown to actively participate in shaping the output to the muscles during different behaviors, where active synaptic components resulting in plateau potentials and persistent inward currents can be activated during motor neuron recruitment. In the present thesis the functional significance of motor neurons during normal and injury states have been examined using a combination of electrophysiology and gene expression profiling.
First the transmitter phenotype of motor neurons was examined. Motor neurons have long been thought to release only acetylcholine at their terminals thus following the central dogma proposed by Dale, stating that a neuron releases the same neurotransmitter from all its terminals. We find that motor neurons release not only acetylcholine but also glutamate at central synapses, whereas we did not discover any sign of glutamate release at the neuromuscular junction. This finding jeopardizes the central dogma, indicating a new level of possible modulation by motor neurons in shaping the motor output through a differentiated release of two fast neurotransmitters at distinct axon terminals.
To further elucidate the functional role of motor neurons in relation to other spinal neuronal populations, the expression profiles of motor neurons and descending commissural interneurons (dCIN) were compared. This task required development of a method, which can be used for reliable gene expression profiling with RNA extracted from as few as 50 fluorescently identified and laser dissected cells. Based on this methodology, we find 49 significantly differentially expressed genes that may relate to the functional differences between motor neurons and dCINs in transmitting and shaping the motor output.
Our method was subsequently used to measure the transcriptional response of motor neurons following spinal cord injury. Injury causes long-term changes in spinal networks located caudal to the injury resulting in maladaptive pathophysiological states including spasticity. In normal animals the expression of plateau potentials caused by persistent inward calcium and sodium currents (PICs) is conditional and depends on the presence of monoamines released from descending pathways. Motor neurons therefore lose the ability to express plateaus immediately after a spinal cord injury as the descending fibers are severed. The ability of motor neurons to express PICs reappears after a few weeks and has been implicated in injury-induced spasticity. We use the expression profiles of motor neurons to examine the molecular underpinnings of this return of plateaus in the late phase of the injury response, 21 and 60 days post injury. We find that the ancillary subunits of the channel complexes conducting the PICs, rather than the pore forming subunits, are subject to extensive regulation. Genes coding for receptors and intracellular pathways relating to the expression of plateau potentials also undergo regulation.
Lastly, we examined the general transcriptional response of motor neurons throughout the injury response; 0, 2, 7, 21 and 60 days post injury and the underlying regulatory control of gene expression. We find that motor neurons are involved in the general injury response with a transient up-regulation of inflammatory and immunologically related processes in the early phase, while developmental pathways are up-regulated in late phases of the injury response. Promoter analysis conducted on expression clusters revealed general targets of regulation for identified transcription factors that participate in the injury response of the motor neurons.
We conclude that the motor neurons engage an extensive molecular machinery to regulate and modulate their electrophysiological properties as a response to injury. This suggests that electrophysiological properties are subject to dynamic regulation that also could be at play in normal states of the spinal cord, thus modulating the functional response of the motor neurons and shaping the motor output. Together, the results presented in this thesis have provided new knowledge about the normal function of motor neurons and a novel insight into the development of spasticity that can help define new therapies for spinal cord injury.

Contents

Preface

List of Publications included in the Thesis

The thesis has been written to fulfill the requirements of the Doctorate of Medicine at Karolinska Institutet. The presented work was carried out at the Mammalian Locomotor Laboratory at the Department of Neuroscience (Kiehn Lab).
The thesis is based on the following four publications, which will be referred to in the remainder by their roman numerals. The manuscripts of these publications are included at the end of the thesis.

I. *Mammalian motor neurons corelease glutamate and acetylcholine at central synapses*, PNAS. 102(14):5245-9, Nishimaru H, Restrepo CE, **Ryge J**, Yanagawa Y, Kiehn O

II. *Gene expression profiling of two distinct neuronal populations in the rodent spinal cord*, PLoS ONE. 2008;3(10):e3415. **Ryge J**, Westerdahl AC, Alstrøm P, Kiehn O

III. *Global gene expression analysis of rodent motor neurons following spinal cord injury associate molecular mechanisms with the development of post-injury spasticity*, in revision J. Neurophysiology, J. Wienecke, A-C Westerdahl, H. Hultborn, O. Kiehn and **J. Ryge**

IV. *Identification of gene clusters in motor neurons under common regulatory control relating to motor dysfunction after spinal cord injury*, Manuscript, **Ryge, J**, Winther, O, Wienecke J, Sandelin, A, Westerdahl, A-C, Hultborn, H and Kiehn, O

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Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
AP	Action Potential
aRNA	Anti-sense RNA
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
bp	base pair
CaM	Calmodulin
CDF	Calcium Dependent Facilitation
CDI	Calcium Dependent Inactivation
CIN	Commissural Interneuron
CNS	Central Nervous System
CPG	Central Pattern Generator
DE	Differential Expression
DNA	Deoxyribonucleic Acid
EPSP	Excitatory Post Synaptic Potential
GABA	Gamma-Aminobutyric Acid
GO	Gene Ontology
IPSP	Inhibitory Post Synaptic Potential
LMD	Laser Microdissection
MLR	Mesencephalic Locomotor Region
MM	Miss Match
MN	Motor Neuron
NA	Nor-Adrenaline
NMDA	N-methyl-D-aspartic Acid
NMJ	Neuromuscular Junction
PIC	Persistent Inward Current
PKA	Protein Kinase A (cAMP-dependent)
PKC	Protein Kinase C
PM	Perfect Match
PWD	Position Weight Matrix
QLT	Quantile Linear Transformation
qqplot	quantile-quantile plot
RNA	Ribonucleic Acid
RMA	Robust Muliti-array Analysis
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TF	Transcription Factor
TTX	Tetrodotoxin
VDF	Voltage Dependent Facilitation

Chapter 1

Introduction

In late 18th century Italy, Luigi Galvani discovered "animal electricity" when he, much to his surprise, found that touching an exposed sciatic nerve of the frog with a metal scalpel caused its leg to kick. Galvani had been conducting a separate series of experiments on static electricity causing the metal scalpel to pick up a charge, subsequently causing the frog leg to kick when a current surged through the nerve in turn activating the muscles. This discovery initiated a series of new investigations as it became possible to stimulate and record muscle and nerve activity in a well-defined manner, previously inaccessible to experimental manipulation. Ultimately, this knowledge led to the understanding that muscles as well as the tissue of the central nervous system (CNS) mediate their actions through electrical impulses, defining electrophysiology as an area of research. Using electrophysiology, the spinal cord was established as the seat of locomotor control and its neuronal components have since been investigated in great detail. It is by now well established that spinal neuronal networks generate the coordinated locomotor output, while sensory feedback modulates ongoing activities. New advances in molecular biology have introduced novel tools to the field of locomotor research, enabling new avenues to be explored complementing the findings based on the discoveries of Galvani three centuries ago. Gene expression profiling is being increasingly used in the field of neuroscience and, in combination with fluorescent markers of neuronal populations, it provides a powerful tool to assess molecular mechanisms in well defined cell populations. In the following I will give a short review on the development of concepts within the field of vertebrate locomotion during the last century and the role of motor neurons in generating the final motor output. The motor neurons of the spinal cord constitute a point of convergence for motor regulation receiving all the necessary input for the generation of movements. Recent findings have involved them in active modulation of the motor output and in the injury response

of the spinal cord leading to subsequent development of spasticity. Gene expression profiling of motor neurons in normal and pathophysiologal states such as spasticity can therefore shed new light on the molecular mechanisms underlying the observed activities in this neuronal population.

1.1 The spinal cord and locomotion

In the this section, the basic mechanism of spinal locomotor control will be outlined, with particular emphasis on the history of reflexes and their contribution to motor control as well as on recent advances used to dissect out the individual neural components of the locomotor network and reflex circuits.

1.1.1 Reflexes and the motor program: Early studies of locomotion

The term reflex describes a stereotypical action executed in response to a distinct stimulus. Motor reflexes have been studied since the time of Decartes (1594-1660). In his time they were initiated by crude mechanical manipulations and it was only with Galvani's discovery of bio-electricity that a new era of experimental exploration was initiated. It was soon discovered that the spinal efferent (ventral) nerves conduct the signal from the spinal cord to the muscles, while spinal afferent (dorsal) nerves carry the sensory signals from the body to the spinal cord (Francois Magendie and Charles Bell [29]). Through a series of experiments in the 19^{th} century in what can be termed early electrophysiology, the spinal cord became unequivocally identified as the organ that associates the sensory input of the limbs with their motor output, i.e. the seat of simple reflexes. With the discoveries of Pavlov first communicated in 1903 describing conditioned reflexes of more general behavior, reflexes became associated with higher brain function as well [29]. Reflexes became established as the most fundamental behavioral output of the CNS and the founding principle of brain function. This framework of action and re-action through simple reflex mechanisms gave great emphasis to the role of the sensory system in the behavioral repertoire, including locomotion.

Sherrington's work in the early 20^{th} century on motor reflexes identified the motor unit and the stretch reflex: Stretching of a muscle activates intrinsic stretch receptors leading to feedback excitation of the synergist muscle via afferent projections to the spinal cord, paralleled by a concomitant inhibition of the antagonising muscle through inhibitory components intrinsic

to the spinal cord. (Figure 1.1 A, Ia loop). It was extrapolated that these

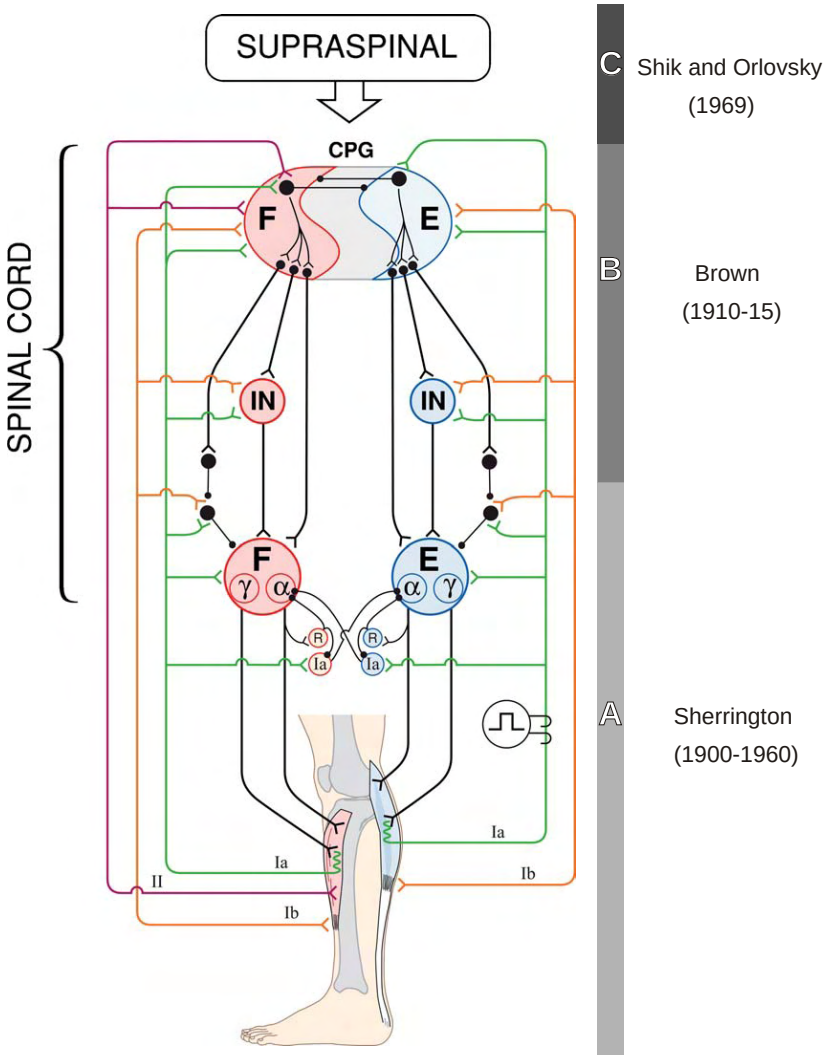


Figure 1.1: Conceptual diagram showing reflex and locomotor circuits in mammals. Timeline A, B and C indicated the development of concepts in motor control. **A.** The basic reflex circuitry. The basic mechanisms were originally described by Sherrington, though the neural correlates were detailed later. **B.** Brown first suggested that intrinsic spinal networks could generate well-coordinated rhythmic activity typical of locomotion. The concept of central pattern generator (CPG) was coined later in the 1960s and has since then been generally accepted. Ia and Renshaw cells are arguably part of the CPG though illustrated here as separate neural units for historical reasons. **C.** Descending activation of the CPG was first described by Shrik and Orlovsky. Adapted from Frigon and Rossignol, Prog. Brain Res. (2006)

reflex mechanisms were hardwired in the spinal cord, such that the activation of a reflex through sensory stimuli caused a short stereotypical pattern of muscle contraction comprising the basic substrate for movements (bending and stretching around a joint). The direct experimental validation of this hypothesis had to await the technical advance of single cell recordings of spinal neurons conducted by Eccles and co-workers in the early 1950s, since the integrative components of the reflex response located in the spinal cord coordinating excitation and inhibition were experimentally inaccessible at the time. However, Sherrington's findings led him to propose that it was the sequential activation of reflexes mediated by the sensory feedback to the spinal cord that generates locomotor behaviors such as walking or running (reflex stepping), an idea that persisted until the 1960s [30, 176].

Spinal locomotor control: Early spinal transection studies

In contrast to Sherrington, experiments conducted by Graham Brown in 1910-15 led him to conclude that the spinal cord was an important autonomous source of coordination for locomotion independent of sensory input. Though Sherrington acknowledged these experiments, little credence was given to these seminal findings at the time, which largely remained in obscurity for more than 40 years [30, 176]. Brown showed that a completely de-afferented cat, with a thoracic transection of the spinal cord, depriving the lumbar spinal cord from descending fibers from the brain, was capable of generating hind limb locomotor-like activity [63]. These experiments demonstrated that the spinal cord was capable of generating coordinated patterns of activity and identified intrinsic spinal networks as key components of locomotion. In sharp contrast to the reflex chain theory, Brown suggested a flexor-extensor half-center model with mutual inhibition as a possible mechanism for this intrinsic network, generating coordinated alternating patterns of muscle contractions (Figure 1.1 B). This idea was not picked up before the 1960s and 1970s, when the group of Anders Lundberg and his colleagues in Sweden gave it new attention [65, 176].

1.1.2 The central dogma of neuronal synaptic transmission and its implications on motor function

Though the cell had become a well established concept by the turn of the 20^{th} century as the basic building block of plant and animal tissue, the cellular organisation of the brain still sprouted heated debate. Early anatomical studies during the 19^{th} century identified well-defined structures in the brain, resembling an extensive reticulum pervading the whole CNS. While Golgi de-

veloped his staining technique in 1873 for the first time visualizing individual neurons, he remained a supporter of the idea that the CNS was composed of a gigantic syncytium. This was also supported by the fact that separate connections between the cellular structures of the CNS could not be visualized with the resolution available at the time. Ramón y Cajal used the technique developed by Golgi to conduct a series of studies on the anatomy of the CNS around the transition to the 20th century. Though he was faced with the same problem of visualizing cellular connections, he on the other hand was a strong proponent of the neuron theory, i.e. the idea that the CNS was composed of individual cells (neurons) as any other tissue. But the identification of connections between neurons remained circumstantial until the 1950s with the advance of the electron microscope [128].

In the mean time, growing evidence supporting synaptic transmission between neurons started to emerge from another field, as experimental advances in electrophysiology allowed increased cellular resolution. Loewi first showed in the early 1920s that the ganglionic cells of the peripheral autonomic nervous system transmit signals through chemical release [126, 127], but he himself doubted that a similar mechanism existed in the CNS. It was instead Henry Dale who showed, through a series of experiments in the early 1930s, that acetylcholine is in fact also the chemical transmitter of pre-ganglionic cells originating from the spinal cord of the CNS [40–42] (reviewed in [180]). While the debate about the cellular composition of the CNS had subsided at this time, despite Dale's experiments, it still remained a debated issue how the individual neurons transmitted their electrical signals within the CNS. Intracellular recordings of the neuromuscular junction (NMJ), the synaptic innervation of the muscle fiber by spinal motor neurons, was subsequently used to study the effect of synaptic transmission of the voluntary CNS, as this structure is large and easily accessible. Though the NMJ is a peripheral synapse it was considered an acceptable model for neuronal synaptic transmission as the motor neuron itself belong to the voluntary CNS. Experiments conducted by the group of Bernard Katz in the early 1950s unequivocally showed that a cell of the CNS, the motor neuron, used acetylcholine as a chemical transmitter to conduct its signal to the muscles [52]. He also showed that this transmitter was released in a quantal fashion, indicating the vesicular mechanism of transmitter release [53].

At the same time Eccles pioneered electrophysiological single cell recordings of neurons in the spinal cord, including motor neurons. The motor neuron is the principle cell of the spinal cord located in the ventral horn, transmitting the signal from the CNS to the skeletal muscles. Though initially skeptical of the idea of chemical release as the means of synaptic transmission, Eccles' own intracellular recordings from motor neurons undoubtedly showed

the existence of two kinds of graded potential responses in post-synaptic elements: excitatory and inhibitory post-synaptic potentials (EPSP and IPSP, respectively) [14]. This observation could only be explained by the effect of chemical neurotransmitters acting on the post-synaptic cell. These results also elegantly showed the existence of the inhibitory component of neural transmission as predicted by Sherrington from his reflex studies (Figure 1.1, e.g. Ia interneuron).

Following these early studies it became a widely accepted dogma that neurons only release one type of neurotransmitter, known as Dale's principle [175]. But electrophysiological studies on the peripheral release of chemical transmitters as well as evidence from invertebrates suggested already in the 1960s that some neurons could synthesize and co-release more than one chemical messenger [113]. Co-release was suggested as a possible general mechanism of neurons already by the mid 1970s [17] and anatomical evidence for co-expression of neurotransmitters in neurons of the vertebrate CNS started to accumulate in the late 1970s [81, 82]. In these early studies, the co-release of chemical messengers was limited to a combination of a single fast neurotransmitter (GABA, glycine, glutamate or acethylcholine) with a neuromodulator (serotonin, nor-adrenaline, dopamine, ATP, substance-P, etc.). Recently co-release of two fast neurotransmitters has been shown in spinal interneurons [97, 117]. Still, the principle established by the early experiments of Katz stating that the motor neuron only releases acetylcholine, both at the neuromuscular junction and the central synapses connecting to other motor neurons or to Renshaw cells, remained undisputed. Paper I of this thesis clearly challenges this dogma by showing that in fact motor neurons also release glutamate, the major excitatory neural transmitter of the CNS, at central synapses but not in the muscle.

1.1.3 The concept of a central pattern generator for locomotion

Reflexes and sensory feedback remained an important feature in the description of locomotion, until research in the early 1960s on invertebrate nervous systems introduced the concept central pattern generator (CPG) to describe self-sustained rhythmic patterns of activity in isolated preparations of neural networks [16, 88, 190]. Experiments on cats also began to show increasing evidence of central control of movement with a diminished role for sensory feedback in the mammalian system. In the 1960s and early 1970s, Shik, Orlovsky and colleagues in Moscow showed that locomotion could be initiated from areas in the mesencephalon (midbrain), later termed the mesen-

cephalic locomotor region (MLR) [168] (Figure 1.1 C). Concomitantly the group of Lundberg in Göteborg showed that application of a noradrenergic agonist to the spinal cord in cats could alter the reflex response, providing evidence for a segmental interneuronal network that could be the substrate for spinal stepping [95]. This work lead Lundberg and colleagues to suggest a model that included both a sensory and a central component, with a locomotor network consisting of flexor-extensor half-center CPGs whose sequential activation across the joints of a limb was tuned by sensory input. Subsequent work by Grillner and colleagues on spinal cat and kitten preparations established that intrinsic spinal networks could generate coordinated limb movements on a treadmill resembling normal walking in the complete absence of sensory input [55, 56, 64, 68]. The sensory feedback was shown to correct the ongoing locomotion orchestrated by central spinal networks, where the reflex responses themselves were highly state dependent and not as rigidly hardwired as previously assumed [65]. Since the 1980s the central pattern generator of the spinal cord became a well-established concept, which has been further substantiated by a combination of experiments across phyla both *in vivo* and *in vitro* (reviewed in [145]).

The CPG and the role of reflexes in locomotion, a real dichotomy?

Work on the interaction between spinal networks during locomotion and sensory feedback have identified three potential roles for afferent feedback in the production of rhythmic movements, and all three roles involve adapting movement to changes in the internal and external environments [132, 147]. One role is to reinforce CPG activities, particularly those involving load-bearing muscles, such as the hind limb extensor muscles during the stance phase of gait. The second role is a timing function whereby the sensory feedback provides information to ensure that the motor output is appropriate for the biomechanical state of the moving body part in terms of position, direction of movement, and force. The third role is that of facilitating phase transitions in rhythmic movements, purportedly to ensure that a certain phase of the movement is not initiated until the appropriate biomechanical state of the moving part has been achieved.

In short, the activity of the CPG provides the basic pattern for movements, which in turn is shaped and modified by the sensory inputs as the organism maneuvers in its natural environment.

The development of *in vitro* spinal cord preparations: Dissecting out the CPG

It is assumed that the basic principles of the spinal locomotor network is shared across species in the vertebrate family with key features preserved through evolution from fish to mammal. This notion led to the development of a series of spinal cord *in vitro* model systems of various vertebrate species, where locomotor activity can be induced with bath-applied agonists (Glutamate/NMDA and/or 5-HT) enabling direct access to spinal neurons during locomotor activity. These preparations include lamprey, zebrafish, Xenopus laevis (tadpole), salamander, turtle, neonatal rat and mouse (reviewed in [28, 54, 66, 102, 103, 156, 182]). The development of these preparations had a significant impact on the field of locomotor research in identifying the neural substrates of the CPG because they are experimentally more accessible than in *in vivo* experiments.

The lamprey as well as the frog tadpole were among the first of these *in vitro* spinal preparations introduced in the field of locomotion at the transition to the 1980s [31, 99, 100, 151], chosen for their presumed simplicity. The lamprey belongs to the family of the most primitive surviving vertebrates (agnathostoma; jawless fish) and therefore was thought to contain the principal features of spinal networks capable of generating patterned rhythmic activity. The young hatchling tadpole was chosen since it has a very simple nervous system and behavior, while its later development into a limbed vertebrate (e.g. frog or newt) constitutes a fascinating transformation that beautifully illustrates the phylogenetic transition from non-limbed to limbed propulsion within the same animal. The lamprey and the tadpole are probably at present the two most well described vertebrate locomotor model systems. Both preparations have the basic neuronal architecture for burst generation, located in each hemisegment of the cord and coordinated via commissural and ipsilateral interneurons [20, 67, 156]. Development of a mammalian spinal *in vitro* preparation followed later in the mid 1980s contributing with a model of a limbed vertebrate, i.e. the neonatal rodent [111, 170, 171]. Work in this model has highlighted the different layout of the mammalian CPG necessary for limbed coordination, where the network seems more distributed along the ventral part of the lumbar enlargement containing the spinal CPG networks[102, 107, 109].

Work in these preparations firmly established that isolated spinal networks are capable of generating very complex patterned rhythmic activity resembling the activity observed in intact animals. The studies have further helped elucidate the components of the spinal networks and their distribution in the spinal cord. Molecular implements are now being introduced to com-

plement these findings and provide new tools for identifying and analysing the neural components of the locomotor networks.

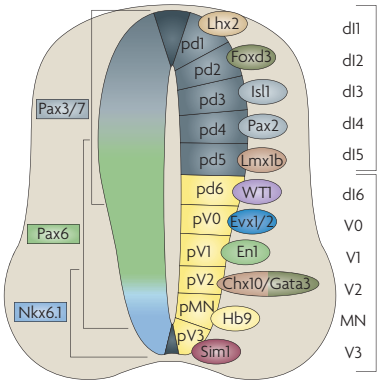
1.1.4 Neural components of the spinal motor network

The idea that neurons can be meaningfully grouped into functionally distinct classes on the basis of their morphology has a history rooted in classical anatomical studies of the brain by Cajal and others. More recently, neurons have been classified according to other defining features such as neurotransmitter, intrinsic firing characteristics, axon projections and afferent inputs. This classification strategy relies on the assumption that cells within such classes are homogeneous and share all their defining properties. The diversity and complexity of the vertebrate brain and its neural components can in this way be reduced to the interaction of neuronal classes rather than individual cells. The same strategy has been used for the spinal locomotor circuits. Basic architectural principles have been established in this way, especially in simpler organisms such as the tadpole, lamprey and zebrafish, identifying major classes of network neurons. Major classes of neurons involved in locomotion have also been identified in the mammalian system, e.g. neurons with ipsilateral, commissural, ascending, descending or bifurcating axon projections in combination with their transmitter identity [102]. But the ipsilateral coordination of joints along a limb makes the spinal network structure of limbed animals more complex than that of simpler aquatic animals. An alternative approach has therefore been to try and understand the progressive assembly of the spinal locomotor networks during development, in this way piecing together the puzzle class by class as the neuronal classes emerge and mature during development.

During development, a few cardinal classes of progenitor cells have been identified along the dorsoventral axis of the neural tube, which are distinguished by their expression of different transcription factors (TF), Figure 1.2. These progenitor classes define a very coarse organisational principle of ipsiplateral and contralateral projecting cells in the spinal cord. Since the expression of the TFs that define these progenitor cell populations subsides later in development as the cell populations diversify, little is known about their cell fate or functional role in the adult animal.

Another way to examine the function of a neuronal class is through their ablation. The effect of their absence provides very strong cues for their role in the intact animal. Different developmental progenitor classes have been ablated to dissect out their functional role in the adult animal [34, 62, 196]. Since the defining features of these progenitor cell classes are very broad (the expression of a single TF), this approach most likely eliminates a range of cell

A



B

		Embryonic	Adult cells
dI6	WT1	CINs, GABA/Glycine	Disynaptic crossed inhibitory INs?
V0	Evx1/2	$V0_V$ and $V0_D$ CINs, 2–4 segments, GABA/Glycine ($V0_D$), Glutamate ($V0_V$)	Disynaptic crossed commissural INs?
V1	En1	Ipsilateral rostral INs, 1–3 segments, GABA/Glycine	Renshaw cells, Ia inhibitory INs
V2	Chx10 Gata3	Ipsilateral caudal INs, > 2 segments V2a (Glutamate), V2b (GABA/Glycine)	Unknown
MN	Hb9 Isl1	Peripheral projections, Acetylcholine	Somatic/visceral motor neurons
V3	Sim1	$V3_V$ and $V3_D$ mixed commissural/ipsilateral > 2 segments (Glutamate)	Unknown

Figure 1.2: Development and molecular determination of spinal locomotor circuits. **A.** Progenitor classes along the dorso-ventral axis of the neural tube are defined by their transcription factor expression at E11. **B.** Each progenitor class diverges into several functional neuronal groups, a few of which are known. Adapted from Goulding, Nature Reviews (2009)

types in the mature system. Strategies to target more specific cell populations are therefore preferred. It is furthermore unlikely that the expression of a single marker such as a transcription factor will identify a homogeneous cell population after the neurons acquire their mature phenotype [139, 140, 177]. Individual gene and protein markers of cell types are relatively abundant in the CNS, and when compared to a small number of cell types within a well-defined anatomical structure of the brain they might help identify coarse classes of cell types. But single markers are likely to be less efficient in identifying distinct cell types when comparing across all cell types. This has already been suggested by the analysis of large-scale expression mapping such as Allen Brain Atlas and Gensat, where the overlapping expression of genes across brain structures clearly suggests that it is the combination of co-expressed genes that define a cell class rather than any individual gene. These mapping efforts will not on their own enable a complete map of brain neuronal cell types to be assembled, as it is hard to discern precise patterns of co-expression with cellular resolution across multiple tissue sections. Gene expression profiling of individual cells (or very well defined cell populations) has on the other hand been a very useful tool for identifying defining features of individual cell types.

A successful strategy to dissect out the defining features of functionally distinct neuronal populations in the mature CNS (including the spinal cord) therefore relies on finding the combination of characteristics that uniquely identifies all the common properties of the cell population, i.e. anatomical

position, axon projection patterns, dendritic innervation, electrophysiological characteristics and gene expression profiles. Individual aspects of the cellular identity such as the electrophysiological properties might define a set of populations, which must be further divided based on their network connectivity to identify distinct functional populations (and vice versa), illustrating that the combination of individual features with network connectivity is crucial for distinguishing neural populations from each other.

We have followed such a strategy to compare two well-defined neural populations of the spinal cord, the commissural interneurons and the motor neurons (Paper II). These two classes of neurons have distinct functions in the locomotor network and can be identified based on their anatomical location, axon projections, afferent input and electrophysiological properties. But their defining features in relation to their gene expression have not previously been described. We used microarrays to examine the difference in gene expression between these two neuronal populations. For this purpose we developed an optimized experimental protocol that allows for the isolation of gene transcripts from fluorescently identified cells in fresh tissue, which can be used to generate amplified aRNA for microarray hybridization from as few as 50 laser microdissected cells. Together with an optimized analysis methodology, this provides a protocol that can be used for gene expression profiling of fluorescently identified neural populations during normal or disease states of the spinal cord, such as development or injury.

1.2 Plateau potentials and persistent inward currents (PICs) in spinal motor neurons

The classic view of the mammalian neuron that emerged after the discovery of transmitter-induced synaptic transmission held that the post synaptic potentials are integrated across a passive membrane. The compartmentalization of the neuron into cell body, axon and dendrites was in this way conceptionally divided into regions with different electrophysiological membrane characteristics: The dendrites and soma were considered passive while only the action potential initiation zone (axon hillock) and axon itself contained active components responsible for the action potential propagation. By the 1970s evidence was mounting that there could be active components in the dendritic membrane of the mollusk [47, 48, 172, 191], and in the late 1970s a similar phenomena was observed in the CNS of vertebrates by Llinás, Schwindt and Crill among others [124, 125, 164].

1.2.1 Active components of dendritic synaptic signal integration

In cat motor neurons, Schwindt and Crill described a persistent inward current (PIC) generated by non-inactivating voltage-dependent Ca^{2+} channels, characterized by a negative slope region in the I-V curves from ramp current injections in voltage clamp [164–167], Figure 1.3 Aii and Bii. Compared to steady-state outward currents, the PIC was large enough to produce a maintained shift in the membrane potential, i.e. a plateau potential in current clamp recordings [163, 166], Figure 1.3 Ai and Bi. In these experiments, penicillin and tetraethylammonium (TEA) was used to block outward currents of Cl^- and K^+ respectively, thereby 'uncovering' the inward Ca^{2+} current. Further investigations carried out by Hultborn, Hounsgaard, Kiehn and colleagues at the University of Copenhagen, showed that the plateau potential could also be unmasked by activation of serotonergic, adrenergic, muscarinic as well group 1 metabotropic glutamate receptors [33, 43, 43, 106, 178] and that these plateaus are TTX resistant [85, 86], Figure 1.3. The Ca^{2+} dependent plateaus are conducted by slow-inactivating L-type Ca^{2+} channels, but electrophysiological studies have not been able to dissect out which of the four known channels ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, $Ca_v1.4$) are responsible for the PIC in motor neurons as no antagonists have been found that specifically target each of these channel subtypes. Anatomical histochemistry studies of these channels suggest that both Cav1.2 and Cav1.3 are expressed in the soma and dendrites of spinal motor neurons [1, 23, 169, 195], strongly implicating these two channels with the expression of PICs in this cell population.

It was later discovered in spinal motor neurons that the PIC can also be carried by a TTX-sensitive sodium component, responsible for up to 50 % of the total PIC in mammals [76, 115, 119]. No separate sodium ion channel that solely conducts persistent inward currents has been described. It has therefore been hypothesized that the persistent inward currents conducted by sodium ion channels most likely is mediated through the same channels that conduct transient currents, which are thought to have an additional slowly inactivating state [185].

1.2.2 Descending control of PICs

The major descending pathways relating to motor control use GABA, glutamate, dopamine, serotonin or noradrenaline as their neurotransmitter [80, 98]. Several early anatomical studies localized the source of the spinal monoaminergic neurotransmitters to nuclei in the brain stem [37, 38, 58, 59, 174], with very little intrinsic release from local cells within

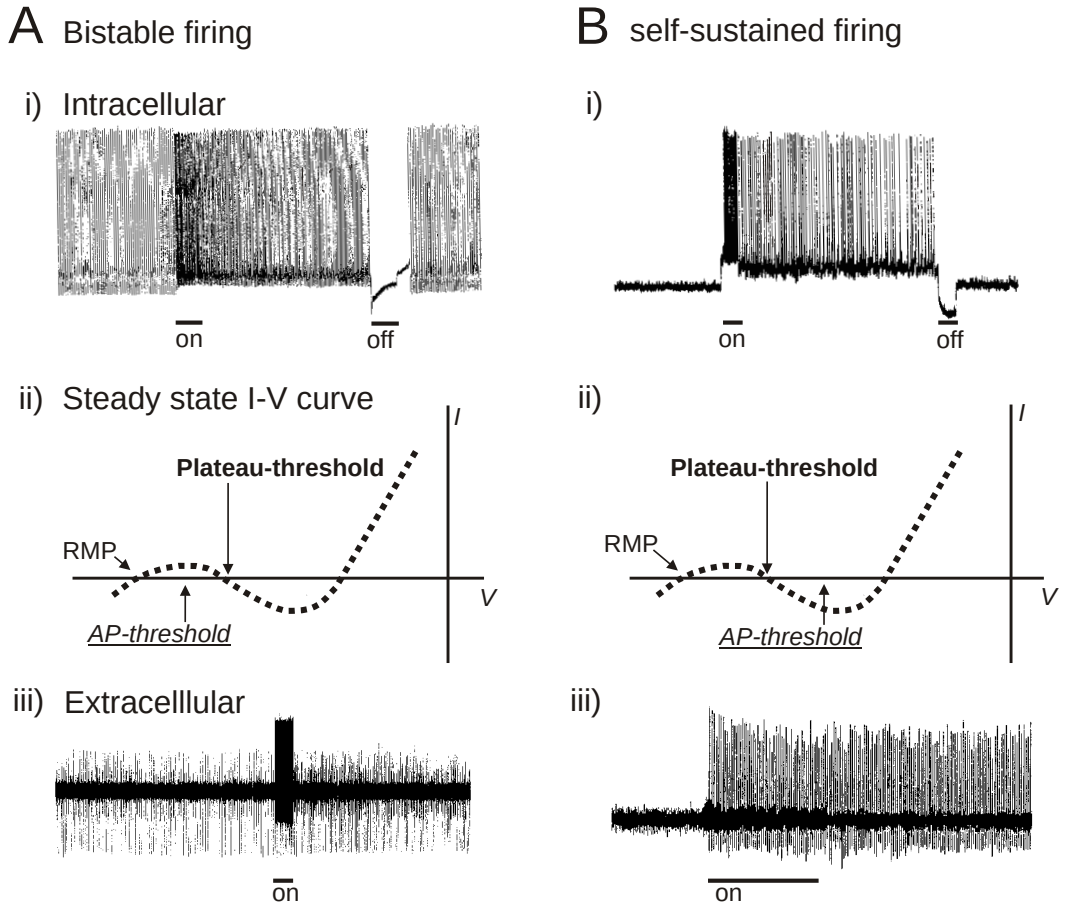


Figure 1.3: Relationship between plateau potential onset threshold, spike threshold, and stimulus-evoked firing behavior. **A**. Bistable firing. **i)** Intracellular recording from a motor neuron in a decerebrate cat[84]. Bistable firing was initiated by a brief synaptic excitation of the motor neuron pool and terminated by a brief synaptic inhibition. **ii)** Theoretical steady-state I-V curve that can explain the bistable firing behavior. The plateau onset threshold is above the one for spike initiation (AP threshold). RMP: resting membrane potential. **iii)** Bistable firing recorded extracellularly in an unrestrained rat[51]. The initial low-frequency firing activity was switched to a stable high frequency by a brief excitation of the motor neuron pool. **B.** Self-sustained firing. **i)** Intracellular recording from a lumbar motor neuron in an *in vitro* preparation of the turtle spinal cord[86]. The membrane bistability was induced by 5-HT, initiated by a brief depolarizing current pulse (on) and terminated by a brief hyperpolarizing current pulse (off). **ii)** Theoretical steady-state I-V curve that can explain the prolonged firing. The plateau onset threshold is below the one for spike initiation. **iii)** Self-sustained or prolonged firing in a human motor unit initiated by a brief excitation of the motor neuron pool[104]. Adapted from Kiehn and Eken, Curr. Opin. Neurobiol. (1998)

the mammalian spinal cord [24, 141, 142]. Transection studies performed in the cat spinal cord showed that these pathways also target motor neurons, as the expression of PICs turned out to be conditional, depending on the presence of either nor-adrenaline or serotonin released from descending fibers [33, 83, 84]. This effect is summarized in Figure 1.4, where an increas-

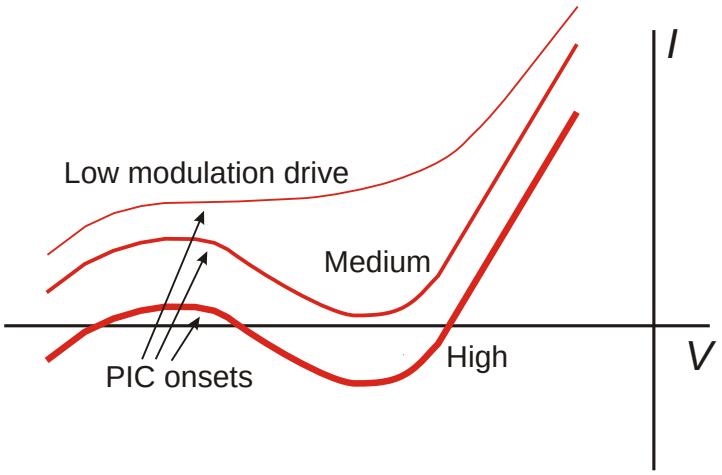


Figure 1.4: The effect of monoamines on the expression of PICs in motor neurons of the spinal cord. Current-voltage relations as a function of monoaminergic input reveals a negative slope region with increasing levels of monoamines indicating an increased weight of the persistent inward current.

ing monoaminergic drive reveals a PIC seen as a negative slope region in the current-voltage (I-V) relationship. The low monoaminergic drive represents the acute spinalized animal deprived of descending input, thus incapable of inducing a PIC. With increasing levels of monoamines the PIC is unmasked, either through an increasing descending drive in normal animals or by application of agonists in the acutely spinalized preparation.

1.2.3 The functional role of PICs in motor neurons

The plateau current is predominantly of dendritic origin. This was first realized by Gutman based on his theoretical analysis of the data of Schwindt and Crill [71] and later validated *in vitro* [23, 87] and *in vivo* [8, 114]. The dendritic localization of the PICs was corroborated by identification of L-type Ca^{2+} channels in the dendrites [1, 23, 169, 195]. The dendrites of motor neurons are especially extensive, containing approximately 95% of the membrane surface area [36, 157, 200]. It is therefore likely that the PIC serves

to amplify synaptic input to motor neurons, which is necessary for signal transduction from their very extensive dendritic tree to the soma [90] and provides much of the current for motor neuron recruitment [77]. The PIC has been described to be sensitive to synaptic inhibition [91, 112] as well as to excitation, and the role of PIC in the presence of monoamines therefore also seems to increase the general sensitivity of the motor neuron to *both* synaptic excitation and inhibition.

The functional role of PICs and plateau potentials in intact behaving animals, including humans, has been the subject of intense investigations. EMG recordings of freely moving animals have shown that the transition between two modes of bistable firing can be evoked by brief stimuli of excitatory Ia afferents or inhibitory cutaneous afferent pathways, suggesting an underlying activation and inactivation respectively of PICs in the involved motor neurons [50, 51, 105], Figure 1.3 Aiii and Biii. Based on these results it was suggested that the plateau potential of spinal motor neurons can support the muscle activity during postural activity, independent on synaptic input. Data obtained from human subjects also suggests a substantial contribution of PICs to normal motor neuron activation [104, 105] (reviewed in [32]). The ability of motor neurons to express PICs disappears in the absence of monoaminergic drive from descending fibers (Figure 1.4). Spinal cord injury thus prevents the expression of PIC in motor neurons, but they are interestingly found to return after 3-4 weeks and have been implicated in injury-induced spasticity (described in section 1.3).

1.2.4 Modulation of PICs

The slow- or non-inactivating Ca^{2+} and Na^{+} channel complexes are subject to extensive modulation, dynamically regulating the expression of PICs. There are two main categories of regulation that should be distinguished from each other:

1. Increased expression of channel complexes in the plasma membrane, increasing PIC amplitude without changing channel kinetics.

2. Modulation of the existing channel complexes in the plasma membrane, changing channel kinetics and/or conductance.

Mechanisms of Ca^{2+} and Na^{+} channel modulation relating to PICs are illustrated in Figure 1.5 and briefly described below together with mechanisms of channel complex trafficking and expression into the plasma membrane.

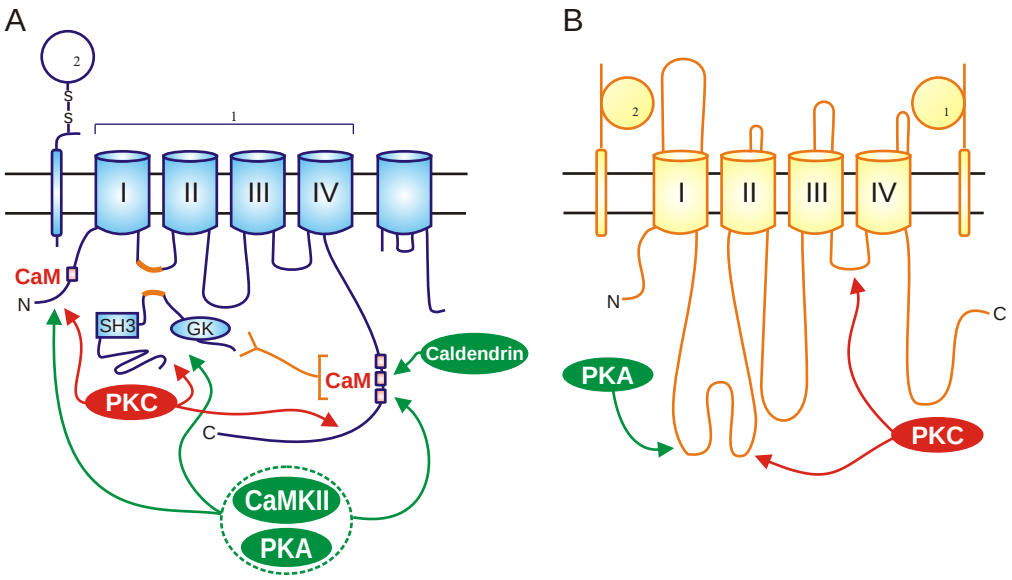


Figure 1.5: Regulation of persistent inward currents. **A.** Modulation of L-type calcium channels $Ca_v1.2/Ca_v1.3$. Protein kinase A (PKA) and calmodulin-dependent kianse II (CaMKII) in green illustrate a primarily facilitating effect on conductivity and/or kinetics, including a possible delay in calcium-dependant inactivation (CDI) depending on the exact location of their action among the potential sites of phosphorylation. Caldendrin (and other calcium sensors) can replace calmodulin (CaM), prolonging the conductive state by depressing CDI (also shown in green). Protein kinase C's (PKC's) direct effect on the L-type channel is predominantly depressing opposing the effect of PKA and CaMKII, illustrated in red. Both the β and $\alpha_2\delta$ subunits influence channel trafficking and expression in the plasma membrane. They can either in combination or independently modulate the activation and inactivation kinetics and conductivity of the channel complex. The β subunit can also interact with the CaM binding area of the C-terminal to CDI. The role of γ subunits is still not clear for $Ca_v1.2/Ca_v1.3$. **B.** Modulation of sodium channels. PKA in green has a fascilitating affect on Na_v channel kinetics and conductivity, possibly prolonging the inactivation states. The inactivation kinetics as well as expression of the channel complex in the plasma membrane are also dependant on the type of β subunit included in the sodium channel complex. PKC illustrated in red has a depressing effect on channel conductivity and kinetics.

L-type calcium channels

Persistent inward calcium currents are conducted by the L-type (Ca_v1) family of Ca^{2+} channels, $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$. The functional L-type

channel complex is comprised of one of the four α_1 pore-forming subunits (α_{1S}, α_{1C}, α_{1D} or α_{1F}) in combination with its ancillary subunits $\alpha_2\delta_{1\text{-}3}$, $\beta_{1\text{-}4}$ and $\gamma_{1\text{-}8}$ in a 1:1:1:1 fashion [2, 186]. The α_1 channel subunit defines the conducting capabilities of the channel, while the other subunits are intimately involved with channel trafficking, gating, kinetics and membrane expression, thus significantly contributing to the conductance properties of the channel complex. From anatomical studies there is evidence that the motor neuron PIC is conducted by $Ca_v1.2$ and $Ca_v1.3$ (see section 1.2.1). However, the exact subunit composition is not presently known for motor neurons in the spinal cord, but it seems clear that the combination of ancillary subunits and their dynamic regulation/substitution are pivotal for the conductance properties of the functional channel complex as well as for its location and expression in the plasma membrane. Since the L-type channels are located in the extensive dendrites of the motor neuron, channel trafficking is of special importance, as the correct localization at or close to post-synaptic innervations is imperative in order for them to exert their effect.

The L-type Ca^{2+} channels are also subject to extensive modulation by interacting proteins. Calmodulin (CaM) is known to be tethered constitutively to the L-type Ca^{2+} channels $Ca_v1.2$ and $Ca_v1.3$ where it works as a calcium detector responsible for both Ca^{2+}-dependent facilitation and inactivation [73, 148, 149]. This balancing effect of positive and negative feedback regulation of the persistent inward current acts on different time scales and probably serves to prevent Ca^{2+} overload as well as to refine channel signaling. CaM can be replaced by other Ca^{2+} binding proteins that alter the Ca^{2+}-dependent inactivation of the channel [19]. CaM kinase II (CaMKII) promotes both Ca^{2+}-dependent and voltage dependent facilitation of $Ca_v1.2$. CamKII also targets and phosphorylates the β subunits causing Ca^{2+}-dependent facilitation, in this way affecting any of the expressed L-type channels. $Ca_v1.3$ is also facilitated by CaMKII but by a different mechanism than $Ca_v1.2$, causing a negative shift in the activation voltage [19]. Protein kinase A interacts directly with $Ca_v1.2$ via binding to A-kinase anchoring proteins (AKAP79/150), causing facilitation upon phosphorylation. Protein kinase C has a more complex effect on $Ca_v1.2$ channels, where its activation through different patways can lead to up-regulation, down-regulation or biphasic effects on conductivity [39]. A general mode of PKC modulation through direct interaction with both $Ca_v1.2$ and $Ca_v1.3$ has been described to oppose the effect of PKA, i.e. PKC inhibits channel activation by phosphorylation of serine-81 located in the N-terminus [39]. Of note is that the effect of PKA or PKC phosphorylation can be counterbalanced by de-phosphorylation of phosphatases.

Sodium channels

Sodium channel complexes are simpler in structure compared to Ca^{2+} channels; they consist of one α subunit in combination with one of its' four β subunits ($\beta_{1\text{-}4}$). There are 9 α subunits belonging to type 1 ($Na_v1.1\text{-}1.9$) and 3 belonging to type 2 ($Na_v2.1\text{-}2.3$) Na^+ channels [61, 186]. Since there are no specific Na^+ conducting PICs as for Ca^{2+} channels, it is rather the association with the ancillary β subunit as well as the phosphorylation state that determines the extent of their slow inactivation [25]. PKA and PKC have been shown to synergistically regulate Na^+ channel activity by reducing peak current and enhancing slow inactivation [25, 39]. It has not been determined which channel complex is responsible for the TTX sensitive PIC in spinal motor neurons.

Indirect modulation of PICs

Apart from direct modulation of the channel complexes conducting the PIC, several other modulatory targets can have an indirect effect on the expression of PIC. PICs are unmasked by activation of serotonergic and nor-adrenergic receptors, likely acting to depress outward currents upon receptor activation, in this way unmasking the inward currents. Modulation of these receptors or the channels they target could therefore have a significant effect on the expression of PIC. For example, the termination of PIC have been linked to Ca^{2+}-activated K^+ channels, thus depression of its conductance could prolong PIC.

1.3 Spinal cord injury and spasticity

The mammalian central nervous system has limited capability for regeneration. Spinal cord injury therefore leads to neurological dysfunctions affecting the motor, sensory and autonomic system [158]. The physical impact of a spinal cord injury is typically restricted to a relatively small area compared to the full extension of the spinal cord, and the pathophysiological state resulting from the injury should therefore likewise be confined to the functions conveyed by the spinal networks located in the damaged tissue. Extensive research has been devoted to examining the mechanisms of the injury response in the spinal tissue directly associated with the physical damage (Figure 1.6 B and C). But, since the spinal cord contains extensive descending and ascending pathways, spinal cord injury also has a large impact on the functional outcome of spinal networks located caudal to the injury, Figure 1.6 D. Spinal

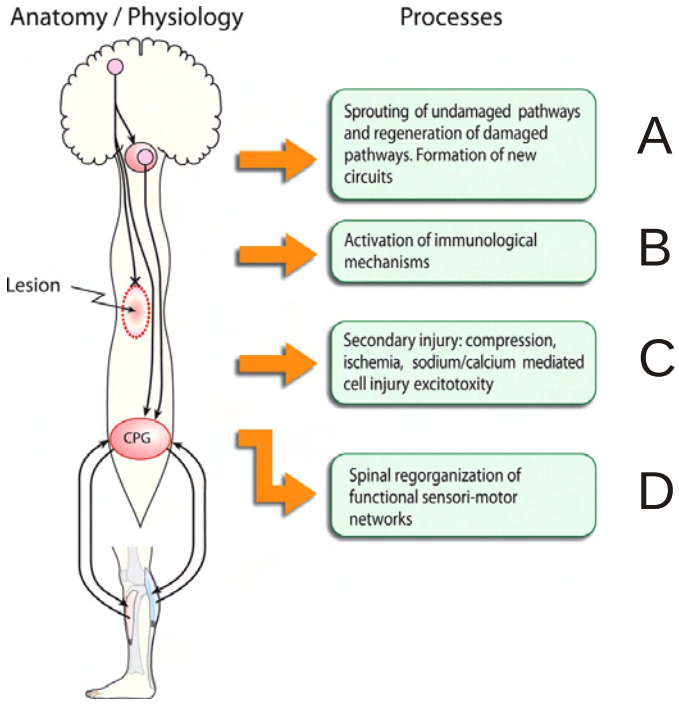


Figure 1.6: General effects of spinal cord injury. Adapted from Rossignol et al., J. Neuroscience (2007)

cord injury can therefore have a detrimental effect on motor control if the descending pathways activating spinal motor networks involved in movements are severed (see section 1.1.3). The limited capacity of the descending as well as ascending fibres to regenerate across the injury site poses severe limits to the recovery of spinal cord injury, causing long term pathological effects in the spinal networks that might not have been physically damaged by the injury itself (Figure 1.6 D). In the following, focus will be on the effect of injury on the control of movements.

1.3.1 The injury response of motor networks located caudal to a spinal cord injury

In the immediate phase following spinal cord injury the excitability of the motor networks caudal to the injury becomes depressed. This initial state of decreased network excitability is often followed by a maladaptive gain of function resulting in spasticity [57, 89, 123]. Spasticity is defined as "velocity-dependent increase in the tonic stretch reflex", characterized by a disturbing

hyper-reflexia causing prolonged muscle activity upon short activation of sensory afferents [12, 143]. It can be described as "a condition in which the stretch reflex that is normally latent becomes obvious. The tendon reflex has a lowered threshold to tap, the response to the tapped muscle is increased, and usually muscles besides the tapped one respond; tonic stretch reflexes are affected in the same way" (P. Nathan quoted in [136]). This pathological state is probably the result of a dynamic response to the aberrant activity in the neural networks that normally coordinate movements, causing a reconfiguration of the entire spinal network, including the reflex circuits accessible to quantification [46, 57] (Figure 1.6 D). Increased excitability of motor neurons observed in the chronic phase of injury have also been implicated with the pathophysiological state of injury-induced spasticity.

1.3.2 PICs and injury-induced spasticity

As described above, the PIC and the ability of motor neurons to generate plateaus are conditional and dependent on the presence of neuromodulators such as noradrenalin and serotonin (section 1.2.2). A complete transection of the spinal cord in animal preparations drastically reduces the PIC and motor neuron excitability due to loss of descending monoaminergic input [84]. Interestingly motor neurons regain the ability to generate plateaus 2-3 weeks post-transection in the complete absence of descending pathways [9, 51]. This reappearance of plateaus correlates with the emergence of hyper-reflexia and spasticity and led to the proposal that the reappearance of endogenously expressed plateaus in motor neurons could be very closely related to the hyper-reflexia observed in chronic patients of spinal cord injury [51].

The rat-tail model of injury-induced spasticity

Since the initial discovery of motor neuron plateaus and PICs and their implication in injury-induced spasticity in the 1980s [51], Bennett and colleagues developed a rat-tail injury model with pronounced injury-induced spasticity in the chronic spinal phase to further investigate this phenomena [7]. In this model the injury is performed by a transection of the sacral spinal cord (S1-S2) and only networks responsible for tail movements are affected, leaving bladder, bowel as well as hind limb functions intact. Another advantage with this model is the ability to perform *in vitro* experiments on the isolated sacral cord, enabling pharmacological manipulations not suitable for an *in vivo* approach.

Following spinal cord injury in the rat-tail model, muscle spasms typical of spasticity can be triggered in the chronic phase by brief stimuli of

sensory afferents [7]. Intracellular recordings of motor neurons during this activity shows that the sensory input causes an unusually long-lasting activity in motor neurons (200-500 ms) caudal to the transection [10, 120]. This exaggerated synaptic response to brief afferent stimuli appears immediately after injury, but in the acute phase the depression of motor neuron excitability prevents these exaggerated sensory inputs from causing muscle spasms. In the chronic phase when the motor neurons regain the ability to express plateaus and PICs, the exaggerated sensory response is capable of triggering plateaus in motor neurons leading to self-sustained motor neuron activity resulting in the observed muscle spasms.

The re-appearance of plateaus and PICs in motor neurons in the chronic phase of injury is therefore intimately related to the observed spasticity, but their expression in the complete absence of descending monoaminergic drive represents a conundrum that seems to relate to the pathology of the post injury phase. There is a wealth of possible mechanisms that could be involved in the expression of plateaus and PICs in motor neurons post injury, including modulation of the channel complexes conducting the PICs and regulation of their expression in the plasma membrane, modulation of the receptors on which the expression of PICs depends, changes in the post-synaptic response to afferent input as well as depression of channels conducting persistent outward currents. Investigations on the return of plateaus and PICs have typically emphasised the role of monoamines, that is the supersensitivity of receptors to these [74, 118, 154] and possible mechanisms of their release in the absence of descending fibres [5, 121, 183]. To address this question we instead use gene expression profiles of motor neurons to examine their global transcriptional response to injury (Paper III). We can in this way investigate the molecular mechanisms underlying the expression of plateaus and PICs of motor neurons in the chronic phase of injury.

1.3.3 Other aspects of injury affecting spinal cord functions

Spinal cord injury results in a wealth of responses at the injury site associated with the physical damage itself, as well as in distal neural networks with projecting fibers severed by the injury, Figure 1.6. Spinal cord transection studies that focus on the functional outcome of spinal motor networks caudal to the injury ignore many of these effects. This approach can be considered relative naive, since chronic spinalized animals suffer from a broad range of secondary effects impairing spinal functions. Apart from the immediate damage to tissue, reduced blood flow often results in substantial ischemic

necrosis [110, 160]. Edema of the cord develops and metabolic disturbances involving intra-neuronal Ca^{2+} accumulation and increases in extracellular K^+ concentration can occur [162]. This translates into a general failure of normal neuronal function and a stage of spinal shock [3]. In the phase of secondary tissue damage, which takes place over a time course of minutes to weeks after injury, increased production of free radicals, excessive release of excitatory transmitters and inflammatory reactions occur. In addition to often massive ischemic necrosis, apoptotic cell death can be observed. Extensive proliferation of astrocytes starts, leading to glial scar formation. Traumatic injury to the spinal cord also triggers a strong inflammatory response, with recruitment of peripheral derived immune cells such as neutrophils (6-24h), macrophages (24h to 2 wk) and T cells [11, 161]. The interaction of the CNS with the immune system is very controlled under normal physiological conditions due to the blood brain barrier (BBB), and the mobilization can have a mixed effect on the recovery of function [129]. The extracellular milieu is also affected by the disrupted BBB, resulting in altered chemical gradients of ions across cell membranes in this way affecting neuronal signaling. In the chronic phase, extending from days to years after injury, apoptotic cell death continues, channel and receptor functions are impaired and scarring and demyelination accompany Wallerian degeneration [181]. These effects of injury are not limited to the injury site but affect the neural networks throughout the spinal cord.

It is therefore crucial to appreciate all the involved factors of the injury response and their mutual interactions, even when the main focus is only on one of these: The interaction at macroscopic level and their consequences at the microscopic level. The effects of injury on motor networks are embedded within these mechanisms of repair and regeneration, and the limited capability of the mammalian CNS to regenerate is one of the main reasons for the observed long term pathological states observed in animals subject to CNS trauma. The plasticity of the CNS simply causes the neural networks to reconfigure as they are confined by the permanent barrier of the damaged tissue that cannot be repaired.

Gene expression profiling of individual neuronal populations in the spinal cord following injury has the potential to provide a unique insight into the underlying molecular mechanisms of individual cell populations resulting in the complex injury response. As part of this thesis we have initiated this process by examining the progressive transcriptional response of motor neurons following injury and the underlying transcriptional regulation by expression clustering and transcription factor analysis (Paper IV). We have in this way broadened the perspective and investigated how the global transcriptional response of motor neurons relates to the general injury response causing a

complex regulation of gene expression resulting in the observed increase of motor neuron excitability.

Chapter 2

Aims

I. Examine transmitter release of motor neurons at peripheral and central synapses to evaluate a property that has been one of the defining features of motor neurons for several decades, their transmitter identity.

II. Compare gene expression profiles of two well-defined spinal neuronal populations, the motor neurons and the commissural interneurons, as a first step towards identifying distinct features of these neuronal types that relate to their functional differences in generating and transmitting the locomotor output.

III. Examine the molecular mechanisms underlying the return of plateaus and persistent inward currents in motor neurons located caudal to a transection of the spinal cord using microarrays.

IV. Examine the general progressive transcriptional response of motor neurons to injury and the underlying transcriptional regulation using expression clustering complemented with transcription factor analysis.

Chapter 3

Methodological considerations

The main techniques used to address the aims outlined in the previous chapter are detailed in the *"Materials and Methods"* sections of Paper I-IV. They can essentially be divided into categories pertaining to electrophysiology, anatomy and gene expression analysis. The methodologies relating to electrophysiology and anatomy are well establised an are not discussed further here. In the following, additional considerations are given only for a few technical problems relating to mRNA quality and the validation of microarray data obtained from specific neuronal populations, that are not specifically addressed in the appropriate papers. Furthermore, there is presently no gold standard for microarray analysis, but instead an abundance of analytical methods from which the user much choose in order to put together the analysis that best fits the design of the experiment. Therefore general features of the microarray technique and the underlying philosophy behind the analysis strategy chosen in the presented work will be outlined shortly in this chapter.

3.1 mRNA: Confounding factors

3.1.1 Retrograde labeling of neuronal populations

In vitro tracing

Retrograde labeling of motor neurons and CINs was performed by application of rhodamine dextran amine to the distal axon stump. The samples were incubated three hours for the tracer to reach the soma. Alternative tracers were tested (Fast Blue, Texas Red), but none proved as efficient as rhodamine dextrane amine. Though some changes in gene expression might occur in this period, normal locomotor activity and the expression of robust electrophysiological properties are well documented in preparations labeled

in this way [102]. Furthermore, confounding factors pertaining to the labeling procedure will be minimized as the two populations are subject to the same procedure prior to expression profiling.

In vivo tracing

To label motor neurons *in vivo* Flurogold was injected directly into the muscle they innervate or intraperitonaelly. The tracer is taken up by axon terminals and is retrogradely transported to the motor neuron cell bodies in a few days. This method is superior to *in vitro* labeling since the spinal cord can be dissected out and immediately frozen preventing RNA degradation as well as spurious gene expression.

3.1.2 Cell extraction

Laser microdissection (LMD) has been developed and used with success for the dissection of small cell samples from thin tissue slices. Several systems are currently on the market, each suited for different purposes. Each of these can be used to extract fluorescently labeled cells, but the protocols are usually dependent on fixation with cross-binding aldehydes to stabilize the fluorescent signal. Fixation protocols with cross-binding aldehydes were therefore initially tested, but as the RNA gets trapped and inaccessible for subsequent extraction, alternatives with alcohol and acetone were tested. Neither of these proved useful in combination with the fluorescent tracers as they have a tendency to wash out the unfixed molecules and/or quench the fluorescent signal. The Arcturus laser capture system relies on dehydrated tissue sections and we therefore found this system to be problematic for fluorescently labeled cell populations in unfixed tissue, as the dehydration steps simply washed out the fluorescent molecules. Leica LMD was instead used to dissect out cells from fresh tissue, though background signal from the necessary polyester membranes posed a problem for the detection of weakly labeled cells. Using the Leica system for laser microdissection we succeeded in dissecting out fluorescent spinal neural populations with tissue sections mounted on metal frame RNAse free POL membranes [1]. Ten μm thick spinal cord cryo-sections were used to minimize contamination from neighbouring cells. Extracting transcripts from as few as 50 LMD cells poses a great challenge as the degradation of mRNA as well as contamination from other sources must be kept at an absolute minimum. Since the fixation protocols were incompatible with the combination of fluorescent identification

[1] Alternative PEN membranes have too high background fluorescence.

and mRNA extraction, we show that good quality RNA can be obtained from laser dissected cells of fresh tissue provided care is taken to minimize RNAse degradation and optimising the work flow to minimize exposure of tissues at room temperature.

3.2 Microarrays

3.2.1 The basic principles

Microarrays comes in two basic flavors, cDNA arrays and synthetic oligo arrays. But they all share the same founding principle: A microarray basically consist of a solid surface upon which single stranded DNA probes targeting individual transcripts are attached. The probe of DNA attached to the surface will bind complementary strings of RNA (or single stranded DNA) in a solution above the plate selectively. The amount of bound RNA will be proportional to the concentration in the solution:

$$[RNA_i]^{bound} \propto f_i([RNA_i]^{aq}) \tag{3.1}$$

The reaction kinetics of the hybridization reaction of the ith RNA transcript determines the calibration curve f_i. If the bound $[RNA_i]^{bound}$ molecules are fluorescent the light intensity illuminated from a single spot, I_i, is considered to be proportional to the concentration of the corresponding $[RNA_i]^{aq}$ transcript in that solution:

$$I_i \propto g_i([RNA_i]^{aq}) \tag{3.2}$$

where g_i is the calibration curve mapping intensities to concentration.

Since the calibration curves for each probe are unknown, a reference is used to which the sample intensities can be compared, in this way providing a relative measure of expression. In the Affymetrix protocol, control and sample are hybridized on two individual chips while cDNA arrays hybridize both sample and control onto the same array with different fluorescent markers to tell them apart. This difference is reflected in the design of experiments as well as in parts of the subsequent analysis. Only the Affymetrix platform was used to measure the expression profiles in present thesis, and the focus will therefore in the remainder be on this system.

Design of Affymetrix microarrays

Affymetrix manufactures synthetic oligonucleotide microarrays. In their design one gene is targeted by several oligomer probe sequences (25 bp), based

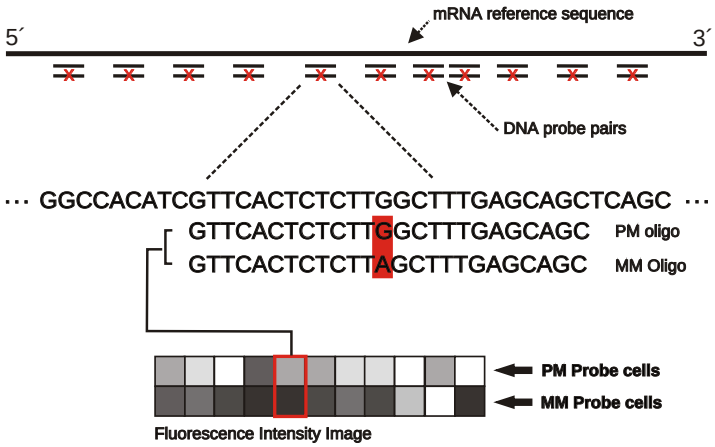


Figure 3.1: Affymetrix probe design targeting one transcript. A transcript is targeted by 11 probes: perfect match (PM) oligo sequences 25 bp long. Miss match (MM) probes have one mutation at the central position to provide a measure of non-specific binding. The probes are attached to a solid surface in well defined areas and the emitted intensity from bound target sequences are proportional to the sample transcript copy number. The 11 PM intensity values are further processed to estimate the transcript expression. PM:Perfect Match, MM: Miss Match

on the known mRNA transcript sequence. These strings are termed the perfect match (PM) probes. This design is illustrated in Figure 3.1, where the PM probes are aligned under the target transcript. In addition to the PM probes, a control set of mismatch probes (MM) are included to get a measure of non-specific background hybridization. These are identical to the PM except for a single base mutation in the central position of the sequence. An example of this is shown in Figure 3.1, where the site of "mutation" is highlighted in red in the two sequences of PM and MM. The PM and MM probe sequences are attached on a plate providing several intensity measures proportional to the concentration of the target transcript, an example of which is shown in the bottom of Figure 3.1. These have to be processed to give an average intensity measure of transcript i, termed expression summary (ES):

$$ES_i = \langle I^j \rangle_i \tag{3.3}$$

where angled bracket represents a measure of average over the j probes targeting transcript i. Spurious signals have been observed for a large proportion of the MM probes giving higher intensities than the PM probes, leading to the conclusion that the use of MM probes to correct for non-specific binding is not always appropriate [93, 138]. We therefore chose to omit MM probes in

our analysis. To calculate expression summaries based on the PM probe sets, we used either of two widely used model-based approaches, robust multi-array analysis (RMA) [92] and Li-Wong expression summaries [116]. Recently an alternative approach to background compensation including subtraction of non-specific binding was proposed, which used information from the MM probes and probe sequence information in combination with RMA, gcRMA [194]. We find that this method introduced dependency structures in the downstream test statistics performed on gcRMA expression summaries and was for this reason discarded in our analysis (not shown). It has further been observed that the noise structure of the microarray intensity values is proportional to the signal, suggesting multiplicative noise rather than additive. To stabilize the experimental error and the expression summary variance for subsequent statistical tests, microarray data is typically log_2 transformed.

3.2.2 Data analysis

Normalization

Microarray normalization is important for making expression values comparable across arrays of different experiments. The key is to identify the biological sources of variability that are of interest, while minimizing the confounding sources of error arising from experimental artifact. Systematic sources of error have also been described as a problem for the DNA microarray technologies and efforts were made to control these. For Affymetrix, data normalization can be carried out directly on the probe values or on the expression summaries. Interestingly the distribution of these can be used to calibrate each microarray independent of the tissue type the individual samples originate from. But, since the correct distributions of the gene expression profiles are in fact unknown, assumptions have to be made about the corresponding microarray signal structure in order to extract the true gene expression signal. Two existing normalization methods, QPN and qspline [13, 193], minimize between-chip variation by nonlinear transformations that map all microarray probe level distributions onto the average distribution of the constituting data set. Both methods rest on the biological assumption that all transcript level distributions are conserved in spite of any experimental perturbations. Hence differences among the observed microarray probe level distributions are attributed to experimental effects and the normalization is designed to equal all these distributions. We developed a pre-processing procedure that avoids such assumptions, re-scaling each array based on parameters obtained from a linear regression to the average distribution of probe intensities. If the gene expression distributions are identical

and the experimental artifact is predominantly linear, this will produce a set of identical probe level distributions with a minimum of underlying assumptions on the nature of the expression distribution.

Identifying differentially expressed genes

After normalization, gene expression summaries are calculated based on the probe set intensities of each transcript. From these, statistical inference can be made about the reliable differences in expression between the experimental samples. T-test and ANOVA analysis can be used to identify the most likely differentially expressed (DE) candidates, but problems with homoscedasticity [2] even after variance stabilizing transformations have sprouted several alternative methodologies. Among these the most widely used include limma [173], SAM [184] and Cyber-T [4]. It has been observed that the variance can be underestimated for non-expressed transcripts, since these will appear to be very tightly distributed simply due to the fact that they are absent and therefore only have variations of background signal leading to false positive classification of these. Both SAM and limma compensate for this through an estimate of global variance which is incorporated into the test statistics. Both methods therefore rely on the assumption of homoscedasticity and any remaining variance-to-mean dependence after the variance stabilizing transformation will affect their test statistics. Cyber-T on the other hand incorporates this dependence into its test statistic as the variance estimate of each transcript includes additional expression summaries in a window defined in the range surrounding the expression values of the tested transcript. Thus the variance stabilization of Cyber-T depends on a local estimate of variance. For the non-expressed genes this strategy still seems to underestimate the variance, and while SAM and limma compensate well for this effect, their lack of flexibility might affect the estimate of individual gene variances in other parts of the intensity range. We therefore developed a conglomerate classifier of DE that combines all three methods to identify the genes of interest.

Clustering of expression patterns

Clustering has been widely used on microarray data to classify the expression profiles of different tumor types and to identify common functionality of genes. Hierarchical clustering has been commonly used for this purpose [49], but is sensitive to outliers. Alternatively, relocation clustering algorithms such as K-means [49] and self-organising maps [179] give results that depend

[2] Homogeneity of variance among genes across the full intensity range.

on initialization of the clustering algorithm. This tendency is even more pronounced when data set increases in size and transcripts with more noisy profiles are included. A practical approach to microarray analysis is therefore to run different clustering methods with different data reduction schemes and manually look for reproducible patterns. Robust alternatives have therefore been suggested. One such strategy is to average over multiple runs of a relocation cluster algorithm with different initialization settings, in this way identifying robust patterns of common expression [69]. This strategy was followed in the present work.

Ontology analysis

The result of microarray experiments consist in most cases of lists of DE genes. These can be further sub-divided into expression clusters. The challenge remains to translate such groups of DE genes into a better understanding of the underlying biological phenomena. The ontology database maintained by the gene ontology (GO) consortium annotate genes according to biological processes (BP), molecular function (MF) and cellular compartment (CC) while INTERPRO and KEGG pathways each classify the proteins they code for according to other aspects of their function. These databases can be used to annotate the list of DE genes from which statistical inference can be performed to identify ontology terms that are particularly enriched in the data set, in this way suggesting that the data contains a subset of genes that engage in or represent the identified ontology. Several strategies have been developed for this purpose [101]. This exploratory approach to mine extensive data sets can yield some insight into the biological processes underlying the biological phenomena under investigation, but it is very dependent on the quality of the ontology annotations and only gives a broad mechanistic description.

Regulatory networks

Transcription factors bind to promoter regions of genes regulating their expression. The identification of transcription factors in a microarray data set can be used to examine the transcriptional regulation underlying the biological phenomena under investigation. TF binding motifs are listed in two data bases, JASPAR [15] and TRANSFAC [135]. Based on the binding motifs of the DE TF their potential gene targets can be identified using the algorithm ASAP on sequences up-stream and down-stream of the coding regions [133]. In combination with expression clustering the regulation exerted by the TF can be classified according to general expression patterns to identify major

targets of regulation.

3.3 Microarray validation

3.3.1 Anatomical validation

It is a possibility to validate microarray data by anatomical examination of proteins located in distal cell structures such as dendrites or axons using microscopy. However, this technique is quite demanding, requiring thorough investigation of distal compartments to determine and quantify the proteins under investigation. It is furthermore difficult to quantify the absolute amount of protein expression necessary for evaluation of expression differences between cells or experimental conditions. For these reasons, this method is not suitable for the validation of microarray expression data obtained from distinct cell populations. Though labeling with *in situ* hybridization of transcripts is limited mainly to the soma (some dendritic localization), it faces the same problem with respect to reliable quantification necessary to evaluate differences in expression between cell populations or experimental conditions. Validation of microarray data was therefore conducted with real time RT-PCR, briefly outlined below.

3.3.2 Real time RT-PCR

Real time reverse transcription PCR (RT-PCR) was used to validate subsets of differentially expressed genes identified from the microarray studies. Real time RT-PCR proved useful for transcripts with relatively high copy numbers in the cell samples, but failed to quantify several transcripts known to be expressed in motor neurons. We conducted a series of test experiments to examine if this was due to substrate depletion or inactivation after > 30 amplification cycles by adding these at cycle 20, but the problem remained. We therefore conclude that factors affecting the primer binding or polymerase accessibility (such as sequence GC content and folding) as well as simple sequence degradation during the PCR cycles, presumably prevent certain transcripts from being detected when extracted from small tissue samples of 50-200 cells. This assumption is not unreasonable, since the abundance of transcripts in larger tissue samples will compensate for these effects, i.e. if amplification initiation is limited due to any of the above mentioned factors, the chance of amplification is reduced at each PCR cycle thus only a large number of transcripts will enable detection of these within a reasonable amount of cycles (< 45).

Chapter 4

Results and Discussion

4.1 The dual transmitter personality of motor neurons

Motor neuron were known since the 1940s to release acetylcholine and only recently did anatomical evidence start to emerge suggesting that glutamate could also be used as a transmitter in this cell type [79, 137]. There was however no electrophysiological evidence to support this. In paper I we examined this question by investigating the transmission at central and peripheral synapses.

Motor neurons have axon collaterals that innervate other motor neurons as well as Renshaw cells, in this way directly exciting other motor neurons while the Renshaw cells provide a negative feedback to the motor neuron pool (Figure 4.1 a). Ventral root stimulation can therefore activate both motor neurons and Renshaw cells (Figure 4.1 b). Selective block of nicotinic acethylcholine and glutamate receptors revealed their individual contributions to synaptic transmission at both of these central synapses. Only by blocking both types of receptors was the post-synaptic signal abolished, clearly showing the that motor neurons release both acetylcholine and glutamate at central synapses. To examine the synaptic transmission at peripheral synapses (the neuromuscular junction, NMJ) electromyograph signals were recorded from quadriceps and gastrocnemius in hind limb attached preparations. Muscle activity was induced by stimulation of cut ventral lumbar roots. Selective block of nicotinic acetylcholine and glutamate receptors showed that there was no measurable effect of blocking glutamate indicating that motor neurons only release acetylcholine at the NMJ. Immunohistochemical visualization of the vesicular glutamate transporter Vglut2 confirmed that it is expressed in some motor neuron axon terminals innervating Renshaw cells. Interestingly

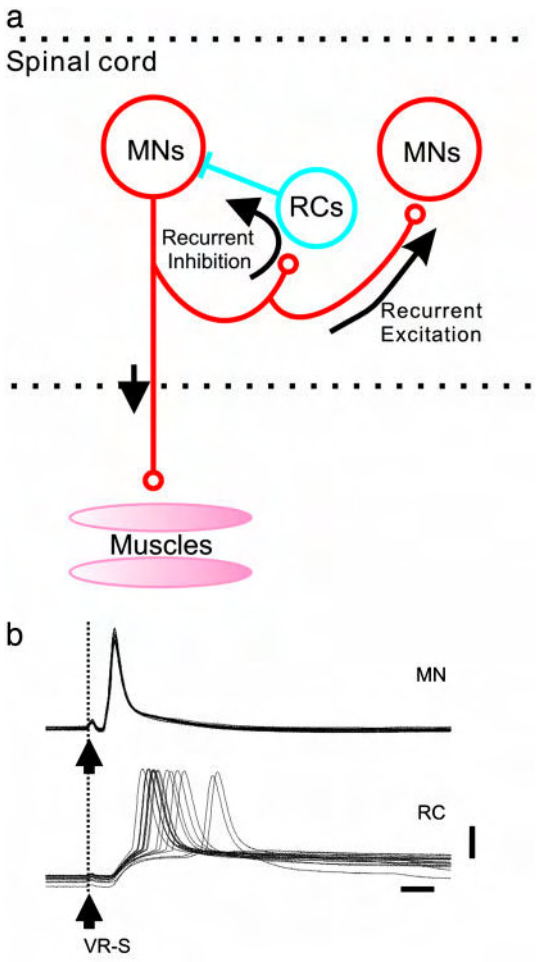


Figure 4.1: Examining motor neuron synapses. **a.** Schematic drawing of synapses formed by motor neurons, **b.** Paired recording of motor neurons and Renshaw cells. Motor neurons are antidromically activated by stimulation of ventral roots, in turn resulting in a response in the Renshaw cells through the motor neuron axon collaterals. Adapted from Paper I.

these terminals very rarely contained the vesicular acethylcholine transporter (VAChT), suggesting that acethylcholine and glutamate are released from separate motor neuron terminals. The functional significance for this segregation and release of two fast excitatory transmitters by motor neurons at central synapses is still not entirely clear. It does point to the fact that motor neurons contain the machinery for adjusting their synaptic output at central synapses, which could be a way to modulate transmission to both Renshaw cells and onto other motor neurons, suggesting an active role in shaping the motor output.

4.2 Gene expression profiling of distinct neural populations in the spinal cord

The microarray technology combined with laser microdissection makes it possible to study the gene expression profiles of identified cell populations. Gene expression profiles from well-defined neuronal populations can in this way help identify a broad set of defining features that distinguish one neuronal population from the other that cannot otherwise be obtained from a single set of experiments.

In this study we wished to establish an experimental protocol that enabled us to compare the gene expression profiles of fluorescently identified neuronal populations of the spinal cord that are directly involved in generating basic motor behaviors, like locomotion. Two populations in the lumbar spinal cord that can be readily identified by fluorescent retrograde tracing and which have been subject to extensive anatomical and electrophysiological characterization were examined: the motor neurons and the commissural interneurons. Though being functionally distinct neuronal groups, it is not known to what extent these cell populations can be distinguished at the transcriptional level. However, differences in gene expression between neural cell types are likely to be relatively small, so both the experimental protocol and subsequent analysis have to be optimized to detect small, but consistent, changes in gene expression.

In vitro retrogradely labeled motor neurons (MNs) or descending commissural interneurons (dCINs) were laser microdissected and their mRNA was extracted (see section 3.1). Twenty-two Affymetrix Rat Neurobiology U34 Arrays (RN_U34) were each hybridized with amplified and biotinylated aRNA obtained from 50-250 laser microdissected cells originating from 22 separate animals, producing the following set of sample arrays: 7 MNs, 7 dCINs and 8 MIX (random sampling in the ventral horn).

4.2.1 Microarray normalization

In the present study, we introduced a new pre-processing method for microarray data that helps with inspection of microarray quality and aides the choice of background compensations, termed quantile linear transformation (QLT). This algorithm operates on the probe intensities, rescaling each array using parameters from a linear regression between quantiles of the probe level distributions and the average probe level distribution, leaving non-linear variations unaffected. Visual inspection of the probe level distributions of each microarray under investigation can be used to illustrate the degree of linear

and non-linear experimental variations in the data[1] given the assumptions of equal gene expression distributions[2]. This means that pure linear experimental variations will produce identical overlapping distribution whereas additional non-linear variations will show up as deviating distributions from the average distribution. The average distribution represents the archetype distribution of the data set. Deviations from this distribution depend on the degree of non-linear artifacts, e.g. saturation alters the tail of a distribution of high intensity values while contamination can alter the shape of the distribution itself. Discrepancies between distributions due to erroneous background compensations manifest themselves as deviations at the distribution tail of low intensity values.

We find this procedure to be a very helpful tool in evaluating microarray data quality, in evaluating the effect of background compensation and aiding in the choice of normalization[3]. Additional normalization is in fact only required if there are substantial non-linear artifacts left after the application of QLT. The algorithm used to compensate for such non-linearities can then be applied on the pre-processed data, minimizing the effect this additional procedure might have on the subsequent statistical analysis compared to its application directly on the raw data where the linear effect still remains.

4.2.2 Detecting differentially expressed genes

To identify differentially expressed genes we introduced a *conglomerate classifier* that for a given significance threshold combines three well-established methods, limma [173], Cyber-T [4] and SAM [184], to produce a set of differentially expressed genes. All three methods agree quite well with each other, but in order to increase the consistency of DE detection the conglomerate classifier based on the average rank of all three statistical tests was used. All three methods are characterized by the way they try to overcome instabilities in the estimate of gene specific variance used to calculate their test statistics. As each variance estimation seems optimal under different conditions (section 3.2.2) they supplement each other to provide robust estimates of significant DE when combined.

Since more than a thousand genes are typically tested in microarray experiments, compensation has to be made for multiple testing. False discovery

[1] Measured signal intensities are assumed proportional to transcript levels but are confounded by unknown experimental artifacts

[2] Actual transcript distribution of the cell

[3] Microarray data generated as a test bed for statistical analysis (not shown) as well as the data described in the subsequent section were analysed using this pre-processing procedure validating its success.

rates have found a broad acceptance in the analysis of microarrays and were used for the present analysis.

4.2.3 Genes differentially expressed between motor neurons and descending commissural interneurons

The analysis identified 49 significantly differentially expressed genes among the 1050 annotated genes on the RN_U34 microarray. Of these 17 were DE in motor neurons and 32 in dCIN. Most of these genes have been reported in either motor neurons or in the spinal cord in general, supporting the quality of our methodology. The DE genes were divided into functional classes to illustrate the functional differences between the two neuronal populations (Table 2 of Paper II).

Five of the 17 genes DE in motor neurons are related to cytoskeletal proteins, perhaps not so surprising as motor neurons are larger and have long peripheral axon projections. Interestingly, the majority of the DE genes relate to neuronal synaptic transmission and signal transduction (channels, receptors, transmitters and their release). The two cell populations are known to release different neurotransmitters, which is confirmed by the microarray results. MNs release acetylcholine supported by the DE of *VAChT* in this population. The dCINs on the other hand constitute a mixed population of both inhibitory and excitatory cells that release either GABA/glycine or glutamate [18, 153, 155], reflected by the DE of *Viaat*, *Gat1* and *EAA4* in these cells. Two genes, *Npy* and *Penk*, coding for neuropeptides that previously have been connected mainly with the regulation of pain perception in the spinal cord, were surprisingly found to be DE in the dCIN population.

One gene coding for a voltage gated K^+ channel is higher expressed in motor neurons, $K_v1.1$, while two are more expressed in dCIN, $K_v3.1$ and $K_v1.4$. $K_v1.1$ and $K_v3.1$ both code for delayed rectifying channels, where the channel encoded by $K_v3.1$ is activated at more depolarized membrane potentials and inactivates faster than the channel encoded by $K_v1.1$ [72]. $K_v1.4$ codes for an A-type fast-inactivating potassium channel that has been shown to induce rapid repolarization of the action potential [72]. These properties of the channels encoded by $K_v3.1$ and $K_v1.4$ suggest that dCINs can produce faster spiking [78, 150] than MNs although such functional tests have not been done systematically in the rodent spinal cord.

It furthermore seems that the dCINs have a more dense and broader range of receptor/ligand gated channels than the motor neurons, which will affect the synaptic integration in the two cell populations. There are 11 genes in

this category that are more expressed in dCINs than in MNs, while there are only 3 genes in this category that are more expressed in MNs than in dCINs. Apart from receptors relating to the synaptic integration mediated by common neurotransmitters such as glutamate, GABA, glycine and serotonin, several genes coding for orexin and somatostatin receptors were DE in dCINs. Both orexin and somatostatin have been suggested to be involved in sensory processing. The DE of the genes coding for both orexin receptor 1 and 2 as well as somatostatin receptor 2 in the dCINs therefore indicates that these cells could be a target for such sensory modulation and integration.

In summary, we identify clear changes in gene expression between the two neuronal populations relating to their distinct functions. Differential expression of voltage gated channels indicates differences in the membrane properties of the two neuronal populations, while the marked over-representation of receptor related genes in dCINs could reflect a more diverse input and signal integration of these cells, which form an integrated part of the motor network that subsequently transmits their processed output to the MNs.

It is also reasonable to assume that many genes are shared in the two neuronal populations, and though they for this reason do not constitute defining features that distinguish them from each other, they are nevertheless important for the functions of each neuronal population. Since the microarray technique doesn't provide absolute quantification of gene expression, it is unfortunately not possible to make any statement about genes that might be present in both populations at equal levels of expression.

4.3 Molecular mechanisms associated with the return of plateaus and PICs in motor neurons following spinal cord injury

Spinal cord injury causes long term neuronal dysfunction of spinal networks located caudal to the injury. This includes pathological changes of the spinal networks involved in the coordination of movements (section 1.3.1). Increased excitability of motor neurons has been implicated in this pathophysiological state. The return of plateaus and PICs have unequivocally been shown to be involved in this injury response and related to the developing spasticity in the chronic phase (section 1.3.2). To examine the molecular underpinnings of this return of PICs in the absence of descending monoaminergic input, we used our method developed for neuron specific gene expression profiling (section 4.2) to follow the progressive changes in gene expression in motor neurons as a result of injury to the spinal cord in a well established animal

model of injury-induced spasticity developed by Bennett and colleagues (see section 1.3.2).

4.3.1 Expression changes in the chronic phase relating to injury-induced spasticity

To determine the transcriptional response of motor neurons in the late phase of spinal cord injury, we compared the motor neuron expression profiles obtained 21 and 60 days after spinal cord transection with their sham operated counterparts. RNA extracted and amplified from laser dissected motor neurons labeled *in vivo* (section 3.1)) was hybridized onto Affymetrix Rat 230 2.0 arrays: spinalized for 21 days (Spi-21, n = 8), spinalized for 60 days (Spi-60, n = 8), sham operated controls for 21 days (ShamC-21, n = 6) and sham operated controls for 60 days (ShamC-60, n = 5). The microarray data was normalized first using linear (QLT) with subsequent non-linear compensation (see section 4.2.1). To identify the significantly DE genes a conglomerate classifier was used based on three statistical methods: limma, Cyber-T and SAM (see section 4.2.2). Extracting genes that fall within a 1 % false discovery rate (FDR) this produces two lists of genes for Spi-21 and Spi-60 that contain 1452 and 1841 DE genes, respectively.

From these two lists, genes were extracted and ordered into functional groups relating to general excitability and PICs (Table 1-3, Paper III). In particular, since the emergence of plateau potentials and increased motor neuron excitability are likely to be influenced by many factors, the discussion of these is divided into three major categories relating to 1) ion channel configuration that defines the intrinsic membrane properties, 2) receptors mediating fast synaptic transmission or acting on neuromodulatory pathways and 3) intracellular pathways modulating membrane excitability and signal transduction.

Ion channels

Regulation of inward currents. Our study shows that the expression of genes relating to both sodium and calcium channels undergoes changes as a response to the injury. We find that of the L-type channels, the gene coding for $Ca_v1.3$ is down-regulated, while we observe no expressional change in the gene coding for $Ca_v1.2$. There were no genes coding for sodium α channel subunits that were up-regulated as a response to injury. It therefore seems unlikely that it is an increased amount of voltage-gated α subunits that is directly responsible for the expression of the plateau in the injury state. Although the persistent inward currents (both Na^+ and Ca^{2+}) are

returning in the chronic spinal state, it has never been demonstrated that the amplitude is larger than in acute control animals when induced by maximal transmitter activation. The reports by the Bennett group focus on the super-sensitivity to monoaminergic transmitters and their agonists following transection [76, 118], rather than on the size of the persistent inward current per se. In light of this it therefore seems more relevant to focus on the modulatory mechanisms affecting the channels conducting the persistent inward current rather than on the expression of the pore-forming α-subunits themselves.

However, the conductance kinetics and activation curves of α subunits can be modulated both through physical modulations such as phosphorylation or through altered composition of the channel complex with its ancillary subunits. We find several genes coding for proteins that can affect the L-type Ca^{2+} channels and the expression of PICs. In particular, we find the genes coding for the calcium channel β subunits to be down-regulated, which could indicate both a regulation of L-type channel conductance dynamics as well as an altered expression in the membrane. The observed up-regulation of calmodulin (*Calm1*) together with the down-regulation of the β subunits could indicate an altered membrane targeting of the Ca^{2+} channels with a potential slower inactivation. The up-regulation of a gene coding for an alternative Ca^{2+} binding protein (*Cabp1*), which affects the inactivation of L-type channels could also play a part in the observed Ca^{2+}-mediated persistent inward current seen in the chronic spinal phase. *Cabp1* codes for a neuronal calmodulin-like Ca^{2+} binding protein that has been described to substitute for calmodulin as the Ca^{2+} sensor of both $Ca_v1.2$ and $Ca_v1.3$, prolonging Ca^{2+} currents by preventing Ca^{2+}-dependent inactivation [35, 197]. We also find an up-regulation of the gene coding for CaM kinase II gamma (Camk2n1) further suggesting that $Ca_v1.2$ and/or $Ca_v1.3$ conductivity may be enhanced. CaM kinase II has been shown to cause a negative shift in L-type Ca^{2+} current activation [19, 60]. Persistent inward Ca^{2+} currents have also been shown to be promoted by stimulation of PI3K [188] and PKA pathways [$Ca_v1.3$; [39, 152]] while PKC inhibits $Ca_v1.3$ [6]. We find concomitant up- and down-regulation of genes associated with PKA and PKC and their counterbalancing phosphatases therefore suggesting a conglomerate modulatory effect by these pathways on the persistent inward calcium current effectively increasing conductance without affecting the channel composition.

No separate sodium ion channel that solely conducts persistent inward currents has been described. Persistent inward Na^+ currents are instead conducted by the same sodium ion channels that conduct transient currents, which are thought to have an additional slowly inactivating state [185].

This additional conductive state is also modulated by the β sodium channel subunits [26, 94, 199]. Several genes pertaining to sodium channels were found to undergo regulation as a response to injury. There were no genes coding for α channels subunits that were up-regulated as a response to injury. The sodium channel $Na_v1.6$ is the major component of the sodium current of spinal neurons of the rodent, but the expression of the gene coding for $Na_v1.6$ was unaltered. PKA and PKC are known to reduce the current in $Na_v1.2$ but they have no reported regulatory effect on $Na_v1.6$ [21, 22]. The only reported regulation of $Na_v1.6$ is by MAP kinase p38-α [192] , which was not affected by spinalization. Thus any effect on this channel appears to reside in the regulation of its subunits.

It is therefore interesting that we found up-regulation of the gene coding for the sodium β_1 subunit (*Scnb1*) together with the down-regulation of the gene coding for the sodium β_3 subunit (*Scnb3*), indicating altered intracellular trafficking of sodium channels as well as modulation of their conductance kinetics. The resulting ostensible larger expression of sodium channels in the plasma membrane could increase overall sodium conductance and a concomitant redirection of channel trafficking to soma and dendrites rather than to axons and terminals could also increase the role of sodium persistent inward currents.

In summary, from previously published results showing non-conditional expression of persistent inward currents in motor neurons through both Ca^{2+} and Na^+ channels in chronic spinal animals [119] we might naïvely have expected to find an up-regulation of the genes coding for the pore-forming α subunits of $Ca_v1.3$ and the Na^+ channels that have a persistent component. Our results certainly demonstrate that the changes in these channel complexes are far more complex and not least targeting modulatory subunits and intracellular pathways.

Regulation of outward currents. We also found significantly DE genes relating to potassium and chloride channels suggesting a rather complex regulation of outward currents in motor neurons post injury. The appearance of plateaus does not seem to be a result of a clear reduction in overall outward K^+ or Cl^- currents. Only the down-regulation of *Kcnn2*, which codes for a small-conductance calcium-activated potassium channel ($K_{Ca}2.2$.) that generates the afterhyperpolarization in motor neurons could signify an increased ability to generate plateaus. This conductance has been shown to be blocked by serotonin in motor neurons leading to increased expression of plateaus [70, 86].

Neurotransmitter receptors

Regulation of excitatory synaptic transmission. Several genes relating to ionotropic glutamate receptors were regulated after injury. Among the most prominent of these was the up-regulation *Cacng2* (γ_2) coding for a transmembrane AMPA receptor regulatory protein (TARP) [27], suggesting an increased expression of AMPA receptors in the membrane and a resulting increased sensitivity to glutamatergic synaptic transmission. Equally interesting among the glutamate receptor regulations was an apparent strong regulation of the NMDA receptor complex. Four genes relating to this complex were regulated as a response to injury: *Grin1* (NR1), *Grin3b* (NR3B), *Grina* (NRA1) and *Grinl1a* (GL1AD). Thus the overall regulation of the NMDA complex suggests a rather intricate regulation, with increased sensitivity to glutamate resulting in increased signal transmission at the same time as the Ca^{2+} permeability decreases, possibly serving a neuroprotective role.

For acetylcholine receptors the up-regulation of the genes coding for the nicotinic receptors *Chrna4* (α_4) and *Chrna6* (α_6) suggests an increased sensitivity to acetylcholine transmission in the motor neurons. Several DE genes relating to the synaptic release of acetylcholine suggest a general increase in the release of this neurotransmitter (*Nrg1*, *Cuta* and *Slc18a3* (VAChT) and *Freq*). Together, these regulations of genes relating to both pre- and post-synaptic cholinergic transmission suggest an increased acetylcholine signaling at central synapses that may enhance the recurrent excitation among motor neurons, both by motor axon collaterals terminating directly onto motor neurons [144] and by a polysynaptic positive feedback loop which was recently described [131]. We also note that this feedback mechanism can work via the peripheral synapses through the reflex loop, where an increased cholinergic release at the neuromuscular junction will result in enhanced muscle tone and activation of Ia afferents projecting directly onto motor neurons.

Regulation of inhibitory transmission. Genes relating to GABA receptors underwent a striking regulation that seems to target $GABA_A$ receptors and their expression in the membrane. All of the DE genes coding for $GABA_A$ receptor subunits are down-regulated; two α subunits (*Gabra1* and *Gabra5*) and a γ subunit (*Gabg2*). The DE genes *Gabrg2*, *Gabarapl1*, *Gabarapl2* and *Trak2* relating to $GABA_A$ receptor trafficking furthermore indicate a strong regulation of $GABA_A$ location and membrane expression. Together, this response points to reduced GABAergic transmission and a substantial regulation of $GABA_A$ receptors. It has been shown in the rat tail model that the reflex response in the chronic phase lacks an initial inhibitory

phase present in normal animals, indicating that part of the reason for the pathological long lasting motor discharge may be due to a lack of inhibition failing to terminate the activity[7, 122]. In combination with our present findings this implies that regulation of the GABAergic system plays a part in the chronic pathological state. Interestingly a recent modeling study including a small network of motor neurons and recurrent inhibition through Renshaw cells has shown that a selective decrease in the $GABA_A$ inhibition can unmask motor neuron plateau potentials [187] suggesting a role for the observed regulation in $GABA_A$ receptors in the expression of plateaus in the injury state.

The effect of inhibitory synapses can be further altered if the reversal potential for chloride is changed, indirectly leading to increased motor neuron excitability. Recent experiments have shown that the K^+-Cl^- co-transporter 2 (*KCC2*) responsible for Cl^- extrusion is down-regulated as a response to spinal cord injury, effectively decreasing the reversal potential for chloride and pushing the effect of chloride channel activation towards more depolarizing potentials [96, 189]. The gene for KCC2 is not included in the Ensembl annotations used to filter the probe sets and could therefore not be investigated in the present study. The gene *Slc4a3* coding for another Cl^- transporter (EA3) shown to accumulate Cl^- in embryonic motor neurons was up-regulation as a response to the injury. The combined effect of *KCC2* down-regulation with AE3 up-regulation could very well shift the reversal potential for Cl^-, increasing the excitability of motor neurons through GABAergic as well as glycinergic synaptic transmission from spinal interneurons.

Regulation of neuromodulatory receptors. Increased sensitivity to 5-HT and noradrenalin have been observed after spinalization. This hypersensitivity is thought to contribute to the expression of plateaus in the chronic spinal phase (section 1.3.2). We find gene regulations suggesting an increased expression of adrenergic and dopaminergic, but not serotonergic receptors. One gene coding for the adrenergic a receptor 1D (*Adra1d*) is up-regulated together with the gene coding for an adrenergic receptor kinase (*Adrbk1*) indicating a role for these in the noradrenaline hypersensitivity. It has recently been shown that the activation of the adrenergic α_1 receptor results in a strong activation of both Ca^{2+} and Na^+ persistent inward currents in the rat tail model [75, 154], suggesting a strong role for the up-regulation of the gene *Adra1d* in increasing the excitability of motor neurons post injury. This gene was also more expressed in MN than dCIN suggesting a very specific role for this gene in motor neurons that could be targeted specifically to reduce the adrenergic supersensitivy.

4.3.2 Identification of temporal expression clusters under common regulatory control

After examining the gene expression of motor neurons in the late phase of the injury response, we expanded on the framework by including additional data from two time points in the early phase 2 and 7 days post injury to investigate the dynamic transcriptional response of motor neurons following spinal cord injury.

To examine the progressive transcriptional response of the motor neurons from early to late post-injury phases, Affymetrix Rat 230 2.0 GeneChips were hybridized with RNA samples originating from motor neurons of uninjured control animals (n=4) as well as animals 2 (n = 6), 7 (n = 5), 21 (n=8) and 60 (n = 8) days post injury. The data was pre-processed with QLT and the subsequent compensation of remaining non-linear artifacts. The previously introduced conglomerate classifier based on limma, Cyber-T and SAM was used to identify significantly DE genes, identifying 3,708 DE genes.

Expression clustering

In order to identify common expression profiles across time among the DE genes, transcripts were grouped into clusters of similar expression patterns using a robust consensus cluster algorithm developed by Grotkjaer et al. 2006 [69]. The consensus cluster algorithm is based on an averaging procedure conducted on multiple runs of K-means clustering. This procedure amplifies common patterns in the expression profiles while suppressing non-reproducible features. To reduce miss-classification due to noise in the expression data (of non-differentially expressed genes) we use the consensus clustering on the most likely DE genes.

This analysis revealed the existence of 12 distinct time profiles each containing 178-574 genes (Figure 1, Paper IV). These 12 time profiles divide into two main groups relating to the late response, down-regulation (time profile 1-4, 12) and up-regulation (time profiles 6-10). Besides these two main categories of late regulation expression patterns, there are 2 cluster with a predominant early response; time profile 5 with an early up-regulation at day 2 and time profile 11 with an early down regulation at day 2 and 7, both of which fall back towards control levels 21 and 60 days post injury.

The consensus clustering of the DE genes in this way reveals gene clusters with distinct expression time profiles. Thus, many of the genes affecting motor neuron excitability share expression patterns: The majority that have an excitatory effect are up-regulated in late stages of the injury response (21 and 60 days post injury) with expression patterns matching cluster 6-10,

while the majority of the genes relating to inhibition are down-regulated in the late phases of the response with expression patterns matching cluster profile 1-4 and 12 (Table 2, Paper IV). Most of these genes are therefore the same as we found and described in the study of the late phase respond (section 4.3.1), but the data on their dynamic regulation now subdivide them into classes of common regulations.

Analysis of ontology over-representation and transcription factor regulation was performed on each cluster to identify potential mechanisms of regulation.

Ontology analysis of expression clusters

Ontology analysis of the genes associated with each cluster profile shows that the motor neurons engage in different biological processes as the transcriptional response evolves over time (Table I, Paper IV).

In particular time profile 5 signifies a marked immunological and inflammatory response of the motor neurons in the early phase after injury, which resides to control levels in the late phases. Cluster profiles 1-4 as well as 12 all describe different patterns of transcript down-regulation. Cell-cell adhesion is clearly down-regulated, suggesting that the direct interaction of motor neurons with their neighboring cells are reduced, possibly reflecting synapse stripping. The down-regulation of genes related to ensheathment of neurons suggests changes in myelination. Another prominent down-regulated mechanism involves mitochondrial related energy metabolism, which has several terms. The translational machinery is also down-regulated (ribonucleoprotein complex, translation, ribonucleoprotein complex biogenesis and ribosome). The down-regulation of chromatin structures (nuclesome assembly) suggests that the DNA could be unfolding towards a more favorable transcriptional state, while RNA splicing suggests a reduction in the mRNA processing.

Among the prominent up-regulated profiles (profile 6-10), pathways relating to neuronal development such as anatomical structure development, axogenesis and systems development and neuron differentiation, suggest that injury induces developmental processes as a late response. This indicates that a differentiated and mature neuronal population in the spinal cord is capable of re-engaging in developmental pathways, presumably attempting to ameliorate the conditions of the damaged spinal cord and compensate for the lack of inputs. It is also clear that plasma membrane transporter activities of various kinds are significantly up-regulated, suggesting a very strong control of the electro-chemical transmembrane gradients, possibly also reflecting the changing chemical requirements of the motor neurons. The motor neurons

also up-regulate processes directly relating to membrane excitability and neural transmission (coated vesicle, gated channel activity, neurological system process, regulation of neurotransmitter levels, synaptic transmission), suggesting that the motor neurons change their synaptic strength, both acting as a presynaptic neuron (central and peripheral synapse) through modulation of axon terminals with increased machinery for acetylcholine release and acting as a postsynaptic neuron through modulation of receptor channels as well as changes in membrane excitability.

Transcriptional regulation of expression clusters

The distinct expression pattern shared by the genes of each consensus cluster and their associated ontology terms suggested a common regulatory control of each gene cluster. This possibility was examined by matching transcription factor binding sites with core promoter sequences of the genes associated with each cluster using ASAP [133] with motifs of two known databases (JASPAR and TRANSFAC). Unfortunately these databases are not yet fully annotated and only 2 and 6 of the DE transcription factors had binding motifs annotated in JASPAR (Sp1 and E2F1) or TRANSFAC (Sp1, E2F1, FOXO1, ATF3, ATF4, Myc) respectively, making it difficult to relate transcription factor expression with their target cluster profiles on a broad scale. However, based on TRANSFAC over-representation analysis we find that the expression pattern of these 6 differentially expressed transcription factors correlates nicely with their putative target gene clusters.

Apart from the 6 differentially expressed transcription factors with know motifs, we note that several of the other differentially expressed transcription factors have been implicated with central nervous system development or its response to trauma. These include the down-regulated transcription factors E2F5 [44], Gtf2ird1 (synonymous with BEN) [45] and Nfia [134] as well as the up-regulated transcription factors Pbx3 [159] and Nrf2f [108]. Olig1 and Nkx6-2 are down-regulated. They have been implicated with motor neuron cell differentiation early in development [130, 146, 198] and their down-regulation from the un-injured state also suggests a role for these TF in the maintenance of normal motor neuron function and identity.

The regulation of Spi1, E2F1 and Myc together with their broad cluster targets suggest that these un-specific activators of transcription enhance the general transcriptional capacity of the motor neurons, while the expression pattern of other more specific regulators of transcription like Olig1 or Nkx6-2 could function to shape the response by relief or activation of targeted suppression of specific sets of genes, supporting the hypothesis of suppressor mediated transcriptional specificity.

The observed combination of up- and down-regulated transcription factors therefore suggests the redirection of the transcriptional program, where the transcription factors of cluster 1-4, 11 and 12 must be involved in the maintenance of normal motor neuron function and their down-regulation together with the transient (profile 5) or permanent (profile 6-10) up-regulation of transcription factors suggest dynamic transitions through new transcriptional states. It is also clear from our analysis that these transitions through different transcriptional states across time are mediated by the intricate interactions of several transcription factors, of which we identified 34.

Chapter 5

Concluding Remarks

From the presented work in this thesis it is clear that the contribution of the motor neurons in shaping the motor output is quite significant in both normal and injury states. It is also obvious from the expression studies that a very substantial arsenal of biological processes are involved in the regulation of the electrophysiological properties of motor neurons in response to spinal cord injury, where especially non-pore forming channel and receptor subunits are subject to regulation. This extensive control of all the different components affecting membrane excitability and synaptic transmission clearly illustrates that the regulation of biological processes affecting neuronal functions is quite complex. It seem reasonable to assume that the same repertoire is available in the normal state, suggesting an extensive capacity for regulation that most likely shapes the electrophysiological properties of a cell during normal as well as injury states.

Paper I We find that motor neurons release both glutamate and acetylcholine at central synapses, possibly released from distinct terminals. Only acethylcoline was released at the neuromuscular junction. This is in contrast to the hypothesis originally proposed by Dale that neurons release the same transmitter at all terminals. It will clearly be of interest to reveal the mechanism that allow such diversified transmitter distribution. The functional significance of this finding is not yet clear, but the presence of two transmitters at central synapses could provide an additional component of modulation and transmission. This would indicate an even more intricate regulation of motor neuron activity at central synapses than previously proposed.

Paper II Expression profiling of motor neurons compared to commissural interneurons showed that the two neuronal populations had differentially expressed genes that may reflect their functional differences in

transmitting and shaping the locomotor signal. For the purpose of this project we developed a method that enabled reliable gene expression profiling from as few as 50 fluorescently identified cells. Together with an optimized analysis methodology, the protocol has a wide applicability and can be used to examine dynamic transcriptional responses of well defined neural populations in both normal and diseased states of the brain and spinal cord.

Paper III Using our method for gene expression profiling, we examine the transcriptional response of motor neurons to spinal cord injury. Motor neurons, with their "mysterious" return of persistent inward currents in the absence of descending pathways, have been strongly implicated in injury-induced spasticity. We find regulation of several gene candidates involved in molecular mechanisms that support the return of PICs. Most pertinent of these was the finding that the pore forming channel subunits of neither calcium nor sodium conducting PICs were up-regulated. Our study clearly suggests that the return of PICs is the result of a very complex regulation that involves a substantial modulation of the ancillary subunits of these channel complexes as well as the regulation of receptors involved in synaptic transmission.

Paper IV Examination of the general transcriptional response of motor neurons and the underlying transcriptional regulation by transcription factors indicated that motor neurons are intimately involved in the general injury response. Pathways relating to inflammatory and immunological response are transiently active early in the injury response while several processes relating to development appear in the late phase of the injury response. Promoter analysis performed on the genes contained in expression clusters furthermore identified mechanisms of gene regulation exerted by DE transcription factors. Since mammals have limited regenerative capacity, spinal cord injury causes permanent changes that result in a maladaptive pathophysiological state with spasticity as one of the functional consequences. Affecting the regulatory pathways through experimental manipulation of the identified TF could provide a new avenue for treatment of injury-induced spasticity by targeting pathways rather than individual genes or proteins. To identify the most relevant pathways to target in this way, additional gene silencing of the most prominent up-regulated candidates affecting motor neuron excitability could be used to dissect out the functional relevance for the expression changes observed in the studies presented in this thesis.

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