

From Department of Neuroscience  
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# Optogenetic Control of Spinal Microcircuits:

## Insights into Locomotor Rhythm and Pattern Generation

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**Karolinska  
Institutet**

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Blue light initiates walking, and yellow light inhibits it, in the transgenic mice used in this thesis.

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“I don't know if I was interested so much in the science as I was in the slime that goes along with it. Snakes and frogs. When I saw how slimy the human brain was, I knew that's what I wanted to do for the rest of my life.”

*Dr. Michael Hfuhruhurr*

from “The man with two brains”, 1983

## **ABSTRACT**

Mammalian locomotion is a complex task in which hundreds of muscles work together in a coordinated fashion. Neural networks in the spinal cord, known as central pattern generators (CPGs), carry all the components necessary to produce the cyclical pattern of muscle activity needed for locomotion. The fact that the locomotor CPG is innate and highly localized makes it outstanding as a subject to study how a complex, but concrete behavior, is produced by a neuronal network.

Two fundamental aspects of the CPG are rhythm generation, and flexor-extensor coordination. These two properties have sometimes been linked together, such as in the half-center model, in which the alternating activity between flexors and extensors are the cause of the rhythm. Other models for rhythm generation have also been postulated, consequently no consensus exists regarding the overall structure of the CPG for locomotion.

Pharmacological investigations have indicated that glutamatergic neurons as essential for locomotion. To further elucidate the function of these neurons, the work presented in this thesis has made use of a set of new tools to target glutamatergic neurons, and elucidate their specific contribution to locomotion.

A mouse was produced that expressed the optically gated ion channel Channelrhodopsin-2, making it possible for the first time to selectively activate a genetically specific sub-population of neurons in the spinal cord. The experiments using this mouse show that glutamatergic neuron activation is sufficient to produce locomotor-like activity, both in the spinal cord, and in the hindbrain.

With the use of another set of recently produced transgenic mice, it was possible to probe deeper into the structure of the CPG, and illuminate several key aspects of the organization of the network. Several proposed network models could be refuted and one in particular was promoted. The results show that the CPG network is build up from intrinsically rhythmic modules.

Furthermore, a mouse without glutamatergic neurotransmission was examined. What was found was that the locomotion deficient mouse could produce locomotor-like activity under special conditions, and this activity depended solely on inhibitory interneurons, specifically, reciprocally connected Ia interneurons.

Overall, glutamatergic neurons are shown to form intrinsically rhythmic modules that are indispensable for rhythm generation, and network function. The use of genetics and electrophysiology is a powerful combination that will continue to provide conclusions about how neural networks produce and control complex motor behavior.

## LIST OF PUBLICATIONS

- I. **Hägglund M**, Borgius L, Dougherty KJ, Kiehn O. Activation of groups of excitatory neurons in the mammalian spinal cord or hindbrain evokes locomotion. *Nat Neurosci.* 13(2):246-52, 2010
- II. **Hägglund M**, Dougherty KJ, Borgius L, Iwasato T, Kiehn O. Optogenetic dissection reveals multiple rhythmogenic modules underlying locomotion. *Manuscript*
- III. Talpalar AE, Endo T, Löw P, Borgius L, **Hägglund M**, Dougherty KJ, Ryge J, Hnasko TS, Kiehn O. Identification of minimal neuronal networks involved in flexor-extensor alternation in the mammalian spinal cord. *Neuron.* 22;71(6): 1071-84, 2011

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## LIST OF ABBREVIATIONS

5HT	5-Hydroxytryptamine (Serotonin)
BAC	Bacterial Artificial Chromosome
BMP	Bone Morphogenic Protein
CAG	Chicken beta-Actin Promoter and Cytomegalovirus Enhancer
ChR2	Channelrhodopsin-2
CPG	Central Pattern Generator
Cre	Causes Recombination
eNpHR	enhanced <i>Natronomonas pharaonis</i> Halorhodopsin
ER	Estrogen Receptor
GABA	Gamma-Aminobutyric Acid
LoxP	Locus of crossing over in P1
MLR	Mesencephalic Locomotor Region
mRNA	messenger Ribonucleic Acid
NMDA	N-methyl-d-aspartic Acid
PCR	Polymerase Chain Reaction
Shh	Sonic Hedgehog
UBG	Unit Burst Generator
Vglut2	Vesicular Glutamate Transporter
VIAAT	Vesicular Inhibitory Amino Acid Transporter
YFP	Yellow Fluorescent Protein



# 1 INTRODUCTION

The most apparent divide within biology is arguably that between plants and animals. The *Cnidaria*, one of the most primitive forms of animals, have complex life-cycles, distinct tissues and organs, and, much more interesting: a nervous system. This allows these animals to move, a property that makes them much less plant-like and more animal-like. Movement in the *Cnidaria*, which incorporates animals such as jellyfish, is very basic and depends on the action of a distributed nerve net that coordinates a jet propulsion system. The power of this system is very weak and does not permit the animals to move against a current. However, the possibility to move away from your site of birth in search for food and sex is an evolutionary success, and the development of the movement repertoire has since been one of the most important traits selected for throughout evolution.

The nervous system in primitive animals can be thought of as an extension of a locomotion system, something that is nicely illustrated in the tunicates. These bizarre animals, also known as sea squirts, eventually gave rise to the vertebrates. They are born motile, and has a nervous system directing its sensations into motor commands. However, the adult animal is sessile, and settles down on a rock to filter feed for the rest of its life. When this happens, it devours its own nervous system, demonstrating that the simplest and most primitive reason for wasting energy on building a brain in the first place is to control movement.

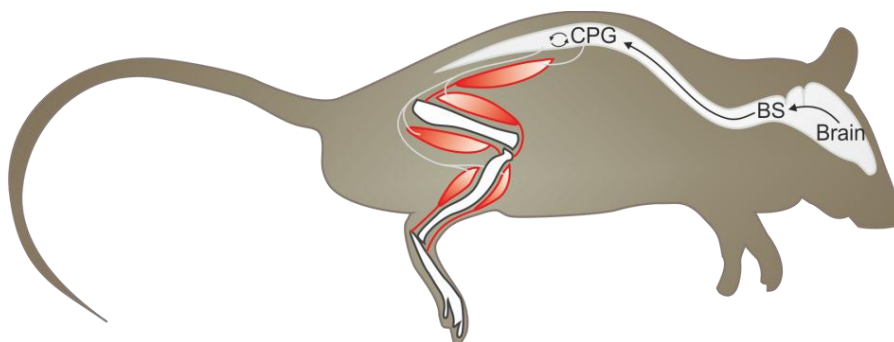
The different ways of connecting neurons in a human brain vastly outnumbers the particles in the known universe. Luckily, they are not wired up randomly, but follow intricate genetically and environmentally directed laws, making the science of understanding the brain a huge endeavor, but a tractable one. Every conscious act is channeled through a very narrow and specific system, in the end relying only on the motor neuron and it is signaling to a muscle to contract. This is true for anything we can consciously do: moving, talking, walking. The final decision to act comes from the motor neurons integration of incoming signals from a wide variety of sources over a great temporal range. To bridge the detailed knowledge we have of the single cell and the level of neuronal networks giving rise to a simple behavior, this thesis have focused on understanding more about the system that generates walking.

When tetrapod animals appeared around 350-400 million years ago (Long and Gordon 2004), the coordination of muscle activation was changed from an undulatory wave of activity seen in ancestral fish, to a stepping movement. This stepping brought about a problem of control; in fish, locomotion depends on a travelling wave of activity that is

quite straightforwardly transformed into the wave of muscle activity seen during swimming (Cohen and Wallen 1980). In modern mammals there is on the order of 50 muscles in each limb. The activity in any one muscle has an impact on every other one, since it shifts the weight and balance of the limb when it is contracting or relaxing. This intractable control problem depends on the combinatorial explosion of possible sequences of muscle activation, caused by the many degrees of freedom in the system. The question of how the nervous system overcomes such a computational hurdle is one of the fundamental issues in the scientific field of motor control.

## 1.1 HISTORICAL BACKGROUND OF THE STUDY OF SPINAL LOCOMOTOR NETWORK

The scientific studies of the spinal cord have brought several big steps forward in our understanding of motor systems, as well as general functions of the nervous system. The British neuroscientist Charles Sherrington studied the spinal cord to describe and elucidate neurons, synapses - a term which he coined - and reflexes, and eventually received the Nobel prize 1932 for this work (Sherrington 1932). Around 20 years later, Sherrington's former student, John Eccles, falsified his own hypothesis about synapses being purely electrical, and showed that they were chemical (Eccles 1957). A Nobel Prize winning accomplishment that might not have happened unless his friend, the science philosopher Karl Popper, had pushed him to it<sup>1</sup>. The Swedish neurophysiologist Anders Lundberg worked with Eccles during his most productive years and later established himself at the Gothenburg University where he made major advancements in the physiology of the mammalian spinal cord and mentored several scientists leading the field today<sup>2</sup>.



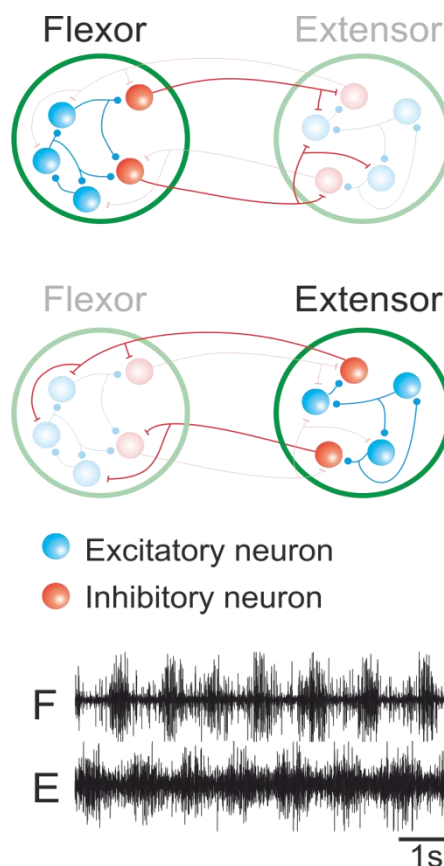
**Figure 1. Locomotion is produced by a Central Pattern Generator (CPG) located in the spinal cord.** Schematic of the fundamental components underlying locomotion. To initiate locomotor activity, a descending signal from the brain, relayed in the brainstem activates the spinal CPG. In turn, the CPG converts the command signal into a rhythmic patterned output that is sent to the muscles.

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<sup>1</sup>Eccles, Neurophysiological basis of experience, 1999, Transaction Publishers, New Brunswick, NJ. P274-275.

<sup>2</sup>Including Hans Hultborn, who later mentored the supervisor to this thesis.

The prevailing view in the early 60s was that stepping was governed by spinal reflexes and was thus relying on sensory feedback from the limbs (Clarac 2008; Grillner et al. 1976). One of Lundberg's accomplishments was to reinstate the idea of a central spinal program that work together with the reflexes to generate and coordinate stepping. The idea had been suggested earlier, particularly by Graham Brown, one of Sherrington's less known students who showed, 100 years ago, that the spinal cord could generate stepping without any descending or sensory input (Brown 1911), a network later known as a central pattern generator (CPG, **Figure 1**)(Delcomyn 1980; Grillner 1981; Stuart and Hultborn 2008). This finding was controversial and didn't surface to the scientific field in earnest until Lundberg brought it forth during the late 1960s. Another of Graham Browns ideas that Lundberg followed up on was the half-center model (Brown 1914).



**Figure 2. The Half-center model.** Two neuronal networks are interconnected via inhibitory neurons (red). When one part is active it inhibits the other part. After some time, fatigue reduces the inhibition, making the other part active, leading to inhibition of the first network. This alternating activity has been stipulated to form the basis of rhythm generation, and flexor-extensor alternation seen during locomotion.

This model is a hypothetical network structure, in which a flexor network is pitted against an extensor network with inhibitory interconnections (**Figure 2**). This connectivity leads to a system in which the activity in one half of the network inhibits the other, and after some fatigue of some sort sets in, the inhibitory signal decreases,

permitting the other half of the network to be active, and inhibiting the former. Lundberg and colleagues did find indirect evidence for such a network in the spinal cord (Jankowska et al. 1967a; b).

The half-center organization is an elegant explanation that can account for both rhythm generation and patterning. However, detailed recordings of muscle activity of adult cats showed that some muscles, notably the semitendinosus, can burst in both flexor and extensor phase during stepping. Furthermore, in developed locomotion, some muscles burst two times per step cycle. This led to the suggestion of a smaller module for the generation of the rhythmicity, that Sten Grillner named the unit burst generator (UBG) which could produce intrinsic rhythmic activity locally (Grillner 1981). The UBG thus generate rhythmicity in a single motor neuron pool without being involved in the global activity of the spinal cord. Within this framework it would also be easier to account for rhythmic movements that do not follow the stereotypical stepping pattern, such as scratching, swimming or backwards stepping. However, the locomotor field is not yet settled on this issue, and several contemporary models of the mammalian locomotor network rely on a half-center-like organization for rhythm generation (McCrea and Rybak 2008).

## **1.2 PROPERTIES OF THE MAMMALIAN CENTRAL PATTERN GENERATOR**

The key features that are needed to produce locomotion *de novo*, i.e. without sensory or descending input as a driving force, is 1) a rhythm generating system, 2) a flexor-extensor patterning system and 3) a system generating left-right alternation (Kiehn 2006) (and also coordinating transitions to alternative modes of locomotion, such as gallop). These essential properties have been supposed to be organized hierarchically, in which the rhythm generation, or the clock, sits on top of the network and distributes a coordinating signal for the next level of the network (McCrea and Rybak 2008). The secondary level is responsible for a subdivision of the motor system of a limb, either focusing on a single muscle, or more typically, a synergistic pool of muscles, such as all flexors or all extensors. It takes the tick from the clock, and delays it, so as to make the muscles it is controlling contract with proper duration and at the correct moment in the cycle. A parallel system coordinates the two sides so that the stepping alternates, as during walking and trotting, or is synchronous, as in a gallop.

### **1.2.1 Hierarchical model of the CPG**

The arguments for the hierarchical structure come from observations that a change in patterning can occur without a change in frequency. An example for this comes from the study of deletions. Deletions are spontaneous or sensory evoked events,

characterized by a loss of one or more bursts of activity in some muscles, while the rest of the muscles may be active (Lafreniere-Roula and McCrea 2005; Stein and Daniels-McQueen 2002; Zhong et al. 2012). Depending on the specifics around the deletion, the rhythm can sometimes continue “in silence”, without an output. The silent rhythm is inferred from noticing that when the bursting resumes, it does so at the proper timing, that is, as it would have been if the deletion had not happened, a so called non-resetting deletion (Lafreniere-Roula and McCrea 2005; Zhong et al. 2012). The interpretation of these deletions that do not alter the underlying rhythm is that the underlying network has one single rhythmogenic center that sits at least one layer up from the motor neurons. During a non-resetting deletion, the pre-motor interneurons fail to transmit the burst, but since the activity resumes at the correct timing as it would have if the deletion had not occurred, it is assumed that there has not been a failure in the rhythm generating machinery. The non-resetting deletions has been taken as evidence that there are at least two levels of interneurons structuring the CPG.

A further point reinforcing the hierarchical model comes from the fact that a change can occur in the patterning or the amplitude of the motor output, without any change in the frequency of locomotion (Burke et al. 2001; Kriellaars et al. 1994). In these cases, it is suggested, that the change in activity happens only on a level downstream from the rhythm generation, similarly to the deletions, and thus not affecting the frequency of locomotion.

### **1.2.2 Left-right alternation**

Left-right alternating activity is an obvious feature of walking in most mammals (Kiehn 2011). The underlying neural substrates has been much studied, both because of the relatively easy definition and characterization of neurons regulating it (commissural interneurons with axons crossing the midline) (Butt et al. 2002; Eide et al. 1999; Kiehn and Kjaerulff 1996; Quinlan and Kiehn 2007), and because of the description of genetic mutations in mice causing a switch from normal walking to rabbit-like hopping (Crone et al. 2009; Kullander et al. 2003). Furthermore, as mentioned above, the phase lag between activity on the left versus right sides is the only parameter determining the alternation, making it far less complex than the patterning of the flexor-extensor activity. However, the flexor-extensor coordination may be less regulated and stricter than the left-right alternation. This is because the left-right can switch totally during different gaits and go from alternating to synchronous, for instance when changing between trot and gallop.

Recordings from commissural interneurons in mammals have resulted in a detailed picture of the left-right connectivity (Butt and Kiehn 2003; Quinlan and Kiehn 2007).

During alternation, a dual inhibitory system is responsible for coordination of the two sides. One pathway sends commissural axons directly to the motor neurons, the other is excitatory, and contact inhibitory interneurons on the other side, that then project onto motor neurons. Another system with direct excitation of contralateral motor neurons is probably responsible for synchronous activity, such as jumping or galloping.

### **1.2.3 Flexor-extensor coordination**

The separation of flexor activity from extensor activity is not as straightforward as that of left from right, since the neurons involved are anatomically intermixed. The ways of inducing locomotor activity *in vitro* have not been focal enough to probe a small part of the spinal circuitry in isolation, and thus conclusions about the circuitry has mostly been drawn about the activity of a whole limb. This may be a reason why many models of the CPG take a global, whole-limb perspective and contribute rhythm generation to the whole network, as in the half-center organization (McCrea and Rybak 2008).

A recurrent theme in the literature is that the rostral lumbar cord is a hotspot for rhythmicity (Cazalets 2005; Delivet-Mongrain et al. 2008; Kiehn and Kjaerulff 1998; Kjaerulff and Kiehn 1996) and has sometimes been taken as the definite site for rhythm generation (Cazalets et al. 1995). Lesion studies of the rodent spinal cord have shown that the rostral, lumbar cord (L1-L2 segments) is most easily recruited into rhythmicity by drugs (Kjaerulff and Kiehn 1996). However, more caudal segments may also display locomotor-like bursting when separated from the rest of the cord, albeit at a lower amplitude, frequency and stability (Kjaerulff and Kiehn 1996). Similar reasoning has been made in the cat, but also there, the more caudal segments can show independent rhythmic activity when only the caudal segments are activated (Deliagina et al. 1983; Grillner and Zangger 1979).

### **1.2.4 Neural substrate underlying rhythm generation**

When searching for basic functions, such as rhythm generation, a fruitful approach has been to study animals with a small number of neurons compared to mammals. The lamprey, a fish, and one of the most ancient vertebrates, has been intensively analyzed with regards to cellular and network properties contributing to rhythm generation in the spinal motor system (el Manira et al. 1994; Wallen and Grillner 1987). These studies have led to a model where the rhythm generating machinery is constituted by ipsilateral, excitatory interneurons that are interconnected to each other, and to motor neurons. These interneurons produce a rhythmic output even when inhibition is inactivated pharmacologically, leading to the conclusion that rhythm generation itself relies only on excitatory neurotransmission (Cangiano and Grillner 2005; Roberts et al. 1998). Excitatory interneurons carry ion channels that make them intrinsically bursting

in response to a tonic excitation, such as a descending stimulation *in vivo*, or a pharmacological cocktail *in vitro*. When stimulated, these neurons depolarize and start spiking to drive each other and the downstream motor neurons. The depolarization also opens voltage dependent calcium channels leading to calcium influx. Calcium activates a second messenger cascade, ending with the opening of potassium channels that terminates the burst and leads to a hyperpolarized state, permitting the cycle to start anew.

Inactivating inhibition pharmacologically in the rodent spinal cord similarly does not preclude rhythmic activity, however, the left-right and flexor-extensor patterning is lost (Cowley and Schmidt 1995). Blocking glutamatergic neurotransmission leads to the disappearance of locomotor output (Talpalar and Kiehn 2010). These pharmacological experiments and the possibility that the burst generator of the lamprey CPG is evolutionary conserved, points to glutamatergic neurons as a convergence point for locomotor control and function (Kiehn et al. 2008).

### **1.3 MOLECULAR NEUROBIOLOGY OF THE MOUSE SPINAL CORD**

#### **1.3.1 The rodent *in vitro* preparation**

Up until the 80s, investigations of the mammalian locomotor system were carried out using *in vivo* experiments, mainly in the adult cat. During the mid 80s, Kudo et al developed the neonatal rodent spinal cord *in vitro* preparation (Kudo and Yamada 1987; Smith and Feldman 1987). The neonatal spinal cord is extra resilient to low oxygen levels because of its small size permits oxygen to diffuse into the tissue, but possibly also because the tissue has adapted for dealing with a lack of oxygen during delivery. These conditions make it ideal as an *in vitro* preparation, since one of the bottlenecks for a successful survival of the preparation is enough oxygen delivery to the tissue. Even though the reason for using neonates is because of the oxygen permeability, it also has the advantage of supplying a genetically hardwired system, without the caveat of needing to take experience of the animal into question. Furthermore, having an isolated spinal cord means that it is easily accessible for lesion studies, intercellular recordings and imaging. But more importantly, using mice presents the possibility to exploit the quickly growing array of genetic tools available.

#### **1.3.2 Spinal neuron classes are defined by master transcription factor expression**

The possibility to assign a specific role to a cell group has permeated the neuroscience field since the molecular revolution started during the 90s. The first transgenic mouse was produced 1981 (Gordon and Ruddle 1981), and the first mouse in which a gene had been deleted (a so called knock-out mouse) was created 1989 (Koller et al. 1989). Since then, a plethora of transgenic mice has been produced and generated lots of data

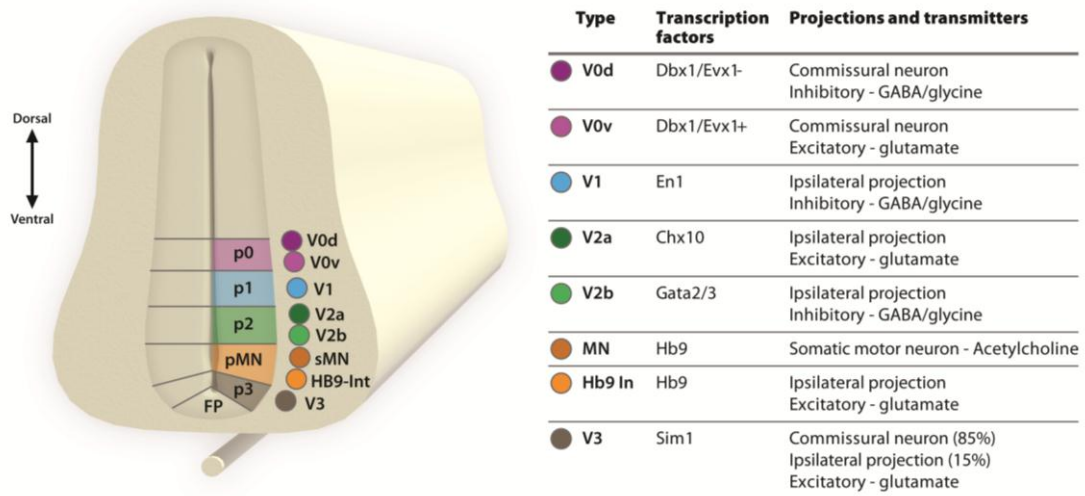
throughout all life sciences. In the spinal cord, master transcription factors expression divide neurons the spinal cord into several classes. During development, the floor plate and notochord, situated ventrally to the spinal cord, and the roof plate, lying dorsally, secrete sonic hedgehog (Shh) and bone morphogenic protein (BMP) respectively, setting up a dorso-ventral gradient that governs which class a neuron will be part of (Goulding 2009; Jessell 2000). Class affiliation is determined by up- or down-regulating a set of master transcription factors, depending on the concentration of the Shh and BMP, that in turn determine the morphology, projection and neurotransmitter phenotype of the cell. In the motor-related ventral cord, progenitor cells become V0-V3 interneurons or motor neurons depending on their dorso-ventral site of birth (**Figure 3**).

The straightforward study of these master transcription factors is to generate knock-out mice, and characterize their phenotype. However, this approach has sometimes given rise to complications in how to interpret the results. The problem is that these master transcription factors are more “symbolic” than functional. They specify which genetic program to run, but in their absence, the neurons do not die or disappear, they adopt another program instead. Therefore, the cells get affiliated with another class, making it hard to attribute a definite function to the master transcription gene and the cell class it defines (Lanuza et al. 2004). Instead, other less developmentally involved techniques are being used to elucidate the function of the neuronal classes. Specifically, the promoter of the master transcription factor gene is used to drive a physiological effector, for instance a toxin killing the cell, making it easier to attribute a certain cell class to a specific function (Crone et al. 2008; Gosgnach et al. 2006; Zhang et al. 2008). Instead of a toxin, ion channels responding to exogenous factors, such as allatostatin, can be used to control neuron activity (Gosgnach et al. 2006; Zhang et al. 2008).

### **1.3.3 V1 interneurons are inhibitory and ipsilaterally projecting**

Among the ventrally born interneurons are the V1 interneurons, defined by their expression of the *Engrailed-1* transcription factor. They are all inhibitory and ipsilaterally projecting, and include two important neuron populations known as the Ia interneurons, and the Renshaw cells (Wenner et al. 2000). The Ia interneurons were functionally described (Hultborn et al. 1976; Hultborn et al. 1971a; b) long before the molecular designations were discovered and may, except for the V1 domain, also stem from the V2b interneurons that are also ipsilateral and inhibitory. The functional definition of Ia interneurons is their afferent input from Ia fibers, coming from the muscle spindle of agonist muscles. They project onto antagonistic motor neurons, and to Ia interneurons related to the antagonistic muscle, and therefore form a plausible substrate for coordinating the activity between flexors and extensors (Hultborn et al. 1976; Hultborn et al. 1971a; b; Miller and Scott 1977; Pratt and Jordan 1987). Renshaw





**Figure 3. Dorso-ventral specification of spinal interneurons.** Diffusible factors along the dorso-ventral axis are deciphered by developing neurons, in turn activating master transcription factors. These factors repress each other mutually, forming sharp boundaries of expression between adjacent cell classes. The final transcription factor expression of a cell determines its neurotransmitter phenotype, projection pattern and consequently function. Some of the V0-V3 interneuron classes have been divided to give room for even further subpopulations, such as the V0d and V0v, and V2a and V2b.

cells are also functionally defined (Renshaw 1946; 1941). They are excited by motor neurons and send reciprocal projections back to them, but they also project to Ia interneurons (Hultborn et al. 1971c).

### 1.3.4 Excitatory spinal interneurons are marked by the Vesicular glutamate transporter 2

Some of the main groups of ventral interneurons develop a mix of both inhibitory and excitatory interneurons, such as the V0 and V2 interneurons. A strategy for catching all excitatory neurons is to target a gene that is selectively expressed in those cells only. Glutamatergic neurons are uniquely defined by their expression of vesicular glutamate transporters (Vgluts)(Aihara et al. 2000). Vglut2 is one of three vesicular glutamate transporters and is expressed in practically all glutamatergic neurons in the spinal cord, and most neurons in the brainstem, midbrain and thalamus (Borgius et al. 2010). Furthermore, the different Vgluts seem to be mutually exclusive, and thus only Vglut2 is found in interneurons in the spinal cord. The gene product is incorporated into vesicle membranes in glutamatergic presynaptic terminals and is instrumental in transporting glutamate into the vesicles. Therefore, it can suit the role of a genetic marker for

glutamatergic neurons (*Paper I* and *Paper II*), and as an effector in the knock out study (*Paper III*).

So far, the reliance on pharmacology, lesions, electrical stimulation and spontaneous events has proven to blunt to concisely settle whether the rhythm and pattern generation of the locomotor networks in the spinal cord are separate entities, and whether they stem from a global network or if they are subdivided into smaller assemblies. The scientific field has thus not reached a consensus on the macro-architecture of the neuronal networks that generate locomotion. Hopefully, this thesis will have an impact on this.

## 2 AIMS

The general aims of this thesis project have been to investigate the causal role that glutamatergic interneurons play in generating locomotion in the mammalian motor system.

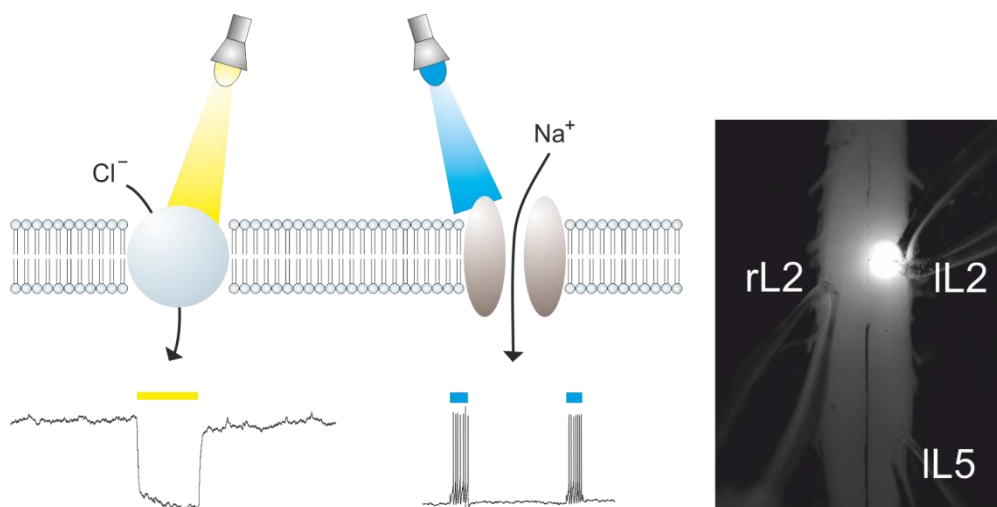
- I Can glutamatergic interneurons, defined by their expression of Vglut2, drive locomotor-like activity when activated? Can descending glutamatergic neurons in the hindbrain initiate locomotion?
- II Is the source of rhythmicity in the spinal CPG distributed, or global?
- III Can the spinal CPG produce locomotion when glutamatergic interneurons are not functional?

### 3 METHODOLOGICAL CONSIDERATIONS

All methods and materials used in the work described in this thesis are detailed in their respective articles (*Paper I-III*). This section will therefore discuss the more advanced technical tools implemented in the work.

#### 3.1 OPTOGENETICS

The main methodological novelties developed and implemented in the work presented in this thesis, comes from the generation and use of transgenic animals carrying optogenetic enhancements. The optogenetics field started to become acknowledged after 2005 and has exploded after 2010. The first paper describing the use of Channelrhodopsin-2 (ChR2) in mammalian cells dates from 2003 (Nagel et al. 2003) and the first time it was implemented in animal preparations was 2005 (Boyden et al. 2005; Li et al. 2005; Nagel et al. 2005). The first transgenic mouse was produced in 2007 (Wang et al. 2007) and the first transgenic mouse with a specific expression was published by us in 2010 (*Paper I*), after starting production in 2007. Optogenetics has had a major impact in the field, and is already a fundamental tool in systems neuroscience.



**Figure 4.** Halorhodopsin is a light-drive ion pump, that mainly pumps chloride ions into a cell expressing it (left), and thus hyperpolarizes the membrane (left, bottom). Channelrhodopsin-2 is an ion channel that depolarizes a neuron expressing it, making it possible to control spiking, with the use of light (middle). To stimulate optogenetic probes focally, we used a laser scanning system permitting activation of a limited set of neurons (right).

The most widely used optogenetic factor is ChrR2 (**Figure 4**). It is a seven-transmembrane molecule that opens a cation channel in response to blue light (Nagel et al. 2003). Several variants with different kinetics and light activation spectras have been developed, but the most prominently used is the codon-optimized H134R version,

also applied in all experiments performed in this thesis (Lin 2011). Complementing ChR2, we also made use of the light-driven chloride ion pump Halorhodopsin (eNpHR, **Figure 4**). This protein was cloned from the *archaea Natronomonas pharaonis* and has a secondary structure similar to channelrhodopsin, although its effect when activated is quite opposite. Upon yellow light illumination, this pump actively pumps chloride ions into the neuron, hyperpolarizing or shunting the cell, and potentially inactivates or reduces its firing (Zhang et al. 2007).

Proper light delivery to the neurons is of utmost importance. Different solutions are required depending on the preparation, but high enough intensity of light is imperative, which is the reason most investigators have used laser sources. For most of our purposes (in *Paper I* and *III*), the mercury lamp from a fluorescence light microscope, led through a 450-490 nm band pass filter, was enough to excite ChR2, while in *Paper II*, a 473 nm laser was used through a galvanic scanner system to create a small, focused spot of light. For eNpHR, the mercury lamp was directed through a 533-553 nm band pass filter.

The production of transgenic mice is costly and time consuming. This has led investigators to use other means of delivering optogenetic probes. In rodents, the most common way is by injection of a viral vector carrying the gene (Cardin et al. 2010; Lin 2012). A few days after injection, the infected cells start to show expression at the site of injection, permitting experiments to be performed. Another practice is the use of *in utero* electroporation, in which a plasmid is injected into the ventricles of an embryo and subjected to a series of electric pulses, that makes small pores in the membranes of the neurons lining the ventricle, facilitating the transfer of the genetic material (Saito 2006). Both of these methods yield high levels of gene products, however, there are drawbacks. Since we use neonatal animals, the wait for high enough expression of the transgene is a problem. The ventricles that make plasmid injection possible for electroporation are in the spinal cord replaced by a very narrow central canal, difficult to target with an injection needle. Furthermore, both techniques are invasive and give rise to variations in expression levels. We therefore opted for the production of a transgenic animal.

### **3.2 BACTERIAL ARTIFICIAL CHROMOSOME TECHNOLOGY**

There are several techniques to produce transgenic mice, all with different pros and cons. The most common ones are homologous recombination in embryonic stem cells, and pronuclear injections. To target a specific gene or region in the genome, it is necessary to use homologous recombination; this is used in knock-out (deleting a genomic region), knock-in (adding a gene) and more subtle changes, such as changing a single nucleotide. When the purpose is to add a gene to the genome, pronuclear

injection is the method of choice. This does not tamper with any specific region of interest; instead it randomly inserts the gene somewhere in the genome. Sometimes this may lead to unwanted effects, since the added material may incorporate into an essential gene or regulatory sequence and disrupt its normal function.

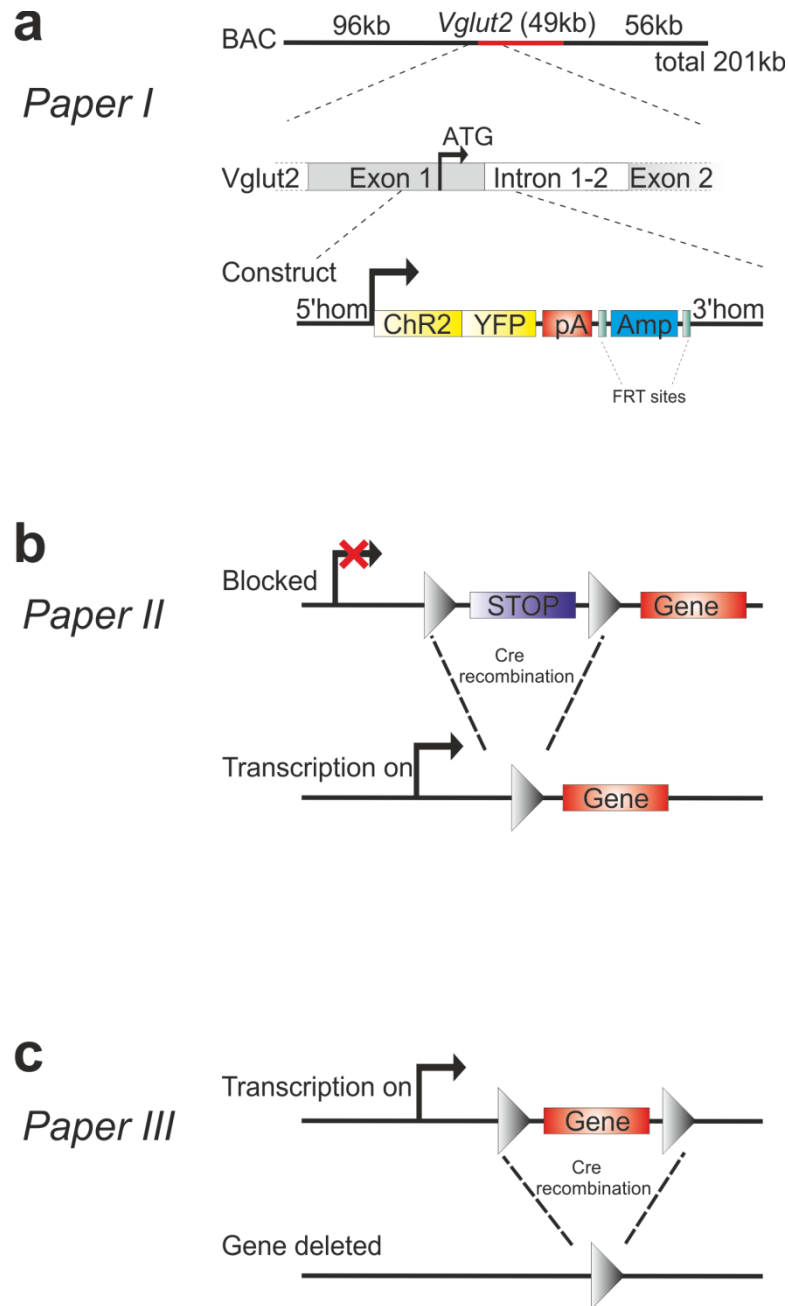
In one of the two founders of the Vglut2-ChR2-YFP mouse generated during this thesis (see Results and discussion, and *Paper I*), heterozygous mice were viable, but the homozygous ones had a strong developmental defect and died during infancy. This may reflect an insertion point into a gene that is essential for survival in at least one copy. More common is the problem that the added material is influenced by neighboring regulatory sequences, and thus changing the circumstances during which the added gene is expressed.

To ensure proper expression of the added gene, it is also necessary to add a regulatory sequence, such as a promoter sequence. The condition in which a gene is expressed is complex and depends on a lot of factors, and for most genes these are not known. Therefore, the pronuclear injection of a normal sized plasmid may not be enough to guarantee correct expression if the promoter sequence is not well characterized. These conditions are why the bacterial artificial chromosome (BAC) technology was developed (Shizuya et al. 1992). In contrast to a bacterial plasmid, which normally is between 4000 and 10 000 base pairs, a BAC is much larger and can carry DNA sequences up to 300 000 base pairs (**Figure 5a**). This procedure makes it possible to insert, into the genome, such a large part of the upstream and downstream regions of a gene of interest that one hopefully gets all of the regulatory sequences governing the expression of the gene (Parkitna et al. 2009). In our case, the expression of the BAC Vglut2-ChR2-eYFP transgene seems to be good in the spinal cord, however, there is a region in the medulla called the preBötzing complex that is composed of Vglut2 positive cells, in which this mouse and the Vglut2-Cre mouse lacks expression (Borgius et al. 2010). Probably this lack of expression reflects a lack of a promoter that is specific for this region, and thus the gene is not expressed there.

### **3.3 SPECIFIC GENE EXPRESSION USING THE CRE/LOXP SYSTEM**

To target specific neuron populations, the Cre/loxP system was used (Nagy 2000). Cre is a viral recombinase that will excise the DNA situated between two loxP sequences, exogenously added to the genome. This method is used in *Paper II* and *III*, to remove a stop signal to facilitate transgene expression (**Figure 5b**), and to disrupt gene expression (**Figure 5c**) respectively. A further enhancement of this system is used in *Paper III*, where Cre is fused to a mutated ligand binding domain of the estrogen receptor (ER) (Ventura et al. 2007) making the activity of Cre dependent on an exogenous factor, tamoxifen. When tamoxifen is not present, Cre is exported out from

the nucleus, and thus hindering it from performing its recombining activity. When tamoxifen is applied, it binds to the ER and transports it back into the nucleus where Cre can come in contact with the DNA and exert its recombination activity, thereby giving access to the temporal as well as spatial domain of expression of a particular gene.



**Figure 5.** (a) To produce the transgenic Vglut2-ChR2-YFP, a BAC was chosen containing the entire Vglut2 gene plus additional upstream and downstream sequences. A targeting cassette containing ChR2 fused to YFP was inserted into the BAC at the startsite of Vglut2 using homologous recombination. (b) The outline of the flox-STOP strategy used in Paper II. In the absence of Cre, a STOP-cassette is blocking transcription of the gene of interest. Upon Cre expression, the STOP-cassette is excised, activating transcription of the gene. (c). In Paper III, Cre is used to delete the gene of interest, to create a knock out mouse.

## 4 RESULTS AND DISCUSSION

Glutamatergic neurons have been implicated as vital for controlling locomotion and for intrinsic rhythm generation in the mammalian CPG. In this thesis, this hypothesis has been validated, expanded upon, and used to elucidate the structure of the networks responsible for rhythm and pattern generation in the spinal cord. What has emerged is a multifaceted picture of a highly flexible network of intrinsically rhythmogenic modules, regulated in a complex manner. Glutamatergic neurons evidently have a controlling function with the possibility to engage the whole lumbar network, or a part thereof. Turning off glutamatergic neuron function disrupts locomotor ability, however, spares a network that can give rise to a locomotor-like patterned output.

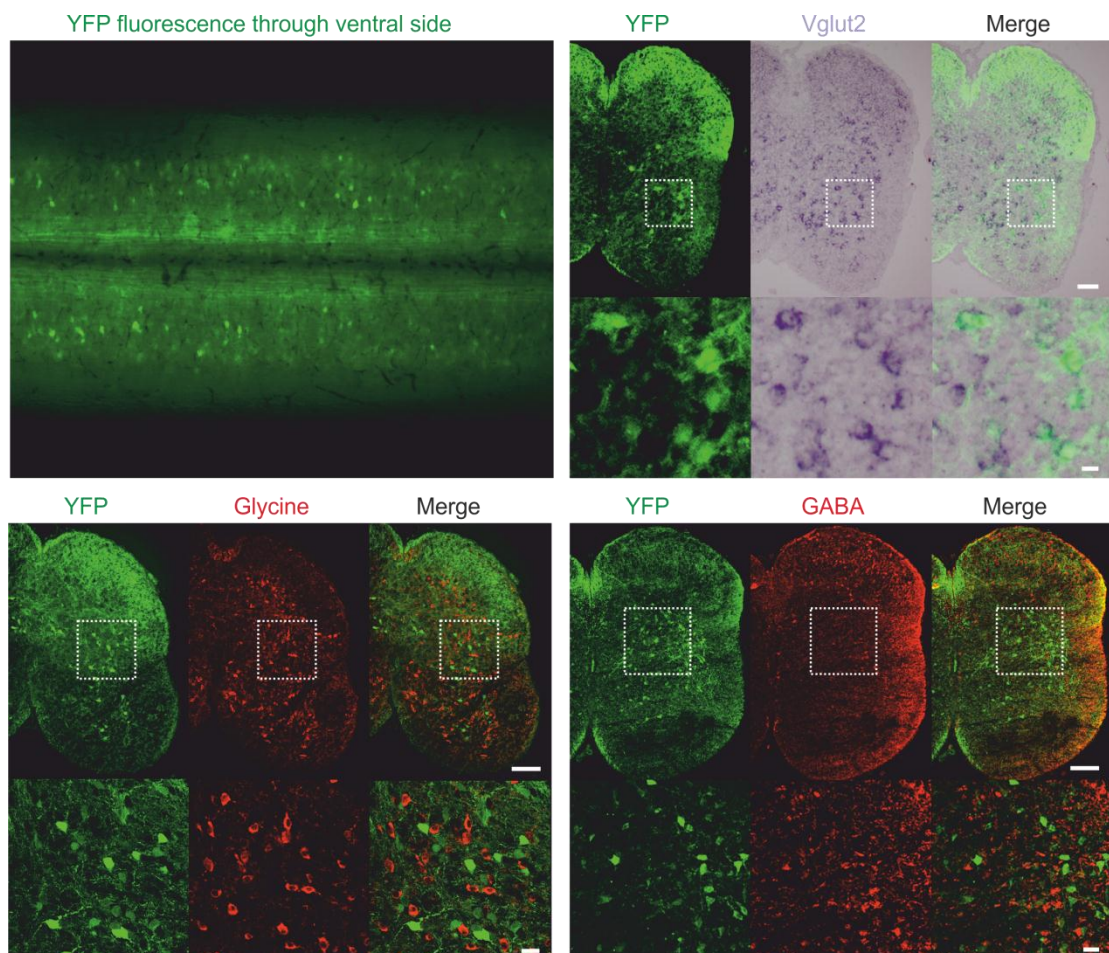
### 4.1 PAPER I: ACTIVATION OF GROUPS OF EXCITATORY NEURONS IN THE MAMMALIAN SPINAL CORD OR HINDBRAIN EVOKES LOCOMOTION

We set out to directly test the contribution of glutamatergic neurons to locomotion, and chose to do this by producing a transgenic BAC-animal, expressing ChR2 in its glutamatergic interneurons. Human codon optimized ChR2 (H134R version), fused to YFP, was inserted into a BAC that carried the whole *Vglut2* gene, plus an additional 96 kbp and 56 kbp upstream and downstream sequences respectively. This was done by homologous recombination in bacteria, and after removal of a selection marker, the BAC-construct was sequenced, purified and injected into pronuclei at the Karolinska Center for Transgenic Technologies. Two founders were positive, verified through PCR and inspection of YFP expression in the nervous system.

#### 4.1.1 ChR2-YFP is exclusively expressed in glutamatergic neurons

To ensure proper expression of ChR2 we made a thorough anatomical investigation of YFP expressing neurons (**Figure 6**). There is no antibody against ChR2 so its expression needed to be detected indirectly by the fusion protein YFP. The YFP expression pattern was compared to antibody-staining against the other two main neurotransmitter systems in the lumbar spinal cord, employing glycine and GABA, of which we found no overlapping expression. *In situ* hybridization showed that 95 % of the YFP positive neurons expressed *Vglut2* mRNA. Smaller cell populations with known glutamatergic phenotypes, the V2a and Hb9 populations were also investigated, and showed 69 and 73% overlapping expression, respectively. The somewhat lower overlap probably stems from the very high variability in fluorescence of the YFP expression.





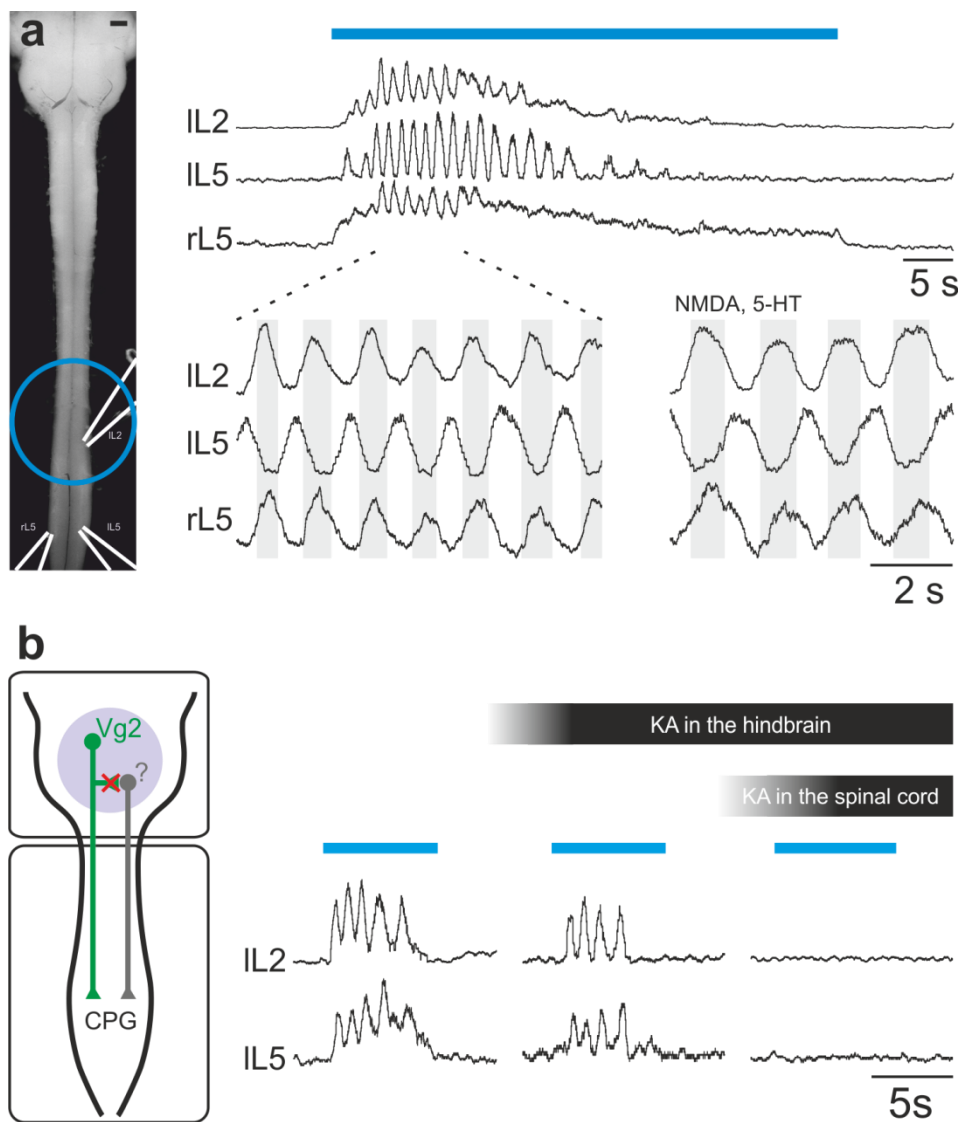
**Figure 6. YFP is exclusively expressed in glutamatergic neurons.** An inspection of the ventral side of the spinal cord of Vglut2-ChR2-YFP mice showed clear somata and fibers (top, right). YFP was expressed only in Vglut2 positive neurons, seen in the *in situ* hybridization (top, left) against Vglut2 mRNA. No expression of YFP was found in glycinergic (bottom, left) or GABAergic (bottom, right) neurons. (Scale bars 100 and 20  $\mu\text{m}$ , in top and bottom parts of the figures, respectively).

The variable ChR2-YFP expression can be attributed to many causes. One would imagine that a cell regulate the expression of their Vglut2 gene depending on the actual glutamate turnover, which in turn depends on how many pre-synaptic terminals it has and the rate of activity within these, i.e. spiking frequency. These variables depend primarily on the axonal but not the dendritic arbor. The size of the dendritic arbor, however, may have a large impact on the size of the cell membrane, and thus, the dilution of ChR2-YFP across it. Altogether we conclude that the mouse demonstrate a specific expression in excitatory neurons only.

#### 4.1.2 ChR2 activation initiates locomotor-like activity

To see that ChR2 was functional, we performed whole-cell recordings from fluorescent cells in slice preparations of neonatal spinal cords. Applying light induced

depolarization in all cells we tested ( $n = 26$ ), however, the amplitude was variable possible because of variable expression levels but also difference in excitability caused by difference in cell size; less current is needed to excite an electrically tight cell. Depolarizations remained after applying blockers to glutamatergic neurotransmission, showing they were not depending on synaptic inputs from other neurons.



**Figure 7. Light stimulation induces locomotor-like activity.** **a.** Tonic light stimulation evokes a patterned rhythmic output, similar to that seen using pharmacological agents. **b.** Light stimulation at the ventral surface of the hindbrain could also induce locomotor-like activity that depended on activation of directly descending glutamatergic neurons, projecting to the spinal cord. This was shown by blocking glutamatergic neurotransmission in the hindbrain only, using kynurenic acid (KA), and demonstrating that the locomotor-inducing effect was retained.

Applying light to the ventral side of the lumbar spinal cord, while recording the lumbar ventral root output, induced well-patterned locomotor-like activity, with a high

frequency compared to locomotor-like activity induced with neuroactive drugs (**Figure 7a**). Flipping the cord over and stimulating the dorsal side with light did not induced any rhythmicity, implicating that we were activating neurons that have direct access to the locomotor machinery, and not unspecifically flooding the whole cord with glutamate. These experiments show that glutamatergic neurons residing in the ventral lumbar spinal cord can quickly convert a tonic input into a well-coordinated rhythmic output.

This is the key property of the CPG concept, the fact that this anatomically distinct part of the spinal cord can produce a cyclical output of bursts to around 100 different muscles (although the preciseness of *all* muscles of the limbs have not been explicitly examined) when activated by a constant, non-rhythmic input.

But what provides this input *in vivo*? A very small, functionally defined area in the midbrain called the mesencephalic locomotor region (MLR) will elicit locomotion when stimulated (Jordan 1998). When increasing stimulation strength, the frequency of locomotion increases and eventually leads to switches in gait, from walking, to trotting, to gallop. The MLR however, does not project directly to the spinal cord. Instead it is connected to a diffuse region of the brainstem called the reticular formation (Noga et al. 2003; Whelan 1996). This area of the brainstem may also initiate locomotion when electrically stimulated, which is a commonly used technique for evoking locomotor-like activity in the rodent *in vitro* preparation. The reticular formation do have neurons that project directly to the lumbar spinal cord (Reed et al. 2009), and is thus perfectly suited to be a relay center for command signals emanating from the MLR and higher brain centers.

#### **4.1.3 Glutamatergic neurons in the hindbrain can evoke locomotor-like activity**

The reticular formation is made up of GABAergic, glycinergic and glutamatergic neurons, but also harbor serotonergic cells that send axons into the spinal cord. Because of the prominent effect 5HT has in initiating locomotion, it has been argued that these have a causal role in activating the CPG (Liu and Jordan 2005). Using the *Vglut2-ChR2-YFP* mouse we were in a perfect position to test this. Illumination of the ventral hindbrain, over the hotspot mostly used for electrical stimulation, led to bursting in the lumbar ventral roots, with similar patterning as that seen during drug-induced locomotor-like activity. However, this experiment could not conclusively demonstrate that the glutamatergic neurons of the hindbrain were the sole source of initiation. Possibly, glutamatergic neurons could be activating another descending system, such as the serotonergic cells. We therefore set up a barrier on the border between the spinal cord and the hindbrain, and perfused the spinal cord with normal artificial cerebrospinal

fluid, while adding a glutamatergic blocker to the hindbrain partition (**Figure 7b**). This would abolish any local glutamatergic activity in the hindbrain. However, the effect of light stimulation onto the ventral hindbrain remained, and thus showed that we were hitting neurons that were directly leaving the hindbrain and projecting into the spinal cord.

It could be postulated that we only activate the axons of these descending neurons when we elicit locomotor like activity by stimulating the lumbar cord. This is unlikely mainly because of two reasons. First, if the axons are responsible for eliciting locomotion in the hindbrain stimulation, a stimulation of the spinal cord at a thoracic region in between the lumbar cord and the hindbrain should elicit a similar response. Stimulating these areas may elicit a few bouts of locomotor-like bursting, but will mostly trigger only tonic activity. Second, the quality and duration of the locomotor activity is so much higher when stimulating the lumbar cord that, even if descending presynaptic terminals are activated, the main effect should be attributed to local spinal neurons.

The result from this study was the first direct demonstration that glutamatergic neurons can activate rhythmicity in the mammalian spinal cord. Since rhythm generation in tadpole (McLean et al. 2008) and lamprey (Grillner 2003) locomotor CPGs have also been stipulated to rely on a glutamatergic neurons on similar grounds as in the rodent, we suggest that this kind of rhythmic core may form a common plan underlying all vertebrate CPGs.

#### **4.2 PAPER II: OPTOGENETIC DISSECTION REVEALS MULTIPLE RHYTHMOGENIC MODULES UNDERLYING LOCOMOTION**

In this investigation, we looked at the contribution of smaller cell populations compared to those in *Paper I*, which made it necessary to switch transgenic strategy. To increase expression level, and manipulate specific cell populations we used a set of newly generated transgenic mice, with a Cre-inducible, high expression of *ChR2-YFP* (*RC-ChR2*) (Madisen et al. 2012). In these mice, the amount of gene expression was governed by a combination of very strong enhancer elements: the CAG promoter, which is a fusion of two powerful promoter elements from the cytomegalovirus and from chicken, plus the woodchuck hepatitis virus post-transcriptional regulatory element. This construct was all incorporated into the ROSA26 locus which also promotes high level of expression. Furthermore, we made use of the functional opposite of ChR2, a mouse expressing eNpHR (*RC-eNpHR*), in which neurons are inhibited in response to yellow light (see **Figure 4**). This functional dichotomy proved to be very rewarding, since it introduced the possibility to probe areas or circuits by both proving the sufficiency of the activity of a cell population for inducing a certain

behavior, and the necessity of this same cell population during ongoing behavior, using ChR2 and eNpHR respectively. To limit expression to our populations of interest, we crossed the optogenetic mice with the well characterized *Vglut2::Cre* line generated in our lab (Borgius et al. 2010), and with a mouse line with Cre expressed in inhibitory neurons only, the vesicular inhibitory amino acid transporter (*VIAAT::Cre*). Since the *VIAAT::Cre* mouse is an as yet unpublished mouse, it was necessary to certify that the expression was truly limited to inhibitory neurons. This was achieved by comparing the Cre-induced reporter gene expression of *ROSA26::YFP* mice with *VIAAT* mRNA expression, and *Vglut2* mRNA expression. The analysis showed a lack of Cre-expression in *Vglut2* positive neurons, and a high (93,7%) overlap with *VIAAT*-mRNA, validating the use of *VIAAT::Cre* mice to target inhibitory neurons.

#### **4.2.1 Glutamatergic neurons are both necessary and sufficient for locomotor-like activity**

In the *Vglut2::Cre; RC-ChR2* cross, light exposure induced a very rapid onset of locomotion, with high temporal acuity, demonstrated by cross correlation and coherence analysis. The frequency of the rhythm was higher, and the duration of locomotor bouts were greatly increased, compared to that seen in *Vglut2-ChR-YFP* mouse.

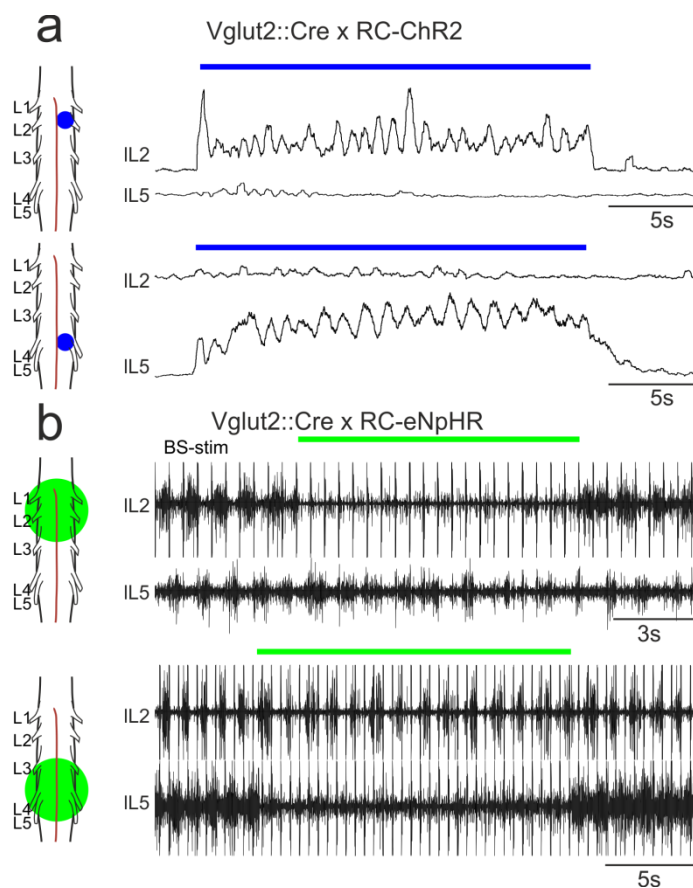
The patterning of the activity recorded from the ventral roots was instantiated directly, further strengthening the hypothesis that light application activates neurons that are critical for directly controlling locomotion. Compared to all other protocols we use to evoke locomotion, in our hands none has as fast onset and offset, and as fast locomotor frequency as that seen in the *Vglut2::Cre; RC-ChR2* cross. The *Vglut2::Cre; RC-eNpHR* was very effective in terminating any ongoing locomotor activity, induced by pharmacological, dorsal root, or brainstem stimulation. Stimulation of the spinal cord in *VIAAT::Cre; RC-ChR2* could not induce any bursting behavior at all, but could potentially turn off locomotor activity induced by other means.

Together these experiments show that direct activation of glutamatergic neurons is sufficient to evoke locomotion and that *Vglut2* positive cells are necessary for locomotion. Inhibitory neurons, on the other hand do not have the capacity to recruit the locomotor machinery, but inhibit any ongoing activity.

#### 4.2.2 Focal activation reveals independent single muscle rhythmicity

The high expression of ChR2 and eNpHR also permitted more focal stimulation. Stimulating with high light intensity could induce global locomotor-like activity, regardless of where in the lumbar cord the light spot was situated.

However, reduced light intensity could evoke locomotor-like rhythmic bursting in a single root, without any activity in other roots. This was true both of the flexor dominated activity recorded from the L2 root, as well as the extensor output from L5 (**Figure 8a**, top and bottom, respectively). One side of the cord could also be stimulated so that it produced locomotor-like activity without the other side of the cord being active. Using *Vglut2::Cre; RC-eNpHR* we saw that during brainstem evoked locomotion, focal stimulation could either turn off flexors or extensors independently, or the left or right side activity (**Figure 8b**, top and bottom, respectively). Recording from nerves projecting to specific muscles furthermore concluded that closely related synergistic flexor muscles could also produce rhythmic bursting in isolation, independent from each other.



**Figure 8.** Focal light stimulation could evoke rhythmic bursting in flexors or extensor (**a**, top and bottom respectively) independently. Similarly, inactivation of either flexors or extensor was also possible using eNpHR during brainstem induced locomotion (**b**).

These surprising findings have a lot to say about the overall organization of the hind limb CPG, and the whole motor system in general. The half-center model, described in the introduction, states that rhythm generation in one limb is inextricably linked to the flexor-extensor patterning (McCrea and Rybak 2008). Similar models, such as the asymmetrical flexor-burst generator, relies on a single burst generator that, during a burst in the flexor-related network, inhibits the extensor-related cells (Zhong et al. 2012). Both of these models could be directly falsified by our experiments. It therefore seems that the smallest common motif in the CPG is an intrinsically rhythmogenic structure, the unit burst generator, targeting a single muscle.

### **4.2.3 The unit burst generator**

*In vivo*, the unit burst generator will not typically be rhythmic in isolation: they are connected in a way as to make them produce a common rhythm, and they need to be regulated with regards to amplitude and burst duration. How this is achieved is only speculation, but following these and others results about the rostral cord being more rhythmogenic (Grillner and Zangger 1979; Ho and O'Donovan 1993; Kjaerulff and Kiehn 1996), a possible explanation is that the rostral cord always lead the more caudal areas in a hierarchical fashion. Proximal muscles govern the whole limb and have a bigger impact on the distal body part, than vice versa, which could prompt for a hierarchical organization. This hierarchical structure could be organized according to the anatomical rostro-caudal plan of the spinal cord, or follow the proximal to distal extent of the limb; both layouts are similarly structured (McHanwell and Biscoe 1981).

Thus, the hypothetical network is hierarchical, but not in the same senses as most CPG models, in which the hierarchy is formed between the different CPG elements (rhythm generation, pattern formation). Rather, it is formed by a network of UBGs, in which proximal UBGs are higher order than distal ones, and synergistic muscles are highly bound to each other, and antagonistic muscles are connected via inhibitory projections. The initial argument for the UBG model comes from observations in cat, showing that the locomotor activity is complex, even in the absence of sensory input and that this activity could not be accounted for in a half-center model (Grillner 1981; Grillner and Zangger 1975). There has, however, not been any direct demonstration of this concept, although there have been hints in the published literature, using pharmacological or lesion studies to isolate parts of the spinal cord (Grillner and Zangger 1979; Kjaerulff and Kiehn 1996). Optogenetics is an excellent tool for dissecting these networks, and the results decisively conclude the capacity for a single motor neuron pool, to be driven by independent rhythmically active networks.

Expanded upon, the modular UBG model gives flexibility for using the limbs in other circumstances than normal forward locomotion, such as scratching or backwards

locomotion (Grillner and Wallen 1985; Grillner et al. 2008). The modularity of the spinal cord configuration has been shown for other motor behaviors as well, such as the nociceptive withdrawal reflex that govern the retraction of a limb from a nociceptive stimuli (Schouenborg 2008). Moreover, the notion of a small intrinsically rhythmic module also fits well with a scenario where motor control of the extremities evolved from a likewise modular network in the fish (Bem et al. 2003; Ijspeert et al. 2007).

### **4.3 PAPER III: IDENTIFICATION OF MINIMAL NEURONAL NETWORKS INVOLVED IN FLEXOR-EXTENSOR ALTERNATION IN THE MAMMALIAN SPINAL CORD.**

The results of *Paper I* and *II*, clearly demonstrates the importance of glutamatergic neurons to locomotor behavior, validating hypotheses built upon a large source of pharmacological experiments. In 2006, Wallén-Mackenzie et al, described an animal in which Vglut2 had been deleted, supposedly rendering all glutamatergic neurons non-functional. These animals were reported to display seemingly normal locomotor-like activity in response to pharmacological agents (Wallen-Mackenzie et al. 2006). The strong clash of this surprising finding, with the general thoughts about the importance of glutamatergic neurons led us to investigate whether there could be another explanation to the locomotion-like pattern seen in these knock out mice.

#### **4.3.1 Glutamatergic neurotransmission is much reduced in the Vglut2-KO mouse**

We used a mouse model, in which exon 2 of the Vglut2 gene was flanked by loxP sites, truncating the gene product after recombination by Cre, making it a Vglut2 knock out mouse (*Vglut2-KO*). First we thoroughly evaluated that Vglut2 transmission was blocked, using whole cell recordings from interneurons and motor neurons. These recordings showed much reduced spontaneous glutamatergic activity, and that evoked synaptic glutamate release was blocked both from motor neurons and interneurons. Crossing the *Vglut2-KO* with the *Vglut-ChR2* mouse generated in *Paper I*, showed no light induced activity, in contrast to controls, and western blots showed no compensatory upregulation of other vesicular transporters. These experiments confirmed the absence of glutamatergic neurotransmission in these mice, and lack of compensation for it.

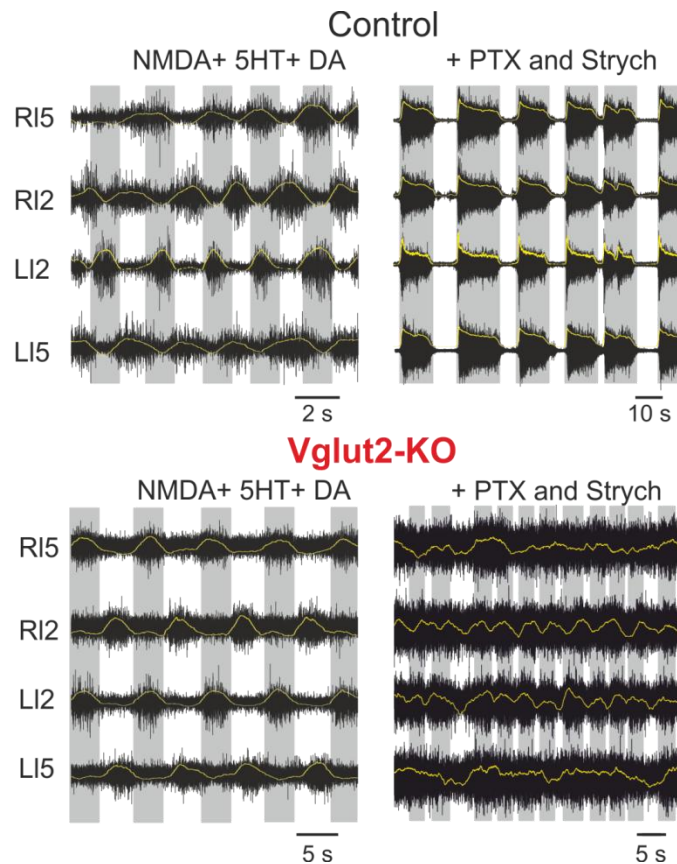
High drug concentrations, and an obligatory addition of NMDA, induced locomotor-like activity with alternating left-right and flexor-extensor activity in the Vglut2-KO. However, the observed rhythm was non-flexible and did not change much in frequency when subjected to different drug concentrations, in contrast to controls. These experiments showed that in the absence of glutamate release from Vglut2-positive neurons a locomotor-like activity could be induced although with a higher thresholds,



similar to that reported in the initial description of the *Vglut2*-KO mice (Wallen-Mackenzie et al. 2006).

### 4.3.2 Locomotor-like activity depends on an exclusively inhibitory network

A more thorough investigation showed that the hypothesis about the importance of glutamatergic neurons was correct, and that the activity in the knock-out depend on a reduced version of the network. The key observation was when inhibitory blockers were added to the locomotion-inducing cocktail of NMDA, 5HT and dopamine. In the control animals, blocking inhibition during ongoing locomotor-like activity led to a synchronous rhythmic activity, in which flexors and extensors were bilaterally phase-locked. In the *Vglut2*-KO, blocking inhibition instead eliminated any coupling, and each root wandered off on its own (**Figure 9**). This observation verified that the activity was only similar in output, but depended on a network, governed entirely by inhibitory neurons. The network that still remains when the synaptic output is removed from the glutamatergic neurons, is organized so that it produces an alternating flexor-extensor output. The most direct interpretation would be that it depends on reciprocal ipsilateral inhibitory neurons that also project to motor neurons. The Ia interneurons show such connectivity, and were thus a likely candidate.



**Figure 9.** PTX and strychnine makes the bursting of all roots synchronous in control animals. In the *Vglut2*-KO, the bursting gets de-coupled, demonstrating that a different network is responsible for the activity.

### 4.3.3 Reciprocal Ia interneuron connectivity is present in the *Vglut2*-KO

Ia interneurons are defined by their activation of low threshold Ia afferents, and they project to antagonist muscles (Wang et al. 2008). In the cat, they have also been reported to connect to antagonist Ia interneurons (Hultborn et al. 1976). Furthermore, they are contacted by Renshaw cells, which are inhibitory neurons that are reciprocally connected to motor neurons (Hultborn et al. 1971a; b). Exploiting the detailed knowledge of this connectivity, we had the possibility to test whether Ia interneurons were responsible for the locomotor-like activity seen in the *Vglut2*-KO.

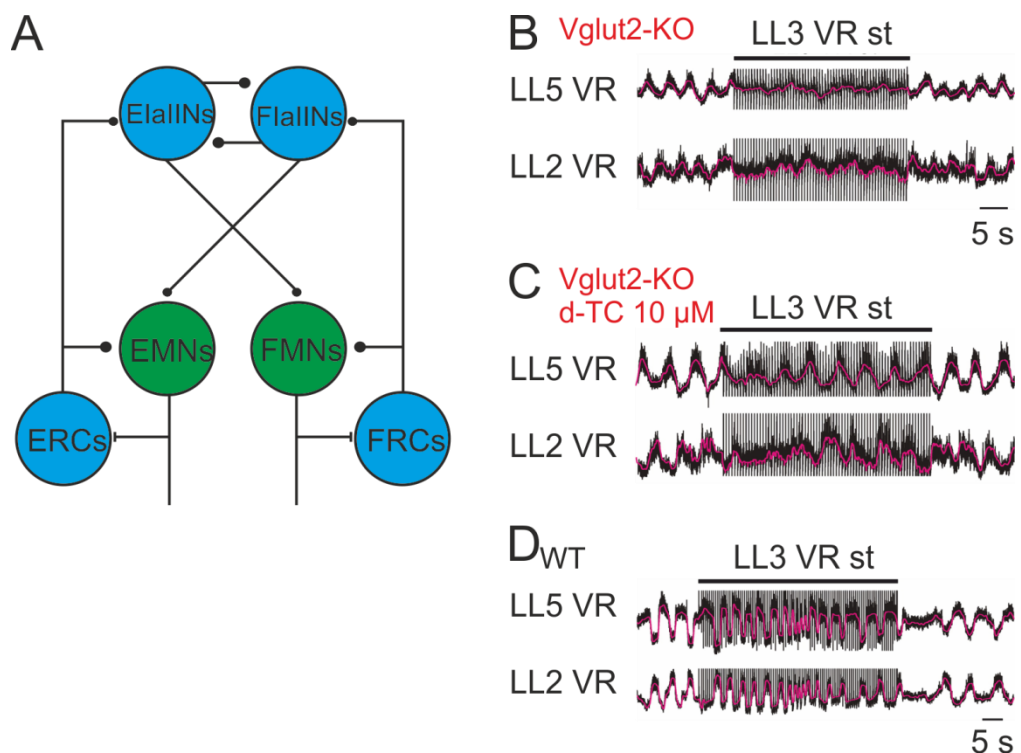
Stimulating the L5 dorsal root evoked monosynaptic reflexes in the L5 ventral root, but very little response in the L3 ventral root. Conditioning L3 dorsal root 10 ms before the L5 dorsal root stimulation reduced the L5 monosynaptic reflex amplitude. This is indicative of the effect Ia interneurons have on antagonistic motor neurons. Renshaw cells can be excited by antidromic stimulation of the ventral root. Furthermore, since the Renshaw cells inhibit Ia interneurons residing in the same segment, preceding the L3 dorsal root conditioning in the previous experiment, with L3 ventral root stimulation, disinhibited the monosynaptic reflex. This concluded that the Renshaw cell projection to Ia interneurons was intact.

Finally, to demonstrate that Ia interneurons were reciprocally connected, we made intracellular recordings from L5 motor neurons. They showed that the disynaptic inhibitory reflex evoked by L3 dorsal root stimulation, could be attenuated by disinhibition through L5 dorsal root stimulation. This concluded for the first time, that Ia interneurons in the mouse spinal cord shares the reciprocal connectivity that has been reported in cats (Hultborn et al. 1976).

### 4.3.4 Ia interneurons produce rhythm and pattern generation

When this connectivity had been established (**Figure 10a**), we could use it to test if the activity of the reciprocal Ia interneurons formed the basis of the flexor-extensor coordination seen in the *Vglut2*-KO. Since the Renshaw cells directly inhibit Ia interneurons, their activity should depress ongoing locomotor-like activity. This was indeed the case; a sustained stimulation blocked rhythmic bursting (**Figure 10b**), and this effect was dependent on Renshaw cell activity, as it could be blocked by nicotinic antagonists (**Figure 10c**). In the control animal, this stimulation instead increased the frequency of ongoing locomotor activity (**Figure 10d**), by an unknown mechanism. The total opposite response between the *Vglut2*-KO and the control animal, emphasizes the differences of rhythm generation in the two cases. These experiments concluded that the reciprocal Ia interneurons form the basis of rhythm and pattern generation, in the *Vglut2*-KO mouse.

It is possible that neurons in the *Vglut2*-KO are wired differently compared to a wild-type animal, because of activity dependent mechanisms, that have been shown to be important for proper development (Hanson and Landmesser 2004). To control for this, we investigated the effect of deleting *Vglut2* after the most crucial developmental time frame. This was done by directing the expression of Cre to a later time point with the help of the tamoxifen/estrogen receptor system. One dose of tamoxifen delivered to the pregnant female when the pups were at E16,5 was enough to reduce *Vglut2* expression at E18,5 by 80-90% and recover the same locomotor phenotype as the full knock-out. This demonstrated that the phenotype did not depend on early developmental re-specification of the inhibitory network, because of a lack of excitatory neurotransmission.



**Figure 10. Activation of ventral roots suppresses drug-induced locomotor-like activity in *Vglut2*-KO mice.** (a) Schematic of flexor- and extensor-related RCs and rla-IN connections. (b–d) Ventral-root recordings showing drug-induced rhythmicity in *Vglut2*-KO. Prolonged trains of stimuli applied to the ventral root totally abolish the rhythm in the *Vglut2*-KO (b). This effect is blocked by nicotinic antagonists (c). In control mice (d) stimulation increases the frequency of locomotor-like activity.

The results from this paper show that ipsilateral inhibitory interneurons have the capacity to induce a rhythmic oscillation with a flexor-extensor and left-right alternation. Since electric stimulation could not induce locomotor-like activity, the conclusion remains that rhythm generation is severely disrupted in the *Vglut2*-KO, as stipulated in the earlier hypothesis about the role of glutamatergic neurons in rhythm generation. The early drawn conclusions regarding *Vglut2*-neurons not being important

for locomotion, is thus debunked. When the underlying network structure was understood, it could be perturbed in a way that unambiguously separated it from control animals. If the animal would survive to adulthood (they die at birth from respiratory failure), I would speculate that it would be totally paralyzed, or else have severely defective motor behavior.

The half-center organization that was so decidedly disproven in *Paper II* is thus shown to exist. But there is no paradox, the original half-center model was responsible for generating rhythmic activity as well as coordinating flexor-extensor alternation. The network structure emanating from the results of *Paper II* would also probably implement ipsilateral inhibitory pathways to coordinate flexor-extensor activity during locomotion. But the same flexor-extensor muscles that are separated by reciprocal ipsilateral inhibitory interneurons, could be synchronously active during another rhythmic motor task. Therein lays the attractiveness of the UBG model, as a smallest common motif in the spinal motor system.

#### **4.4 OVERALL DISCUSSION**

Neurotransmitter phenotype is but one of the many parameters influencing the properties and function of neurons. Location, morphology, projection pattern and ion channel expression are all major determinants of cell function. Still, the simple dichotomy between inhibitory versus excitatory neurons implemented in this thesis have been fruitful, both because of its simplicity and of its physiological relevance. Although the main conclusions in this thesis conform to the generalization that excitatory neurons generate rhythm, and inhibitory neurons generate pattern, the division is a great oversimplification. It would be difficult to envision pure separation of these functions based on neurotransmitter phenotype, since their activity is coupled and complex.

Blocking glutamatergic neurotransmission blocks *all* output from the spinal cord, making it impossible to verify that the patterning is still there. Blocking inhibitory neurotransmission disrupts the pattern, but the remaining activity is not just rhythm generation without pattern. In fact it is very unlike locomotion. The frequency, amplitude, burst duration, interburst duration variance and burst shape is very different (see **Figure 9**), suggesting that the activity seen is more epileptiform than locomotor-like. The lack of decisive conclusions from these experiments stem more from the limitations of the pharmacological techniques, than from poorly stated hypotheses.

Another caveat with pharmacological studies is the causal role attributed to the factor applied. As an example, serotonin has been seen as an initiator of locomotion because of the strong initiating and stabilizing effect it has on the network (Liu and Jordan

2005). Recently, a whole range of other chemicals, collectively known as trace amines have been shown to all activate the locomotor machinery (Hochman, unpublished data). However, the fact that a factor may induce activity does not necessarily mean that all these different compounds do this *in vivo*. More likely, the easily evoked pattern *in vitro*, reflects that the network behaves as a limit cycle attractor, and that any exciting stimulation may push the network into the cyclical attractor domain, but once there, it is stable and difficult to perturb from its trajectory, explaining why so many different kinds of stimulations, especially pharmacological, can evoke the activity. Also a reduced spinal cord will, given time, enter a stable domain of activity when subjected to proper drug concentrations (Kjaerulff and Kiehn 1996). For instance, by increasing extracellular potassium, the general excitability of neurons in the cord is increased. This increase in excitability leads to a great increase in spontaneous locomotor bouts. Obviously this does not lead us to think of potassium as a factor that the nervous system uses to initiate locomotion.

The optogenetic techniques developed and implemented in this thesis have improved greatly on the spatiotemporal accuracy of stimulating neurons, compared to the pharmacological approach. Activation of optogenetic proteins recruit the neuronal machinery in a relatively natural way, by changing the intracellular ion concentrations on a very fast time scale leading to depolarization or hyperpolarization. Adding NMDA and 5HT to the perfusion chamber of a spinal cord preparation will directly act on every neuron that has receptors for these compounds, which is likely every single neuron in the spinal cord. Furthermore, the slow recruitment of neurons by drugs does not mirror their fast activation seen *in vivo*. As evident in *Paper III*, even when all glutamatergic neurons in the spinal cord are taken out of the picture, pharmacological activation of the motor neurons and inhibitory interneurons is enough to start global oscillations mimicking locomotor output, highlighting how difficult interpretations of pharmacological experiments can be.

The combination of molecular developmental biology and electrophysiology has, over the last two decades increased the understanding of spinal microcircuits to a great extent. The connectivity of some spinal microcircuits have been elucidated to a high degree, whereas the complexity of the ipsilateral pattern forming circuits still leaves large gaps in our understanding. To truly understand the system will require a substantial effort. However, the speed with which new techniques and tools are being developed, together with a merging of perspectives from different sub-fields within neuroscience, show great promise in the endeavor to understand how the nervous system generates complex motor behavior.

## 5 BIBLIOGRAPHY

**Aihara Y, Mashima H, Onda H, Hisano S, Kasuya H, Hori T, Yamada S, Tomura H, Yamada Y, Inoue I, Kojima I, and Takeda J.** Molecular cloning of a novel brain-type Na(+)-dependent inorganic phosphate cotransporter. *Journal of neurochemistry* 74: 2622-2625, 2000.

**Bem T, Cabelguen JM, Ekeberg O, and Grillner S.** From swimming to walking: a single basic network for two different behaviors. *Biological cybernetics* 88: 79-90, 2003.

**Borgius L, Restrepo CE, Leao RN, Saleh N, and Kiehn O.** A transgenic mouse line for molecular genetic analysis of excitatory glutamatergic neurons. *Mol Cell Neurosci* 45: 245-257, 2010.

**Boyden ES, Zhang F, Bamberg E, Nagel G, and Deisseroth K.** Millisecond-timescale, genetically targeted optical control of neural activity. *Nature neuroscience* 8: 1263-1268, 2005.

**Brown TG.** The intrinsic factors in the act of progression in mammals *Proc. R. Soc. B*, 84 (1911), pp. 308–319

**Brown TG.** On the nature of the fundamental activity of the nervous centres; together with an analysis of the conditioning of rhythmic activity in progression, and a theory of the evolution of function in the nervous system. *The Journal of physiology* 48: 18-46, 1914.

**Burke RE, Degtyarenko AM, and Simon ES.** Patterns of locomotor drive to motoneurons and last-order interneurons: clues to the structure of the CPG. *Journal of neurophysiology* 86: 447-462, 2001.

**Butt SJ, Harris-Warrick RM, and Kiehn O.** Firing properties of identified interneuron populations in the mammalian hindlimb central pattern generator. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22: 9961-9971, 2002.

**Butt SJ, and Kiehn O.** Functional identification of interneurons responsible for left-right coordination of hindlimbs in mammals. *Neuron* 38: 953-963, 2003.

**Cangiano L, and Grillner S.** Mechanisms of rhythm generation in a spinal locomotor network deprived of crossed connections: the lamprey hemicord. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25: 923-935, 2005.

**Cardin JA, Carlen M, Meletis K, Knoblich U, Zhang F, Deisseroth K, Tsai LH, and Moore CI.** Targeted optogenetic stimulation and recording of neurons in vivo using cell-type-specific expression of Channelrhodopsin-2. *Nature protocols* 5: 247-254, 2010.

**Cazalets JR.** Metachronal propagation of motoneurone burst activation in isolated spinal cord of newborn rat. *The Journal of physiology* 568: 583-597, 2005.

**Cazalets JR, Borde M, and Clarac F.** Localization and organization of the central pattern generator for hindlimb locomotion in newborn rat. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15: 4943-4951, 1995.

**Clarac F.** Some historical reflections on the neural control of locomotion. *Brain research reviews* 57: 13-21, 2008.

**Cohen AH, and Wallen P.** The neuronal correlate of locomotion in fish. "Fictive swimming" induced in an in vitro preparation of the lamprey spinal cord. *Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale* 41: 11-18, 1980.

**Cowley KC, and Schmidt BJ.** Effects of inhibitory amino acid antagonists on reciprocal inhibitory interactions during rhythmic motor activity in the in vitro neonatal rat spinal cord. *Journal of neurophysiology* 74: 1109-1117, 1995.

**Crone SA, Quinlan KA, Zagoraïou L, Droho S, Restrepo CE, Lundfald L, Endo T, Setlak J, Jessell TM, Kiehn O, and Sharma K.** Genetic ablation of V2a ipsilateral interneurons disrupts left-right locomotor coordination in mammalian spinal cord. *Neuron* 60: 70-83, 2008.

**Crone SA, Zhong G, Harris-Warrick R, and Sharma K.** In mice lacking V2a interneurons, gait depends on speed of locomotion. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29: 7098-7109, 2009.

**Delcomyn F.** Neural basis of rhythmic behavior in animals. *Science* 210: 492-498, 1980.

**Deliagina TG, Orlovsky GN, and Pavlova GA.** The capacity for generation of rhythmic oscillations is distributed in the lumbosacral spinal cord of the cat. *Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale* 53: 81-90, 1983.

**Delivet-Mongrain H, Leblond H, and Rossignol S.** Effects of localized intraspinal injections of a noradrenergic blocker on locomotion of high decerebrate cats. *Journal of neurophysiology* 100: 907-921, 2008.

**Eccles J.,** *The Physiology of Nerve Cells*, 1957, The Johns Hopkins Press, Baltimore MD

**Eide AL, Glover J, Kjaerulff O, and Kiehn O.** Characterization of commissural interneurons in the lumbar region of the neonatal rat spinal cord. *J Comp Neurol* 403: 332-345, 1999.

**el Manira A, Tegner J, and Grillner S.** Calcium-dependent potassium channels play a critical role for burst termination in the locomotor network in lamprey. *Journal of neurophysiology* 72: 1852-1861, 1994.

**Gordon JW, and Ruddle FH.** Integration and stable germ line transmission of genes injected into mouse pronuclei. *Science* 214: 1244-1246, 1981.

**Gosgnach S, Lanuza GM, Butt SJ, Saueressig H, Zhang Y, Velasquez T, Riethmacher D, Callaway EM, Kiehn O, and Goulding M.** V1 spinal neurons regulate the speed of vertebrate locomotor outputs. *Nature* 440: 215-219, 2006.

**Goulding M.** Circuits controlling vertebrate locomotion: moving in a new direction. *Nature reviews Neuroscience* 10: 507-518, 2009.

**Grillner S.** Control of locomotion in bipeds, tetrapods, and fish. *Handbook of Physiology—The Nervous System II* American Physiological Society, Bethesda, MD: 1179–1236, 1981.

**Grillner S.** The motor infrastructure: from ion channels to neuronal networks. *Nature reviews Neuroscience* 4: 573-586, 2003.

**Grillner S, Perret C, and Zangger P.** Central generation of locomotion in the spinal dogfish. *Brain research* 109: 255-269, 1976.

**Grillner S, and Wallen P.** Central pattern generators for locomotion, with special reference to vertebrates. *Annu Rev Neurosci* 8: 233-261, 1985.

**Grillner S, Wallen P, Saitoh K, Kozlov A, and Robertson B.** Neural bases of goal-directed locomotion in vertebrates--an overview. *Brain research reviews* 57: 2-12, 2008.

**Grillner S, and Zangger P.** How detailed is the central pattern generation for locomotion? *Brain research* 88: 367-371, 1975.

**Grillner S, and Zangger P.** On the central generation of locomotion in the low spinal cat. *Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale* 34: 241-261, 1979.

**Hanson MG, and Landmesser LT.** Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules. *Neuron* 43: 687-701, 2004.

**Ho S, and O'Donovan MJ.** Regionalization and intersegmental coordination of rhythm-generating networks in the spinal cord of the chick embryo. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 13: 1354-1371, 1993.

**Hultborn H, Illert M, and Santini M.** Convergence on interneurons mediating the reciprocal Ia inhibition of motoneurons. I. Disynaptic Ia inhibition of Ia inhibitory interneurons. *Acta Physiol Scand* 96: 193-201, 1976.

**Hultborn H, Jankowska E, and Lindstrom S.** Recurrent inhibition from motor axon collaterals of transmission in the Ia inhibitory pathway to motoneurons. *The Journal of physiology* 215: 591-612, 1971a.

**Hultborn H, Jankowska E, and Lindstrom S.** Recurrent inhibition of interneurons monosynaptically activated from group Ia afferents. *The Journal of physiology* 215: 613-636, 1971b.

**Hultborn H, Jankowska E, and Lindstrom S.** Relative contribution from different nerves to recurrent depression of Ia IPSPs in motoneurons. *The Journal of physiology* 215: 637-664, 1971c.

**Ijspeert AJ, Crespi A, Ryczko D, and Cabelguyen JM.** From swimming to walking with a salamander robot driven by a spinal cord model. *Science* 315: 1416-1420, 2007.

**Jankowska E, Jukes MG, Lund S, and Lundberg A.** The effect of DOPA on the spinal cord. 5. Reciprocal organization of pathways transmitting excitatory action to alpha motoneurons of flexors and extensors. *Acta Physiol Scand* 70: 369-388, 1967a.

**Jankowska E, Jukes MG, Lund S, and Lundberg A.** The effect of DOPA on the spinal cord. 6. Half-centre organization of interneurons transmitting effects from the flexor reflex afferents. *Acta Physiol Scand* 70: 389-402, 1967b.

**Jessell TM.** Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* 1: 20-29, 2000.

**Jordan LM.** Initiation of locomotion in mammals. *Annals of the New York Academy of Sciences* 860: 83-93, 1998.

**Kiehn O.** Development and functional organization of spinal locomotor circuits. *Curr Opin Neurobiol* 21: 100-109, 2011.

**Kiehn O.** Locomotor circuits in the mammalian spinal cord. *Annu Rev Neurosci* 29: 279-306, 2006.

**Kiehn O, and Kjaerulff O.** Distribution of central pattern generators for rhythmic motor outputs in the spinal cord of limbed vertebrates. *Annals of the New York Academy of Sciences* 860: 110-129, 1998.

**Kiehn O, and Kjaerulff O.** Spatiotemporal characteristics of 5-HT and dopamine-induced rhythmic hindlimb activity in the in vitro neonatal rat. *Journal of neurophysiology* 75: 1472-1482, 1996.

**Kiehn O, Quinlan KA, Restrepo CE, Lundfald L, Borgius L, Talpalar AE, and Endo T.** Excitatory components of the mammalian locomotor CPG. *Brain research reviews* 57: 56-63, 2008.

**Kjaerulff O, and Kiehn O.** Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord in vitro: a lesion study. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 16: 5777-5794, 1996.

**Koller BH, Hagemann LJ, Doetschman T, Hagaman JR, Huang S, Williams PJ, First NL, Maeda N, and Smithies O.** Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 86: 8927-8931, 1989.

**Kriellaars DJ, Brownstone RM, Noga BR, and Jordan LM.** Mechanical entrainment of fictive locomotion in the decerebrate cat. *Journal of neurophysiology* 71: 2074-2086, 1994.



**Kudo N, and Yamada T.** N-methyl-D,L-aspartate-induced locomotor activity in a spinal cord-hindlimb muscles preparation of the newborn rat studied in vitro. *Neurosci Lett* 75: 43-48, 1987.

**Kullander K, Butt SJ, Lebret JM, Lundfald L, Restrepo CE, Rydstrom A, Klein R, and Kiehn O.** Role of EphA4 and EphrinB3 in local neuronal circuits that control walking. *Science* 299: 1889-1892, 2003.

**Lafreniere-Roula M, and McCrea DA.** Deletions of rhythmic motoneuron activity during fictive locomotion and scratch provide clues to the organization of the mammalian central pattern generator. *Journal of neurophysiology* 94: 1120-1132, 2005.

**Lanuza GM, Gosgnach S, Pierani A, Jessell TM, and Goulding M.** Genetic identification of spinal interneurons that coordinate left-right locomotor activity necessary for walking movements. *Neuron* 42: 375-386, 2004.

**Li X, Gutierrez DV, Hanson MG, Han J, Mark MD, Chiel H, Hegemann P, Landmesser LT, and Herlitze S.** Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin. *Proceedings of the National Academy of Sciences of the United States of America* 102: 17816-17821, 2005.

**Lin JY.** Optogenetic excitation of neurons with channelrhodopsins: light instrumentation, expression systems, and channelrhodopsin variants. *Progress in brain research* 196: 29-47, 2012.

**Lin JY.** A user's guide to channelrhodopsin variants: features, limitations and future developments. *Experimental physiology* 96: 19-25, 2011.

**Liu J, and Jordan LM.** Stimulation of the parapyramidal region of the neonatal rat brain stem produces locomotor-like activity involving spinal 5-HT<sub>7</sub> and 5-HT<sub>2A</sub> receptors. *Journal of neurophysiology* 94: 1392-1404, 2005.

**Long JA, and Gordon MS.** The greatest step in vertebrate history: a paleobiological review of the fish-tetrapod transition. *Physiological and biochemical zoology : PBZ* 77: 700-719, 2004.

**Madisen L, Mao T, Koch H, Zhuo JM, Berenyi A, Fujisawa S, Hsu YW, Garcia AJ, 3rd, Gu X, Zanella S, Kidney J, Gu H, Mao Y, Hooks BM, Boyden ES, Buzsaki G, Ramirez JM, Jones AR, Svoboda K, Han X, Turner EE, and Zeng H.** A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. *Nature neuroscience* 15: 793-802, 2012.

**McCrea DA, and Rybak IA.** Organization of mammalian locomotor rhythm and pattern generation. *Brain research reviews* 57: 134-146, 2008.

**McHanwell S, and Biscoe TJ.** The localization of motoneurons supplying the hindlimb muscles of the mouse. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 293: 477-508, 1981.

**McLean DL, Masino MA, Koh IY, Lindquist WB, and Fetcho JR.** Continuous shifts in the active set of spinal interneurons during changes in locomotor speed. *Nature neuroscience* 11: 1419-1429, 2008.

**Miller S, and Scott PD.** The spinal locomotor generator. *Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale* 30: 387-403, 1977.

**Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, and Gottschalk A.** Light activation of channelrhodopsin-2 in excitable cells of *Caenorhabditis elegans* triggers rapid behavioral responses. *Current biology : CB* 15: 2279-2284, 2005.

**Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, and Bamberg E.** Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proceedings of the National Academy of Sciences of the United States of America* 100: 13940-13945, 2003.

**Nagy A.** Cre recombinase: the universal reagent for genome tailoring. *Genesis* 26: 99-109, 2000.

**Noga BR, Kriellaars DJ, Brownstone RM, and Jordan LM.** Mechanism for activation of locomotor centers in the spinal cord by stimulation of the mesencephalic locomotor region. *Journal of neurophysiology* 90: 1464-1478, 2003.

**Parkitna JR, Engblom D, and Schutz G.** Generation of Cre recombinase-expressing transgenic mice using bacterial artificial chromosomes. *Methods in molecular biology* 530: 325-342, 2009.

**Pratt CA, and Jordan LM.** Ia inhibitory interneurons and Renshaw cells as contributors to the spinal mechanisms of fictive locomotion. *Journal of neurophysiology* 57: 56-71, 1987.

**Quinlan KA, and Kiehn O.** Segmental, synaptic actions of commissural interneurons in the mouse spinal cord. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 27: 6521-6530, 2007.

**Reed WR, Shum-Siu A, Whelan A, Onifer SM, and Magnuson DS.** Anterograde labeling of ventrolateral funiculus pathways with spinal enlargement connections in the adult rat spinal cord. *Brain research* 1302: 76-84, 2009.

**Renshaw B.** Central effects of centripetal impulses in axons of spinal ventral roots. *Journal of neurophysiology* 9: 191-204, 1946.

**Renshaw B.** Influence of discharge of motoneurons upon excitation of neighboring motoneurons *Journal of neurophysiology* 4: 167-183, 1941.

**Roberts A, Soffe SR, Wolf ES, Yoshida M, and Zhao FY.** Central circuits controlling locomotion in young frog tadpoles. *Annals of the New York Academy of Sciences* 860: 19-34, 1998.

**Saito T.** In vivo electroporation in the embryonic mouse central nervous system. *Nature protocols* 1: 1552-1558, 2006.

**Schouenborg J.** Action-based sensory encoding in spinal sensorimotor circuits. *Brain research reviews* 57: 111-117, 2008.

**Sherrington C.** Nobel Lecture: Inhibition as a Coordinative Factor". Nobelprize.org. 17 Apr 2013 [http://www.nobelprize.org/nobel\\_prizes/medicine/laureates/1932/sherrington-lecture.html](http://www.nobelprize.org/nobel_prizes/medicine/laureates/1932/sherrington-lecture.html))

**Shizuya H, Birren B, Kim UJ, Mancino V, Slepak T, Tachiiri Y, and Simon M.** Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proceedings of the National Academy of Sciences of the United States of America* 89: 8794-8797, 1992.

**Smith JC, and Feldman JL.** In vitro brainstem-spinal cord preparations for study of motor systems for mammalian respiration and locomotion. *J Neurosci Methods* 21: 321-333, 1987.

**Stein PS, and Daniels-McQueen S.** Modular organization of turtle spinal interneurons during normal and deletion fictive rostral scratching. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22: 6800-6809, 2002.

**Stuart DG, and Hultborn H.** Thomas Graham Brown (1882--1965), Anders Lundberg (1920-), and the neural control of stepping. *Brain research reviews* 59: 74-95, 2008.

**Talpalar AE, and Kiehn O.** Glutamatergic mechanisms for speed control and network operation in the rodent locomotor CPG. *Front Neural Circuits* 4: 1-14, 2010.

**Wallen-Mackenzie A, Gezelius H, Thoby-Brisson M, Nygard A, Enjin A, Fujiyama F, Fortin G, and Kullander K.** Vesicular glutamate transporter 2 is required for central respiratory rhythm generation but not for locomotor central pattern generation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 26: 12294-12307, 2006.

**Wallen P, and Grillner S.** N-methyl-D-aspartate receptor-induced, inherent oscillatory activity in neurons active during fictive locomotion in the lamprey. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 7: 2745-2755, 1987.

- Wang H, Peca J, Matsuzaki M, Matsuzaki K, Noguchi J, Qiu L, Wang D, Zhang F, Boyden E, Deisseroth K, Kasai H, Hall WC, Feng G, and Augustine GJ.** High-speed mapping of synaptic connectivity using photostimulation in Channelrhodopsin-2 transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* 104: 8143-8148, 2007.
- Wang Z, Li L, Goulding M, and Frank E.** Early postnatal development of reciprocal Ia inhibition in the murine spinal cord. *Journal of neurophysiology* 100: 185-196, 2008.
- Wenner P, O'Donovan MJ, and Matise MP.** Topographical and physiological characterization of interneurons that express engrailed-1 in the embryonic chick spinal cord. *Journal of neurophysiology* 84: 2651-2657, 2000.
- Ventura A, Kirsch DG, McLaughlin ME, Tuveson DA, Grimm J, Lintault L, Newman J, Reczek EE, Weissleder R, and Jacks T.** Restoration of p53 function leads to tumour regression in vivo. *Nature* 445: 661-665, 2007.
- Whelan PJ.** Control of locomotion in the decerebrate cat. *Prog Neurobiol* 49: 481-515, 1996.
- Zhang F, Wang LP, Brauner M, Liewald JF, Kay K, Watzke N, Wood PG, Bamberg E, Nagel G, Gottschalk A, and Deisseroth K.** Multimodal fast optical interrogation of neural circuitry. *Nature* 446: 633-639, 2007.
- Zhang Y, Narayan S, Geiman E, Lanuza GM, Velasquez T, Shanks B, Akay T, Dyck J, Pearson K, Gosgnach S, Fan CM, and Goulding M.** V3 spinal neurons establish a robust and balanced locomotor rhythm during walking. *Neuron* 60: 84-96, 2008.
- Zhong G, Shevtsova NA, Rybak IA, and Harris-Warrick RM.** Neuronal activity in the isolated mouse spinal cord during spontaneous deletions in fictive locomotion: insights into locomotor central pattern generator organization. *The Journal of physiology* 590: 4735-4759, 2012.

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