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Neurotransmitter Phenotypes of Neurons in the Spinal Cord and Their Functional Role in the Mouse Locomotor Network

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To my son Gaël Restrepo Barman

Abstract

Walking is a special case of movement because it is rhythmic. An important area in current neuroscience research, is to better understand the different organizational levels (molecular, ion channels, neurotransmitters, connectivity etc.) that implement the function of rhythmic movements. The isolated spinal cord from rodents can generate rhythmic patterns that resemble many aspects of walking. The networks that support this autonomous function are called central pattern generators or CPGs. In this thesis, we use the mouse spinal cord as a model system for studying walking. We describe the neurotransmitter phenotype, development and functional organization of populations directly involved in the normal and abnormal function of spinal locomotor networks. Work presented in this thesis show compelling evidence that motoneurons, the final output cell in the spinal cord, releases glutamate in addition to acetylcholine (ACh) at the central synapses but not at the neuromuscular junction. This discovery changed a long held view that motoneurons only release ACh and challenge "Dales" principle according to which the same neurotransmitter is released from all terminals in a single neuron (paper I). We also investigate the neurotransmitter phenotypes of the commissural interneurons (CINs) that have axons crossing in the midline. The CINs system is essential for walking because CINs are required to coordinate rhythmic activity between left and right side of the body. We used a number of transgenic mice to show that CINs located in the ventral spinal cord are glycinergic, GABAergic and glutamatergic and these neurotransmitters are expressed in almost equal numbers without any evident topographic segregation (paper II). The study of the murine CIN systems confirm the presence of a well-know strong glycinergic inhibitory crossing in vertebrates but also reveal the existence of important excitatory glutamatergic and inhibitory GABAergic components. During development a number of axon guidance pathways are active to set up the organization of the nervous system. One of these is the Eph and ephrin system. Knockout of one of the Eph receptors, the EphA4, or its ligand ephrinB3 leads to a hopping gait. We show that this hopping gait is due to a reconfiguration of the spinal CPG possibly caused by an increased over-crossing of excitatory spinal neurons (paper III). In continuation, we investigated candidate populations as source of the abnormal crossing. One of these are the V2 interneurons that we showed are all ipsilaterally projecting and composed of V2a excitatory neurons expressing the transcription factor Chx10 and V2b inhibitory neurons that express the transcription factor GATA2/3. Although many V2 interneurons express EphA4 we could not find evidence of abnormnal crossing in the V2a population in the EphA4 knockout mice (paper IV). Finally, we investigate the proportion of excitatory and inhibitory neurons crossing in the EphA4 knockout mice. We show that there is a significant increase in the proportion of excitatory crossing neurons accompanied by a decrease in proportion of inhibitory glycinergic crossing neuronsin homozygous EphA4 knockout mice (paper V). This study confirms that the hopping gait seen in the EphA4 mutants may be the consequence of an abnormal change in the balance between excitation and inhibition of crossing neurons. However, the study also showed how changing the assembly of the nervous system may have unpredictable consequences. The study furthermore suggests a much more complex regulation of axon guidance imposed by the EphA4 and ephrinB3 system than previously believed. The work presented in this thesis have used a panoply of different techniques, to reach a comprehensive description of the neurotransmitter phenotypes of a number of spinal neuronal populations and their role in the walking CPG.

Contents

Preface

List of Publications included in the Thesis

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

I. *Mammalian motor neurons corelease glutamate and acetylcholine at central synapses*, PNAS. 2005 102(14):5245-9, Nishimaru H, **Restrepo CE**, Yanagawa Y, Kiehn O.

II. *Transmitter-phenotypes of commissural interneurons in the lumbar spinal cord of newborn mice*, J Comp Neurol. 2009 10;517(2):177-92, **Restrepo CE**, Lundfald L, Szab G, Erdlyi F, Zeilhofer HU, Glover JC, Kiehn O.

III. *Role of EphA4 and EphrinB3 in local neuronal circuits that control walking*, Science. 2003. 299(5614):1889-92, Kullander K, Butt SJ, Lebret JM, Lundfald L, **Restrepo CE**, Rydstrm A, Klein R, Kiehn O.

IV. *Phenotype of V2-derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing mouse spinal cord*, Eur J Neurosci. 2007 Dec;26(11):2989-3002, Lundfald L, **Restrepo CE**, Butt SJ, Peng CY, Droho S, Endo T, Zeilhofer HU, Sharma K, Kiehn O.

V. *Transmitter-phenotypes of crossed neurons lacking EphA4 in the lumbar spinal cord of newborn mice*, Manuscript, **Restrepo CE**, Borgius L, Lundfald L, Kiehn O.

List of Publications NOT included in the Thesis

- *Retinofugal projections following early lesions of the visual cortex in the ferret.*, Eur J Neurosci. 2002 Nov;16(9):1713-99, **Restrepo CE**, Manger PR, Innocenti GM.

- *Immature cortex lesions alter retinotopic maps and interhemispheric connections*, Ann Neurol. 2003 Jul;54(1):51-65, **Restrepo CE**, Manger PR, Spenger C, Innocenti GM.

- *Activity of Renshaw cells during locomotor-like rhythmic activity in the isolated spinal cord of neonatal mice*, J Neurosci. 2006 May 17;26(20):5320-8,Nishimaru H, **Restrepo CE**, Kiehn O.

- *Excitatory components of the mammalian locomotor CPG*, Brain Res Rev. 2008 Jan;57(1):56-63, Kiehn O, Quinlan KA, **Restrepo CE**, Lundfald L, Borgius L, Talpalar AE, Endo T.

- *Genetic ablation of V2a ipsilateral interneurons disrupts left-right locomotor coordination in mammalian spinal cord*, Neuron. 2008 Oct 9;60(1):70-83, Crone SA, Quinlan KA, Zagoraiou L, Droho S, **Restrepo CE**, Lundfald L, Endo T, Setlak J, Jessell TM, Kiehn O, Sharma K.

- *Transmitter phenotypes of commissural interneurons in the lamprey spinal cord*, Neuroscience. 2009 Dec. 15;164(3):1057-67, Mahmood R, **Restrepo CE**, El Manira A.

- *A new transgenic mouse line for the molecular genetic analysis of excitatory Vglut2 positive neurons in the brain and spinal cord*, manuscript, Borgius L, **Restrepo CE**, Leao R, Kiehn O.

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Abbreviations

AP	Action potential
ACh	Acetylcholine
CIN	Commissural Interneuron
CN	Crossed neurons
CNS	Central nervous system
CPG	Central pattern generator
CST	Cortico spinal tract
EIN	Excitatory interneurons
EPSP	Excitatory post synaptic potential
EPSC	Excitatory postsynaptic current
EphA4	Eph receptor A4
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GFP	Green fluorescent protein
GlyT1	Glycine transporter 1
GlyT2	Glycine transporter 2
IHC	Immunohistochemistry
MN	motoneuron
MLR	Mesencephalic locomotor region
NMDA	N-methyl-D-aspartic acid
NMJ	Neuromuscular Junction
RC	Renshaw cells
RNA	Ribonucleic Acid
VACht	Vesicular acetylcholine trasnporter
Vglut2	Vesicular Glutamate transporter type 2

Chapter 1

Introduction

1.1 *De Motu Animalium*

"All animals impart movement and are moved for the sake of something" Aristotle [84] This sentence connote all sort of important problems. The most evident of these is from my point of view, first what purpose drives the animals to move? and how do animals move? This thesis aims to contribute to the resolution of the problem opened with the last question, namely how do animals move?. This introductory chapter includes a very brief description of how my small contribution to the study of movements fits in a very general historical context.
Aristotle calls animals self-movers because "if everything that is in motion is moved by something, and the first movement is moved but not by anything else, it must be moved by itself" [4]. Here Aristotle is arguing for the existence of a basic principle as source of movements. Because animals move, and the movement was a property of the soul, animals were self movers. Hence, if animals move by a internal source "that which first moves the animal must necessarily be in some origin" [84]. As the initial source of the movements in the body he assigned the heart. When describing the movements of animals, Aristotles compared this with puppets: "the movement of the animals is like that of automatic puppets, which are set moving when a small motion occurs: the cables are released and the pegs strike against one another" [84]. Although Aristotle described in detail the faculties of the soul and its relation to movements I will say that Aristotle's general approach is what could be called the "outside-in" approach, it is a more phenomenological description of movements and general mechanical principles [101]. The "inside-out" approach is a reductionist project of explaining general properties from the constituent parts (in our time the neuron properties). The

reductionist project is the one created by the atomist school of Democritus. Aristotle oppose atomism because at his time explanations were inconsistent with the properties of what the world was made of, for instance "it requires the existence of void...and it asserts the law of inertia" [31]. At that time was no evidence for any of these assertions[1]. A contemporary interpreter suggested that in order for Aristotle solve the problem of rejecting the primacy of matter from explanations (as Democritus did) he assumed that form (in the sense of shape) is the most basic explanation for a "thing" being what it is and acting as it does than matter [84]. When Aristotles define the soul he link it to the form (in the sence of function) of the body and at the property of being alive. This intrincate explanation was needed in order to escape from a sort of platonic dualism but at the same time away from a type of reductionism (Democritus school) [74]. Most of the history of science of the mind (neuroscience now) continued to be rooted in this conflicts, trying to escape from dualism but at the same time trying to explain complexity as made of basic constituents parts.
Aristotelian and Greek philosophical debates in general did not advance as the result of the new ideological requirements of the time to come [95]. After ancient Greece the most notable exceptions was Galen who developed a very intricate philosophical-physiology explanations of the soul based in syncretic principles from Greek philosophy and his own [32]. The contemporary science of mind was initiated by Descartes philosophy, who was also influenced by Galen[2]. Descartes supposed that the body was an automata (machine), but the soul an immaterial substance. In contrast to Aristotle and the predecessors Descartes emphasized that the deterministic properties of the matter *res extensa* should be distiguish from the properties of the mental substance *res cogitans*. The last of course we cannot study, but by exorcising the common bodies from intrinsic invisible powers he open the conceptual possibility of understantding the human and animals bodies as machine [78]. This idea evolved during the times of Descartes with Borelli and more radically with Le Metrie among others. With the richness of the atomistic ideas and mechanics, the analysis of functions is carried out by assuming that the organism is made of a hierarchy of components. In this way the project to study the

[1]Furthermore, how is it that things change, but it is possible to recognize them as being the same thing? "The conservation of elements (from the atomistic school) does not explain how new properties can appear in a mixt, giving origin to new properties or how the previous properties inherent to the components of the mixt disapear."[81].

[2]Descartes use the Galenic tradition of the psychic pneuma (*espiritus animale* in latin) [32] to explain how the soul from its place in the head controled the muscles. The *espiritus animale* is supposed to be a subtle type of "wind, a flame, or ether" [24] and its role was to reach the muscles and by inflating them to cause movements.

body should by carried out as Nicolaus Steno in 1669 (a comtemporary of Descartes) declared, "There are two ways only of coming to know a machine: one is that the master who made it should show us its artifice; the other is to dismantle it and examine its most minute parts separately and as a combined unit... and...since the brain is a machine (Descartes, 1664), we need not hope to discover its artifice by methods other than those that are used to find such for other machines...I mean the dismantling of all its components, piece by piece,and consideration of what they can do separately and as a whole" (Cited by Larry W. Swanson [11]).
Later on with the discovery of the electrical properties of matter and their influence on the living, the study of the nervous tissue converged to another old idea. For Galen, Descartes and many others, the effects of soul in the body were caused by a subtle, highly refined type of air or nervous fluid. The discovery of the relation between the nervous fluid and electricity[3] fit again with some of the vague expectations of the Cartesian mechanics, though the living machine turned out to be far more advanced than any existing mechanical device. The development of the evolutionary theories make it unreasonable to separate humans from animals[4] and consequently that the human soul itself was also a type of mechanism, a property of matter [67]. With the discovery by Ramon y Cajal [15] that neurons were distinct units and not a continuum of intersticial networks, the Cartesian machine could finally start to be dismantled into its most basic components.
Above, I have outlined a personal historical perspective of the study of movements. The first part more as a curiosity that reveals the deep rooted elements of the problems of movement. The second part, opened with Descartes and extended until now. In the next part of this work, I will address in more detail some of the developments in the study of movements, in particular the reflex and the central pattern generator.

1.2 The reflex

The developments of the cellular theory was not necessary to the discovery of the reflex. The modern concept of reflexes appears between the works of Willis (1670) and the first work by Marshall Hall (1873) [17]. Sherrington

[3]The frog used by Galvani was one of the more useful preparations in the experimentation and resulted in the discovery of the electrical properties of the nerves. Although attributed to Galvani, he was not the only one to suggest it in his time [89]

[4]Descartes accepts that animal and human bodies were machines but that humans differ from animals in having a soul, *res cogitans*. Le Mettrie, a brilliant but less well positioned intellectual at that time offered very interesting arguments against this but he was never influential.

and Fearing [29] incorrectly developed the idea that the concept of the reflex was already in the works of Descartes. Here I have translated a few of the solid arguments exposed by Canguilhem [17] against Sherrington and Fearing. First, "the essential of the concept of reflex is not only to include elements of a mechanic explanation. It is also to admit that stimulation of part of the periphery of the organism that suffers a shocking stimulus, independently of its nature, after the reflection in a center, will return through the same periphery. For Descartes, movements in the periphery of the organism take their origin from a center, the organic center, the heart."(my translation). Second, "The image that suggest the invention of the word reflex (Prochaska G. 1784 cited by Joules Soury [99] is the ray of ligh reflected by a mirror, imposing a homogenous movement between the incident movement and the reflected movement"(my translation).
The ideas and experimentation that support the development of the concept of reflex start as a process that passes by reinterpretations of the Cartesian *espiritus animale* by Willis and his conceptual explanation of basic movements [17]. It is not important for this work to develop the details but Canguilhem[17] elegantly, described the fundamental historical contributors to the concept of reflex in the 1800 century: "the movement of reflex (Willis) is immediately due to a previous sensation (Willis) caused by physical laws (Willis, Astruc, Unzer, Prochazka), [and in relation to the instincts(Whytt,Prochazka)] of reflexion by the action of sensitive nerves in motricity (Whytt, Unzer, Prochazka) at the level of the spinal cord (Whytt, Prochazka, Legallois) with or without concomitant consciousness (Prochazka)".
Soon after Francois Magendie and Charles Bell [99] rediscovered that the dorsal afferents were the source of sensory inputs and the ventral efferents the source of motor output. With this knowledge and the new developments in electrophysiology, the study of the reflex entered to a new phase. While in the end of the 1800s the phenomenon was correctly defined and basic principles established, the new anatomical and electrophysiological techniques allowed for the dissection of its parts.

The components of the reflex

The physical laws suggested by Willis, Astruc, Unzer and Prochazka [17] were indeed the electrical properties of the neurons. The action potentials can be generated by any neuron and this signal is transmitted through the synapses to other neurons. The nature of the synaptic transmission was later discovered to be chemical, in the case of motoneurons by release of

the neurotransmitter Acetylcholine at the neuromuscular junction (NMJ)[5]. The reflex is initiated by the conversion of a external stimulus (for instance by activation of the stretch receptors) that carry an electrical signal (the action potential) through the dorsal nerves and through its synapse onto local motoneurons activating the corresponding muscle. Concomitantly with the activation of the muscle the antagonist muscle is relaxed, coordinated by the crossed activation of interneurons that inhibit the respective motoneuron. Because the reflex offers a possibility of isolating a simple behaviour, the circuit reflex and its components became a fundamental tool in order to investigate specific properties of neurons.

Motoneurons and Renshaw cells

Renshaw and later Eccles, Fatt and Koketsu showed that antidromic stimulation of the ventral roots while recording from motoneurons resulted in a inhibitory volley of high frequency activity in the motoneurons [92]. Because the hyperpolarizing effect was very short (3-10 msec) it was suggested that these inhibitory neurons (later termed Renshaw Cells (RCs) by Eccles) were directly contacted by the motoneuron collaterals. In elegant work by Eccles, Fatt and Koketsu [27] it was shown that the inhibitory volley was affected by the application of local acetylcholinergic blockers. Finally, due to the fact that the inhibitory signal from RC to motoneurons could be depressed by strychnine it was shown that RC used the neurotransmitter glycine.
One important functional property that was assigned to neurons from this early research by Eccles was the suggestion by Dale that a single neurons release the same neurotransmiters from all its terminals [100][6]. This was something corroborated by the experiments of Eccles, Fatt and Koketsu who showed that motoneuron central colaterals release acetylcholine like their terminal endings in the muscle. Dale's principle has since been challenged several times. The first neurottransmitters discovered to be co-release with classical neurotransmitters like catecholamines, serotonin and GABA were neuropeptides [46]. Later small fast transmiters such as glycine and GABA were shown to be co-released from interneurons in the spinal cord [52]. Interestingly, most of these discoveries were preceded by imunohistochemistry studies [86]. With the discovery of the proteins responsible for the filling of the excitatory neurotransmitter glutamate into the synaptic vesicles (vesicular transporters), immunohistochemistry and *in situ* hibridization pointed to

[5]Although it seems that the final evidence, came from the work of Fatt and Katz, the idea was suggested by Elliot and continued by Dixon (according to Dale. [22])

[6]Nicoll and Malenka [82] gave an interesting explanation of why Eccles was so keen to elevate Dale's rumnation to the rarified level of "principle".

the possibility that motoneurons contained these transporters and therefore use glutamate at the synapses [43].
Since the time of Eccles, Fatt and Koketsu, motoneurons were considered to only release ACh. In PAPER I of this thesis we show compelling evidence that motoneurons release glutamate from their collaterals synapsing onto RCs and motoneurons but not at the neuromuscular junction. Furthermore, and more strikingly is the possibility that such release may not be confined to the same synaptic terminals.

1.2.1 From the reflex concept to the central pattern generator

The most important achievements of the Cartesian program was the success of being able to explain a basic behaviour (at least isolate one). As Sherrington put it: " Experiments to-day do ...put within reach of the observer a puppet-animal which conforms largely with Descarte's assumptions"[7].
If the reflex was a basic component, then movements such as walking could be seen as a chain of reflexes[8]. And if sensory information is fundamental for movement, the absence of any sensory inputs will preclude any form of elaborate muscle output. Then, if an animal is deafferented of its dorsal roots, it is not possible to elicit organized movements. Nonetheless, Graham Brown in 1910 presented evidence that the nervous system could self-generates complex patterns of activity independently of sensory input [37]. Browns work was largely ignored for many years[9], but between 1960 and the 1970, Lundberg and coworkers discovered some of the properties in the spinal network predicted by Brown model. This model suggests the existence of antagonizing centers (ipsilaterally providing excitation to respective motoneurons) that are regulated by reciprocal inhibition. Lundberg and collaborators showed that the cat lumbar spinal cord when isolated from the brain could generate rhythmic locomotor-like activity when exposed to neuroactive drugs [48]. With the discovery by Orlovsky and coworkers, of the structures in the midbrain (called the mesencephalic locomotor region, or MLR) [85] capable of initiating locomotion it became possible to investigate in detail the interaction between descending drive and the self-generating centers in the spinal cord (later called Central Pattern generators, or CPGs[42]).
In contrast to the mechanical puppet that requires an external push to be activated, the nervous system was now shown to be able to maintain a self-

[7]Foreword to the 1947 edition of 'The integrative action of the nervous system' [97]
[8]Walking is a special case of movement because of its rythmic nature.
[9]Maybe because Eccles was never really keen to elevate Brown's results !?

organized pattern of activity. The reflex chain did not explain complex rhythmic movements but its components are part of more elaborate networks. The spinal cord contains reflex networks but also networks with CPG properties. The main focus of this thesis is the study of the CPG components.

1.3 The CPG

The concept of a central network generating rhythmic activity was origiginally based on studies in cats [37], but the concept Central Pattern was developed by Wilson based on studies in insects [104] and finally stablished by Grillner as Central Pattern Generator (CPG) [42]. CPGs properties[10] have since then been observed in a rich diversity of species and phyla [23]. Since CPG properties appear within different structures localized in many different regions of the nervous system, this preclude evident evolutionary homologies among them. However, CPGs properties that implement analogous rhythmic movements from fish to mammals [38] have been shown to share anatomical similarities [85]. Furthermore molecular biology studies have shown commonalities between the molecular genetic pathways[11] used early in developmental in vertebrates [30].
The simplicity of the nervous systems and easy maintenance of the *in vitro* lamprey and tadpole spinal cords make both very well characterized locomotor models [40, 87]. The studies in tadpole and lamprey strongly suggest that the rhytmic activation is independently generated by each side of the cord [16] with coordination between the two sides mediated by reciprocal glycinergic inhibition.
In the model of lamprey [39], swimming is initiated by activation of a descending drive from the brain to ipsilateral excitatory interneurons EINs. The EINs transform this input to a excitatory rhythmic output, produced by the intrinsic properties of neurons and excitatory interconnectivity among excitatory interneurons[12]. During the bursting the excitatory drive of the EINs

[10] "Strictly speaking, the term CPG should only apply to activity generated in completely spinalized (i.e., total spinal section that removes all descending pathways from the brain stem and telencephalon) and paralysed (e.g.,curarization) animals, a condition that abolishes all phasic sensory inputs associated with limb movements [94]."

[11] A note of caution: Evidence in the generalized case for CPG function came from studies in the development of larval crawling in *Drosophila*. In a first study it was reported that absence of sensory neurons was not necessary for the the rhythmic larval crawling [102]. However, another recent study has shown that this may not be the case [98].

[12] The NMDA based oscillations explain slow rhythmic patterns less than 1 Hz, higher frequency oscillations are supposed to be produced by interconnectivity of ipsilateral EINs [16].

is converted to inhibition to the other side of the cord by the activation of commissural glycinergic inhibitory neurons. This inhibitory crossing is the basic component of the control of left-right alternation and downregulation of frequency [16].
Some of the above propeties from lamprey studies have also been described in the mammal spinal cord [55]. However while CPGs properties appear through all segments in the lamprey, it is not the case in the rodent spinal cord. Hindlimb CPGs in mammals appear localized in the ventro-medial region of the spinal cord from the lower thorasic spinal cord to the caudal lumbar cord [18, 61], with the flexor related segments L1-L2 being the most rhytmogenic region[18, 59]. A common aspect of all vertebrate species is the role of ipsilateral excitatory interneurons in the generation of the intrinsic rhythm [57], however see [93]. Another common property between the fish and the mammal CPGs is the role of the glycinergic CINs in the maintainence of left-right alternation [14, 41, 60]. However, the overall organization of the commissural inhibitory system in rodents seems more complicated because it includes indirect and direct pathways and excitatory pathways [57]. The aim of the work presented in PAPER II was to thoroughly investigate the relative proportion, location and organization of commissural interneurons in mouse. This study shows that GABAergic and glutamatergic CINs are expressed in almost equal numbers with a small proportion of CINs potentially having a combined glycinergic/GABAergic phenotype.
Despite these advances, the study of the CPG components in mammals has been more complicated for several reasons. First, the complexity of limb movements is associated with an increased number of cells [8] compared to lamprey and tadpole. Second, the organization of this increased number of elements is arranged in a very rich manner. The most dorsal horn lamina appear organized in layers, while the deeper and ventral horn appear as clusters of cells. The undefined clustering of the ventral region makes it extremely complicated to distinguish cell identities based on position alone[13]. A fundamental part of this thesis is to expose some of the new approaches that have been use in order to deal with these problems.

1.3.1 The rodent spinal cord in vitro preparation

A fundamental tool in the study of locomoto networks is the neonateal rodent spinal cord [62]. Despite the early stages in which it is possible to maintain the isolated spinal cord, fundamental properties of the CPG elements can

[13]For instance in the cerebral cortex, the evident layer structure and neuron morphologies have been a classical initial criteria for classification.

be investigated. With the use of transmitter agonists (NMDA, 5-HT and Dopamine) it is possible to induce a reproducible patternt of activity that resembles some fundamental aspects of adult locomotion [19] however see [53]. The accessibility of the preparation makes it suitable for fine dissections and pharmacological studies. Under fictive locomotion induced with transmitter agonists the spinal cord can be divided by using barriers between the segments, or surgically. In this way it have been shown that the more rhythmogenic regions are localized in rostral segments L1 and L2 [18] with the rhythmicity of the network distributed rostrocaudally along the entire lumbar enlargement [56, 61, 103]. Using the same logic, fine microsurgical experiments have indicated that the most rhythmogenic regions are localized in the ventromedial areas of these lumbar segments [59]. The use of transmitter agonists as a way of inducing normal-like locomotor patterns can also be combined with the use of antagonistic drugs in order to investigate the organization of the connectivity [47]. Similarly, with the use of genetic tools it is feasible to disrupt or affect the function of a desired neuronal population. All of the PAPERS presented in this thesis make use of the neonatal rodent spinal cord preparation.

1.3.2 The problems of classification

In neuroscience the interest to classify neurons is based on the prospect of understanding their functionality. In order to classify cells it is necessary to decide what properties are to be included as criteria for the respective grouping. A classic approach has been to classify neurons in relation to their anatomical shapes and connectivity (axonal length, dendritic branching, body size etc). Unfortunately, the intricate connectivity of the nervous system has made it complicated to reach any consensus about the general organization of connectivity [11]. The spinal cord is not less complicated, since neurons are intricately connected with many others brain regions [49]. However because, of the knowledge of the specificity of the functionality of the spinal cord and because CPGs are networks that can operate without sensory input, this functional definition gives us a heuristical framework to evaluate the role of components of the CPG. Therefore if a neuron (or another organization level) is part of the CPG, its absence necessarily should affect the capacity of the CPG to operate autonomously or disturb the intrinsic properties. In this way molecular biology techniques can be used as a tool to disrupt specific cell types or molecules suspected to be involve in the development or function of the CPG circuitry. The discovery of the hopping gait of the EphA4 knock-out mouse was the opening precedent in this line of thinking (PAPER III). Subsequently, approaches have made use of

trascription factors to explore neuronal properties.

1.3.3 The EphA4 mutant

The importance of this study resides in the discovery of a molecule that alters the CPG itself. The genetics here can be seen as a tool in order to alter a component in a level of organization of the network. This study combined the basic knowledge about the functioning of the spinal cord in vertebrates with the new available genetic tools.
The interest in axonal guidance molecules the Ephrins and its Eph receptors in the study of CPG resulted from the discovery of the abnormal gait of the EphA4 receptor and ephrinB3 knock-out mice [25]. In both mutants hindlimbs move in synchrony in a hopping gait. Initially, it was thought that this behavior was a consequence of abnormal cortico spinal tract (CST) axon guidance defects [65]. However electrophysiological and anatomical studies in the neonate lumbar spinal cord showed that the abnormal synchrony resulted as a consequence of local spinal cord changes associated with perturbations in the overcrossing of neurons (Paper III). As stated before, CINs are a fundamental component of the CPG because of their role in mediating coordination between both sides of the spinal cord. Therefore, any developmental factor playing a role in the establishment of the cross connectivity should result in consequences for CPG functioning.
The Eph receptors comprise a complex family of tyrosine kinases receptors divided into two major groups A, and B. The ephrins A anchored the membrane by glycosylphosphatidylinositol (GPI) and bind to EphA receptors. The ephrinB ligands, have a transmembrane domain and bind to EphB receptors. However, the EphA4 receptor can bind to ephrinB ligands (e.g. ephrinB3) [64]. In the spinal cord the EphA4 receptor is expressed by interneurons, while its ligand (ephrinB3) is expressed in the midline of the cord [63]. When neurons expressing EphA4 contact ephrinB3, a cascade of internal signals results in the depolymerization of actin, causing the retraction of the axonal filopodia. Because ephrinB3 is localized in the midline, axons expressing EphA4 will mostly be unable to cross the midline. Physiologically, the EphA4 and the ephrinB3 knockouts are very similar. In both animals the abnormal hopping gait can be rescued to normal alternation when inhibition is boosted (PAPER III) and abnormal branching is seen with the use of fluorescent tracers. In the case of the EphA4 mice, a major proportion EphA4 positive interneurons which cross the midline (pseudo-commissurals) are present in the knockout when compared with the normally alternating heterozygous EphA4 mouse. Furthermore some EphA4 neurons express the vesicular glutamate transporter Vglut2. From

these lines of evidence and the known role for EphA4, it was suggested that in the normal CPG, ipsilateral neurons that express this receptor are predominantly excitatory. Therefore, in the mutant an ipsilateral excitatory population might be abnormally crossing, resulting in a change in the excitatory balance. In order to evaluate this hypothesis, we have investigated if normally excitatory ipsilateral interneurons (e.g. Chx10 and Hb9 interneurons) abnormally crossed in the EphA4 knockout (PAPER IV). We also investigated the nature of the entire crossing population (both excitatory and inhibitory) using a number of transgenic mice. Surprisingly, we discovered that the anatomical basis of this abnormal gait resulted from an increased proportion of excitatory crossing neurons, and a simultaneous decrease in the inhibitory proportion of glycinergic crossing interneurons (PAPER V). However, neither the Hb9 nor the Chx10 interneurons abnormally crossed in the EphA4 knockouts.

1.3.4 Spinal cord development

The spinal cord develops as the rest of the nervous system from the folding of the neural plate. Once the neural tube is formed by the closing of the neural folds, dorsal and ventral signals, trigger the expression of differential genes in distinct cell populations depending of their positions [34]. The dorsal or ventral signals are gradients of diffusible morphogens. Ventrally, Sonic hedgehog (SHH) is secreted by the notochord and dorsally bone morphogenetic protein (BMP) is secreted by the roof plate [34, 51]. The different concentrations and bimodal expression of the morphogens are detected by the cells in the ventricular zone during their early developmental stage and transformed into the activation of distinctive sets of genes. Some of the genes early activated by morphogens are transcriptor factors that can activate or inactivate the transcription of many more genes. The assumption behind these developmental studies is that the subsequent intrinsic properties of a cell population are unfolded from an intrinsic precedent genetic program particularly defined by the transcription factors. The sets of genes that a cell expresses establish the possible environmental and internal alternatives and hence the next steps that it can take. Therefore, an alternative for investigating the general role of a population in the functioning of a network is to use the distinctive expression of transcription factors to investigate specific topographic location of a cell group as defined by the expression of the transcription factor itself (PAPER IV). The genetic marker can also be use to selectively knockout a population [20] or to depress its electrical activity [35].

In relation to their genetic expresion the dorsal horn is divived into six domains pd0-6 and the ventral cord into four domains called pv0-3 [51]. Our main interest relates with the progenitor domains localized in the ventral part where the main core of the CPG has been localized [56]. V3 interneurons are the most ventral of all of the populations. They are identified by the expression of Sim1. This population is composed of excitatory glutamatergic CINs and ipsilaterally projecting neurons. Based on its migratory positions the population have been divided into a dorsal and ventral part [36, 83]. Knocking out the population resulted in changes in the burst duration (extended bursting) [106] combined with co-activation. The later suggesting abnormal left-right alternating patterns. V2 interneurons, are identified by the expression of Lhx3 [88] and are divided into V2a neurons, expressing Chx10, and V2b neurons expressing GATA2/GATA3. In Paper IV we aimed at characterizing this populations and showed that both populations are ipsilaterally projecting with short (segmental) and long range projections (several segments)[26]. The Chx10 population is glutamatergic and the V2b are inhibitory, GABA and glycinergic [1, 72]. The ablation of the Chx10 population results in abnormal left-right drifting and alternation. Anatomical evidence suggest that this result is a consequence of a direct connectivity between Chx10 neurons and inhibitory CINs systems [20]. The Chx10 population has also been related to the control of the cycle period and burst amplitude [21]. V1 interneurons are idenfitied by the expression of En1 [96]. This group of neurons is composed of the Ia and Renshawn cells (inhibitory GABA and glycinergic), but these neurons only make approximately 25% of the total V0 population [2]. The selective ablation or silencing of the En1 population caused a reduction in the duration of the cycle period [35]. V0 interneurons are identified by the expression of Dbx1. In relation to their position, they are futher subdivided into a ventral group V0v and a dorsal group V0d[68]. Labeling studies in E12.5 have shown that these neurons are mainly CINs projecting rostrally [79, 90][14]. This population has been related to the control of left-right alternation [69]. Motoneurons populations are identified by the expression of the transcription factor Hb9. Hb9 is also expressed in ipsilaterally projecting excitatory interneurons [3, 45, 105]. The Hb9 interneurons have been proposed as being components of the rhythm generator part of the CPG [12], however see [66].

The use of transcription factors is a necessary alternative to the clasification of cell groups. However it is my opinion, that there are aspects of the phenotype of cells that do not depend just on the unfolding of an intrinsic

[14]However the V0 population also projects caudally, and ipsilaterally [79, 90].

genetic program. For instance it has been shown that during the development of the spinal cord of *Xenopus laevis* the general balance of excitation is changed by manipulating neuronal Ca^{2+} spikes resulting in a compensatory balance in the spinal cord by increasing inhibition. Thus the neuron transmitter phenotype switches from excitatory to inhibitory [10][15]. This shift can be seen metaphorically as a top-down effect, while the transcription factors are part of the bottom-up component. The specification of the final neuronal properties could be the result of the interplay between the intrinsic program and state of activity of the surrounding environment. In this way activity dependent processes could bee seen as a source of stochastical variability in the final phenotype while transcription factors are restricting this variation [5].

Although I cannot advance any reasonable explanation of how a neuron reaches its final state, I do not agree with the claim that "Each of the spinal CPG interneuron [16] classes exhibits a unique phenotype" Goulding M. [36]. Because first, even if we know that the CPG is localized in the ventral region of the spinal cord [56], this does not mean that all cells there are part of the CPG. Second, it is not clear if the final phenotype is exclusively defined by the transcription factor themselves [17]. Third, the characterization of the cell properties in the ventral domain (V0-V3) in the spinal cord is far from being known. Many of the properties so far described are the result of experimentation at embryonic stages[18].

The transcription factors are a good way to advance on the classification. Their expression may relate to aspects of the phenotype of neurons that compose the spinal locomotor circuits. This allowed us to advance interpretations in the possible wiring and phyiological causal effect of the CPG components. However, it is very possible that populations can be further subdivided in relation to physiological and anatomical properties and not necessarily by the expression of another transcription factor. In a future classification system the transcription factors may become just one aspect of the phenotype.

[15] Also, increasing evidence has shown that ion channels can activate intracellular signalling pathways and this action could sometimes require ion flux [54].

[16] As defined by the expresson of the transcription factors.

[17] We do not know what the final phenotype look like. For instance could we have predicted that motoneurons use glutamate as neurotransmitter?

[18] A common aspect of the populations described by the expression of transcription factors is that the patterns of projection have been examined mostly in embryonaric stages. This is due to the restricted expression window of some trasnscrition factors, making it impossible to follow embryonic defined population to adulthood or even to newborn stages.

Chapter 2

Aims

I. To investigate the possible co-release of glutamate from motoneurons and the nature of the transporter used.

II. To describe the relative proportions and anatomical locations of inhibitory and excitatory CINs in the lumbar spinal cord of newborn mice.

III. To investigate the role that the axon guidance molecule EphA4 play in the development and organization of the CPG.

IV. To investigate the neurotransmitter phenotype and general anatomical aspects of the V2 interneurons and to evaluate if this population abnormally crosses in the absence of EphA4 signaling.

V. To describe the relative proportions and anatomical locations of putative inhibitory and excitatory CINs in the lumbar spinal cord of newborn EphA4 knockout mice.

Chapter 3

Methodological considerations

The methods used in this thesis are described in great detail in the papers. Here follows a short account of some issues.

3.1 Animals

All of the experiments presented in this thesis were carried out in neonatal mice. We make use of wild type C57BL/6 and genetically or transgenic modified mice. The majority of mouse lines used in these studies contained neuronal populations with intrinsic markers for transcription factors or specific neurotransmitters. In none of the lines used in our experiments have we noted abnormal behaviours or any physiological distinction from wild type mice. The homozygote mice EphA4$^{lacZ/lacZ}$(knockout or null) mice and the homozygote mice ephrinB3 (knockout or null) mice are the only animals that showed an abnormal phenotype. In the heterozygous mice condition these two lines presented a normal walking gate.

3.2 *In situ* hybridization and himmunohistochemistry

It is important to mention that we refrain from using immunohistochemistry in order to investigate neurotransmitter content of neurons because we discovered that glycine immunoreactivity was drastically weakened in the neonatal spinal cord by the axotomy required to retrogradely label CINs. The depletion of glycine immunoreactivity was not a consequence of the *in situ* incubation because depletion did not occur in non-axotomized interneurons in the same sections. Therefore, *in situ* hybridization was carried out

to detect the expression of GlyT2 mRNA in paper II. In the case of the glutamate transporters Vglut1 and Vglut2 *in situ* hybridization the patterns observed in paper III correspond perfectly to previous studies [43]. In the case of Vglut2 *in situ* hybridization excessive developing times (more that 30 hours) with 5-bromo-4-chloro-3-indolyl phosphate, p-nitroblue tetrazolium chloride (BCIP/NBT) may result in unspecific labeling. Therefore we took great care to keep developing times as short as possible (6-24 hours), thereby avoiding any background signal in our preparations. In general the antibodies and mRNA riboprobes used in these studies have been rigorously evaluated when reliable information has not been presented in the literature, otherwise basic assays and controls were always carried out in order to optimize antibody concentrations and visualization.

Analysis and visualization

In the case of papers II, III, IV and V, high resolution images of individual sections from confocal and normal microscopy were analysed in Adobe Photoshop to estimate the number of double or triple labeled neurons. We used the midline and the most ventral part of the section outline as reference landmarks for the alignment of sections. Schematic section maps were drawn of each image and latter superimposed to illustrate the relative positions of the different neuronal phenotypes. Although all sections came from the L2 segment in age-matched animals there is expected to be some variation in section size. We did not attempt to correct for this variation when superimposing the schematic maps (for example by stretching or shrinking them). This means that the maps do not provide a precise quantitative but rather a qualitative assessment of relative neuron positions as obtained by the profile locations. In the case of paper V, for comparison we counted the profiles in all laminae and then divided the cord into two parts, ventral and dorsal. The ventral spinal cord was, in this case, defined as the part of the cord ventral to a horizontal line extending laterally from the dorsal border of the central canal. For each putative neurotransmitter marker, counts were made in three to four different animals, in three to five sections from each animal. Neuronal profiles labeled with each of the neurotransmitter markers, Texas-Red Dextran Amines (TxRDA) or Biotinylated Dextrane Amine (BDA)-labeled cell profiles, EphA4 positive neurons, and double or triple labeled profiles were identified using the zoom and multiple layer functions. Neuronal profiles were not counted within the smear of red fluorescence from TxRDA that was often seen near the midline, and which is probably due to contamination from TxRDA that may spread along the midline and central canal after application. In paper I, colocalization of Vglut2 in the axon

terminals of motoneurons was examined with confocal microscope (LSM510, Zeiss) after making optical sections consisting of stacks of 0. 6μm-thick sections. Noise was removed from the images using wavelet software kindly provided by Jacques Boutet de Monvel (Karolinska Hospital, Stockholm).

3.3 Analysis of electrophysiological experiments

Locomotor experiments

For the locomotor experiments presented in PAPER III, we used standard extracellular recordings from ventral roots to monitor the locomotor-like activity in isolated spinal cords from mice. The drugs used to induce locomotion were a combination of 5HT and NMDA in low doses (5-20 μM and 4-7.5 μM, respectively). The period and the phase of the evoked rhythmicity were the fundamental parameters used for the analysis. The period is a measure of the interval between bursts of ventral root activity. The phase is a measure of the degree of coordination between the ventral root burst on one side for instance left L2 root (lL2) and the other side, rL2. By sampling recordings during the stable phase of pharmacologically induced locomotor like activity and by using of significant number of animals circular statistics [59] can be applied to rigorously evaluate the degree of left-right coordination (synchrony or alternation). In such analysis, the phase is represented as a circle in which 0.5 denotes alternation and 0 represents synchrony.

Motor neuron and Renshaw Cell recordings

To record from Renshaw Cells we performed whole-cell recordings from visually identified GABAergic neurons in the lumbar ventral horn close to the motor neurons using isolated spinal cord preparations taken from newborn (P0-P4) GAD67::eGFP heterozygous knockin mice. Electrical stimulation of the ventral roots was done by a glass suction electrode placed in close proximity to the exit point of the root. Neurons recorded in this way were identified as RCs by showing that electrical stimulation of the nearby ventral root evoked short-latency excitatory postsynaptic potentials, at the stimulus threshold that also caused antidromic activation of MNs. MNs were identified by the following criteria (i) They were characterized as being large neurons located in the ventral horn that could be activated antidromically from the ventral root. In accordance with classical criteria, the antidromic spike followed high stimulus frequencies (10-20 Hz) with no jitters in the activation

latencies,(ii) MNs had more hyperpolarized membrane potentials and lower input resistance than interneurons and a characteristic depolarizing "hump" on the falling phase of the action potential. Neurons were filled afterwards to confirm their identity and to perform anatomical visualization of their terminals.

Chapter 4

Results and Discussion

4.1 PAPER I: Dual neurotransmitter release of acetylcholine and glutamate from motoneurons

In this paper we use a multidisciplinary approach to describe, that mouse spinal motoneurons, in addition to acetylcholine, corelease glutamate to excite Renshaw Cells and other motor neurons but not to excite muscles.
In situ hybridization studies have suggested that rodents motoneurons contain a small amount of the RNA that could be translated into the active protein Vglut2 [43]. Immunohistochemical localization of the protein in axon terminals provided further evidence that the protein itself might be active in the synapses. Through the use of electrophysiological techniques, we finally show that glutamate is not only present but it indeed is actively released by motoneurons onto their classically known central targets, Renshaw cells (RCs)[92] and motoneurons. Interestingly, the release of glutamate did not seem to occur in the same synapses because immunohistochemical analysis of VACht and Vglut2 only showed sparse co-localization events. Furthermore, we did not observe the presence of the glutamate transporter in the neuromuscular junction and there was no electrophysiological evidence of release of glutamate by motoneurons in the muscle. The electrophysiological studies were carried out by ventral root stimulation and recording of visually identified GAD67::GFP interneurons localized close to the motoneuronal pool. Calbindin visualization in GFP positive recorded cells was used as a positive confirmation that the recordings were from RCs. The amplitude of the excitatory postsynatptic component (EPSC) in the RC was between 20-30%. However, in motoneuron-motoneuron contacts, the EPSPs were

sometimes mainly glutamatergic. The stimulation of ventral roots while recording muscle activity and applying blockers of nicotinic receptors blocked all muscle activity.
Together in this work, we have shown by electrophysiological recordings and immunohistochemistry that motor neurons contain and release glutamate in addition to ACh. These findings change a long-held notion that motor neurons, the principal neurons in the spinal cord, only use ACh as their transmitter. The experiments also suggest that motor neurons are exceptions to "Dales" principle that the same transmitter is released from all terminals in a single neuron. Apparently a complex intracellular regulation is able to selectively direct the synthesize and accumulate transmitters into vesicles in different terminal branches of a neuron (Figure 4.1).

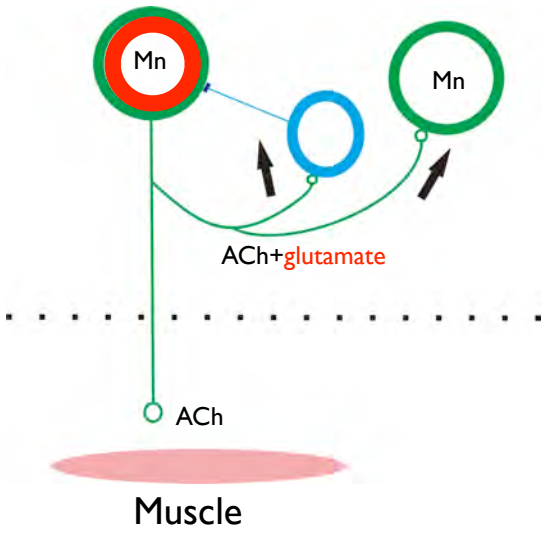

Figure 4.1: Schematic drawing of synapses formed by motor neurons. The colateral axons from motoneurons relase ACh and glutamate onto RCs and other MNs but at the neuromuscular junction only ACh is released.

Although glutamate release from motoneurons has been shown from other studies [77] the nature of the glutamate transporter is controversial since others have not seen its presence in motoneurons [71, 77]. A possible explanation for these results could be that glutamate release from motoneurons is restricted to a subpopulation of cells. We have obtained evidence for this possibility from labeling studies in a transgenic animal that express the protein Cre under the control of the Vglut2 promotor [9]. In this study we injected fluorogold intraperitonally in order to label motoneurons and performed immunohistochemistry against the nuclear protein Cre to inspect the groups of

neurons that express Vglut2. Figure 4.2 shows that some motoneurons express Cre and that the expression is low. The relative low expression of Cre in motoneuons is in accordance with low reactivity of the *in situ* riboprobes and the small component of glutamate released at the synapses. Other studies in adults cat and rats have not shown the presence of Vglut2 [71]. Alternative explanations for this results could be that glutamate is developmentally regulated. Finally, it is possible that the small sampling in these studies did not allow the visualization of the restricted population expressing Vglut2.

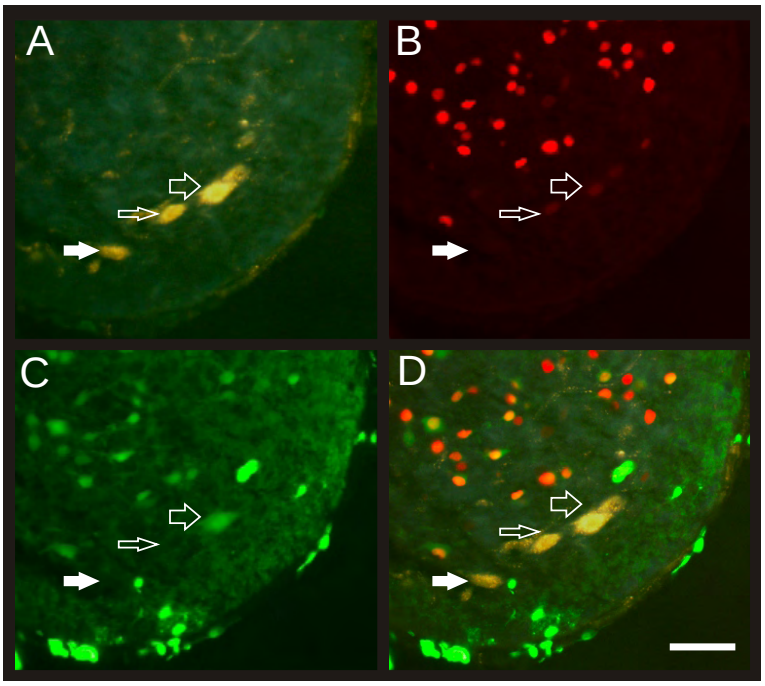

Figure 4.2: Labeling of L2 motoneurons with fluorogold in a BAC-Vglut2::Cre mouse crosssed with a YFP reporter mouse. **A.** Fluorogold labeling of motoneurons, arrows. **B.** Cre revealed weak expression of Vglut2 in two motoneurons (open arrows). **C.** The expression of YFP is only present in one of the neurons, big open arrow. **D.** Merge of previous images. Scale bar 25 μm.

4.2 PAPER II: The neurotransmitter phenotypes of commissural interneurons (CINs)

In this paper we described the distribution and relative prevalence of different CIN neurotransmitter phenotypes in the ventral region of the mammalian

spinal cord lumbar (L) segment L2. We visualize putative CIN neurotransmitter phenotypes by combining retrograde labeling of the entire CIN population in lumbar segments of the ventral spinal cord of newborn mice with *in situ* hybridization and neurotransmitter-specific GFP expression in transgenic mice. We find that putative glycinergic and GABAergic CINs comprise about 60% of the transmitter-identified CINs in the ventral spinal cord while the remainder is comprised of putative glutamatergic CINs. CINs expressing only glycinergic markers (30%) are slightly more abundant than those expressing only GABAergic markers (21%). The putative glycinergic and GABAergic phenotypes appear to be co-expressed in only a minor population of the transmitter-identified CINs (9%). When comparing the relative contribution of phenotypes obtained by electrophysiological sampling of CINs with the data obtained from the anatomical sampling here the results appear very different. Thus, in the electrophysiological studies most of the CINs were determined to be glutamatergic both for intrasegmental [91] and for intersegmental [13] connections. The most likely explanation for the discrepancies between our anatomical study and the electrophysiological studies is that they sample different pools of CINs. The entire population of CINs in the L2 segment is labeled by the midline tracer injections used here, whereas only those CINs which have direct or indirect synaptic connections to motor neurons are detected electrophysiologically. The electrophysiological sampling of motoneurons therefore excludes CINs that terminate exclusively on interneurons, whereas the population of CINs labeled retrogradely from the midline includes CINs that project to motor neurons as well as to inteneurons only. In this paper we also compare the present results with previous studies in other species. From this comparison it is clear that, with the notable exception of the Xenopus tadpole, all investigated species have both inhibitory (glycinergic and/or GABAergic) and excitatory (glutamatergic) CIN populations in the cord. However, the relative sizes of putative inhibitory and excitatory CIN populations vary. The strong GABAergic component of the CIN system found in rodents clearly deviates from what is found in all other vertebrates investigated. The GABAergic component might be an early developmental phenotype in mammals that is replaced by a purely glycinergic component later in adult life. The difference between neonatal rodents and the aquatic vertebrates, however, seems not to reflect a developmental difference but rather a fundamental difference in spinal cord organization, since putative GABAergic CINs were absent both in zebrafish embryos, Xenopus tadpoles and adults lampreys. Together, the comparison of the reported data suggests that most vertebrate phyla independent of their developmental stage use a combined inhibitory and excitatory CIN system to coordinate left-right spinal cord activity, the former mediating left-right alternation and

the latter mediating left-right synchrony [14]. However, in rodents it has been shown that segmental crossed inhibition is not only mediated by glycinergic CINs but also indirectly by excitatory CINs connecting through inhibitory interneurons located on the contralateral side [91]. This segmental dual inhibitory system may also be present in the cat [49]. Recent experiments from the lamprey have also shown that the CPG controlling fin movements contains a dual inhibitory pathway [76], suggesting that such a dual inhibitory CIN system might have evolved to control limb or limb-like (fin) movements. In addition, it has been shown that putative GABAergic CINs also constitute part of the inhibitory crossed projections and might be involved in left-right alternation in the spinal cord of neonatal rodents [44]. Excitatory CINs are also active in binding left-right motor synergies along the length of the lumbar cord during locomotion in rodents. Although glutamatergic CINs are present in cat [50] and lamprey [73] their role is unknown [7, 49]. However, populations of glutamatergic CINs found in both lamprey and cat seem to be directly involved in coordinating the left-right synchronous activity observed after blocking all glycinergic and/or GABAergic synaptic transmission in the cord [39, 49]. Interestingly, in the case of zebrafish, ablation of a type of glutamatergic CINs has been shown to affect slow frequency movements but not fast movements [75].
The present study shows that CIN projections located in the locomotor CPG area of the upper lumbar spinal cord in the neonatal rodent are heterogeneous, with at least four different putative neurotransmitter phenotypes. The relative proportions of putative excitatory and inhibitory projections suggest that both glycinergic and GABAergic CIN systems may play major roles in coordinating left-right motor activity in neonatal rodents.

4.3 PAPER III: The Role of EphA4 and EphrinB3 in the functioning of the CPG

In this paper a specific molecule is shown to affect the normal function of a CPG network. In EphA4 and ephrinB3 mutants the synchronous pattern of activity of the hind limbs can be induced in isolated spinal cord preparations. These observations suggest that EphA4 plays a role in the configuration of the spinal CPG network. An alternalive explanation is that the hopping phenotype was the result of the aberrant CST projections [25, 63]. This explanation cannot account for the results observed in the *in situ* preparation at earlies stages of development (P0-P4) because the CSTs do not reach the lumbar regions until P7-P9 [33].

Induction of rhythmic activity with NMDA and/or 5-HT in the isolated mouse spinal cord leads to locomotor-like activity with left-right and flexor-extensor alternation. This pattern can be shifted to left-right and flexor extensor synchrony if glycinergic or GABAergic inhibition is disrupted. In contrast, when the EphA4 receptor is knocked out or its normal function is impaired [6, 28] 5-HT and NMDA application leads to a hopping gait with segmental left-right synchrony and preserved flexor-extensor alternation in the isolated spinal cord. When the inhibition is boosted (by applying sarcosine or nipecotic acid) or the excitation is slightly reduced (small doses of CNQX) (unpublished Kiehn) the spinal cord activity in these animals is shifted from segmental left-right synchrony to an left-right alternation (normal walking). It was reasoned that the abnormal rhythmic patterns seen in the EphA4 knockout mice exposed a functional reconfiguration of the CPG and that the abnormal phenotype of the EphA4 knockouts was due to an abnormal crossing of excitatory neurons overriding the normal inhibitory alternating crossing system. However the inhibitory crossing system is maintained because the normal alternation can be rescued. Considering that the EphA4 molecule is suppose to play a role in the control of axonal crossing, the natural conclusion was that the abnormal phenotype was related to abnormal crossing of a population of excitatory neurons.
The above pharmacological and electrophysiological studies were suported by anatomical studies. By applying dextrans in the midline, we were able to visualize the existense of exuberant crossing processes in the EphA4 and ephrinB3. In adition it is shown that the proportion of crossing EphA4 positive interneurons in the EphA4 knockout is higher compared to the EphA4 heterozygote (normal walk). Both results seems in accordance with the role suggested for EphA4 and ephrinB3 in repelling processes away from the midline (however see paper V). The absence of one or another molecule could result in the inability of a process to maintain its ipsilateral conectivity. Finally, with the use of *in situ* hybridization against Vglut2, evidence is presented supporting the excitatory nature of some of the EphA4 neurons. Together these results suggest that the hopping phenotype in the EphA4 and ephrinB3 mutants are consequence of a change in the balance of the excitatory crossing.

4.4 PAPER IV: Phenotype of V2-derived interneurons

From the studies on the EphA4 knockout it was suggested that the abnormal gait resulted from aberrant crossing of normally ipsilateral excitatory interneurons (PAPER III). The work presented in PAPER IV aimed to investigate the possibility that the aberrant crossing in the EphA4 knockout originated in the V2 population. We were also interested in closely characterizing this population as a prelude to a physiological study of the V2 population in the functioning of the CPG [20, 26].
The V2 population is localized in band-like pattern in laminae VII and X. The V2 population is subdivided into two major groups by the differential expression of two transcription factors Chx10 expressed in the V2a and Gata2/3 expressed in the V2b population. We found that the V2a population is exclusively excitatory and glutamatergic while the V2b population is exclusively inhibitory, expressing GABA and glycine. In this paper we show that there is a developmental shift in the ipsilateral projections of V2a interneurons, from being both rostrally and caudally projecting in E13.5, to becoming mainly caudally projecting later in development. Meanwhile, V2b interneurons shift from being ipsilateral at E13.5 to having at least some processes extending over the midline at the time of birth. In all ipsilateral retrograde tracing experiments only a small proportion (10-20%) of the V2 population was retrogradely labeled, even close to the site of dye application and even when the entire hemicord was filed with dye. This means that we were not able to trace more than a small fraction of the total number of ipsilaterally projecting V2 cells and therefore we were unable to accurately determine their projection patterns. At the time we did not have a clear explanation for the relatively low percentage of retrogradely labeled ipsilaterally projecting V2 cells. However recently it was shown that most of the Chx10 neurons indeed have short local axons [26].
We also found that the V2a cells do not cross the midline aberrantly in EphA4-null mice. Despite intensive efforts to label V2a cells from the midline in EphA4-null mice, we never saw any crossing axons from V2a cells. Furthermore, as the aberrantly over-crossing axons observed in EphA4 knockout mice were labeled by the same types of dextrans as used in this study, we feel confident that V2a interneurons are still confined to the ipsilateral side and do not cross aberrantly in EphA4 knockout mice.
Taken together, this evidence suggests that there is another ipsilateral excitatory EphA4 population which does not overlap with the V2a population that has aberrant commissural projections in the EphA4-null mice. Given that

we were unable to determine the identity of 20% of all the EphA4-positive cells in the ventral half of the spinal cord and that the development of the transcription factor profile is time dependent, this would appear plausible. At the moment the only other population of excitatory ipsilateral interneurons that we have excluded is the Hb9-positive interneurons located close to the midline in the upper lumbar spinal cord, as none of these neurons express EphA4.
In summary, the work presented in PAPER IV has described the anatomical characteristics of V2 cells located in the lower thoracic and upper lumbar spinal cord, although V2 cells are distributed along the entire spinal cord as well as in supraspinal structures [70, 80]. The location of the V2 population in the ventral spinal cord, where the CPG network are concentrated, suggested that V2 interneurons may be members of the CPG networks, a situation similar to that was described for V2a-like Alx neurons in zebrafish [58]. The characterization of the V2 interneurons opens up the possibility of exploring the role of these cells in the CPG network. Such experiments, that were not included in the present thesis, showed that the V2a neurons might be involved in coordinating both left-right alternation, motor neuron amplitude modulation, descending drive and sensory drive to the CPG [20]. The data also suggest that there are further Chx10-negative, EphA4-positive excitatory interneuron populations that are yet uncharacterized.

4.5 PAPER V: Neurotransmitter phenotypes of crossed neurons in the hopping EphA4 knockout mouse

This study is an extension of the problems opened up by PAPER III and IV. In the present study we investigated the nature of the entire excitatory and inhibitory crossing populations in the lumbar spinal cord using a number of transgenic mice.
To specifically identify glycinergic, GABAergic or glutamatergic neurons, we used transgenic mice that express GFP under the promotor of the neuronal glycine transporter, GlyT2, mice where eGFP is knocked into the gene of the GABA synthesizing enzyme GAD67, or mice that specifically express Cre under the promotor of the vescicular glutamate transporter 2, Vglut2 (Borgius et al. 2010). These three mouse lines were crossed with EphA4$^{lacZ/lacZ}$ mice, which result in mice where glutamatergic, GABA-ergic and glycinergic neurons are intrinsically labeled in both EphA4$^{lacZ/+}$ heterozygous and EphA4$^{lacZ/lacZ}$ knockout mice. Neuronal processes which crossed the midline,

were visualized by applying dextran amines in the midline in the L2 segment of the spinal cord in newborn animals. We were surprised to find that the number of crossed neurons (CNs) in sections from L2 was not significantly different between the EphA4 knockout (hopping gait) and the EphA4 normal heterozygous. If this result is a true reflection of the absolute number of EphA4 positive cells, we face a conflict in relation to the assumed role of the EphA4 molecule for axon guidance in the spinal cord, namely that axons expressing EphA4 are repelled away from the midline where ephrin B3 is expressed [63]. Thus, if the EphA4 molecule is preventing the axonal crossing, why is there not an abnormal increase in the number of crossing neurons? Although aberrant crossing of spinal neuronal processes has been seen in all published studies either as a consequence of the absence of the EphA4/ephrineB3 molecules or the absence of downstream signaling of EphA4 [6, 28] we did not find an increase in the number of local-segmental crossed neurons. This suggests that the aberrant fibres originate from axons crossing away from the segments where the somata are located.

We also found that the proportion of excitatory crossing neurons increased in the EphA4 knockout and the proportion of inhibitory glycinergic crossing neurons decreased. In both cases the changes were associated with the ventral horn and are linked to the expression of EphA4. The only changes associated with the dorsal horn were an increase in the total number of CNs and a reduction in the population of glycinergic EphA4 positive crossed neurons with respect to the entire population of CN-EphA4. Together these results indicate that the increase in the EphA4 positive population seen in knockout mice is specifically related to the glutamatergic population and that this increased excitatory over-crossing may be directly involved in the switch from alternating to hopping in the knockout. The concomitant decrease of the inhibitory glycinergic crossing adds to the change in the excitatory/inhibitory balance in a direction that will favor a hopping gait.

It will be of great interest to find out which population of glutamatergic neurons is involved in the increased over-crossing. As described before, we focused on the V2a interneurons that are all excitatory and ipsilaterally projecting. However, we were not able to show any aberrant over-crossing in this population in PAPER IV, suggesting that another group of ipsilaterally projecting neurons is involved in the increased over-crossing. The cause of the changes in the balance of excitation/inhibition of the CN population and the hopping gait of the EphA4 knockout can be reasonably explained. However, we cannot explain the paradoxical results in relation to the total number of CN based on the known role for the EphA4 molecule. The results from this study have been an enlightment in this thesis because we discovered that changes in EphA4 cannot be seen as having a linear predictable effect.

The basic knowledge of the function of EphA4 is not enough to predict the complex interactions in which the molecule is involved. In experiments with knockouts mice it is therfore imperative never to assume that a change is isolated. The lesson should be : it is wrong to assume that is possible isolate changes, while "holding" the surrounding constant. Although it is an ideal for experimentalists the *ceteris paribus* assumption is a complicated one for biologists.

4.6 PAPER III and V: considerations in relation to the tracing

We discovered that the hopping gait of the EphA4 mice can be explained by local changes in the spinal cord itself. While the electrophysiology and pharmacology provided the initial data to reach this conclusion, anatomical tracing of crossed neurons was fundamental in order to investigate the substrate for the changes. In PAPER III it was shown that the exuberant branching in L2 can be seen after applying dextrans tracers in the L4 segment (see Figure 2A, from paper III; 4.3A). In this study it was also shown that the proportion of EphA4 crossing neurons was higher in the knockout mice compared to the heterozygous EphA4 mice. Since electrophysiological and pharmacological experiments showed that the synchronous activity could be changed to alternation by boosting the inhibition or slightly decreasing the excitation, it was suggested that together these results supported the existence of an aberrant excitatory crossing in the EphA4 knockout. PAPER IV showed that the abnormal crossing of EphA4 positive neurons did not originate in the V2 population in the EphA4 knockout mice. In PAPER V we therefore decided to investigate the complete population of excitatory and inhibitory crossing neurons in the EphA4 knockout mice. In this study, crossed neurons were labeled by careful application of a dextran tracer in the midline of the L2 segment (4.3B). Surprisingly, we discovered that besides an expected increase in the proportion of excitatory crossing neurons, there was a concomitant decrease in the proportion of inhibitory glycinergic crossing neurons. More striking was that the total number of crossed neurons was the same in the knockout and the heterozygous mice. Therefore, the aberrant fibers described in PAPER III posed a conundrum because there was a increase in the total number of crossing neurons.

In unpublished experiments, we have been trying to investigate the source of the aberrant crossing of fibers reported in PAPER III. The analysis shows that aberrant crossing of fibers is most clearly seen when the dextran tracer

is applied caudal (e.g. L4) to the site of investigation (e.g. L2) (4.3A). That is the protocol normally used to label descending commissural interneurons. A close examination of the labeling revealed that there is not a correspondent labeling of cell bodies at the projection area of the abnormal branching (Figure 4.3a). Therefore, the exuberant branching might not explain changes in the segmental crossing as reported in PAPER III and V but rather it explain crossing of branches from neurons located outside the segment. We have carefully injected dextran tracers into the dorsal horn in L2 in EphA4 knockout mice (4.3c) and EphA4 heterozygous mice (4.3d). With this labeling we were able to see the same abnormal exuberant fibers as reported in PAPER III. The examination of sections away from the injection site revealed few crossing neurons, suggesting that the source of the fibers is not local and might be distributed in to neurons located in several segments. Hence, the abnormal fiber crossing seen in L2 when labeling in L4 could have been a consequence of two things: i) the application site extended into the dorsal horn, and/or ii) the crossing fibers originated from ascending neurons (possibly in the dorsal horn) that have abnormal midline crossing in rostral segments in the knockouts.

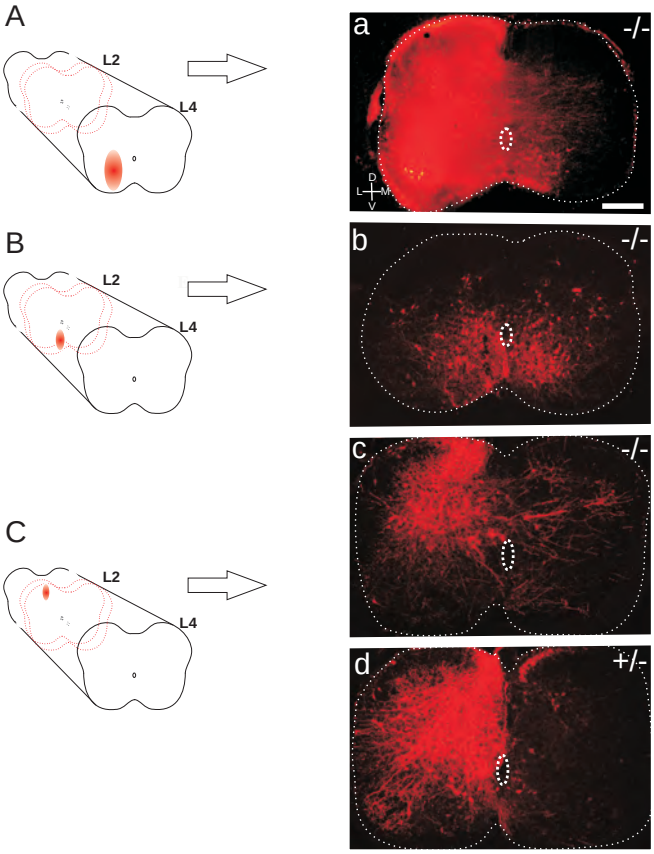

Figure 4.3: Labeling protocols. **A.** Application of tracer in caudal segments (L4) resulted in the labeling of numerous contralateral abnormally exuberant projecting fibers in the L2 segment of the EphA4(-/-) knockout (a). **B.** Careful application of tracer in the most ventral part of the cord in L2, resulted in the labeling of CNs but the exuberant projecting fibers are not evident in the EphA4 knockout (b). **C.** Application of tracer in the dorsal horn of L2, result in the labeling of of numerous contralateral abnormal exuberant projecting fibers in the EphA4(-/-) knock-out (c) but not in the normal walking heterozygous mice (+/-) (d). Scale bar 200 μm.

Chapter 5

Concluding Remarks

Anatomy, electrophysiology and molecular biology have been used in the course of this thesis as tools to work towards a better understanding of the function of locomotor networks. Although the main emphasis during this theses have been the study of the neurotransmitter phenotype of neurons localized in the spinal cord, this work was carried out to gain insights into the overall organization of the locomotor network in the spinal cord and the CPG.

Paper I Motoneurons release acethylcoline and glutamate at central synapses but not at the neuromuscular junction. Although at the central synapses motoneuros released glutamate, when contacting other motoneurons this component was in some cases mainly mediated by acetylcholine. Although, other laboratories have carried out similar studies, there is no consensus as to how glutamate is used functionally in the synapses. Together these findings change a long-held notion that motor neurons, the principal neurons in the spinal cord, only use ACh as their transmitter.

Paper II Our results show that putative glycinergic, GABAergic and glutamatergic CINs are found in almost equal numbers with a small proportion of CINs potentially having a combined glycinergic/GABAergic phenotype. In none of these populations were we able to distinguish a topographic segregation. The presence of GABAergic CINs seems specific to rodents. This study showed that CIN projections in the locomotor CPG area of the upper lumbar spinal cord of the neonatal rodent that contains circuits are heterogeneous, with at least four different putative neurotransmitter phenotypes. The relative proportions of putative excitatory and inhibitory projections suggests that both glycinergic and GABAergic CIN systems may play major roles

in coordinating left-right motor activity in neonatal rodents. The low incidence of co-expression of putative glycinergic and GABAergic phenotypes suggests that these inhibitory CIN transmitter pathways may be controlled independently.

Paper III This paper is the first description of a specific molecule that has consequences for the normal development of the mammalian locomotor network, the CPG. The abnormal hopping gait seen in EphA4 and ephrinB3 mutants is explained by the abnormal excitatory over crossing of spinal EphA4 positive neurons.

Paper IV This paper is a description of the entire V2 neuron population in lower thoracic and upper lumbar spinal cord. The characterization of the V2 interneurons opened up the possibility of explore a preliminary classification based in the expression of this trascription factor.

Paper V This study shows that there is a significant increase in the proportion of excitatory neurons crossing the midline accompanied by a decrease in inhibitory crossing in homozygous EphA4 mice as compared to heterozygous EphA4 mice. These results suggest that the hopping phenotype observed in EphA4$^{lacZ/lacZ}$ mice is due to a change in the balance between excitation and inhibition crossing the midline. The results also indicate that the Epha4 receptor is required for the normal development of crossed interactions in the rodent CPG and suggest a more complex and dynamic regulation of axon guidance via the EphA4/ephrineB3 system in the spinal cord than previous studies have proposed.

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