Neuromodulation Within a Spinal Locomotor Network

Role of Metabotropic Glutamate Receptor Subtypes

Petronella Kettunen

Stockholm 2004
All published papers were reproduced with permission from the publisher.
© 2000, 2002 Society for Neuroscience
© 2003 The American Physiological Society
Publishing and Layout: Petronella Kettunen
Printed by Larseric Digital Print AB
© 2004 Petronella Kettunen
ISBN 91-7140-079-6
ABSTRACT

The metabotropic glutamate receptors, mGluRs, are G-protein coupled receptors. Eight subtypes have been cloned and divided into three groups depending on the amino acid sequence similarity, pharmacology and their signal pathways. In the lamprey spinal cord, group I mGluRs are located postsynaptically, while group II and III are presynaptic and depress synaptic transmission. The goal of this thesis has been to elucidate the mechanisms by which the two subtypes of group I mGluRs, i.e. mGluR1 and mGluR5, modulate the firing properties of single neurons, the synaptic interactions and the overall activity of the spinal locomotor network in the lamprey.

mGluR1 activation by endogenously released glutamate increases the frequency of the locomotor rhythm induced by NMDA in the isolated lamprey spinal cord preparation. This increase in the frequency is the result of a number of cellular and molecular mechanisms that have been studied in detail. Firstly, mGluR1 potentiates the NMDA-induced current, and enhances NMDA-induced TTX-resistant membrane potential oscillations known to occur during locomotion. Mathematical simulations of the interaction between mGluR1 and NMDA receptors reproduce the modulation of the NMDA-induced oscillations and the increase in the locomotor frequency.

Secondly, mGluR1 activation depolarizes the membrane potential of neurons and consequently induces repetitive firing. These effects are due to an inhibition of a leak current responsible for setting the resting membrane potential. Interestingly, mGluR1 activates different signaling pathways to modulate NMDA current and leak conductance. Both effects require activation of G-proteins. The mGluR1-mediated inhibition of leak current requires PLC activation and release of Ca\(^{2+}\) from internal stores, as well as tyrosine kinase activation. The potentiation of NMDA current is not, however, dependent on an increase in intracellular Ca\(^{2+}\) or on tyrosine kinases.

Thirdly, activation of mGluR1 receptors gives rise to a synthesis and release of endocannabinoids from postsynaptic neurons. The released endocannabinoids act as retrograde messengers which bind to presynaptic receptors and reduce glycinergic synaptic transmission. The reduced inhibitory transmission will result in an increase in the locomotor frequency. Hence, mGluR1-activation triggers the release of endocannabinoids which thus contribute to the mGluR1-mediated modulation of the locomotor network operation.

Finally, endogenous activation of mGluR5 during locomotion decreases the burst frequency and produces long-lasting oscillations of the intracellular Ca\(^{2+}\) concentration. These oscillations are mediated through PLC and Ca\(^{2+}\) release from internal stores. Furthermore, they are also dependent on Ca\(^{2+}\) influx through voltage-dependent L-type Ca\(^{2+}\) channels. Thus, mGluR5 seems to modulate the locomotor frequency via mechanisms involving oscillations of intracellular Ca\(^{2+}\) concentration.

In conclusion, the two group I mGluRs subtypes, mGluR1 and mGluR5, use separate signaling pathways and mediate opposite effects on locomotor activity. The modulatory effects of mGluR5 seem to involve Ca\(^{2+}\) oscillations. Those of mGluR1 depend on three different cellular and synaptic mechanisms which act in concert to regulate the locomotor frequency.

Key words: mGluR1, mGluR5, spinal cord, modulation, glutamate, NMDA, leak channel, locomotion, endocannabinoids, lamprey.
ISBN 91-7140-079-6
LIST OF PUBLICATIONS

This thesis is based upon the following articles:


III Kettunen, P. and El Manira, A. Signal transduction pathways mediating the cellular effects of mGluR1 in spinal cord neurons. Manuscript.

IV Kettunen, P., Kyriakatos, A., Hallén, K. and El Manira, A. Neuromodulation via conditional release of endocannabinoids in the spinal locomotor network. Submitted manuscript under revision.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS ................................................................. 11
INTRODUCTION .................................................................................. 13
AIMS .................................................................................................. 23
METHODS ......................................................................................... 25
RESULTS AND DISCUSSION ........................................................... 29
CONCLUSIONS AND FUTURE PERSPECTIVES ............................. 37
ACKNOWLEDGEMENTS ................................................................. 41
REFERENCES .................................................................................. 43
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>acetoxymethyl</td>
</tr>
<tr>
<td>AMPA</td>
<td>amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>APV</td>
<td>DL-2-amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>CC-IN</td>
<td>crossing caudally projecting interneuron</td>
</tr>
<tr>
<td>CPCCOEt</td>
<td>7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester</td>
</tr>
<tr>
<td>CPG</td>
<td>central pattern generator</td>
</tr>
<tr>
<td>DHPG</td>
<td>(R,S)-3,5-dihydroxyphenylglycine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EPSP</td>
<td>excitatory postsynaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>IPSP</td>
<td>inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>mGlurR</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MN</td>
<td>motoneuron</td>
</tr>
<tr>
<td>MPEP</td>
<td>2-methyl-6-(phenylethynyl)pyridine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PK</td>
<td>protein kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase</td>
</tr>
</tbody>
</table>
INTRODUCTION

Networks underlying behavior

The basis of all behavior resides in the neuronal networks that interact with sensory inputs to produce motor responses. To understand and predict a behavior, simple or complex, one has to understand the underlying components of the network, ranging from the cellular and synaptic levels, down to its molecular components. The connectivity between neurons within the network, the receptors and channels that they possess and the neurotransmitters that are released, can all be modulated and alter the overall performance of the neuronal network.

Central pattern generators, CPGs, are networks of neurons that produce a specific motor behavior: i.e. a reflex, such as coughing; rhythmic movements like breathing, flying, walking or swimming; or more complex movements, for example speaking (Grillner, 2002). Examples of well studied model systems for behavior with varying levels of complexity in invertebrates and vertebrates include the mollusk aplysia gill withdrawal reflex (Kandel 2000; Pittenger and Kandel, 2003), the stomatogastric ganglion of the lobster and the crab (Harris-Warrick, 1988; Katz and Harris-Warrick, 1990; Marder et al., 1998; Nusbaum et al., 2001), the frog embryo (Dale and Kuenzi, 1997, Roberts et al., 1998, Sillar et al., 1998) and the neonatal rat spinal cord (Clarae et al., 2004; Kiehn et al., 2000, Kiehn and Kullander, 2004). The relatively simple preparations from invertebrates and from vertebrates have shown us many cellular mechanisms that also exist in higher organisms and that the network structure and physiology of lower and higher vertebrates is highly conserved (Grillner, 1985, Grillner et al., 1997).

The lamprey

The eel-like, jawless lamprey (among others: Lampetra fluviatilis, Petromyzon marinus and Ichtyomyzon unicuspis, cf. Hardisty and Potter 1982), together with the hagfish, is the most primitive living vertebrate, evolving from the main vertebrate line 450 million years ago. The larval lamprey, ammocoete, dwells burrowed in the bottom sand of freshwater streams for 3-12 years before it undergoes metamorphosis. Over a few months it develops its eyes and the characteristic suction mouth with its bony teeth (Rovainen, 1979a, b). The adult lamprey is freely migrating, with most species being parasitic using the suction cup to attach to the prey.
Fig. 1. The lamprey. The picture illustrates two stages in development in the lamprey. To the left is an adult *Lampetra fluviatilis* used for the in vitro spinal cord preparations and to the right a larval *Petromyzon marinus* suitable for primary cultures of spinal cord neurons.

The lamprey CNS has been an appreciated model system due to several advantages. Firstly, the spinal cord and brain can be easily isolated and survive for days in a cooled recording chamber. The thin, ribbon-like spinal cord is unmyelinated and fairly transparent with easily visible cells enabling electrophysiological and/or optical recordings (Bacskai *et al.*, 1995). This preparation is also accessible for pharmacological treatment due to its thin structure. Secondly, the lamprey CNS is similar anatomically and physiologically to the nervous system of higher vertebrates, but contains fewer cells making feasible to couple the properties of single neurons to simple behavioral responses. By now, the network has been mapped in some detail so that there are a number of known cell types with defined connectivity and membrane properties, making this preparation attractive as a model system (Buchanan 1982; Buchanan and Grillner 1987; Buchanan, 1996; 2001; Grillner *et al.*, 2001; Grillner, 2003).
The locomotor pattern generator in the lamprey spinal cord

Swimming is the lamprey behavior that has been most extensively studied (Cohen and Wallén, 1980; Poon, 1980). The intact lamprey swims by alternating activity (1-8 Hz) from myotomal muscles on either side of the body, forming almost one full wave at any given time (Wallén and Williams, 1984). The neuronal network underlying locomotion consists of ipsilaterally projecting excitatory (glutamatergic) interneurons that excite motoneurons, lateral interneurons and contralaterally projecting inhibitory (glycinergic) interneurons, responsible for reciprocal inhibition (Fig. 2; Buchanan, 1982; Buchanan and Grillner, 1987; Buchanan et al., 1989, Grillner et al., 1991; 1998). The network is activated by descending reticulospinal neurons from supraspinal networks in the brainstem and forebrain (McClellan and Grillner, 1983, El Manira et al., 1997, Sirota et al., 2000), acting through NMDA, AMPA/kainite and metabotropic glutamate receptors (mGluRs; Ohta and Grillner, 1989; Hagevik and McClellan, 1994; Krieger et al., 1998). The spinal cord consists of about 100 segments with around 1000 neurons per segment (Rovainen, 1979). The motor output associated with swimming can be monitored through extracellular recordings from ventral roots.

In the isolated spinal cord/notochord preparation, the locomotor activity can be elicited either by electrical stimulation of reticulospinal axons (McClellan and Grillner, 1984), by stimulation of sensory inputs (McClellan and Grillner, 1983) or even more easily by the pharmacological activation of network neurons with glutamatergic agonists, such as D-glutamate, N-methyl-D-aspartate (NMDA) or kainate (Cohen and Wallén, 1980; Poon, 1980, Grillner et al., 1981; Brodin et al., 1985). The frequency of this so-called fictive locomotion in the spinal cord/notochord preparation ranges between 0.5 and 8 Hz depending on the agonist used (Brodin et al., 1985). In the lamprey spinal cord, as in other CPG-preparations (cf. Marder and Calabrese, 1996), this rhythmic behavior can be maintained without sensory feedback and corresponds to swimming in the intact animal. This finding implies that the functional CPG resides in the spinal cord, but evidently sensory and descending activities are able to affect the performance of the CPG (Grillner et al., 1981, McClellan and Grillner, 1983; McClellan and Sigvardt, 1988; di Prisco et al., 1990; 1997)
Fig. 2. The lamprey CPG. The reticulospinal (RS) neurons excite all classes of spinal interneurons and motoneurons. The excitatory interneurons (E) excite all types of spinal interneurons, i.e. the inhibitory glycinergic interneurons (I) that cross the midline and inhibit neurons on the contralateral side, the lateral interneuron (L), which inhibit ipsilateral I interneuron, and motoneurons (M). The stretch receptor neuron are of an excitatory type (SR-E) that excites neurons on the ipsilateral side, and an inhibitory type (SR-I) that inhibits neurons on the contralateral side. RS neurons receive excitatory synaptic inputs from cutaneous afferents, the ventral thalamus (VTH) which in turn receives inputs from the basal ganglia.

Computer modeling

Even if the neurons forming a neuronal network are identified, their ion channels and receptors known and their neurotransmitters defined, their complex network function and properties might be difficult to understand or predict. However, the collected detailed information about the building-blocks of the network from the experimental process can be used in mathematical modeling to expand the understanding of network function.

A computer based model for realistic simulation of neurons and synaptic connections within the lamprey spinal locomotor network has been developed.
based on the knowledge obtained from experimental data (Ekeberg et al., 1991). These model neurons possess voltage-gated ion channels, Ca\(^{2+}\)-dependent K\(^{-}\) channels and the main ligand-gated ion channels for synaptic conductances. The model neurons were included in a model network of the lamprey CPG that reflects its known synaptic connectivity (Hellgren et al., 1992; Trävén et al., 1993). Alternating bursting activity and intersegmental coordination could be simulated with the model network. The model CPG was further developed to a neuromechanical model, including muscle tissue and the surrounding environment (Ekeberg and Grillner, 1999). This mutual exchange of information, involving predictions based on mathematical modeling and experimental verifications of the hypotheses, has proven successful for our understanding of the function of neuronal networks and has become an integrated part of the research process.

### Modulation of the lamprey CPG

Modulation of a neuronal network is required to enable a variety of behavior using the same hard-wired anatomical network when the external or internal environment is changing (cf. Harris-Warrick, 1991; Katz, 1995; Nusbaum et al., 2001; Sillar 1991; El Manira et al., 2002; Grillner, 2003). In the lamprey, there is a plasticity in the locomotor behavior from changing the speed and strength of ongoing swimming to crawling and bending the body around a local support (Rovainen, 1979). To allow the production of these diverse behaviors by the same network neurons, the motor system is subject to neuromodulation involving extrinsic and intrinsic transmitters.

A number of aminergic and peptidergic modulatory systems and metabotropic GABA\(_B\) and glutamate receptors are present in the lamprey CNS. These modulatory systems serve to fine tune the activity of the locomotor network to meet varying demands. Immunohistochemical studies have shown the existence of cells below the central canal that are immunoreactive to 5-HT, DA and tachykinins. They give rise to a dense ventromedial plexus in which the dendrites of locomotor network neurons are distributed. In this plexus, modulators are released in a paracrinic fashion because these neurons do not form conventional synaptic contacts with dendrites of spinal neurons. The frequency of the locomotor bursts is reduced either by activating 5-HT and dopamine receptors, or by blocking the re-uptake of these amines during fictive locomotion (Harris-Warrick and Cohen, 1985; Christenson et al., 1989; Schotland et al., 1995). 5-HT, through activation of 5-HT\(_{1A}\)-like receptors, blocks K\(_{Ca}\) channels mediating the AHP either directly or indirectly via an action of Ca\(^{2+}\) channels (Hill et al., 2003; Wallén et al., 1989; Wikström and El Manira, 1998). This modulation reduces the spike frequency adaptation in single neurons, which in turn delays the burst termination and results in an increased ventral root burst duration and a
decrease of the locomotor frequency. Dopamine inhibits calcium channels via activation of D$_2$ receptors, thereby indirectly reducing the amplitude of the AHP (Schotland et al., 1995).

The spinal locomotor network is also modulated by activation of GABA receptors. In the lamprey spinal cord, there are three types of GABA immunoreactive neurons (Brodin et al., 1990). Bipolar GABAergic neurons in the dorsal horn also co-localize NPY and form close appositions with axons of sensory neurons (Christensson et al., 1990; Parker et al., 1998a) to mediate presynaptic inhibition. Small neurons surround the central canal while multipolar neurons are found in the lateral grey column. The multipolar neurons make both axo-axonic and axo-dendritic synaptic contacts with network interneurons. Application of GABA re-uptake blockers reduces the burst frequency during NMDA-induced fictive locomotion (Tegnér et al., 1993). This effect is partially counteracted by GABA$_B$ receptor antagonists. Similarly, application of benzodiazepine agonists, which potentiate the activation of GABA$_A$ receptors, also reduces the locomotor frequency. There is thus an endogenous release of GABA in the spinal cord which acts on both GABA$_A$ and GABA$_B$ receptors to modulate locomotor activity. The mechanisms underlying GABAergic modulation have been analyzed in detail, GABA$_A$ receptors appear to act both presynaptically by depressing synaptic transmission from network interneurons via presynaptic inhibition (Alford et al., 1991) and postsynaptically by hyperpolarizing neurons in the spinal cord. GABA$_B$ receptors inhibit HVA Ca$^{2+}$ channels activated during the action potential and the LVA channels responsible for the postinhibitory rebound (Matsushima et al., 1993; Tegnér et al., 1993). A decrease in N- and P/Q-type HVA Ca$^{2+}$ current results in a decrease of transmitter release at the presynaptic axon terminals, while at the soma-dendritic levels it results in a decrease of the activation of KCa channels and thereby a reduction of the AHP. This modulation will reduce the spike frequency adaptation in single neurons and delays the termination of the locomotor burst. The inhibition of LVA Ca$^{2+}$ channels by GABA$_B$ receptor activation will reduce the ability of neurons to fire action potentials on the postinhibitory rebound, delaying the initiation of a new ventral root burst.

The endogenous peptide Substance P causes an increase in the locomotor burst frequency, which lasts for 24 hours or more (Parker et al., 1998b). The initiation of this effect is mediated through activation of protein kinase C that leads to potentiation of NMDA-mediated synaptic transmission. The prolonged maintenance of an increased burst frequency requires protein synthesis (Parker et al., 1998b).

Pre- and postsynaptic G-protein-coupled glutamate receptors are also important for modulating the properties of the lamprey locomotor network, and are the subject of this thesis.
Glutamate receptors

The main excitatory transmitter in the vertebrate CNS is glutamate (Watkins and Evans, 1981; Monaghan et al., 1989, Nakanishi et al., 1992). This is also the case in the lamprey spinal cord. Glutamate activates both fast-acting ionotropic (ion channel coupled) receptors, as well as slower-acting metabotropic (G-protein coupled) glutamate receptors, mGluRs.

Ionotropic receptors

Three classes of ionotropic glutamate receptors exist and were named after the agonists NMDA, AMPA and kainate. AMPA receptors are the main, glutamate receptors responsible for fast synaptic transmission (Dingledine et al., 1999). AMPA receptors are permeable to Na⁺ and K⁺ ions and in some case to Ca²⁺ (Burnashev, 1992). Kainate receptors share many molecular properties of AMPA receptors, they are activated synaptically during high frequency stimulation (Castillo et al., 1997; Vignes and Collingridge, 1997). Kainate receptors are also found on presynaptic axons where they modulate transmitter release (Kullmann, 2001; Lerma, 2003).

NMDA receptors give rise to slow EPSPs. These channels have a high single channel conductance and are permeable for Ca²⁺ ions in addition to Na⁺ and K⁺ ions (Curtis et al., 1959; Mayer and Armstrong, 2004). The NMDA channel contains a binding site for Mg²⁺ that is occupied and blocks the channels at resting membrane potentials. When the membrane potential is depolarized Mg²⁺ is relieved and the block removed (Nowak et al, 1984; Asher and Nowak, 1988). Another specific property of the NMDA receptor is the need for glycine as a co-agonist (Laube et al., 1998).

NMDA activation during fictive locomotion induces oscillations of the membrane potential in spinal neurons with an amplitude of 15-40 mV. These oscillations persist in TTX and have been suggested to be important for maintaining a slow rate locomotor rhythm (Sigvardt et al., 1985; Wallén and Grillner, 1985; 1987). The oscillations show four phases: a trough phase when the membrane potential is slowly depolarized due to voltage-activated Ca²⁺ currents, and a decrease in Ca²⁺-activated K⁺ channels (KCa). When the membrane potential reaches the level where the Mg²⁺ block of NMDA channel is relieved, the cell is rapidly depolarized. Ca²⁺ influx, through NMDA receptors and voltage-dependent channels, activates at this point KCa channels that in turn hyperpolarize the cell. When the membrane potential is returned to the level where Mg²⁺ can block the NMDA channels, the cell is rapidly hyperpolarized (Grillner and Wallén, 1985; Hill et al., 1989).
Metabotropic receptors

mGluRs are part of the family of G-protein-coupled receptors, CPCRs, possessing a 7-transmembrane domain motif and a large extracellular N-terminus with the ligand binding site and an intracellular C-terminus with binding regions for intracellular signaling molecules and phosphorylation sites (Masu et al., 1991).

Eight mGluRs have been cloned in mammals (Houamed et al., 1991; Masu et al., 1991; Abe et al., 1992; Tanabe et al., 1993; Okamoto et al., 1994; Duvoisin et al., 1995) and those found in fish and invertebrates show high sequence homology (Pin and Duvoisin 1995; Pang et al., 1994; Parmentier et al., 1996; Bargmann, 1998). mGluR1-8, have been divided into three groups, group I, II, and III depending on receptor sequence, pharmacological profile and intracellular signaling pathways (Nakanishi 1992; Pin and Duvoisin, 1995). Group I consists of mGluR1 and mGluR5, group II contains mGluR2 and mGluR3; while mGluR4 and mGluR6-8 belong to group III. Group I mGluRs have been found to be located in the peripheral parts of the postsynaptic densities (Baude et al., 1993), the group II and group III mGluRs are located presynaptically and depress synaptic transmission.

The most striking difference between the three different groups of mGluRs the underlying intracellular signaling pathway. Group I mGluRs activate phospholipase Cβ (PLCβ; Rhee, 2001), and intracellular Ca²⁺ signaling via phosphoinositide hydrolysis (Sladecek et al., 1985; Nicoletti et al., 1986), while group II and III are negatively coupled to adenylyl cyclase.

The group I mGluRs, mGluR1 and mGluR5 were originally thought to have the same functions and properties since they are both coupled to PLC and production of IP₃. However, both cellular and network studies have shown distinct functions of the two subtypes of group I mGluRs. When expressed in oocytes, the activation of mGluR1 and mGluR5 produce different patterns of fluctuations in the intracellular Ca²⁺ concentration. The pattern of mGluR1 activation consisted of one single peak while mGluR5 induced intracellular Ca²⁺ oscillations (Kawabata et al., 1996; Nakanishi et al., 1998).

Physiological effects of mGluRs in the lamprey

The three groups of mGluRs have been shown to exist in the lamprey spinal cord and their distribution and pharmacological profile is similar to that of mammals, suggesting for a conserved functional role (Fig 3.; El Manira et al., 2002). The modulation of the lamprey CPG involves activation of both presynaptic and postsynaptic receptors. Studies of the effects of mGluR activation on glutamatergic synaptic transmission have shown that group II and III mGluR agonists can act presynaptically and depress the amplitude of
EPSPs elicited by stimulations of reticulospinal axons (Krieger et al., 1996). The intraspinal and sensory synaptic transmission can also be inhibited by group II and III antagonists (El Manira et al., 2002). The presynaptic inhibition does not appear to involve a reduction of Ca\(^{2+}\) influx since mGluR agonists that reduce synaptic transmission do not reduce the Ca\(^{2+}\) current, nor do they affect the Ca\(^{2+}\) influx in reticulospinal axons. These results indicate that mGluRs depress synaptic transmission by acting directly on the release machinery. The postsynaptic effects of mGluRs in the lamprey spinal cord will be the primary focus of this thesis.

**Fig. 3. Localization of mGluRs in the lamprey spinal cord.** Group I mGluRs (mGluR1 and mGluR5) are located postsynaptically and can be activated by the specific antagonist DHPG. The antagonist CPCCOEt blocks specifically mGluR1, while MPEP blocks mGluR5. Group II and III mGluRs are presynaptic and depress synaptic transmission.

### mGluR pharmacology

An indispensable tool for carrying out this project has been the collection of specific agonists and antagonists. The field of mGluR pharmacology is rapidly developing, but unfortunately, there is still a lack of a few subtype specific ligands with high affinity. In addition, the newly developed agonists and antagonists still need extensive testing to establish their specificity in different model systems and species.

The Group I mGluRs are both activated by the agonist (R,S)-3,5-dihydroxyphenylglycine, DHPG (Ito et al., 1992). Unfortunately, the process of developing mGluR1 and mGluR5 specific agonists has not been completely successful. (RS)-2-Chloro-5-hydroxyphenylglycine, CHPG is an mGluR5 selective agonist (Doherty et al., 1997; Salt et al., 1999a), but an EC50 value of 750 µM makes it difficult to use in practice.
Among the group I mGluR antagonists, there are subtype specific compounds: 7-(Hydroxyimino)cyclopropa[b]chromen-1α-carboxylate ethyl ester (CPCCOEt; Annoura et al., 1996; Litschig et al., 1999) and (S)-(+)–α-Amino-4-carboxy-2-methylbenzeneacetic acid, LY 367385 (Clark et al., 1997) for mGluR1 and 2-Methyl-6-(phenylethynyl)pyridine hydrochloride, MPEP, for specific inhibition of mGluR5 (Gasparini et al., 1999; Salt et al, 1999b). Also in this case, drug concentrations above 100 µM are required to ensure a selective blockade of either subtype.

**Intracellular signaling of G-protein coupled receptors**

mGluRs regulate the activity of a network via the modulation of synaptic transmission; and ion channels and receptors (Anwyl 1999). This modulation is accomplished by multiple signal transduction pathways downstream of the mGluRs that are still not fully understood.

The G-protein-coupled receptors mediate their function via a GTP-binding protein. The heterotrimeric G-proteins consist of three subunits: α, β and γ. In the inactive state, the α subunit binds to GDP and the three subunits are attached together. When the α subunit binds to GTP, its affinity to the βγ subunits is decreased, resulting in their dissociation. The separated α and/or βγ subunits can then interact with their effectors, such as PLC, adenylate cyclase or PLA₂. The different G-proteins responsible for mGluR activation are Gq/11 for the PLC activated by group I mGluRs (Masu et al., 1991; Abe et al., 1992) and Gi for the group II and III receptors inhibiting adenylate cyclase (Schoepp et al., 1992; Thomsen et al., 1992; Kemp et al., 1994).

There are two types of intracellular Ca²⁺ channels residing in the endoplasmatic reticulum, ryanodine receptors, and IP₃ receptors. IP₃ receptors are activated by IP₃ produced by PLC when the membrane phosphoinositides PIP₂ is cleaved to IP₃ and DAG. IP₃ receptors are also activated and modulated by Ca²⁺. Ryanodine receptors are activated by Ca²⁺ and are often found in connection with other Ca²⁺ channels inserted in the plasma membrane. Both receptors are part of the Ca²⁺-dependent Ca²⁺ release from the ER, a mechanism that increases the cytosolic Ca²⁺ levels. The activation of intracellular Ca²⁺ receptors have been shown to play an important for patterning of fast, local Ca²⁺ sparks to bigger Ca²⁺ waves, traveling through the whole cell. Free cytosolic Ca²⁺ is a powerful signal which has a number of functions such as exocytosis and regulation of gene transcription. Intracellular Ca²⁺ homeostasis is kept fine-tuned by a number of channels, transporters, buffers and Ca²⁺ dependent enzymes (Berridge et al., 2003).
AIMS

The aims of this thesis have been to study the role of metabotropic glutamate receptors (mGluRs) in the lamprey spinal cord. I have focused on the cellular and signaling mechanisms used by the two subtypes of the group I mGluRs, i.e., mGluR1 and mGluR5, to modulate the frequency of the locomotor rhythm in the lamprey spinal cord.

The specific questions were:

- What are mechanisms behind the mGluR1-mediated modulation of the locomotor rhythm? (Paper I)
- How does mGluR1 activation affect the membrane properties of spinal neurons? (Paper II)
- Does mGluR1 use separate signaling pathways to modulate different cellular targets? (Paper III)
- Does the increase of the locomotor frequency by mGluR1 involve release of endocannabinoids within the locomotor network? (Paper IV)
- How does mGluR5 activation modulate the locomotor frequency and what are the underlying intracellular signaling pathways? (Paper V)
METHODS

A variety of experimental methods have been used to study the role of group I mGluRs in the lamprey spinal cord. Different techniques have been suitable for studying the mechanisms at different levels ranging from the network/behavioral level, through synaptic and cellular properties down to molecular mechanisms (Fig. 4). Further details about the methods can be found in the publications included in this thesis.

**Fig. 4. Lamprey brainstem-spinal cord preparation as a model system.** Left: Motoneurons and interneurons can be labeled in the intact spinal cord with FDA or RDA and dissociated. The labeled neurons can be recorded from using patch-clamp techniques to identify the subtypes of ion channels they possess Right: The spinal cord can be maintained *in vitro* and exhibit locomotor activity.

**In vitro spinal cord preparation**

The dissected spinal cord from adult lampreys (*Lamproptera fluviatilis* and *Ichtyomyzon unicuspis*) can be used for two different experimental preparations: extracellular recordings from ventral or dorsal roots; and intracellular recordings from motoneurons, interneurons and reticulospinal axons. The two dissection procedures are fairly similar except from the case that the spinal cord is fully removed from the notochord when intracellular recordings are performed. Before dissection, the lampreys were anesthetized with tricaine methane sulfonate (MS-222). The dissected spinal cord was pinned down in a sylgard-lined recording chamber with circulating physiological solution at a temperature of 8-12 °C.
Extracellular recordings from ventral roots

To study the generation and regulation of locomotor rhythm, fictive locomotion can be induced in the spinal cord/notochord preparation by bath application of NMDA (75-100 µM). The NMDA induced alternating bursting activity was recorded from opposing ventral roots with extracellular suction electrodes filled with physiological solution. Drugs were applied directly to the physiological solution perfusing the preparation. The analysis of the recorded locomotor rhythm was performed with DATA-PAC (Run Technologies, Laguna Hills, CA).

Intracellular recordings from network neurons

The fully isolated spinal cord is a good preparation for studying the synaptic connections within the CPG since the cells in the thin, ribbon-like spinal cord are easily visualized with a light microscope. The spinal cord was pinned ventral side up in the recording chamber and intracellular recordings were made from network neurons with sharp electrodes. Motoneurons can be identified by recording their axonal action potentials in a one-to-one fashion from the corresponding ventral root using an extracellular suction electrode. Crossing network interneurons were identified by recording extracellularly their axonal action potentials 2-3 segments caudal and contralateral to the intracellular recording site. Then a stimulating extracellular glass electrode was placed on the contralateral gray matter rostral to the recorded motoneuron to activate contralaterally projecting descending inhibitory interneurons. Receptor ligands or signal transduction blockers were applied to the physiological solution and non-permeable drugs could be dialyzed to the cell through the sharp recording electrode.

Isolated cells in culture

Dissociated lamprey neurons are suitable to study molecular mechanisms and cellular properties. The dishes with primary cultures can easily be pre-incubated with receptor ligands or intracellular blockers the activity of the neurons can be studied using electrophysiological recordings or measurements of intracellular Ca$^{2+}$ levels.

Dissection and cell culture

Larval lampreys (*Petromyzon marinus*) were anaesthetized with MS-222. To be able to identify motoneurons within the cell culture, fluorescein-coupled dextran amine was injected into the muscle on both sides of the body to allow
Methods

retrograde transport of dye along ventral root axons. The dorsal roots were lesioned to prevent transport of the dye in sensory neurons before dissection. The isolated spinal cord was treated with collagenase and protease before a total trituration of the tissue. The solution with the dissociated tissue was then distributed into culture dishes and incubated at 10°C for 2-4 days (cf. El Manira and Bussières, 1997).

Whole cell patch-clamp

Whole-cell patch-clamp recordings from motoneurons, interneurons and dorsal cells in the primary cultures can be performed. The setup was equipped with an Axopatch 200A amplifier. The dissociated cells were continuously perfused using a gravity-driven multibarreled perfusion system with the tip positioned close to the recorded neuron to give a distinct application of the tested drugs. The pre-labeled neurons were identified and chosen for the recordings. Voltage clamp recordings were used to monitor changes in currents over time and current clamp recordings to record fluctuations in membrane potentials in the patch clamped cells. Cells were either pre-incubated with the drugs or received them through the perfusion system. Membrane impermeable chemicals were introduced in the patch pipette.

Real-time laser scanning microscopy and Ca²⁺ imaging

Ca²⁺ imaging of primary cells in culture, pre-incubated with cell permeable Ca²⁺ indicators is a good way to study the Ca²⁺ dynamics. The membrane-permeable acetoxymethyl (AM)-ester form of the indicator (Fluo-3/AM and Fluo-4/AM) was added to the culture medium for pre-incubation with the cells. Imaging was performed with the real-time laser scanning Noran confocal setup. The Ca²⁺ indicators were excited with a 488 nm line of an argon laser and an emission filter passing wavelengths of >515 nm was used. Brightness-over-time plots were generated by sampling (7-15 Hz) the averaged intensity within a manually specified region of interest within the cell soma. Several cells could be visualized and recorded from at the same time. Changes in fluorescence (ΔF), which is a measure of changed intracellular Ca²⁺ concentration, were normalized to the resting fluorescence levels (Frest) of the cells, and the fluorescence from a region that did not include dye-filled neurons (Fbackground) was subtracted. The fluorescence data was presented as

\[ \frac{\Delta F}{F} = \frac{\Delta F}{F_{\text{rest}} - F_{\text{background}}} \]
RESULTS AND DISCUSSION

Mechanisms of group I mGluR-dependent potentiation of locomotor frequency (Paper I)

The mechanisms behind the increase in the frequency of the NMDA-activated fictive locomotion was studied at different levels of complexity, from ventral root recordings in the isolated spinal cord/notochord preparation to measurements of NMDA-induced currents and Ca\(^{2+}\) influx in isolated neurons in culture. In the intact spinal cord, the fictive locomotor frequency is increased by the group I mGluR agonist DHPG and decreased by the mGluR1 specific antagonist CPCCOEt indicating that this receptor subtype is activated by endogenously released glutamate during locomotor activity.

It has been suggested that the effect on locomotion of group I mGluRs is mediated presynaptically and involves facilitation of synaptic transmission via Ca\(^{2+}\) release from ryanodine-sensitive stores (Cochilla and Alford, 1998). Therefore, we tested whether these effects were the result of an activation of ryanodine receptors. However, blockade of intracellular Ca\(^{2+}\) release by ryanodine receptors neither altered the DHPG-mediated increase of motor activity, nor blocked the CPCCOEt-mediated decrease of the locomotor frequency.

Activation of group I mGluRs in isolated spinal cord neurons in culture by DHPG potentiated the NMDA-induced current. This effect was blocked by inhibiting G-proteins with the non-hydrolysable GDP analog GDP-\(\beta\)-S. However, loading the cells with the Ca\(^{2+}\) chelator EGTA to buffer cytosolic Ca\(^{2+}\) did not affect the potentiation of NMDA current by DHPG.

Ca\(^{2+}\) imaging on cultured cells showed that DHPG also potentiates NMDA-induced calcium signals, a potentiation which could be blocked by the mGluR1 specific antagonist CPCCOEt. Interestingly, DHPG was still able to potentiate the NMDA responses at saturated concentrations of NMDA, indicating that the active modulation of NMDA channels by mGluR1 will result in stronger NMDA responses than a mere increase in NMDA concentration.

The mechanisms underlying this intracellular modulation of the NMDA receptor was further studied. The influence of L- and N-type Ca\(^{2+}\) channels on the potentiation of NMDA channels was tested with specific channel blockers, leading to a decrease in the overall NMDA responses but leaving the potentiation of NMDA responses by DHPG unaffected. These results indicate that the source of the NMDA-induced Ca\(^{2+}\) increase is in part caused by the activation of L- and N-type Ca\(^{2+}\) channels evoked by the depolarization of the neuron. The remaining part corresponds to Ca\(^{2+}\) influx through NMDA channels.
Further examination of the signal transduction showed that the DHPG induced potentiation of NMDA Ca\(^{2+}\) responses was neither affected by blockade of protein kinase C, A or G, nor by inhibition of ryanodine receptors. These results differ from what have been seen in other preparations (Aniksztejn et al., 1991; Bleakman et al., 1992; Harvey and Collingridge, 1993; Fitzjohn et al., 1996).

The functional consequence of the interaction between mGluR1 and NMDA receptors was then examined on NMDA oscillations and locomotor frequency. NMDA application induced membrane potential oscillations that are TTX resistant (Sigvardt et al., 1985; Wallén and Grillner, 1987). These oscillations are thought to contribute to the generation of fictive locomotion and the modulation of the oscillations can explain how the locomotor rhythm is regulated (Tegnér et al., 1998). Group I mGluR activation increased the duration of the plateau phase and decreased the duration of the hyperpolarizing phase. This modulation of the NMDA-induced membrane potential oscillations could be counteracted by the mGluR1 antagonist CPCCOEt, but persisted after inhibition of protein kinase C.

To determine if the modulation of NMDA-induced oscillations by mGluR1 can account for the increase in the locomotor frequency, we used mathematic modeling of the neuronal network (Ekeberg et al., 1991; Hellgren et al., 1992; Wallén et al., 1992; Tegnér et al., 1998). The NMDA-induced TTX-resistant membrane potential oscillations have previously been studied in a cell model (Brodin et al., 1991). The effect of mGluR1 on the oscillations was simulated in the cell model and the experimental data could be reproduced. It was then tested whether the potentiation of NMDA channels is sufficient for mGluR1 to mediate the increase in the locomotor frequency. A network model of the lamprey CPG (Hellgren et al., 1992) was used to show that the NMDA receptor activation gives rise to rhythmic alternating activity and that simulation of the potentiation of NMDA receptors by mGluR1 resulted in an increase in the locomotor frequency.

This study shows that mGluR1 is located postsynaptically on motoneurons and interneurons. Its activation potentiates the NMDA-induced currents, modulates membrane potential oscillations and thereby increases the frequency of the locomotor rhythm in the lamprey spinal cord.

**Modulation of membrane properties by mGluR1 (Paper II)**

The modulation of neuronal excitability by group I mGluRs was further investigated using whole cell patch-clamp recordings from isolated cells in culture. Application of the group I mGluR agonist DHPG to cells held at resting membrane potential (between -50 and -65 mV) resulted in only a slight depolarization of the recorded membrane potential. When the cell was
depolarized to -40 mV, DHPG induced sufficient depolarization to induce firing of action potentials.

The currents involved in the depolarization by group I mGluRs were examined in voltage-clamp. In cells held at -40 mV, DHPG induced an inward current associated with a decrease in membrane conductance. However, in cells held at -60 mV DHPG did not induce any significant inward current even though the membrane conductance was decreased. The decreased membrane conductance implies that the inward current induced by group I mGluR activation results from closing of ion channels in the membrane.

The current blocked by DHPG corresponded to a leak conductance. When leak currents were present application of DHPG always induced an inward current, but when leak currents were subtracted DHPG did not induce any significant inward current. The nature of the current blocked by DHPG was tested with modified K⁺ and Na⁺ concentrations showing that the current flowing through leak channels is carried mainly by K⁺ and to a lesser extent by Na⁺ ions. The group I mGluR subtype responsible for the block of the leak current was investigated using subtype specific antagonists, showing that the blockade was mediated by mGluR1, and not mGluR5.

Finally, we examined the signal transduction underlying the mGluR1-induced modulation of the leak channels. The inhibition of leak current by mGluR1 is mediated via phospholipase C, which produces IP₃ to release Ca²⁺ from internal stores. Such mGluR-mediated release is supposed to occur since dialysis of neurons with the fast-acting Ca²⁺ chelator BAPTA and depletion of intracellular Ca²⁺ stores with thapsigargin totally abolished the effect of mGluR1 on the leak current.

These results show that mGluR1 increases the excitability of network neurons by acting on a leak conductance. This effect of mGluR1 together with its interaction with NMDA receptors will result in an increase in the locomotor frequency.

**mGluR1 activates separate signaling pathways for different molecular targets (Paper III)**

In this paper we have compared the signal transduction mediating the effects of mGluR1 on leak channels and NMDA receptors. This study adds to the understanding of the intracellular signaling mechanisms of mGluR1 in lamprey spinal cord neurons. Activation of mGluR1 potentiates NMDA receptors and inhibits a leak current which leads to the increase in the locomotor frequency seen in the isolated spinal cord. The intracellular pathways activated by mGluR1 were compared for these two cellular targets. As described previously (Paper I), G-proteins are involved in the mGluR1-mediated potentiation of NMDA currents. G-protein activation is also
involved in the case of mGluR1-induced inhibition of leak currents. What
differs between the two pathways is that the inhibition of leak channels
requires release of Ca$^{2+}$ from intracellular stores this is not the case for the
potentiation of NMDA current and Ca$^{2+}$ responses. The potentiation of
NMDA currents by DHPG was present in neurons dialyzed with the chelator
EGTA. Furthermore, NMDA Ca$^{2+}$ signals are potentiated by DHPG in spinal
cord neurons in which intracellular Ca$^{2+}$ stores were depleted with
thapsigargin.

In other model systems, the potentiation of NMDA receptors has been
shown to be mediated by Src, a tyrosine kinase phosphorylating the NMDA
receptors (Yo et al., 1997; Zheng et al., 1998). The broad-spectrum tyrosine
kinase kinase antagonists genistein and lavendustin A were used to examine if
tyrosine kinase phosphorylation is required for either the mGluR1-induced
NMDA potentiation or the block of leak current. The results show that in the
lamprey spinal cord, tyrosine kinase is required for the mGluR1-mediated
inhibition of leak currents, but does not seem to be involved in the
potentiation of NMDA receptors.

These experiments indicate that mGluR1 uses two separate intracellular
signaling mechanisms to potentiate NMDA receptors and to inhibit leak
channels. These cellular mechanisms may act synergistically to modulate the
activity of the spinal locomotor network.

**Endocannabinoids are released by mGluR1-activation to
modulate locomotion** (Paper IV)

The role of endocannabinoids in modulating the activity the lamprey
locomotor network was studied. First, the cannabinoid receptor agonist
WIN55,212-2 was applied to the in vitro spinal cord preparation, resulting in
an increase in the burst frequency recorded from ventral roots. This increase
in burst frequency resembles of that produced by mGluR1 activation with the
agonist DHPG: Conversely, application of the cannabinoid receptor
agonists SR141716A and AM251 decreased the frequency and burst
duration of the NMDA-induced locomotor rhythm. This result indicates that
cannabinoids are endogenously released during ongoing locomotion and
participate in setting the baseline burst rate.

To ascertain that the agonist WIN55,212-2 and the antagonists
SR141716A and AM251 act on the same cannabinoid receptor subtypes, we
tested the effect of WIN55,212-2 on the locomotor burst frequency in the
presence of these antagonists. The cannabinoid receptor antagonist prevented
the increase in the locomotor burst frequency induced by WIN55,212-2.

The results above show that endocannabinoids are released within the
spinal locomotor network (Fig 5A) and that a specific agonist mimics the
increase in the frequency produced by mGluR1 activation. To determine if
endocannabinoids contribute to the increase in the locomotor frequency induced by mGluR1, the group I mGluR agonist DHPG was applied in the absence and presence of cannabinoid receptor antagonists. The fact that the cannabinoid receptor antagonists SR141716A and AM251 blocked the increase in locomotor frequency induced by DHPG indicates that mGluR1-induced release of endocannabinoids is necessary to mediate the effects and mediating the increase in frequency of the locomotor rhythm.

The mechanisms underlying the increase in frequency mediated by endocannabinoids were then examined. Endocannabinoids act retrogradely and a possible target is the crossing glycinergic transmission responsible for the left-right alternation of locomotor activity. When glycinergic transmission is blocked, either by surgical methods or pharmacologically, the decreased inhibition leads to an increased locomotor frequency in the spinal cord preparation.

![Fig. 5. The effect of endocannabinoids on the spinal locomotor network. (A) Glutamate acts on NMDA and AMPA receptors to produce excitation; the inhibition is mediated by glycine released from crossing-interneurons. Glutamate also activates mGluR1 and induces release of endocannabinoids, which act as retrograde messengers to depress inhibitory synaptic transmission. (B) Spinal network neurons receive excitation (grey) alternating with crossing inhibition (black). The release of endocannabinoids (endocann., dashed trace) within the locomotor network can decrease the crossed inhibition as compared to control (solid trace) and increase the burst frequency.](image)
To examine if activation of the postsynaptic mGluR1 affected crossing-inhibitory synaptic transmission, intracellular recordings of the inhibitory postsynaptic potentials, IPSPs were made from identified motoneurons and crossing interneurons while interneurons on the contralateral side were stimulated extracellularly. The amplitude of IPSPs was significantly reduced by DHPG in control, but not when the spinal cord was pre-treated with the cannabinoid receptor antagonists SR141716A and AM251. Application of the agonist WIN55, 212-2 also directly decreased the IPSP amplitude and completely occluded the effect of DHPG (Fig 5B).

Blockade of G-proteins by GDP-β-S was used to examine whether endocannabinoids are released from motoneurons and crossing interneurons following the activation of mGluR1. In neurons loaded with the G-protein blocker, DHPG failed to decrease the IPSP amplitude. This observation suggests that activation of mGluR1 can induce release of endocannabinoids from motoneurons and interneurons that act as retrograde inhibitors to depress inhibitory synaptic transmission contributing to locomotor pattern generation and regulation in the lamprey CPG.

**Endogenous activation of mGluR5 during locomotion and its signaling pathways (Paper V)**

The group I mGluR-induced Ca\(^{2+}\) dynamics in cultured cells were studied with real-time laser scanning microscopy. The group I mGluR agonist induced long-lasting intracellular Ca\(^{2+}\) oscillations that were blocked by the mGluR5 antagonist MPEP, but not by the mGluR1 antagonist CPCCOEt. It was tested whether Ca\(^{2+}\) fluctuations were connected to any changes in membrane potential in patch-clamped cells. In cells treated with the mGluR1 antagonist and kept at rest, no fluctuations in membrane potential could be recorded when DHPG was applied. This observation indicates that the mGluR5-induced cytosolic Ca\(^{2+}\) fluctuations did not activate membrane conductances to affect the membrane potential of neurons. The intracellular pathways underlying the Ca\(^{2+}\) oscillations were examined using a phospholipase C blocker and depleting of intracellular Ca\(^{2+}\) stores by thapsigargin. Both treatments abolished the Ca\(^{2+}\) oscillations in the neurons studied.

To determine which of the two intracellular Ca\(^{2+}\) receptors in the endoplasmatic reticulum that was responsible for the release of intracellular Ca\(^{2+}\), the ryanodine receptor or the IP\(_3\) receptor, cells were pre-incubated with ryanodine, which blocks ryanodine receptors at high concentrations. The oscillations were not blocked with the ryanodine treatment which implies that Ca\(^{2+}\) release is mediated via activation of IP\(_3\)-receptors.

The role of extracellular Ca\(^{2+}\) in the generation of the Ca\(^{2+}\) oscillations was studied by using Ca\(^{2+}\)-free solution and antagonists of L-type and N-type
voltage-gated Ca\(^{2+}\) channels. In both Ca\(^{2+}\)-free solution and after pre-incubation with the L-type Ca\(^{2+}\) channel antagonist, the Ca\(^{2+}\) oscillations induced by mGluR5 were completely blocked. These mGluR5-induced oscillations did not involve activation of PKC.

The role of mGluR5 was tested in the in vitro spinal cord preparation during NMDA-induced fictive locomotion. The mGluR5 antagonist MPEP increased the swimming frequency, indicating that mGluR5 is activated by endogenously released glutamate during locomotion. However, the DHPG induced potentiation of the swimming frequency was not reduced by MPEP, indicating that mGluR5 is not involved in this mechanism. This further supports the conclusion that DHPG-induced potentiation of NMDA current and the increase in the locomotor frequency are mediated by mGluR1 (paper I).

This study presents the unique properties of mGluR5 in comparison with mGluR1; activation of either mGluR1 or mGluR5 produces opposite effects on the locomotor frequency. mGluR5 induces intracellular Ca\(^{2+}\) responses, while mGluR1 interacts with NMDA receptors. The difference in signaling pathways of mGluR1 and mGluR5 might be the result of differential efficiency in the coupling to PPI hydrolysis, which has been reported to occur (Casabona et al., 1997).
CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis is summarizing the project of elucidating the functional role of group I mGluRs in a locomotor network. In conclusion, mGluR1 and mGluR5 are both activated endogenously by glutamate during locomotion, and serve separate roles. These differences are determined at the molecular level and are reflected at the network level (Fig. 9). mGluR1-activation increases the excitability of the network neurons by potentiating NMDA current, depolarizing the neurons by blocking leak channels and releasing endocannabinoids that decrease inhibitory synaptic transmission. These three effects of the mGluR1 activation in spinal cord neurons will lead to an increase in the locomotor. In contrast, mGluR5 activation induces oscillatory release of Ca\(^{2+}\) from intracellular stores, with no detectable change in membrane potential. The effect of mGluR5 on the locomotor rhythm is the opposite of that of mGluR1 – mGluR5 decreases burst frequency.

In this project, we have also studied the molecular mechanisms underlying the modulation of network neuron by group I mGluRs and pointed out some similarities and differences with other model systems. However, regardless of the intracellular mechanism used or channels activated or blocked, the final output is often common whether it is occurring in the lamprey or for example in the neonatal rat. As an example, group I mGluRs excite motoneurons in spinal respiratory neurons (Dong and Feldman, 1999) while group II and III
mGluRs depress synaptic transmission, this is well comparable with the effects seen in the lamprey spinal cord (Krieger et al., 1998, 2000). The role of mGluRs in locomotion has also been studied in intact animals. In knock out animals of mGluR1 show motor deficits (Aiba et al., 1994; Conquet et al., 1994) and micro-injections of mGluR agonists or antagonists show the role of mGluRs in the modulation of locomotor activity (Vezina and Kim, 1999)

Still, most mammalian studies examining the role of mGluRs on locomotor behavior are limited and do not go further into the complexity of molecular mechanisms. In this perspective, the spinal cord preparation and the cultured neurons of lamprey have been valuable in linking the different levels together.

To further understand the role of mGluRs in the lamprey spinal cord and to use this knowledge to expand to other fields, there are in my opinion a few points that need further attention.

The study of the interaction between mGluRs and NMDA receptors (Paper I, III) is interesting in the sense that it concludes that the effect of mGluRs is postsynaptic and not presynaptic. This is further supported by the emerging number of studies demonstrating the intricate network of postsynaptic proteins that physically couple mGluRs to it target channels and receptors (Xiao et al., 1998; Tu et al., 1999; Grant and O’Dell, 2001). The physiological studies performed in intact neuronal networks and in intact cells have helped to describe the function of mGluR-mediated modulation on NMDA receptors. The examination of the underlying mechanisms will need the fruitful interaction between the fields of molecular biology and physiology to map the last pieces in the signal transduction puzzle.

Paper II points out the distinct differences between mGluR1 and mGluR5 regarding their possibility to modulate neuronal excitability. When compared with the findings of paper V, the two receptor subtypes use signaling mechanisms that are strikingly separate in performance and function. mGluR1 modulates while mGluR5 elicits intracellular Ca\(^{2+}\) oscillations. In addition, the description of the modulation of non-selective leak channels adds to revealing the function of the fairly novel and growing field of TASK and TREK background channels. The role of these channels in neuronal and network functions needs to be further characterized.

The discovery of the involvement of endocannabinoids in a network underlying a behavior (Paper IV) and the presentation of the idea of neumodulation can be triggered on demand invites to a new way to look upon the modulation of neuronal networks. In vertebrates, motoneurons have been considered as mere output neurons, which do not often directly modulate the synaptic input they receive. The novel mechanism using on-demand release of endocannabinoids by activation of mGluR1 suggests that also motoneurons have a modulatory potential, allowing them to contribute to locomotor pattern generation. The proposed mechanism of activating a modulatory endocannabinoid capability through activation of specific
receptors like mGluR1 may possibly be widespread in different vertebrate locomotor networks. It is still not known if the action of endocannabinoids is fast enough to act on synaptic transmission to motoneurons and crossing network interneurons in a phasic manner during fictive locomotion or if it serves to mediate a tonic modulation of synaptic efficacy. Future experiments are required to determine the importance of endocannabinoid retrograde signaling in regulating synaptic transmission during the different phases of the locomotor cycle and the role of endocannabinoids for excitatory transmission.

Paper V brings up a phenomenon that still remains enigmatic – the description of long-lasting prominent Ca\(^{2+}\) oscillations as a result of mGluR5 activation. Despite the ambitious attempts to map the Ca\(^{2+}\) dynamics in neurons during the last 20 years, the complexity of this signaling system is striking. In the case of mGluR5-mediated long-lasting Ca\(^{2+}\) oscillations, the possible functional roles could be coupled to long-term modulation of the performance of the network, including activation of genes and structural changes. Among others, Greenberg (1992) have reported the activation of genes upon Ca\(^{2+}\) influx. The timing of the Ca\(^{2+}\) waves to the ongoing locomotion as well as their subcellular localization are subjects that would be of great importance for solving the question of their significance for cellular, synaptic and network activation.
ACKNOWLEDGEMENTS

I would like to thank a number of people that have been of great support for me during my time as a graduate student:

My supervisors: Abdel El Manira and Sten Grillner.

My inspiring mentors: Carl Johan Sundberg (Fyfa/CMI at KI), Shahid Islam (SöS/KI); Jan-Olov Höög (MBB at KI); B.O. Molander (The Stockholm Institute of Education), Henrik Mickos (SU), Wen-Biao Gan (NYU School of Medicine), Don Faber (Albert Einstein College of Medicine) and Jeff Lichtman (Washington University School of Medicine).


Helpful people: Monica Bredmyr, Iris Sylvander, Tommy Nord, Lars Flemström, Katarina Eriksson and Helene Axegren.


Dan Larhammar with research group, among others Robert Fredriksson

My friends from the Biomedicine programme at the Karolinska Institutet: Tony, Andrea, Ola, Emma, George, Vendela, Teresa, Pontus, Patrik, Marianne, Anna, Mikael, Al, Janne and Kristina.

My friends at Neurobiology 2000 at the MBL


Carl Carlheim-Gyllensköld with family: Susanne, Lennart and Siri.
REFERENCES


Doherty A.J., Palmer M.J., Henley J.M., Collingridge G.L. and Jane D.E. (1997) (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) activates mGlu5, but no mGlu1, receptors expressed in CHO cells and...


Thomsen C., Kristensen P., Mulvihill E., Haldeman B.and Suzdak P.D. (1992) L-2-amino-4-phosphonobutyrate (L-AP4) is an agonist at the type IV metabotropic glutamate receptor which is negatively coupled to adenylate cyclase. Eur J Pharmacol. 227:361-262.


