Using Optogenetics and Fictive Locomotion to Investigate the Effects of Renshaw Cell Inhibition on Normal Locomotion in P3 Mice

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# Table of Contents

ABSTRACT .............................................................................................................................. 2  
INTRODUCTION .................................................................................................................... 3  
  Locomotion .......................................................................................................................... 3  
  The CPG .............................................................................................................................. 3  
  Cell Populations in the Spinal Cord .................................................................................... 5  
  Renshaw Cells ................................................................................................................... 5  
  Electrophysiology and Fictive Locomotion ....................................................................... 7  
  Optogenetics ....................................................................................................................... 7  
  Aims .................................................................................................................................... 8  
METHODS ............................................................................................................................. 8  
  Animals ................................................................................................................................. 8  
  Genetics ............................................................................................................................... 8  
  Dissection Protocol ........................................................................................................... 9  
  Fictive Locomotion ........................................................................................................... 9  
  Analysis ............................................................................................................................... 11  
RESULTS .............................................................................................................................. 12  
DISCUSSION ......................................................................................................................... 14  
  Caveats ............................................................................................................................... 16  
ACKNOWLEDGEMENTS ..................................................................................................... 17  
REFERENCE LIST .............................................................................................................. 17  
SUPPLEMENTARY INFORMATION ......................................................................................... 20
Abstract
The circuit of recurring inhibition between motor neurons and Renshaw cells in the spinal cord has been known for around 70 years, though no determined function has been outlined as of yet. Renshaw cells are thought to be part of the central pattern generator in the spinal cord establishing them as an important part of the animal’s locomotive properties. In this study we aimed to investigate the role of Renshaw cells in locomotion with the help of optogenetics and electrophysiology. Halorhodopsin was inserted into the genome of mice and driven to expression with Cre recombinase in Renshaw cells. The spinal cord of P3 mice was extracted and by inducing fictive locomotion with appropriate neurotransmitters we could inhibit the Renshaw cells in action with a green laser, opening the halorhodopsin channels for Cl⁻ ions. In previous experiments where the ability of Renshaw cells to release inhibitory neurotransmitters was inactivated, no effect was observed in either behavioral experiments or electrophysiological experiments. In a system where the effect of Renshaw cells was knocked out acutely with optogenetics there was no discernible change in fictive locomotion cycle length, frequency or amplitude. Nor was there an effect on alternation. The access of light to the Renshaw cells area might have been limited during the experiment considering the angle of light delivery and strength of the laser. Furthermore, the maturity of Renshaw cells at P3, the exclusive ability of the marker used to target Renshaw cells and the observed nature of neonatal inhibitory neurons acting as excitatory neurons could all be called into question about whether they contributed to these results or not.


**Introduction**

All higher mammals have a vast network of communication between the central nervous system (CNS) and the peripheral nervous system (PNS) in order to move and perceive their surroundings. Every cell population is communicating with a select few other populations in both the CNS and/or PNS in this network. Complications increase further when the possibilities of transmission are considered; a cell can either inhibit or promote the signal in the receiving cell, and it is only the sum of these inputs to the receiving cell that is important for the continuing signal. A few cell networks are there to coordinate all these transmissions into actions, thoughts or memories. It is cells in the CNS of the spinal cord that help control signals that trigger locomotion through the PNS.

**Locomotion**

Locomotion is the activity where an animal transports itself with a purpose to move from point A to point B. In quadrupeds the habitual procedure is to move both hindlimbs and forelimbs in an alternating pattern within the pair (left/right) and laterally (forelimb/hindlimb). The limbs would move in two phases each step cycle; the swing phase when the step is initiated and the limb is swung forward, and the stance phase when the limb makes contact with the ground. During the swing phase the limb is flexed and in contrast it is relaxed and extended during the stance phase (Muybridge, 1979). The more speed the animal wishes to achieve, the more strength is needed to complete the step cycle. To increase the strength with which the limb moves a higher number of motor neurons need to be recruited. Only the number necessary for each movement is recruited to conserve energy, but the recruited number can increase at any time during the movement. A good example would be to walk normally towards a flight of stairs exerting a continuous strain on the muscles specific to that movement. When one reaches the stairs however, more motor neurons would have to be recruited to carry the body in an upwards direction. The same is true when increasing the speed of the stride. Recent studies have found that along with the motor neurons, the appropriate interneurons are also recruited to modulate movement, enabling us finer motor control and a balanced posture. The interneurons recruited are specific for varying speeds; interneurons active at lower speeds are silenced at higher speeds and so on (Ampatzis et al., 2014; McLean et al., 2008; Zhong et al., 2011). Research on locomotion has been performed on laboratory animals of many species, with varying methods ranging from pure observational studies to experiments carried out after optogenetically altered cell populations are stimulated or inhibited, as is the case in this study.

**The CPG**

Activities in mammalian bodies are sometimes not consciously controlled by the animal. Small neuronal networks are in charge of running automated tasks like breathing, feeding in for example infants or peristaltic movements. These are necessary for our survival and the control we are permitted in each instance varies. For example, one cannot hold their breath until suffocation, the impact of the neuronal network is too strong for that. However, holding ones breath does create a pause in the automated action. There is no such way to will your
Peristaltic machinery to pause. The networks are there to perform all necessary tasks that should not require active thought. They can be vastly complicated, for example the networks controlling memories, or they can be simpler, like the ones regulating locomotion. Even those simpler locomotor networks have remained difficult to outline in mammals. Nevertheless, understanding neuronal networks can help elucidate brain and spinal cord function and suggest new treatments for neuronal diseases.

During past experiments involving cats walking on a treadmill while the connections between the brain and the spinal cord were severed it was clear that the connections from the brain are not needed for coordination of locomotion unless changes concerning speed, direction or posture need to be made. In succession the brainstem and sensory neurons of the dorsal spinal cord were ruled out in the same manner as units controlling locomotion. This led to the deduction that the network had to lie in the ventral spinal cord. The current theory involves several populations of interneurons in the lumbar segments of the ventral and dorsal spinal cord that are collectively referred to as the central pattern generators (CPG). The CPG rhythmically sends out signals coordinating locomotion with the help of several cell populations in the spinal cord. Inhibitory and excitatory interneurons help fine tune the output, recruiting the correct number of motor pools, and commissural and ipsilateral interneurons control left-right alternation (Goulding and Pfaff, 2005; Grillner, 2006, 2003; Kiehn and Kullander, 2004). In invertebrates and lower vertebrates the CPG have been sufficiently mapped out, but in higher mammals it has proved to be tougher to pin down (Butt and Kiehn, 2003). In higher mammals and primates it is unconfirmed exactly where in the spinal cord these clusters of regulatory neurons reside and indeed which cell populations is part of them. However, there are candidates that garner more support than others. The Dmrt3 neurons and Renshaw cells are examples of populations for which there is strong suspicion of their involvement. In the schematic in Figure 1 we see how the putative CPG in mice communicates with the motor neurons and the contralateral side to influence the motor output in a coordinated manner (Adapted from (Memic, 2012)).

![Figure 1: A schematic of the putative CPG in a transverse section of the lumbar spinal cord.](image)

**Figure 1**: A schematic of the putative CPG in a transverse section of the lumbar spinal cord. 
Ia=Ia inhibitory interneuron, R=Renshaw cells, EI=Excitatory interneuron, MN=Motor neuron, CIN=Commissural interneuron
Cell Populations in the Spinal Cord

Apart from the main long-distance communicators in the CNS, like the motor neurons and primary afferents, there are myriads of interneurons in the spinal cord that modulate all the input and output to these communicators. The dorsal spinal cord is divided into 6 cell groups; dI1-dI6, of which the dI6 progenitor cells differentiate into cells participating in motor circuits while dI1-dI5 are involved in the sensory circuitry (Goulding, 2009). dI6 interneurons are mainly commissural inhibitory interneurons that communicate with motor neurons as showed in a study by (Andersson et al., 2012), in which a gene was inactivated controlling the expression of the transcription factor Dmrt3 in mice. The homozygous mutant showed considerably altered locomotive synchrony and stride length in the hind legs. When monitoring cell population density of neurons rich in the Dmrt3 transcription factor during development, a shift in the ontogeny of cells was observed. When compared to wild type mice the Dmrt3−/− mutants were shown to have a large increase of neurons positive for Wilms Tumor 1 transcription factor (WT1) while exhibiting a general decrease of spinal commissural interneurons. This indicates that cells marked with Dmrt3, being only one of the populations involved, nevertheless plays a significant part in overall cell population development in the spinal cord, and shows that every part of the network communicating for successful locomotion must be balanced and functional. In the ventral mouse spinal cord which harbors the motor control of the body the interneurons are divided into four separate groups; V0, V1, V2 and V3 (Goulding, 2009). V0 interneurons are commissural and migrate to the ventral horn settling medial to the somas of motor neurons. Their axons extend 1-4 spinal cord segments rostrally through the ventral funiculus, a phenotype that does not appear when deprived of the Evx1 transcription factor (Moran-Rivard et al., 2001). V2 interneurons are both inhibitory and excitatory, and reside in ventral lamina VII and may extend their axons caudally through several spinal cord segments ipsilaterally (Al-Mosawie et al., 2007; Goulding, 2009). The varied expression of Lhx3, Chx10 and Gata2/3 characterizes the inhibitory or the glutamatergic phenotype (Li et al., 2005). V3 interneurons project glutamatergic axons contralaterally and express Sim-1 (Zhang et al., 2008). V1 interneurons are inhibitory and project ipsilaterally and rostrally (Higashijima et al., 2004). The group contains Renshaw cells and Ia interneurons but 75% of V1 interneurons do not belong to these cell populations and are unidentified as of yet (Goulding, 2009). It has been suggested that V1 interneurons are not involved in flexor/extensor patterns, but that they are involved in motor neuron burst duration and length of step cycle periods (Gosgnach et al., 2006).

Renshaw cells

One cell population that has garnered much attention due to its unique position of communication with motor neurons through recurrent inhibition is the Renshaw cells (RCs), named so after their discoverer Birdsey Renshaw (Renshaw, 1946). RCs are inhibitory interneurons situated in ventral lamina VII that receive excitatory collaterals from motor neurons and inhibit them in turn (see figure 2) (Bhumra et al., 2014). They also communicate with other RCs, contralateral proprioceptive neurons and Ia inhibitory interneurons. The embryonic V1 interneurons that later differentiate into RCs express a specific transcription factor; engrailed-1. Several other transcription factors play a role in the
development of RCs as well, specifically Pax6 as the silencing of the Pax6 transcription factor during vital periods of embryonic activity will cause a complete loss of RCs in the adult (Sapir et al., 2004).

Recognizing Renshaw cells morphologically is somewhat dependent upon the staining of a scaffolding protein called gephyrin that clusters around inhibitory postsynaptic densities (PSDs). The phenotype of RCs has a higher density of inhibitory PSDs on the dendrites proximal to the soma, which are typically larger than the average PSD. Another trait which is used as a marker of RCs is having a high concentration of the calbindin Dk28 calcium buffering protein, though this is not as unique to RCs as the size of the PSDs (Alvarez and Fyffe, 2007). Renshaw cell synapses are organized proximally to the motor neuron soma (Fyffe, 1991).

The function of recurrent inhibition of motor neurons has long been debated. Some argue that the function is simply to adjust excitatory outputs from the motor neurons enabling finer coordination of movements or posture by preventing excessive discharge of the motor neurons (Eccles et al., 1954; Mazzocchio and Rossi, 1997). However, in experiments where knockouts targeting VIAAT (vesicular inhibitory amino acid transporter) have been performed on the RC population preventing inhibitory transmission, no significant change in posture or movements has been observed. This was investigated through behavioral studies, fictive locomotion, and observational studies of gate parameters and motor coordination (Perry et al., 2015a). It has been difficult though to be sure of targeting only the RCs and no other interneuron populations as well, due to the similarity between different interneuron populations in the spinal cord. All of the specific traits of Renshaw cells taken together could help us distinguish the correct cells with confidence, but one such marker alone could misclassify some interneurons as RCs which are in not fact RCs. A new marker for RCs has been confirmed however that lets us target only the RCs; namely the cholinergic nicotinic receptor alpha 2 (chrna2) (Perry et al., 2015b). This marker appears in a few places in the CNS but has been limited to the RCs in the ventral mouse spinal cord. This discovery opens up for new possibilities for studying Renshaw cells, including the above mentioned knockout of RCs ability to fire inhibitory bursts to motor neurons in contact with them.

Discussions regarding the function of Renshaw cells have suggested their involvement with not only the motoneurons, but also possible modulation of neighboring interneurons (Alvarez and Fyffe, 2007). Additionally they have been suggested to play a role in motor neuron recruitment, synchronization of motor output as well as the modulation of synergistic and

Figure 2: A: Schematic depicting recurrent inhibition, B: Diagram of electrophysiology experiment showing the effect of Renshaw cell inhibition on Motor neurons while continuously stimulated.
antagonistic motor nerves (reviewed in Hultborn et al., 1979; Jankowska, 1992; Windhorst, 1990) (researched in Maltenfort et al., 1998; Mattei et al., 2003).

Electrophysiology and Fictive Locomotion

Neurons communicate with each other by transmitting an electrical signal along the membrane. At the cleft between two neurons the signal can either be transmitted directly by the membranes of the two cells linking through gap junctions, or by the release of chemicals from one end that ligate to receptors on the other side leading to the opening of channels that either let ions in or out. These openings determine whether the signal is transmitted further or if the neuron ceases to fire. When the ion flux over the membrane raises the membrane potential to a certain threshold the neuron fires, which is called a depolarization and the signal is transmitted further. At the point of depolarization the membrane potential has effectively been raised around 100 mV from -70 to +30. This voltage change can be measured on a cellular level and used to deduce the activity in neurons or neuronal networks. The scale can vary from single cell measurements, to measurements of the activity of an entire nerve.

Fictive locomotion is a method that can be used when studying locomotion in vitro. Adding certain neurotransmitters will induce an in vitro spinal cord to commence a firing pattern in the α-motor neurons of the ventral horn that in the animal would trigger locomotion (Rossignol, 1996). The pattern is generated at lumbar segment L2 by the central pattern generator (CPG) cells, and the alternating pattern is believed to be coordinated by commissural interneurons that cross the midline (Andersson et al., 2012). By the application of the appropriate neurotransmitters this pattern is induced. Using optogenetics the effects of acutely silencing a specific neuronal cell population can be observed in the locomotive pattern. In vitro testing of mice spinal cords is only viable up to a certain age, as the thicker the spinal cord grows, the more difficult it is to supply it with sufficient nourishment and reactant ions. In this study we have chosen to utilize P3 animals as they are the optimal choice for the electrodes and glass pipettes, and are established in electrophysiology meaning they can be compared easily to other studies.

Optogenetics

Optogenetics is a method that uses genetics to insert the genetic information required to translate light sensitive proteins in the nerve cell membrane. These proteins, called opsins, have the capacity to either open Na\(^+\) channels depolarizing the cell or Cl\(^-\) channels hyperpolarizing the cell. The proteins channelrhodopsin and halorhodopsin have respectively been derived from light sensitive bacteria (Zhang et al., 2011). The wavelength needed to activate these proteins is supplied by blue light for the channelrhodopsin (Boyden et al., 2005) and yellow light for the halorhodopsin (Zhang et al., 2007). When used correctly one can insert one of these opsins into the genome and control where it is expressed using a driver gene, for example Cre recombinase (Cre). Then it is possible to acutely inhibit or excite the particular cell/cells with temporal control. However, optogenetics can do so much more. By engineering these opsin molecules and inserting other elements we can actually control the conditions in which the cell/cells then fire organically (Tye and Deisseroth, 2012). With optogenetics we can cause the cell/cells to illicit a long subthreshold pulse, generating an
ultrasensitive environment (Berndt et al., 2009). Or we can activate an opsin coupled to a G-protein that triggers a signaling cascade making the cell more or less receptive depending on the needs of the experimental setup (Airan et al., 2009). These new methods of using optogenetics are capable of creating an environment closer to the physiological norm, which can be advantageous in some cases, like in in vivo behavioral experiments. In the present study we crossed mice carrying the silenced gene for halorhodopsin with mice carrying the cre gene fused to the chrna2 gene. This creates mice that transcribe cre recombinase along with the chrna2 subunit. This in turn leads to floxing out of the inhibitor of the halorhodopsin promoter only in cells transcribing chrna2. In the experiments employing chronic knockouts the results have been inconclusive perhaps because of the incredible ability of the nervous system to compensate for losses in cell populations. In this experiment we will direct a laser at the ventral side of the isolated spinal cord and deliver continuous light for 30-60 second periods several times over a period of 15 minutes, as was previously done in (Dougherty et al., 2013). When the system is already intact and the cell population is acutely inhibited the instant consequences should be able to be observed. The transgenic animals are their own best control, although experiments will also be performed on litters from wild type breedings.

Aims

The aim of this study is to determine whether the acute inhibition of Renshaw cells will affect the locomotive properties of P3 mice pups. This was tested by removing the spinal cord and measuring the electrical activity of the ventral roots while shining a light inhibiting the activity of Renshaw cells. The hypothesis to be tested is that Renshaw Cells affect the locomotive pattern of P3 mice during fictive locomotion through recurring inhibition.

Methods

Animals

Animals were kept in accordance with Swedish legislation and procedures were performed under the permit afforded by the local Swedish ethical committee (C135/14). The Halorhodopsin mouse was ordered from The Jackson Laboratory (B6;129S-Gt(Rosa)26SorCAG-hop/EYFP)Hze/J mouse line), while the Chrna2-cre mouse was created transgenetically in the Kullander lab by introducing the cre gene in the middle of exon two of the Chrna2 gene with the help of a Bacterial Artificial Chromosome (Leão et al., 2012; Perry et al., 2015b). The animals were crossed using heterozygous or homozygous Halorhodopsin mice and heterozygous Chrna2-cre mice. The litter mates used as wild types in the study were any members of the litter without the cre driver, whatever the results of the halorhodopsin genotyping.

Genetics

The Chrna2-cre mouse had the Cre gene under the same promoter as the cholinergic nicotinic receptor alpha 2 subunit ensuring simultaneous transcription. The Halorhodopsin mouse was created with a gene cassette of an inhibitor coupled to the halorhodopsin gene, where the inhibitor was surrounded by loxP sites. In the cells that transcribe chrna2, there will also be
transcription of cre, floxing the inhibitor of halorhodopsin. The genotype of the experimental animals was confirmed using a polymerase chain reaction (PCR) on Bio-Rad Thermal Cyclers S1000™ and T1000™, and electrophoresis nucleotide separation on a 3% agarose gel at 140 V for 25 min (250 mA, 50W) (for primers see Supplementary Table S1).

Dissection Protocol

The animals were sedated in a chamber with isofluorane and decapitated with scissors. The tips of their tails were taken for genotype confirmation. They were pinned to a Styrofoam plate by their hind limbs and fore limbs dorsal side down and the ventral skin was removed. Using scissors the rib cage was cut laterally on both sides and removed. The heart, lungs, intestinal organs, and kidneys were removed. After this the animal was moved to a petri dish with Sylgard® (Dow Corning) and pinned dorsal side down. Chilled dissection solution (see Supplementary Table S2) was added to the petri dish to cover the spinal cord of the animal. The solution was sufficiently aired prior to dissection with medical oxygen (95%, 2 bar) and carbon dioxide (5%, 2 bar). Using smaller dissection scissors connective tissue, fat and muscle was cleaned off the spinal column. The vertebral column was cut in the cervical section, and the ventral side of the vertebrae was then cut off in a horizontal lengthways motion starting at this cut. The dissection solution was discarded and refilled. The spinal cord was then cut in the sacral section. Using smaller angled scissors the dorsal and ventral roots were cut as distally to the cord as practicable while taking special care not to pull and damage the lumbar section. Any residual connective tissue anchoring the cord to the dorsal vertebrae was cleaned off. The spinal cord was moved to a chamber containing artificial cerebrospinal fluid (aCSF, see Supplementary Table S3) which was aired with medical oxygen and carbon dioxide (95%, 2 bar and 5%, 2 bar respectively) continually during both dissection and experiments as a storage space. Prior to the experiments the spinal cords were left to equilibrate in this room-tempered solution for 30 minutes.

Fictive Locomotion

Post dissection the spinal cords were transferred to a chamber (Zeiss, West Germany 47 50 52 - 9901) supplied with a continuous stream of aired aCSF via a pump (Gilson, Minipuls 3). Four small pipettes of varying opening circumferences from 9-13 μm, containing electrodes both interiorly and exteriorly (A-M SystemsInc Suction electrode Catalog #573000) (See Figure 2) were manipulated into the vicinity of the ventral left L2, L5 and right L2, L5 roots. The glass capillary pipettes were pulled using borosilicate glass (GC150F-10; Harvard Apparatus) and a PC-10 gravitational pipette puller (Narishige, Japan). Suction was used to stopper the roots into the pipettes, wherein the exposed axons of the roots were able to send a chemical signal via the aCSF through the interior of the pipettes to the interior electrode. Every wave of this chemical signal is called a burst, and it represents the part of the step cycle where the muscle would be contracting. The burst and the interburst together make up one step cycle. The signal was amplified (A-M Systems Differential AC Amplifier Model 1700) and digitized (Axon Instruments, DIGIDATA 1322A 16-bit Data acquisition system) to be read by the computer using Clampex software (10.3). The signal from the exterior pipette measuring the chemical signal of the container was used as a reference. Before the start of the
experiment excitatory drugs were added to induce locomotion, including NMDA, serotonin (5-HT) and dopamine (see Supplementary Table S3 for concentrations). A baseline was established to let the locomotion signals stabilize for at least 10 minutes from the start of locomotive signals first being measured. A green laser (wavelength 532 nm) was rigged above the chamber with the spinal cord, aimed at the L2 area and switched on and off at different time points during the experiment (for schematic see figure 3, for time table see Supplementary Table S4).

Figure 3: Schematic of lumbar section of spinal cord and light delivery. Darker green dot represents the aim of the laser.
Analysis

The frequency, length of cycles and peak amplitude was calculated using a customized program in Matlab (Mathworks, R2015b) constructed by Fabio Caixeta in the Kullander lab. Additionally the program constructed Rose diagrams from circular statistics. An arrow length limit was set displayed as a smaller circle within the diagram for significance, using the Rayleigh test as done previously in (Kjaerulff and Kiehn, 1996) for all cycles either during the periods with the light on or off. Every subject group was assembled into one rose diagram. The groups were then overlayed together to display all groups in one diagram. All other statistical tests were performed in GraphPad Prism Software (GraphPad Software inc., San

Figure 4: a: Schematic of suction electrode, b: Picture of nether part of suction electrode, c: Picture of aCSF chamber with positioned suction electrodes
Diego, USA); Wilcoxon matched-pairs signed rank test for statistical significance within the group, Mann-Whitney test between baselines of \textit{Chrna2^{Cre};Halorhodopsin}^{+/−} litter mate controls, and a Kruskall-Wallis test for the baselines of all three groups in the burst amplitude and burst area measurements.

\textbf{Results}

Renshaw cells are inhibitory interneurons situated in ventral lamina VII close to alpha motor neurons, which they communicate with through recurrent inhibition. At excitation of alpha motor neurons they send contraction signals to muscles on one hand, but they also excite Renshaw cells on the other hand, which inhibit the excitation of motor neurons. To investigate the effect of removing recurrent inhibition from the circuit we activated a halorhodopsin gene via a \textit{Cre} driver in Renshaw cells. Activating these membrane channels with light should effectively inhibit the activity of RCs.

With fictive locomotion neurotransmitters we induced rhythmic locomotive signals measured in the ventral roots of L2 and L5. The locomotive rhythm of control cords was visually distinguishable from \textit{Chrna2^{Cre};Halorhodopsin}^{+/−} cords during light delivery periods (figure 5). However, when regarding images of rose diagrams assembled in Matlab they do not show a discernible difference between the groups. Rather, most cords whether control or experimental showed some tendencies towards abnormal alternation at some point in the experiment (figure 6).

Figure 5: Raw traces of \textit{wt/wt} control cords, \textit{n}=1, \textit{wt/wt} litter mate control cords, \textit{n}=1, and \textit{Chrna2^{Cre};Halorhodopsin}^{+/−} experimental cords, \textit{n}=1. Arrows indicate start and finish of light delivery.
As alternation was in fact not expected to be affected we wanted to investigate other parameters more related to Renshaw cell function. If RCs inhibit motor neurons to prevent excessive firing or to simply modulate the output to the muscles, then the effect could be expected to be stronger bursts (higher burst amplitude) or longer bursts (longer cycle length or burst area) during light ON periods. During subsequent analysis of the measurements from the L2 roots the change in locomotive rhythm during periods of light delivery appeared to be statistically significant regarding amplitude within the Chrna2Cre;Halorhodopsin+/− group and litter mate control group, but not the wt/wt control group (figure 7C-7D, p(Chrna2Cre;Halorhodopsin+/−)=0.0002, p(litter mate controls)<0.0001, p(wt/wt)=0.2858). Cycle length measured during light on periods reached statistically significant differences compared to light off periods for the Chrna2Cre;Halorhodopsin+/− group, but not the litter mate control group (p(Chrna2Cre;Halorhodopsin+/−)<0.0001, p(litter mate controls)=0.9663). In burst area the Chrna2Cre;Halorhodopsin+/− group was significantly different during light on periods from light off periods, and so was the litter mate control group, while the wt/wt control group was not (p(Chrna2Cre;Halorhodopsin+/−)=0.0057, p(litter mate controls)<0.0001, p(wt/wt)=0.9217). When comparing the baseline, i.e the light Off periods, of each of the groups to each other the baseline of Chrna2Cre;Halorhodopsin+/− and the litter mate control group were statistically different from each other regarding cycle length (p < 0.0001), and all three groups were statistically different from each other in the baseline of amplitude and burst area (p<0.0001, p<0.0001 respectively).

In the L5 roots the results were similar. In amplitude the Chrna2Cre;Halorhodopsin+/− group and litter mate control group obtained significant results, while the wt/wt control group did not (p(Chrna2Cre;Halorhodopsin+/−)<0.0001, p(litter mate controls)=0.0022, p(wt/wt)=0.4140). In cycle length the Chrna2Cre;Halorhodopsin+/− group obtained significant results, while the litter mate control group did not (p(Chrna2Cre;Halorhodopsin+/−)=0.0391, p(litter mate controls)=0.8524). In burst area the Chrna2Cre;Halorhodopsin+/− group and the litter mate control group obtained significant results, while the wt/wt control group did not (p(Chrna2Cre;Halorhodopsin+/−)=0.0266, p(litter mate controls)=0.0009, p(wt/wt)=0.7725). When comparing the baselines of the groups the Chrna2Cre;Halorhodopsin+/− group was significantly different to that of the litter mate control group regarding cycle length. The three groups were also significantly different from each other regarding both amplitude and burst area (p(cycle length)=0.0002, p(amplitude)<0.0001, p(burst area)<0.0001).

Figure 6: Rose diagrams depicting the alternation between left and right in the forelimbs in experimental cords, litter mate cords and wt/wt cords. On the left is the alternation when the light is ON and on the right is the alternation when the light is OFF. Black: Experimental cords, n=6, Blue: Litter mate cords, n=3, Red: wt/wt cords, n=2. Dots represent individual cord angles.
Discussion

Visually a few differences between the independent wt/wt group, litter mate wt/wt group, and Chrna2\textsuperscript{Cre};Halorhodopsin\textsuperscript{+/−} group do appear. In figure 5 a marked change in rhythm occurs in these sample snapshots of the raw traces. However, the mutant traces look slightly less rhythmic than the wild types both before and after the light is on as well. In individual rose diagrams we see tendencies of change in the light period also. However the alternation was not expected to be affected at all as the study by (Gosgnach et al., 2006) determined that there was no involvement of V1 interneurons in left/right alternation or flexor/extensor alternation. All data taken together masks these few tendencies and statistically there is no change in cycle length from before the light is switched on compared to during (figure 6).

RCs are able to inhibit excited motor neurons when stimulated (Bhumbra et al., 2014; Eccles et al., 1954; Renshaw, 1946). The fact that this occurs with the help of recurrent inhibition indicates a close connection between these two cell types. The expectations for this study depended on this supposition of close cooperation. That there is a recurrent inhibition whereby motor neurons inhibit themselves upon their own excitation indicates that the function is a fine tuning of locomotion, movement or posture. The ventral roots that have been recorded in this study are affiliated with locomotion. Without this self-inhibition the motor neuron could be expected to fire more freely and perhaps for longer or stronger bursts. This in turn could affect the rhythm of movement in locomotion, leading to completely synchronous or asynchronous walking, as was the case for Dmrt3 mutants in (Andersson et al., 2012) when
the extension phase of the hind legs was prolonged. More likely though is a simple change in cycle length and/or amplitude, maintaining alternation. When regarding the results of all the experiments however, the indication is that Renshaw cells do not affect locomotion in this setup.

True, the results of the statistical tests implemented show that there is a difference between light on and light off periods in the Chrnα2Cre;Halorhodopsin+/− group. However, this same significance was often seen in the corresponding fashion within the litter mate control group. Another unexpected piece of information delivered by the statistical analysis was the fact that all baselines were in fact significantly different from each other in all three measurements and all three groups. Theoretically there should be no difference between mutants and wild types in the baseline since the halorhodopsin protein should only be active when light is delivered to the Renshaw cells and its presence in the genome should not disrupt the normal function of the mouse. From this the conclusion can be made that not enough subjects were used in the study as the individual variance can be quite large; the number of wild types was lower than the number of Chrnα2Cre;Halorhodopsin+/− animals. This also seems to be the issue when considering the results of the alternation analysis of the wt/wt group. That the alternation is more stable for the Chrnα2Cre;Halorhodopsin+/− group than the wt/wt group is something that strengthens the conclusions made in (Gosgnach et al., 2006) wherein they exclude RCs from having an effect on flexor/extensor alternation. Unfortunately it here also demonstrates the difficulties of achieving stable results through this method on which to build an argument upon without using larger batches of experimental animals.

According to the study by (Bui et al., 2008), the positioning of Renshaw cell synapses onto motor neurons and their strength suggest that their function is to ameliorate the excitatory input from mainly Ia primary afferents. It then follows that in this experimental setup, in which the cell bodies of primary afferents are missing, no effect concerning these would be seen. Additionally, Renshaw cells might not have reached full maturity at the time of the experiments as the branch connecting RCs to motor neurons has only been observed to form by P4, introducing the possibility of missing synapses and faulty firing mechanisms in our P3 subjects (Alvarez et al., 2013; Mentis et al., 2006). Furthermore, several papers have found that inhibitory neurons can function in an excitatory manner in neonates while cytosol concentrations of calcium have not stabilized (Owens et al., 1996; Wu et al., 1992). However, performing fictive locomotion experiments on mice of age P7 or over would be significantly more difficult and would involve perfusing the tissue first and risking cell death of motor neurons both during perfusion and during experiments, when the penetrance of the aCSF might not be enough. While all these difficulties are daunting, to use this same setup to investigate the link between RCs and locomotion in P7 or older animals could be more enlightening if successful.

The fact remains that a few convincing experimental spinal cords showed a tendency towards both having longer cycle lengths during the light on periods, and having a slightly skewed alternation pattern in both the L2 ventral roots and the L5 ventral roots. The above mentioned developmental characteristics of Renshaw cells apply to this situation, but it is important to note that the switch from excitatory properties to inhibitory properties is a gradual change...
rather than a sudden happenstance. Therefor one can argue that the spinal cords that respond as we expected could be spinal cords that have matured faster or have become more inhibitory compared to its litter mates and the other samples in the study.

**Caveats**

To fully understand why our results were so far from our expectations, we must investigate further the accuracy of the Cre-line. In fact, it is known that another population in the spinal cord is also affected in this breeding (Perry et al., 2015b). This population lies at the dorsal midline and its effects on the locomotive properties of the spinal cord are not known. However, the possibility remains that this population somehow compensates for the acute inhibition introduced for the RCs. In such a case the population could be an excitatory cluster of cells itself, and the inhibition of them cancels out the effect of the RCs, coincidental though it may seem. Additionally it is of course possible that other cells communicating with RCs or motor neurons compensate for the sudden loss in a fail-safe type of mechanism.

Another explanation for these unstable results may lie in the slight differences in damages made during the dissections from cord to cord, or the sensitivity of the instruments which could vary depending on the layer of chloride ions coating the silver electrodes, the fit of the pipettes to individual roots, and the exactness of the concentration of the administered drugs, which could affect the frequency. With these possible sources of variation one could argue that spinal cords that respond out of character when measuring even the baseline could be disregarded, as the instability of the baseline would affect the overall “light off”-period. In fact many such cords were excluded prior to final analysis. However, the cut might not have been specific enough.

The most important caveat of this study is the effectiveness of the light delivery system. Not only was the strength of the laser varied over the days of experimentation due to the battery fading and replacement, but the accuracy was debated. Since the RCs lie close to the surface of the ventrolateral spinal cord it was assumed that the strength of the laser would be enough to activate the halorhodopsin channels in most of them. The midline population also targeted by the gene modification was assumed to not influence the output of the ventral roots. However, the laser was directed at the midline between the ventral roots of L2, meaning it had to be strong enough to transmit the light waves through more cell layers than would have been necessary if the laser was directed from the lateral side (Figure 3). Perhaps a fiber optic laser trained directly on the RC area, directed from the lateral side, of each of the ventral roots measured would have been advisable. To fully rule out the possibility of the RCs playing an important part in locomotion one such study should be conducted.

However, if we want to look further and speculate that Renshaw cells do not affect locomotion in either alternation or shape of the burst, there are other theories that could be interesting to investigate. In the study by (Mazzocchio and Rossi, 1997) they discuss how Renshaw cells could be implicated in posture. Perhaps an acute knockout could be performed here as well to elude compensation as posture was investigated on the rotating beam test in (Perry et al., 2015a) for the VIAAT knockout. If the method for delivering light to the spine in
vivo is ever fully developed, then personally I believe the future of Renshaw cell research could be on the way to exciting new discoveries.

Acknowledgements

Firstly, I am very grateful for Klas Kullander as he again welcomed me into his lab and found me a great project. I am also very thankful to Fabio Caixeta for valuable input on the method of fictive locomotion and for somehow finding time during the holidays to help me analyze my mountains of data. I would also like to thank Magnus Lindgren, Jennifer Jagdmann, and Steven Edwards for support and opinions relating to my material. Finally I must thank my extraordinary supervisor Sharn Perry, for teaching me so much about life in a lab, for demonstrating more techniques than was required to my extreme delight, and for invaluable input towards writing my thesis.

Reference List


Li, S., Misra, K., Matise, M.P., Xiang, M., 2005. Foxn4 acts synergistically with Mash1 to specify subtype identity of V2 interneurons in the spinal cord. PNAS 102, 10688–10693.


Supplementary Information

**Supplementary Table S1**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Halorhodopsin</th>
<th>Chrna2-Cre</th>
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<tbody>
<tr>
<td>Wt forward</td>
<td>5'-TCC CAA AGT CGC TCT GAG TT-3'</td>
<td>Forward</td>
</tr>
<tr>
<td>Wt reverse</td>
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<td>Reverse</td>
</tr>
<tr>
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**Supplementary Table S2**

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**Supplementary Table S3**

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**Supplementary Table S4**

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