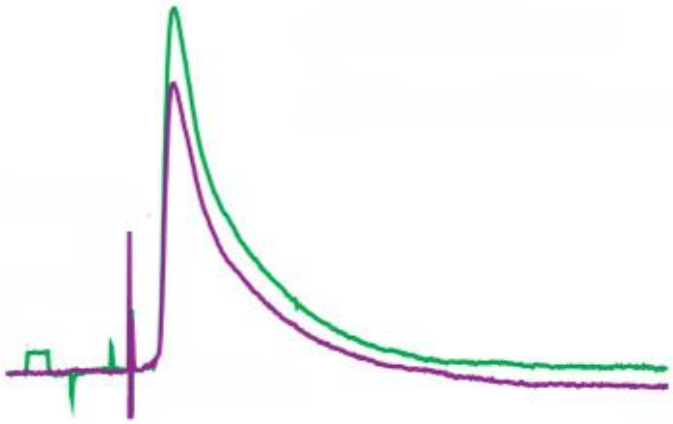
FACULTY OF SCIENCE
UNIVERSITY OF COPENHAGEN

Bachelor Thesis

By Anne Hedegaard

Measuring Post Activation Depression in adult mice in vivo

In normal and transgenic mice with motoneurone disease



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Resumé

Dette bachelor projekt undersøger om fænomenet Post Aktiverings Depression (PActD) findes i mus, og i så tilfælde, hvorvidt det ændres i lidelser hvor spasticitet er et karakteristika. PActD påvirker en rygmarvsrefleks som kontrollerer bevægelser, dvs. det sensoriske input fra en muskel til en motor neuron som kontrollerer den muskel påvirkes.

PActD opstår som følge af gentagen stimulering (inden for en tidsramme af sekunder) af sensoriske afferenter, hvilket aktiverer den monosynaptiske Ia refleks. Depressionen ses som en reduktion af det resulterende Ia EPSP's (Excitatorisk PostSynaptisk Potentiale) størrelse. Denne effekt forekommer i raske spinale motorneuroner og er blevet undersøgt indirekte i mennesker ved målinger af H-refleksen samt ved direkte intracellulære målinger af Ia EPSP'er i katte.

Det er tidligere blevet vist at en reduktion i PActD er involveret i forskellige menneskelige lidelser som har symptomet spasticitet til fælles. Den nylige udvikling af transgene musemodeller som afspejler disse lidelser gør det fordelagtigt at studere lidelserne og PActD i mus. Det primære formål i denne opgave var at undersøge om det overhovedet er muligt at måle PActD i voksne mus in vivo ved brug af intracellulære (elektrofysiske) metoder.

Det lykkedes at bekræfte at PActD er til stede i raske mus samt at vise at størrelsesordenen og varigheden af formindskelsen af EPSP'erne kvantitativt svarer til resultaterne fra forsøgene med katte. Jeg bekræftede en signifikant reduktion i PActD effekten i forsøg udført på mus med motorneuron sygdomen Amyotrophic Lateral Sclerose (ALS) som involverer spasticitet, sammenlignet med de raske mus. Derudover eksperimenterede jeg med teknikken 'voltage clamp' med det formål at stabilisere membran potentialet i de celler der måles fra.

Abstract

This bachelor thesis investigates whether the phenomenon of Post Activation Depression (PActD) exists in mice and if so, whether it changes in disorders characterized by spasticity. PActD affects a spinal reflex that controls movement, i.e. it affects the sensory input from a muscle to a motoneurone innervating that muscle.

PActD arises from repetitive stimulation within seconds of sensory afferents, which activates the Ia monosynaptic reflex pathway. The depression is seen as a reduction in the size of the resulting Ia EPSP (Excitatory PostSynaptic Potential). This effect is found in normal spinal motoneurones and has been explored indirectly in humans, measuring H-reflexes, and by direct intracellular measurements of the Ia EPSPs in cats.

Reductions in PActD have been shown to be implicated in different human disorders involving spasticity. Given the recent development of transgenic mouse models of such disorders it would be advantageous to be able to study this in mice. The primary aim of this thesis was to see if it was even possible to measure PActD in adult mice in vivo using intracellular (electrophysiological) recording.

I was not only able to demonstrate that PActD is present in healthy mice, but also that both the magnitude and the time course of the phenomenon appear to be quantitatively similar to that which has been shown in cats. Using the transgenic mouse model of the motoneurone disease Amyotrophic Lateral Sclerosis (ALS), of which spasticity is a symptom, I confirmed a significant reduction in PActD in this mutant. Furthermore, I also demonstrated that changes in membrane potential can be controlled for using voltage clamp.

Preface

The present study serves as a thesis for the bachelor degree in Physics with a specialization in Biophysics at the University of Copenhagen, Faculty of Science. The work was carried out between February and May 2012 in the Mouse Laboratory of Neural Control of Movement, Department of Neuroscience and Pharmacology, Faculty of Health Science, University of Copenhagen. Internal supervisor was Thomas R. Heimburg (Niels Bohr Institute) and external supervisor was Claire F. Meehan (Panum Institute).

Acknowledgements

I have been granted guidance beyond ordinary expectations and would like to thank Claire Meehan for being such an engaging supervisor who always provided extensive and comprehensive answers to my many questions. She made learning difficult skills enjoyable. Furthermore, I thank the following people for their contribution to this project:

- Lillian Grøndahl and Claire F. Meehan who provided technical assistance with the necessary surgery
- Stefan Marklund and Karin Graffmo, Umeå University, Sweden, who created the mutant SOD1^{G127X} mice.
- Mihai Moldovan who was responsible for introducing and maintaining the SOD1^{G127X} colony in Copenhagen and who gave me helpful advice on statistics
- Hans Hultborn who gave critical feedback on early drafts of my thesis
- Nicolas C. Petersen who helped program the configuration we used with the Signal software

In this thesis I was involved in the original design and implementation of the experimental protocol. I performed the electrophysiology experiments under the guidance of Claire F. Meehan and I was responsible for the analysis of all the data presented, including the statistical analysis.

Abbreviations

AP: Action Potential
ALS: Amyotrophic Lateral Sclerosis
CDP: Cord Dorsum Potential
CP: Common Peroneal
DCC: Discontinuous Current Clamp
ECG: ElectroCardioGram
EPSP: Excitatory PostSynaptic Potential
IPSP: Inhibitory PostSynaptic Potential
PActD: Post Activation Depression, this abbreviation is chosen to avoid confusion with the more commonly used PAD referring to Primary Afferent Depolarization.
PBD: Post Burst Depression
SEVC: Single Electrode Voltage Clamp
SOD1: SuperOxide Dismutase-1 enzyme, mutated with a TGGG insert
Tib: Tibial
V_m: membrane potential

1 Introduction

Being able to obtain reliable intracellular measurements of even small events in adult animal models in vivo has many uses in the neuroscience field. Specifically for this project, subtle changes in the effect known as Post Activation Depression (PActD) are investigated in both healthy and mutant mice because the effect may be part of the pathophysiology underlying the human disorder spasticity.

The actual electrophysiological experiments utilize the way that motoneurones (neurones innervating muscle movement) communicate, so firstly, a brief outline of how nerve signals arise and are conducted is given. Then follows a more explicit insight into the background of this study, presenting the spinal pathway in which the PActD can be measured and an explanation of how PActD is believed to be linked to motor disorders.

1.1 Origin of membrane potentials

A neurone composes of a cell body or 'soma', which receives signals from other neurones via its dendrites and from the soma an axon extends which is isolated with myelin except for exposed points named nodes of Ranvier. The neurone passes on signals from the soma through its axon to the axon terminals where synapses with other neurones are formed.

According to the conventional view, the signalling of neurones depends on the flow of certain ions between the intracellular and extracellular regions of the neurone. The concentrations of cations and anions are considered equal within the bulk of the inside and outside media, but close to either surface of the neural membrane, a small excess of ions are accumulated in such a way (cations outside, anions inside) that a potential difference of about -50 to -80 mV is maintained while the neurone is at rest, i.e. not conducting signals.

In general the membrane potential V_m is calculated as a difference between the intracellular and extracellular potentials: $V_m = V_{in} - V_{ex}$ but it is common practice to regard the extracellular potential as zero.

At rest, each ionic species involved in neuronal signalling (Na^+, K^+, Ca^{2+} and Cl^-) diffuse passively over the membrane through permeable ionic channels (trans-membrane proteins). The direction of this diffusion of each ionic species is determined by both an electrical gradient and a concentration gradient which in combination are known as the electrochemical gradient. Electrically, the ions are attracted to potentials of the opposite sign, i.e if $V_m < 0$, cations flow inside and anions out, and vice versa for $V_m > 0$. Chemically, the ions flow down their concentration gradient, from high to low concentration. This flow is passive, but ion pumps (running on ATP) actively transport the ions back again to maintain the concentration gradient, with the result that the chemical force on the ions is constant[1].

With the chemical force being constant, the passive flow of the ions vary with the membrane potential, and at which value of V_m the flow reverses can be calculated for each ionic species from the Nernst equation[1]:

$$E_{rev} = \frac{RT}{zF} \cdot ln\left(\frac{[ion]_{ex}}{[ion]_{in}}\right)$$

R = gas constant, T = temperature in Kelvin, F = Faraday constant, z = valence of ion

The reversal potential, 'E_{rev}' thus resembles the V_m necessary to oppose the constant chemical force. The electrochemical gradient for each ionic species, also known as the driving force 'U', can then be expressed as the difference between actual membrane potential and reversal potential: $U = V_m - E_{rev,ion}$

Under resting conditions, $E_{rev,Na^+} = +58$ mV and $E_{rev,Ca^{2+}} = +116$ mV which, in combination with open ion channels, will cause an inward flow of these cations until a depolarization of the membrane potential is reached. Conversely, $E_{rev,K^+} = -84$ mV which drives an outward flow of this cation to hyperpolarize the membrane potential if it is in a depolarized state. As previously mentioned, the usual $V_{m,rest}$ is between -50 to -80 mV, i.e. normally close the reversal potential of K^+, which point to the fact that the permeability, 'p_{ion}', of Na^+ and Ca^{2+}

channels is much lower than that of K^+ channels. $V_{m,rest}$ can be calculated according to the Goldman-Hodgkin-Katz equation[1]:

$$V_{m,rest} = \frac{RT}{F} \cdot ln\left(\frac{p_K \cdot [K]_{ex} + p_{Na} \cdot [Na]_{ex} + p_{Cl} \cdot [Cl]_{in}}{p_K \cdot [K]_{in} + p_{Na} \cdot [Na]_{in} + p_{Cl} \cdot [Cl]_{ex}}\right)$$

A length of neuronal membrane, e.g. the axon, can be viewed as a cable through which ionic currents travel. The current encounters resistance inside the 'cable' both in terms of the intracellular fluid, which has poor conduction properties, as well as when it flows between inside and outside. Here the charged particles encounter resistance in shape of the ionic channels which are not just open pores in the membrane, but ports with a limited size and selectivity that restricts which type of ion can pass and how many at a time.

The ionic current is strongest when the driving force is largest, thus when the actual membrane potential is furthest away from the reversal potential of the ionic species. The membrane separates oppositely charged pools of ions, hence functioning as a capacitor. Under the influence of a constant current, the biological membrane also takes time to charge and discharge when the current is taken off[1], just like its electrical counterpart.

All these electrical properties of the neuronal membrane can be measured from 'outside' with an intracellular electrode (inside a glass micropipette) through which ionic currents and their resulting field potentials can be recorded.

1.2 Neuronal signalling

The actual signalling of neurones is conducted via Action Potentials (APs), which are characterized by a fast depolarization phase followed by a repolarization and hyperpolarization of the axonal membrane making it resemble a spike. APs are initiated from the axon initial segment (a segment of axon with high Na^+ channel density proximal to the soma), and travel down the axon via passive conduction between the nodes of Ranvier (non-myelinated parts of the axon, with large clusters of Na^+ channels) where the AP gets 're-activated' and travels onwards in one direction.

Most neuronal axons are 'isolated' with myelin which reduces ion current leak and thus increases the passive travel distance and conduction velocity considerably[1]. Re-activation happens due to opening of voltage gated Na^+ channels, and the depolarizing rise phase is fast because a few open Na^+ channels will increase the initial depolarization and trigger more Na^+ channels to open, thus reinforcing the Na^+ inflow, until the time when the channels inactivate.

The one-directionality of the AP is an effect of the refractory period of the ion channels, in which they cannot be re-activated within a certain timespan. Repolarization occurs when the depolarization reaches a level where voltage gated K^+ channels open to let K^+ out of the nerve cell thus restoring the balance between cations and anions. Because the K^+ channels inactivate fairly slowly, the resting potential is usually exceeded and the membrane becomes hyperpolarized after the AP has passed[1].

The onset of depolarization and hence voltage change happens at the synapses between neurones. Neurotransmitter released from the pre-synapse (axon collaterals or terminals) bind to the receptors of ligand gated cation channels[2] on the post-synapse, thus letting primarily Na^+ into the signal-receiving neurone, depolarizing the intracellular membrane slightly.

The post-synapse is usually a dendritic process extending from a neuronal soma, or it can be the soma itself, within which small depolarizing potentials known as: Excitatory Post Synaptic Potentials (EPSPs) travel passively towards the soma[3].

[1] Axons without myelin have different types of voltage gated Na^+ channels spread out along their whole length, enabling them to re-activate APs like other axons, but their conduction velocity is much lower.

[2] There exist both ionotropic channels with receptors for nACh, AMPA and NMDA and metabotropic channels with $GABA_B$ or mGluR receptors, but the difference between them is not relevant in this context.

[3] Passive conduction depends on the nerve's cable properties, e.g. diameter and length constant, which determine how far the signal can travel before dying out.

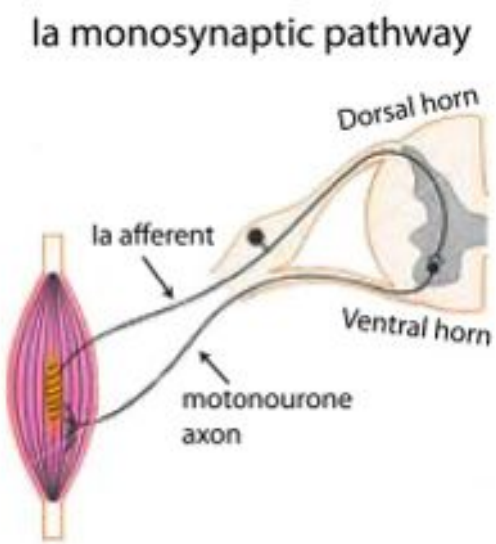

Figure 1: Physiologically, the Ia monosynaptic pathway is initiated if the muscle spindles (seen at the connection between Ia afferent and muscle) are stretched, which will result in an AP travelling up the Ia afferents which enter the spinal cord through the dorsal roots. As seen on the figure, the Ia afferents extends all the way down through the grey matter of the dorsal horn in the spinal cord and terminates in the ventral horn, where they form excitatory synapses with motoneurones. If the signal arriving from the Ia afferents is sufficient (i.e. the EPSP summation takes V_m to firing threshold), an action potential will be initiated from the motoneurone and travel down its axon to the neuromuscular junction causing the muscle to contract. In reality, multiple Ia monosynaptic connections between each muscle and several motoneurones innervating the same muscle group exist, but the figure shows the simplest schematic pathway. The picture is from http://www.kmle.co.kr

The size of the depolarization depends, among other factors, on how much neurotransmitter is released, which again depends on different presynaptic factors like the amount of Ca^{2+} intake and the size of the readily releasable pool of transmitter[4].

All signals are of course not depolarizing, i.e. excitatory, hyperpolarizing signals also occur and their function is inhibitory (Inhibitory Post Synaptic Potentials, IPSPs). Inhibition arise when the neurotransmitter binds to an anion channel (with e.g. $GABA_A$ or Gly receptors), letting in Cl^- which hyperpolarize the post-synaptic membrane, hence making it difficult for the dendrite to conduct succeeding EPSPs.

When the dendritic signals, i.e. EPSPs and IPSPs, arrive at the soma a summation occurs and sufficiently depolarizing potential changes will open the Na^+ channels located in the axon initial segment, initiating an AP which will be fired along the nerve axon.

The above describes the orthodromic pathway of nerve signals, but actually signals can also travel in the opposite direction, antidromically (from axon to the initial segment), when external stimulation is given at the axon[5]. This ability to backfire APs will in this thesis be utilized in motoneurones to identify which nerve branch they belong to.

1.3 The specific neuronal pathway of interest

Various spinal reflex pathways have been identified, the simplest of which being the Ia monosynaptic reflex, illustrated on figure1.

The cell body of the motoneurones are situated in the ventral part of the spinal cord and their axons extend out to the muscles which they synapse onto in so called neuromuscular junctions. Motoneurone axons going to the same group of muscles are located in a nerve bundle together with the sensory Ia afferents belonging to those muscles. Just before reaching the spinal cord, the mixed nerve bundle separates into sensory and motor fibres and these two branches enter the dorsal and ventral horns of the spinal cord through the dorsal and ventral roots respectively.

[4]The function of Ca^{2+} in the pre-synapse is to activate the SNARE complexes (proteins that fuse the synapse membrane with the membrane of the vesicles containing neurotransmitter) which will result in exocytosis (neurotransmitter release). The higher the Ca^{2+} intake the more SNAREs are activated and more transmitter is released, but if the vesicles are not docked i.e. readily releasable, a high Ca^{2+} intake has no effect.

[5]This is not a physiological situation, only experimental, described in review[2]

Inside the spinal cord, the Ia afferents extend all the way from the dorsal horn down to the ventral horn where they connect directly (monosynaptically) with the motoneurones whose axons lay together with the Ia afferents in the mixed nerve bundle - these are called homonymous motoneurones. This connection from a muscle to the motoneurone innervating that muscle is of excitatory nature and it gives rise to monosynaptic EPSPs which can be measured intracellularly in the motoneurone cell body.

In addition to the described monosynaptic connection to homonymous motoneurones, the Ia afferents also make di-synaptic inhibitory connections to antagonist motoneurones via Ia interneurons[6], as well as general polysynaptic connections to arbitrary motoneurones via other intermediary neurones. Regarding the EPSPs originating from the Ia monosynaptic pathway, various factors can influence the size of it:

1. As described earlier, a membrane potential far away from the Na^+ reversal potential will give rise to a larger driving force causing more Na^+ to pass through an open Na^+ channel on the post-synapse – hence a very negative V_m should increase the size of the EPSPs.

2. Repetitive stimulation of the Ia afferents have been demonstrated to diminish the resulting EPSP, measured intracellularly in the motoneurone cell body[3][4]. If repeated stimulation is given within 400 ms of the initial stimulation, the decrease is believed to be due to the combination of two effects:

   a) Presynaptic inhibition, which hyperpolarizes the Ia terminal (i.e. the pre-synapse) via $GABA_A$ergic interneurons[5].

   b) Post Activation Depression, PActD, which is believed to be a presynaptic phenomenon, but what exactly mediates it is yet unknown, see elaboration in review[6].

3. Repeated stimulation of the Ia afferents with intervals ranging from 400 ms to 10 s still diminish the EPSP size, but since the pre-synaptic inhibition is considered to be over by 400 ms[7], this depression is regarded as being due to PActD[4], which is the focus of this thesis.

In animals it is possible to perform intracellular in vivo recordings and directly measure the size of the summating EPSPs arriving in individual motoneurone cell bodies from the Ia afferents. Using this method in cats, the maximum amount of depression has been estimated to be around 25% and gradually returning to normal levels over a time course of $1 - 5$ sec.[4].

1.4 PActD and motor disorders

Since motoneurones control the output given to muscles, changes in PActD due to a disease or injury would be expected to have functional consequences. Changes in PActD influence the spinal reflex pathways that may contribute to the development of spasticity, see further description in review[6]. Spasticity is characterized by hyperreflexia and exaggeration of the stretch reflex[7], and reduction of PActD is believed to contribute to the latter[7], if not both.

Various human motor disorders have the symptom of spasticity as a feature, so it has been of interest to investigate whether they all have different levels of PActD. The human studies conducted in this field have used measures of decreases in the H-reflex (the electrical counterpart of the stretch reflex) as an indirect measure of PActD, see[8] and review[9]. To evoke the H-reflex, the experimenter will repetitively stimulate the mixed nerve bundle cutaneously and record the monosynaptic response, i.e. the H-reflex, in terms of an ElectroMyoGram in the muscle.

[6]This makes good sense in our arms and legs for example, where every movement is an interplay between an antagonist pair of flexor and extensor muscles, doing the opposite of each other: If a flexor muscle is contracted, the corresponding extensor muscle must be relaxed, inhibited.

[7]The stretch reflex is activated if a muscle is involuntarily stretched (e.g. if the experimenter moves the subject's limb instead of the subject's brain giving the message) and works to rectify the movement by activating that muscle to contract. The stretch reflex is initiated by stretch sensitive muscle spindles; these spindles are the Ia afferents' terminals on the muscle.

Using this method, reductions of H-reflex[8] – and hence PActD have been shown in spastic patients suffering from:

- Multiple sclerosis[8]
- Spinal cord injury[8]
- Hemiplegia as a result of a stroke[7]

Therefore reduced PActD seems to be a recurrent feature of spasticity, and there are speculations as to whether PActD should be considered part of the pathophysiology of spasticity, see arguments in review[6] and[8].

Spasticity is also a feature of diseases affecting the motor system including the motoneurone disease Amyotrophic Lateral Sclerosis (ALS). The ability to record PActD the direct intracellular way in animal models of the disease would allow us to explore this phenomenon. Intracellular recordings are done with electrophysiological methods and the main technical constraint to consider is the stability of the motoneurone cell; including the penetrating electrode's ability to stay inside the cell for a longer period of time and achieving a steady membrane potential. Stability in vivo is mostly affected by how well the glass microelectrode penetrates the cell and respiratory and cardiovascular movement.

Motoneurones in the spinal cord are less affected by the above mentioned movements in larger animals like cats, which is why they have been the preferred choice of animal model. But within recent years, transgenic mouse models for a number of motoneurone diseases, which are known to cause spasticity, have been developed. Hence it would be an obvious advantage to move the studies of features of motoneurone diseases and of spasticity to the mouse model.

The cat experiments were carried out in vivo, but measurements in mice in vivo will be affected by the stability problems mentioned above to a much higher degree, as well as the mice being very sensitive to stress and blood loss during surgery. This is why many labs have chosen to focus on a mouse in vitro preparation, but they on the other hand are limited by the fact that adult motoneurones die quickly because of lack of oxygen, and are therefore restricted to operate with mice in the near natal stage[10]; which means they cannot show developmental and degenerative changes happening during the time course of a disease.

During the last couple of years it has been shown that it is in fact possible to obtain relatively stable intracellular recordings of intrinsic properties of motoneurones in adult mice in vivo[11], hence the aims of this thesis concerned with PActD in the mouse in vivo model can be summarized as:

1. **Determine whether it is possible to accurately measure subtle changes in EPSP size by electrophysiological methods, and hereby determining whether PActD is actually present in healthy adult mice in vivo.**
2. **Determine whether the time course of the depression is similar to the one seen in the cat.**
3. **Determine whether the magnitude of the effect is comparable with what has previously been measured in the in vivo cat preparation.**
4. **Investigate what features of the mouse model could affect PActD, for example age or motoneurone type.**

If PActD turns out to be accurately measurable in the adult mouse in vivo preparation, the results will then be used as comparison to test whether PActD in transgenic mouse models of human motor disorders with spasticity as a feature is reduced, as one would predict from the human work on H-reflexes.

[8]Since the H-reflex is recorded at the muscle, it measures whether APs were initiated from the collection of motoneurones activated by the stimulation. If every EPSP reaching these motoneurones are 25% depressed it will result in a lot of cells not reaching firing threshold, and therefore the reduction of the H-reflex exceeds 25%.

One of the disease models available to this lab is the SOD1^{G127X} (SuperOxide Dismutase-1 enzyme) mutant which develops ALS, a progressive neurodegenerative decease that preferentially attacks motoneurones. The general cause of ALS is unknown, but for the cases where ALS is inherited (Familial ALS), which accounts for about 10% of all cases, it has been discovered that in $15-25\%$ of those instances, point mutations in the gene encoding the SOD1 enzyme cause ALS[12].

2 Methods

2.1 Surgery

All mice, control C57BL/6J and mutant SOD1^{G127X}, undergo the following surgical procedures before intracellular recording can be carried out. Anaesthesia is provided in several stages, first a volatile gaseous anaesthetic, isoflurane is given to ensure a painless/stress-free needle-injection of a mixture of 1 : 1 hypnorm and midazolam[9]. 0.15 ml of this mixture is injected intraperitoneal for induction pr. 25 gram mouse-weight, and during surgery, the same cocktail is given at least every $15-20$ minutes but in a dose of 0.3 ml, or it is given as required if the mouse is judged to be light.

Atropin is given to reduce mucal secretions and hereby hinder blockage of the tracheal cannula which is inserted for ventilation during the experiment. 3 intraperitoneal cannulas are inserted for giving drugs during the experiment, one tube for the anaesthetic hypnorm/midazolam mixture, one for a neuromuscular blocking agent, pavulon (to be given before the measurements begin), and one for the emergency drug Ephedrine, given in response to an unexplainable drop in heart rate. A rectal probe monitors the mouse's temperature and turns on a heatlamp and a heatblanket if the temperature drops below 37°C.

In the mouse leg, the sciatic nerve is dissected and this is further dissected into its two main branches: Common Peroneal (CP) and Tibial (Tib) [10].To expose the spinal cord, a hemi-laminectomy is executed at vertebrae level T12-L1. Only doing the laminectomy on the ipsi-lateral side to the dissected leg helps keep the spinal cord stable and minimizes movement due to breathing, which improves measurements. The mouse is then transferred to a stereotactic frame; its head is put in a headholder and vertebrae-clamps are placed on vertebras immediately rostral and caudal to the exposed segment to support the spinal cord.

The mouse's back-skin is retracted upwards and tied to the frame with silk ties to make a pool around the spinal cord which is filled with paraffin oil. The dissected leg is placed on custom-made legholder and the skin is pinned to the holder to make an oil pool that will protect the nerves against drying out. A pair of hook electrodes are placed under each dissected nerve branch with as much distance between them as the amount of separation of the nerve branches achieved allows. Sometimes it was not possible to obtain sufficient distance between one hook electrode and the junction of the CP and Tib branches to prevent stimulus spread, but the interference was controlled for during the experiment. Pictures from the surgical procedure can be seen on Figure 2.

Finally the mouse is hooked up to a ventilation machine that ensures 71 breaths pr. minute and monitors the CO_2 level in the exhaled air. Changes in CO_2 levels indicate changes in state of health of the mouse. Clips for measuring the mouse's ElectroCardioGram (ECG) are placed on the skin and on the ear. The output is registered both on an oscilloscope and can be listened to over speakers and is another way of controlling the condition of the mouse while recording. A unipolar electrode with two branches (one branch is technically the ground) and little silver balls at the tips is used for recording the mouse's CordDorsumPotential (CDP), also known as the incoming volley. It is placed on the spinal cord, and the mouse is paralyzed via injection of the

[9] the dosages were: 0.5 ml hypnorm, 0.5 ml midazolam diluted in 1 ml distilled water

[10] Common peroneal containing axons from motoneurones innervating mainly flexor muscles along with their respective sensory Ia afferents coming from these muscles, and the same for tibial, just mainly extensor

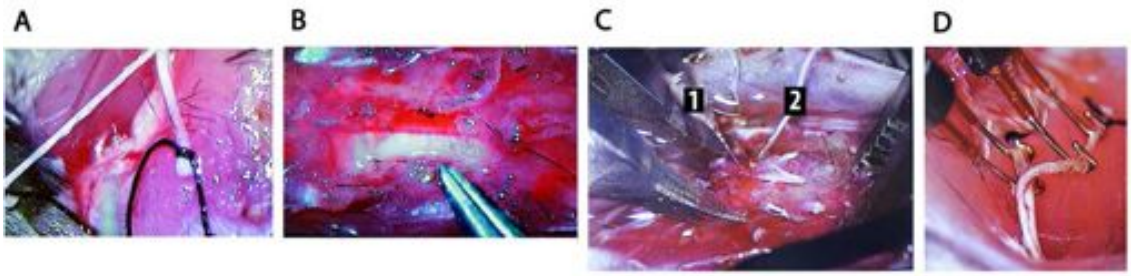

Figure 2: A: Dissection of the sciatic nerve into the common peroneal (left) and tibial (right) branches. B: A pair of forceps is pointing at the exposed spinal cord after the hemi-laminectomi. C: Close up picture of the setup in the stereotactic frame with vertebrae clamps holding the spinal cord, 1: the glass microelectrode and 2: the CDP electrode at the exposed spinal cord. D: Hook electrodes with the dissected nerves lying across.

before mentioned neuromuscular blocker pavulon before measurements begin.

2.2 Experimental setup and design

The nerve branches stimulated by the hook electrodes contain both motoneurone axons belonging to motoneurones in the ventral horn of the spinal cord, and sensory afferents entering the spinal cord through the dorsal roots. Out of all the different sensory afferents we are interested in a particular type, the Ia afferents, which along with forming lots of synapses with intermediary neurones has a direct monosynaptic excitatory connections with homonymous motoneurones in the ventral horn. Only this single synapse connection is shown on Figure 3, and it is this connection which gives rise to monosynaptic EPSPs, which the experiments of this thesis aim to measure.

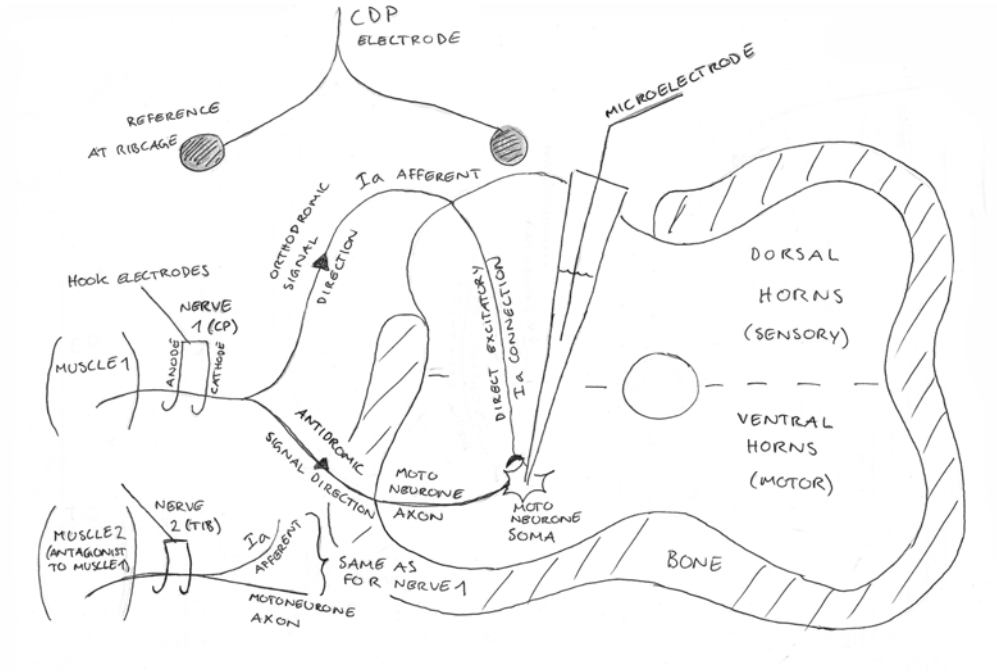

Figure 3: The cartoon shows the basic intracellular setup which enables us to measure EPSPs initiated by the Ia monosynaptic pathway and arriving in the motoneurone soma. All electrodes are shown and where they will be located in the spinal cord and on the nerves.

During the experiment, the nerve branches will be stimulated through the hook electrodes and APs will travel orthodromically up the Ia afferents but also backwards – antidromically down the motoneurone axons and into the motoneurone cell body as indicated on Figure 3. In the soma, the AP is recorded as an antidromic 'spike'. The AP travelling the regular way (orthodromically) through the Ia afferents will arrive later in the motoneurone cell body than the backfired antidromic AP, because the antidromic signal circumvents the one synapse of the regular pathway.

Both signals will be measured intracellularly with a glass microelectrode driven down through the exposed spinal cord and into the motoneurone. The incoming volley measured with the CDP electrode is utilized to distinguish the arrival times of the APs at the spinal cord, how this is done will be explained in detail later. To avoid the antidromic spike and only measure the monosynaptic EPSPs from the Ia afferents, which are known to have a lower firing threshold, the stimulus intensity is reduced to a level below the motor axon's threshold, but still above the Ia afferent's threshold.

2.2.1 Electronics setup

The equipment setup can be divided into 3 individual circuits, one for stimulation, one for intracellular recording and one for other recordings as ECG and CDP, each shown on a diagram and on pictures, see Appendix A. Stimulation of the nerve branches is conducted through a 'stimulator box' and during tracking (search for a motoneurone) stimulators A and B are set to pass current through the hook electrodes to nerve branches CP and Tib respectively. When a motoneurone has been located, stimulator C is turned on and set to whichever nerve branch the motoneurone is found to belong to and stimulators A and B are turned off. Stimulator C gets input from the computer programme Signal via the digital output on the 'CED 1401', an analogue to digital converter, whereas stimulators A and B were controlled from the 'Analogue delay box'. When triggered from the main delay, Signal instructs stimulator C to give a train of pulses followed by a single pulse, whose distance to the train can be varied[11].

The recordings are conducted in current clamp mode, where the axoclamp (a differential amplifier that briefly can be described as the control centre for intracellular recordings) is used in 'Bridge' mode. Bridge mode allows for the glass microelectrode to accurately record the potential changes happening in the spinal cord when the neurones respond to current stimulation from the hook electrodes. When a motoneurone cell has been found and penetrated with the microelectrode, it records the membrane potential intracellularly[12].

The output from the intracellular glass microelectrode passes through the headstage amplifier on its way to the axoclamp which calculates the membrane potential by subtracting the extracellular zero (which has been adjusted before penetrating the cell) from the actual output received from the headstage amplifier. This continuous measure of the membrane potential is then further amplified by passing it through 2 separate custom made amplifiers, both amplify the signal and one delivers band filtering as well. One provides low amplification and the signal is not filtered, allowing one to measure the changes in membrane potential as they occur during the tracking and intracellular recording. This signal will be referred to as IC Low (intracellular low amplification). This low amplification enables viewing of action potentials (usually around 80 mV in size) on the oscilloscope screen.

The second amplifier is set to a much higher amplification and uses a combination of low pass and high pass filtering, hereby selecting the biological frequency range of interest (5 Hz – 5 kHz). This signal will be referred to as IC High (Intracellular high amplification). The high pass filter selects everything above 5 Hz thus getting rid of cyclic changes in membrane potential due to respiratory and cardiovascular movement, since these happen at lower frequency. Hereby the IC High signal stays in the same place on the oscilloscope screen and in combination with

[11] See the section 'improvements' below, which explains why this stimulation arrangement was necessary.

[12] The axoclamp has other modes such as DCC (Discontinuous Current Clamp) and SEVC (Single Electrode Voltage Clamp) which are advantageous if the glass microelectrode is used for passing current and recording at the same time. But in the above mentioned setup, the microelectrode is primarily used for recording, and stimulating current is passed through the hook electrodes, so it does not constitute a problem.

being highly amplified it enables viewing of small events like EPSPs (around a few mV in size) on the oscilloscope during the experiment. Thus, during the experiment, on IC Low the full action potential spikes can be seen, aiding the antidromic identification of motoneurones, whereas the EPSPs, which can be as small as a tenth the size of an AP, can be observed on IC High simultaneously, allowing for comparison of latencies of the events.

Both signals are passed through an analogue to digital converter (CED 1401) so that they can be recorded using the Signal software. The IC Low channel is set to a higher sampling rate and since it shows the exact membrane potential without filtering, it is used for data analysis rather than IC High. The CDP is also amplified, filtered, digitised and recorded at the same time.

2.2.2 Stimulation

Stimulation is performed with 2 pairs of hook electrodes, each pair slid underneath the dissected nerve branches. One hook is a cathode the other an anode and the current passing between them is picked up by the nerve lying across both hooks with the effect that an AP is initiated (the AP will then travel both orthodromically up the Ia afferent, and antidromically down the motor axon as mentioned before).

Because the resistance in the extracellular fluid is much lower than the resistance inside the nerve, stimulation must be very strong, usually 50μA-2 mA[13], to ensure sufficient activation of all axons and Ia afferents in order to obtain maximum AP output from the nerve. The stimulation intensity needed to ensure this differs between nerves, but each nerve branch's minimal firing threshold is found by turning down the stimulation until the CDP is barely seen – this corresponds to only the lowest threshold axon(s) and afferent(s) being activated. The intensity is then set to 5 times this found threshold to get maximum output. If the stimulation outcome is poor, interchanging the polarity of the hooks can improve it because an AP having to travel past the anode might fail because of anodal block. Therefore it is worthwhile to ensure that the cathode is nearest the active end of the nerve branch.

Once an antidromically identified motoneurone is penetrated, the stimulators used for tracking (A&B) are both turned off and stimulator C is set to stimulate the nerve from which the motoneurone was antidromically identified. The intensity of stimulation is manually reduced to a level below threshold for the antidromic spike from the motor axon, but still sufficient for activating the lower threshold Ia afferents, causing a monosynaptic EPSP in the motoneurone. The stimulation protocol is controlled by the programme Signal which records in frames triggered by the equipment, leaving an 8 sec. delay between each triggering, allowing the nerve to be completely relaxed before next round of stimulation.

Each frame begins with a train of 4 very brief current pulses (with widths of 0.5 ms) over 200 ms, which is equivalent to 20 Hz, this will be referred to as the **'conditioning train'**. After a variable time interval, a single **'test pulse'** of the same width and strength is given. Every pulse will cause a monosynaptic Ia EPSP, but the EPSP resulting from the first pulse of the conditioning train, referred to as the **'conditioning EPSP'**, is anticipated to have the same size every time, and is therefore used for comparison with the **'test EPSP'** following test pulse. The time interval between the last pulse of the conditioning train and the test pulse is varied throughout the experiment, either $\Delta t = 0.5$, 1, 2, 3, 4, or 5 sec., tested in different orders.

2.2.3 Antidromic identification via CDP recording

The CDP electrode consists of two separate branches; one located in the vicinity of the ribcage which provides a baseline/reference for the other branch, which measures the electric field potential at the dorsal surface of the spinal cord in response to stimulation at the peripheral nerves CP and Tib, with respect to the reference, see Figure 3. As mentioned in the introduction, field potentials arise because of the electric activity of neurones in the surrounding area as they de-, re- and hyperpolarize. The CDP is negative when a majority of adjacent neurones depolarize, which

[13]Which are large values in physiological terms, for comparison, only $1 - 10$nA is usually necessary to obtain the same effect if stimulation was performed intracellularly

depletes the area around the electrode of positive charges, see part II and IV of Figure 4. And when neurones repolarize, cations will flow out and anions in, resulting in a positive field potential, see parts I and III on Figure 4.

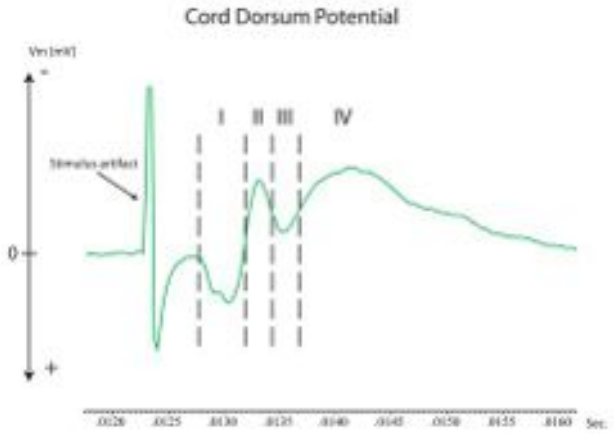

Figure 4: The figure reflects an actual recording of the Cord Dorsum Potential and every turning point (marked in between the dashed lines) specifies where the action potential is located relative to the CDP electrode, in terms of ionic flow. Notice that the scale for membrane potential is upside down, the reason being that it has become convention to show the CDP like this; if the polarity of the electrode was switched, the CDP and the scales would be the other way around.

The first tri-phasic part of the CDP show the orthodromic action potential in the sensory axons (afferents), as it arrives in and passes through the dorsal root beneath the CDP electrode. Whereas the fourth part reflects the summation of synaptic activity in the dorsal horn of the spinal cord where the Ia afferents make polysynaptic connections with intermediary neurones[14]. Because the CDP shows the position of the orthodromic APs during a triggered time course as they arrive at the spinal cord, it provides a timescale reference which can be used in the search for motoneurones and for antidromic identification of them.

A glass microelectrode is used for tracking and it is driven up and down through the spinal cord using an electronic microdrive while recording its surrounding field potential. When in the tissue above the motoneurones, a negative intermediate field is seen; it is broad and has the same timing as part IV of the CDP and reflects excitatory synaptic activity in the vicinity. When closing in on a motoneurone, the negative potential should increase, arrive earlier and get narrower with respect to another place in the spinal cord. Large and narrow negative field potentials that have about the same latency as part II and III on the CDP indicate a lot of antidromically arriving action potentials in the area (of course triggered by and timelocked to the stimulation), which is expected around the motoneurones.

When the microelectrode is thought to be in the vicinity of a motoneurone cell, its potential measurement, $V_{m,ex}$, must be set to zero (calibration), and penetration of the cell membrane is attempted. If the penetration is successful, a sudden decrease in the potential recorded with the microelectrode is expected, preferably down to -50mV or lower, corresponding to the membrane potential of the found cell. Figure 5 illustrates tracking and penetration of a cell and is made from recorded frames in one of my experiments.

The APs seen in a penetrated motoneurone are antidromic if they appear at a similar latency to part I and II of the CDP and orthodromic if they arrive at similar latency as the beginning of part IV. For and EPSP to be monosynatic is must appear **after** the antidromic spike at a latency allowing for a single synapse. The timing of the monosynaptic EPSP with respect to the antidromic spike according to the CDP is illustrated on Figure 6, which comprise of an example taken from one of my experiments.

[14]The CDP electrode registers the synaptic activity at a distance and the different synapses activate with different latencies, which is why part IV is so wide.

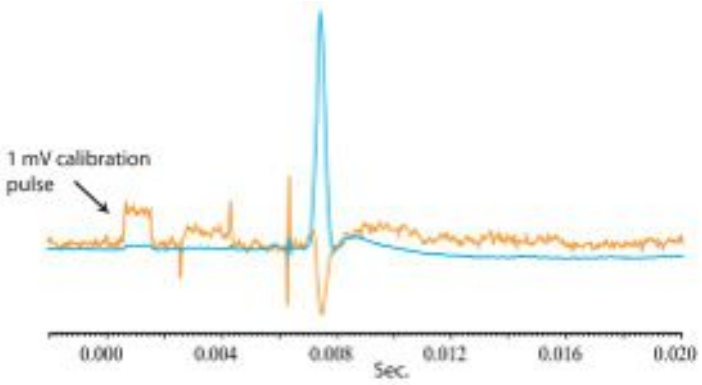

Figure 5: This figure illustrates what is seen on the oscilloscope during tracking. When approaching a motoneurone, a field like the dip on the yellow line is seen on the IC High channel, and if the cell is successfully penetrated, a (hopefully) antidromic spike (blue) will appear on the IC Low channel. Notice that IC High (yellow) is filtered, hence it does not show the actual V_m which obviously change when the electrode goes from extracellular to intracellular. How the antidromic nature is confirmed is shown on Figure 6. From the size of the calibration pulses on the two superimposed traces it can be seen that they do not have the same amplification, this was for the purpose of illustration.

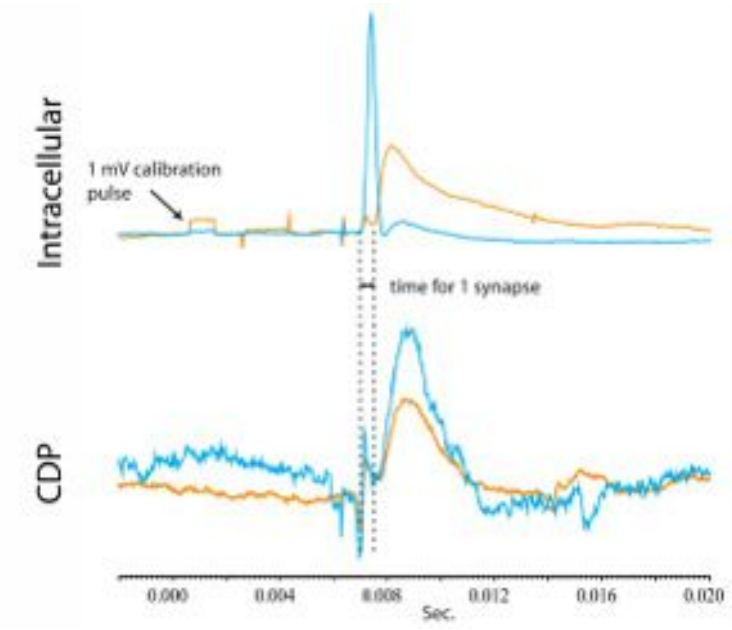

Figure 6: This figure illustrates antidromic identification of motoneurones. On the top half of the figure, the antidromic AP is seen in blue and its corresponding CDP is shown on the bottom half. After recognising the antidromic spike, the stimulation threshold is turned down until only the Ia monosynaptic EPSP is seen, represented with the upper yellow line. The amplitude difference between the two CDPs makes the lowering of threshold visible. For the top half of the figure, amplification between the spike and the EPSP was changed like on Figure 5 for the purpose of illustration.

The intracellular microelectrode is used for recording (most of the time) but can also pass current if needed. A need for passing negative current arises if the axon of a motoneurone has very low stimulation threshold and therefore will keep firing antidromic APs at so low stimulus

that the EPSPs can hardly be distinguished. Negative current will hyperpolarize the cell and stop the firing of APs, and vice versa positive current can bring a high threshold hyperpolarized cell towards firing threshold so that APs occur, enabling antidromic identification. During tracking up and down through cells the electrode can become blocked, which increases the electrode's resistance and makes the recording unreliable; this is taken care of by briefly passing a large amount of current through it.

2.3 Improvements

Initially stimulator A and B were used for delivering the conditioning train and the test pulse respectively, their timing determined by the analogue delay box. During analysis of the first experiments we discovered however that even when set to the same intensity, the actual stimulus output given by stimulator A and B were not equal. The delayed test pulse (measured as the size of the stimulus artifact from stimulator B) was permanently lower than the conditioning train (from stimulator A), so it was not valid to compare the two EPSPs. We tried switching the stimulators around to see if it had something to do with the stimulator box, hence the test pulse artifact should then be higher than the conditioning train with the stimulators switched around. But the test pulse was still lower, which means that the problem lies somewhere between the delay box and the stimulator box.

To solve this problem we used our data acquisition software (Signal) to trigger both the conditioning train and the test pulse from the same stimulator, stimulator C instead of A and B as described above. This ensures that the intensity is the same every time which in turn ensures against false-positive findings when comparing EPSP sizes. We wrote a configuration file for Signal which allows us to switch between different time delays by simply clicking a button instead of turning nobs on the delay box. Stimulators A and B are still used for tracking and antidromic identification of the motoneurones.

2.4 Data analysis

Only cells with a V_m of -45 mV or lower were accepted for analysis. Raw data consists of multiple frames from each penetrated cell and each frame contains one recording of the conditioning train of EPSPs and a test EPSP. Since measurements of small events like EPSPs require very stable cells, and good stability is a difficult to achieve, a single frame cannot be trusted to tell the true magnitude of the EPSPs. The solution to this is averaging.

Averaging serves to equal out all other random events and fluctuations that are not time locked throughout the frames as the EPSPs. To obtain a reliable measurement, as many frames at approximately the same membrane potential for the same time interval, are included in the average. This implies that a selection process takes place, and the criteria for excluding frames were: large changes in the cell's general membrane potential, local changes in the membrane potential in the vicinity of the conditioning or test EPSP due to respiration or cardiovascular movement or a noteworthy difference in membrane potential at which the conditioning and test EPSPs were initiated[15].

The actual averaging was done with the Signal software, and the ranges (continuous error bars) could be viewed for each average. For each average it was checked whether the ranges at the conditioning EPSP were the same size as at the test EPSP because equal sized ranges signifies that the magnitude of PActD is approximately the same throughout the included frames, i.e. the depression is consistent and present in every single frame. A few cells were discarded based on their ranges being huge – about the size of the EPSPs themselves, which made the values untrustworthy. Statistical tests and plots were performed with the GraphPad Prism 5 software and statistical significance was established at $P < 0.05$

[15]Sometimes there can be large periodic movement and one of the EPSPs can sit on a hilltop of movement while the other sits in a valley. Such a frame would get excluded, but if both were e.g. in the valley, the frame might be includable.

3 Results

3.1 PActD in normal mice

Measuring the time course of PActD in healthy control mice was the first objective of this thesis in order to compare it with the work done in cats. Sufficient stability was achieved in 8 cells from 3 mice to measure all or at least 3 different time intervals while inside the same cell. The amount of depression will always be expressed as the test EPSP size in percentages of the corresponding conditioning EPSP. The following Figure 7 illustrates how some good intracellular measurements looked.

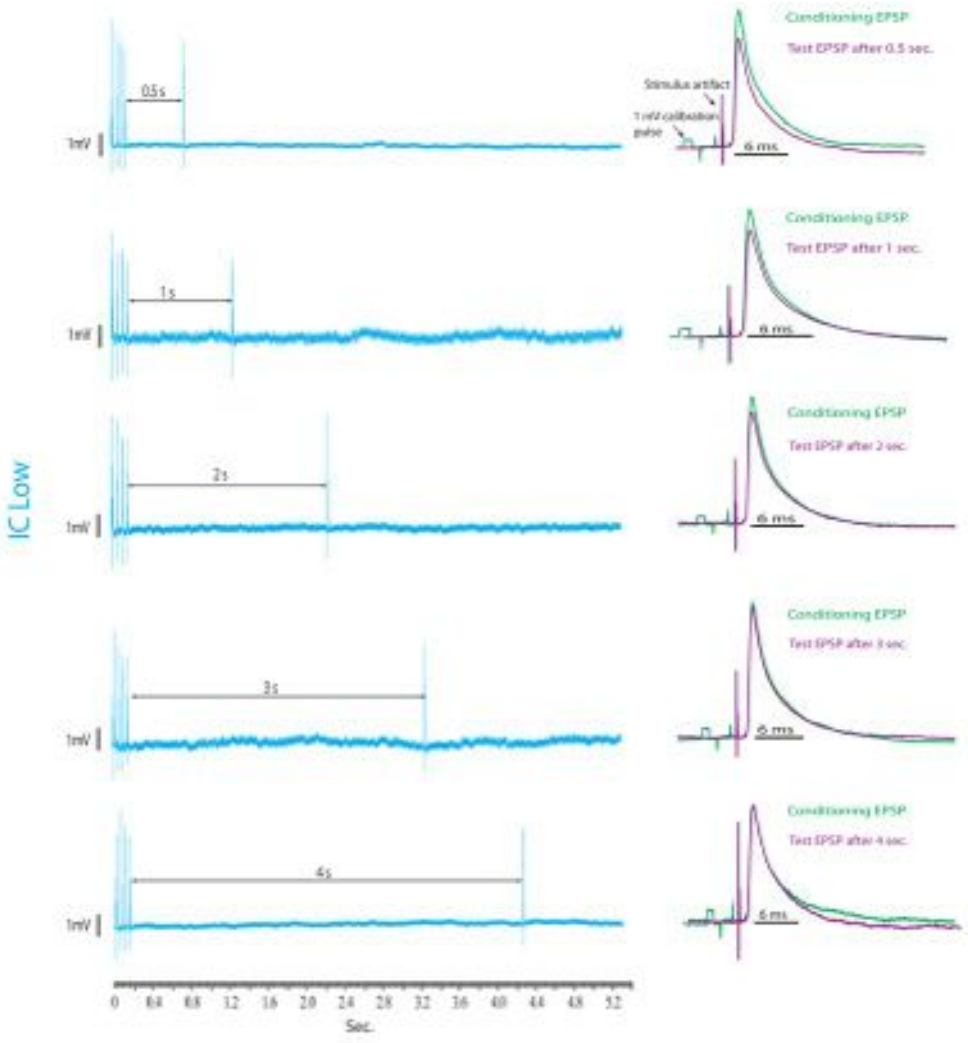

Figure 7: The blue lines recorded from the IC Low channel displays the varying of the time interval between the conditioning train of 4 pulses at 20 Hz and the test EPSP in one very stable cell. The timescale has been cut off at about 5 sec. since the remainder of the frames are without events. Because the averages used are so zoomed out, it may not be all vertical lines that represent EPSPs, some could be large stimulus artifacts overshadowing the EPSPs, but it illustrates the timing. To the right of each time interval, an overlay of close-ups of the conditioning EPSP (green) and the test EPSP (purple) from a corresponding average (though from another cell than the one used for the time intervals) shows how the test EPSP size gradually returns to 100% of the size of the conditioning, while the duration of the EPSPs stay the same.

All 8 time course measurements are plotted in Figure 8A below, and Figure 8B shows the time course obtained in the cat.

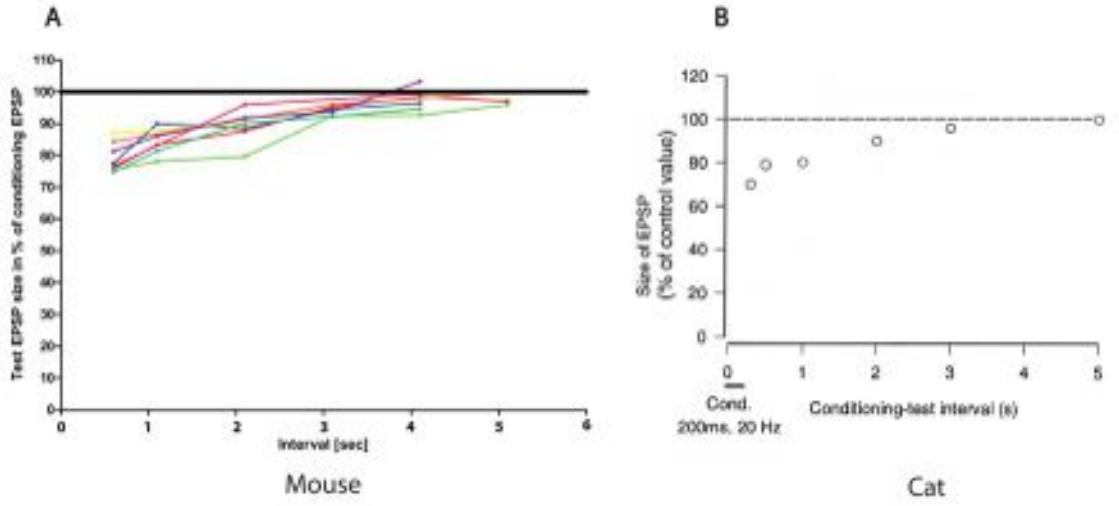

Figure 8: A: Displays a plot of the different time intervals with the corresponding depression obtained in the mouse experiments. Each coloured line represents a different cell of which there were 8 in total. The thick black line indicates 100% (i.e. no depression of test EPSP) and it is visible that the post activation depression is virtually over by 4 sec., which made more measurements at 5 sec. irrelevant. With the exception of one outlier (light green dot at 2 sec.) the spread of PActD values at each time interval is practically the same throughout the time course. B: Here an edited (showing only the PActD measured by the same method) version of the graph illustrating the time course for PActD formally obtained in the cat preparation is shown[4].

From this it can be seen that the time course of PActD appears to be very similar in cat and mouse and it appears to be a similar phenomenon, hence it is feasible to carry on investigations of PActD in mice.

3.2 Variables affecting PActD

The majority of the measurements were performed at the 0.5 sec. interval setting since this setting will provide a constant measure of the amount of depression caused by PActD unaffected by presynaptic inhibition[16]. Data was acquired from 6 control mice from 2 different age groups and averages were made from 26 individual cells – including the 8 cells involved in the time course plot on figure8. The next objective was then to see if the amount of depression at 0.5 sec. is dependent on the following features:

- Age
- Muscle group
- Membrane potential
- Conditioning EPSP size

I performed non-parametric, unpaired Mann-Whitney tests on all data first, to avoid assuming a Gaussian normal distribution. But a D'Agostino & Pearson normality test (recommended by the

[16] As mentioned in the introduction, presynaptic inhibition is surely over by 0.5 sec. and PActD should be the effect dominating the depression of the test EPSP size

Prism software) on all data from the control and mutant SOD1 mice showed that it is acceptable to assume a normal distribution for both groups ($P_{Control} = 0.9963$ and $P_{SOD1} = 0.7654$), so when the Mann-Whitney test returned a statistically significant P value, I followed up the non-parametric test with a unpaired t-test, which has greater power. Age turned out not to be a significant influencing factor, as illustrated on Figure 9 with a P value of $P = 0.7953$, so I did no further tests. The young mice used were about 112 days old [110 , 114] days, and the older ones were around 220 days [210 , 227], so approximately twice the age of the young ones.

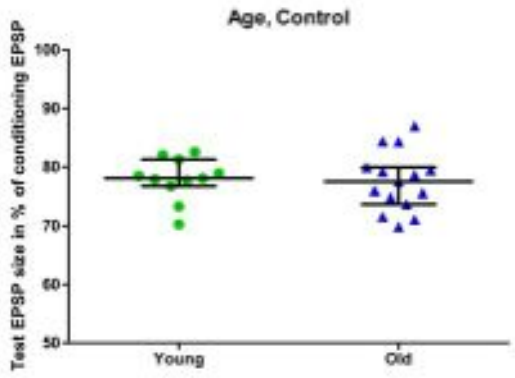

Figure 9: From this figure it can be seen that the magnitude of depression differs little between the young and the old control mice. When data points are spread out horizontally it implies that they represent values of depression which are nearly equal. Data consists of 11 cells from 2 young mice, and 15 cells from 4 older mice. The medians, 78.05 and 77.49, for young and old respectively are shown along with the 25% and 75% quartiles – the interquartile range.

The two different nerve branches dissected in the experiments, the common peroneal and tibial, differ on features like diameter and which group of muscles they innervate (flexor versus extensor), so it was tested whether difference in nerves innervating different muscle groups could influence the maximum amount of depression, see Figure 10. According to analysis of the amount of data obtained, muscle group appears not to be a significant influencing factor with a P value of $P = 0.1462$ calculated from the non-parametric Mann-Whitney test.

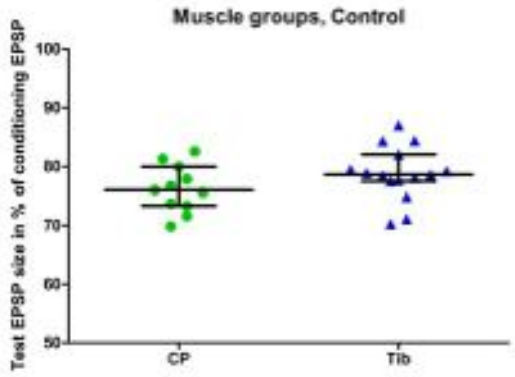

Figure 10: Medians are shown along with the interquartile range and visibly, the medians seem slightly different for the two types of nerves innervating different muscle groups, but as seen from the Mann-Whitney test this was not statistically significant. For Tib it is seen that the bottom 25% of measurements are clustered really close to the median and lots of measurements have near equal values, whereas the data points for CP are evenly spread out. Data comprise of 11 cells from CP with a median of 76.0 and 15 cells from Tib with a median of 78.6.

Theoretically it is expected that membrane potential will affect the size of the EPSP, see introduction, but whether these two parameters, membrane potential and conditioning EPSP size, will affect the amount of PActD is not known. Plots of the depression versus either parameter should reveal whether a correlation is present, Figure 11 A and B.

I tested for the slight linear tendency seen on Figure 11A with a Pearson correlation test (2 tailed) and the resulting P value did not reach statistical significance ($P = 0.078$), but it is possible that it might change if more data was acquired. But the conclusion from the current results must be that there is no linear relationship present on either graph, which indicates that although EPSP size is dependent on membrane potential, PActD is not. From the way the experiment is

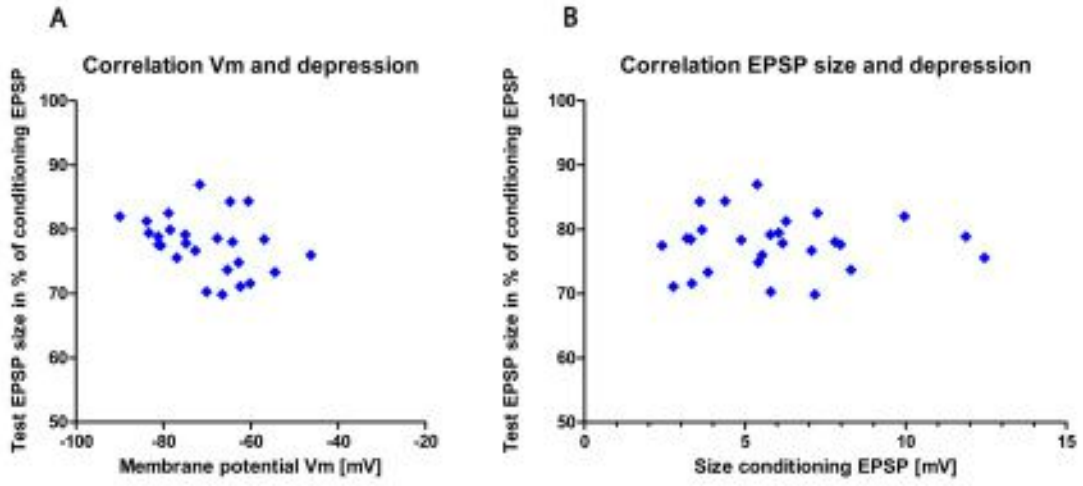

Figure 11: The size of the test EPSP (expressed in percentages of the conditioning EPSP) is plotted against A: the membrane potential at which each measurement was obtained and B: size of the conditioning EPSP in mV. Both plots contain all 26 measurements performed at the 0.5 sec. interval. One could argue that there is a slight linear tendency present on A, but no correlation is apparent on B.

conducted, it can be argued that size of the EPSPs is more dependent on the threshold at which we are able to get the Ia monosynaptic EPSP without the cell firing APs, than directly dependent on the value of membrane potential.

3.3 PActD in SOD1 mice

The data from SOD1 mutants comprised of 22 cells from 3 mice all at the pre-symptomatic stage. Since there was a slight, although not significant, tendency of V_m correlating with the magnitude of PActD, I checked whether the average V_m obtained in cells from SOD1 mutant mice were comparable with the average membrane potentials in control cells. The mean membrane potentials turned out to be nearly equal: mean $V_{m,control} = -70.4 \pm 10.49$ mV (26 cells) and mean $V_{m,SOD1} = -69.98 \pm 12.93$ mV (22 cells), signifying that there should be no problem comparing the 2 groups[17].

Since neither age, muscle group, membrane potential or conditioning EPSP size had a significant influence on the amount of depression I included all data from both the control and the SOD1 mice groups in the last comparison of control versus SOD1, shown in Figure 12.

Firstly I performed a nonparametric Mann-Whitney test, which reported a highly significant P value of $P = 0.0008$, and since this non-parametric test was significant but has low power, I performed an unpaired, one tailed t-test with Welch's correction to avoid assuming equal variances. I selected one-tailed based on the results of the human work on H-reflexes, mentioned in the introduction. From those results it is reasonable to set up the experimental hypothesis:

The SOD1 mutant model suffering from ALS will have reduced PActD

The P value of the Welch correction stated that the variances indeed should be considered to be significantly different, $P_{Welch} = 0.0396$. The result of the unpaired t-test rejected the null-hypothesis of equal means for the control and SOD1 groups down to a 0.02% chance ($P = 0.0002$)

[17] A non-parametric Mann-Whitney test provided a P value of P = 0.9917, confirming the conclusion of comparability originally made from the near equal means

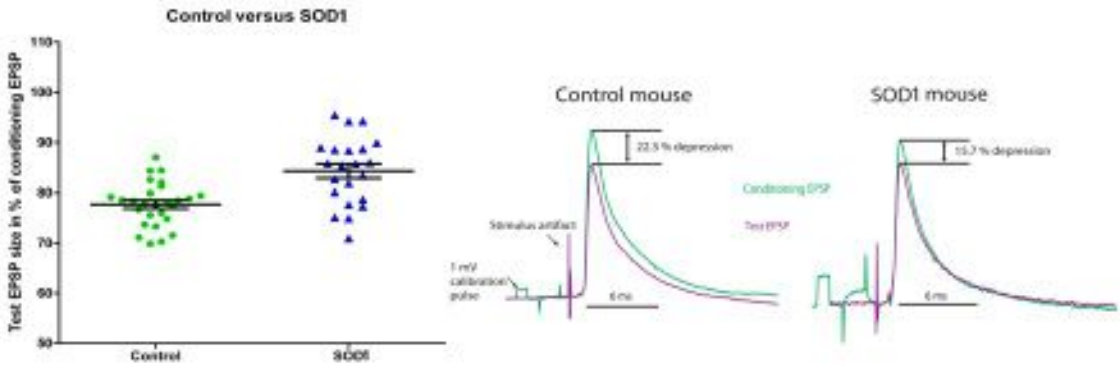

Figure 12: Left: Since an unpaired t-test was performed on these data, the means with corresponding SEM are shown on this figure. The control group include 26 data points and the mean is 77.69 ± 0.8696. The SOD1 group include 22 data points and the mean is 84.27 ± 1.423. A substantial difference of 6.581 ± 1.668 between the means can be seen, and a P value of $P = 0.0002$ implies high statistical significance. Right: the mean depressions (100% minus mean value) are illustrated with overlays of averaged data from a control and a SOD1 mouse, where green lines indicate the conditioning EPSP and the purple lines indicate the test EPSP.

of observing the difference of means due to random sampling from identical groups, this implies acceptance of the experimental hypothesis with a 99.98% confidence.

Practical aspects of intracellular recording in mice affect how reliable results one is able to obtain. Because only sweeps with similar membrane potentials can be included in the averages, stability problems due to penetration and movement complicates the data selection process. Due to this the includable frame number from which averages are made can often be rather lower than ideally desired or the range of the average will be very big. However, the electrophysiological equipment has an in built possible way of eliminating membrane potential changes: voltage clamp.

3.4 Voltage clamp

All measurements for this thesis were conducted in current clamp (axoclamp set to "Bridge" mode), which essentially is about controlling the current injected, in this case through the hook electrodes, and measuring the resulting changes in intracellular membrane potential. In voltage clamp, the concept is reversed; you set which value of membrane potential you want the axoclamp to keep the cell clamped at, $V_{m,command}$, and then calculate the amount of current the axoclamp needs to inject through the intracellular electrode to keep $V_m = V_{m,command}$ [13]. If the cell receives an excitatory signal like an EPSP, negative current corresponding to the positive potential change needs to be injected, and this will show up on the current channel as an Excitatory Postsynaptic Current, EPSC. Meanwhile, the IC Low channel normally recording membrane potential changes ought to be flat in voltage clamp mode, if the clamping is perfect.

Voltage clamp has never been performed in the mouse spinal cord in vivo and there are technical constraints concerned with it, especially because one is restricted to Single Electrode Voltage Clamp (SEVC) in the mouse preparation[18]. This implies that the same electrode must be used for passing current and measuring the actual membrane potential almost at the same time, which cannot be done in Bridge mode. The voltage clamp setup works as a feedback loop, where the axoclamp measures an actual V_m, compares it to $V_{m,command}$, calculates how much current is needed to take V_m to the level of $V_{m,command}$, and at the end injects the calculated amount of current.

[18]It is not technically possible to get two electrodes into the same motoneurone cell at the same time to do two-electrode voltage clamp, but the benefit would be that one electrode can be used for passing current and the other for recording actual membrane potential

The axoclamps Discontinuous Current Clamp (DCC) mode allows the electrode to switch quickly between recording potential and passing current, thus allowing fast repetition of the feedback loop. The frequency of switching essentially determines how good the clamping gets, but it cannot be turned up indefinitely because of the capacitor properties of the electrode and the membrane. One must make sure that the current returns to zero before switching, i.e there should be no capacitance discharge while recording, otherwise the axoclamp would record a false value of V_m including the current discharge, and then inject the wrong amount of current and so on[13].

If high switching frequency is not obtainable (the electrode might start oscillating so much that the cell ruptures), a complimentary way of improving the clamping is to increase the gain. Researchers Robert Lee and CJ Heckman have designed and built an external feedback loop for the axoclamp which can increase the gain 11 fold, commonly known as the "Bob box"[14]. What it basically does is add another calculation loop to the already existing loop inside the axoclamp. My external supervisor asked CJ Heckman for help with setting up voltage clamp in her lab, and he came to Denmark and donated a Bob box to the lab and helped set it up. The Bob box circuit is described in[14] and a schematic of it can be seen in Appendix B. The increased gain allows for much more precise estimations of the current need to keep the membrane potential stable at $V_{command}$.

Following CJ Heckman's visit we made attempts at clamping EPSPs and record EPSCs in our mouse in vivo preparation, and it worked, although not perfectly. An example of a switch from current clamp to voltage clamp (from DCC to SEVC mode on the axoclamp) is seen on Figure 13.

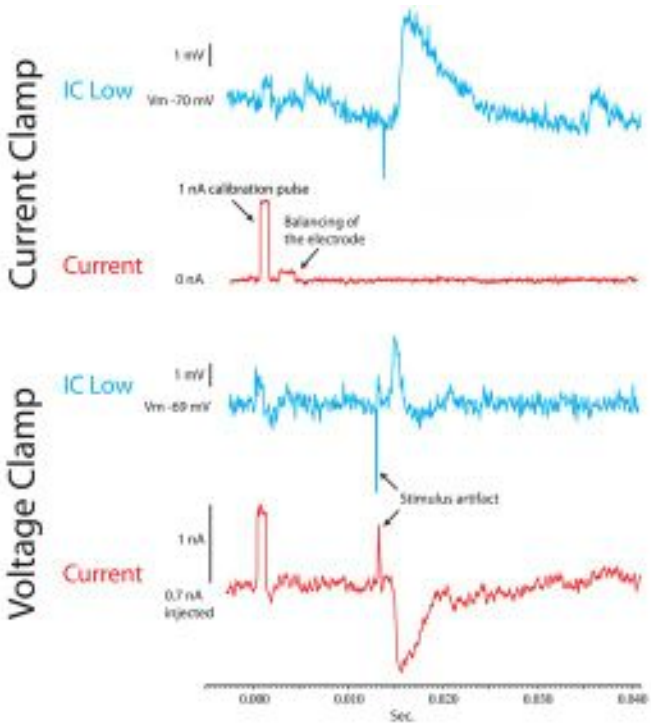

Figure 13: On the top half of the figure, the unclamped EPSP is seen on the IC Low channel (blue) along with a flat line (with exception of the 1 nA calibration pulse and balancing due to a small blockage of the electrode) on the current channel (red). The bottom half is one frame later where the switch from current clamp to voltage clamp has taken place. Here the EPSC is seen on the current channel, and the membrane potential is clamped at −69 mV. Since a bit of the EPSP is still protruding on the IC Low and it is not completely flat, it signifies that the clamping needs improvement. +0.7 nA is injected to clamp the membrane potential at −69 mV instead of −70 mV

Right at the onset of voltage change when the EPSP arrives in the soma, our clamping is not fast enough, but by tweaking switching frequency and gain, we achieved a much better clamping of another cell, shown up close on Figure 14, and were actually able to measure PActD in voltage clamp mode, see Figure15.

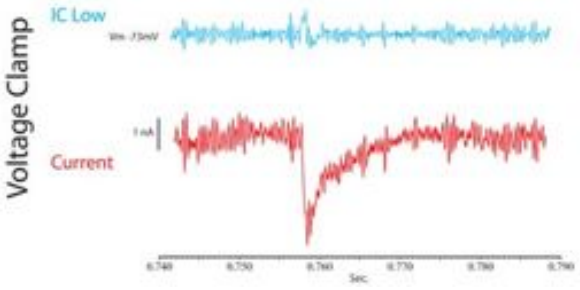

Figure 14: A single sweep of a clamped EPSC is shown on the current channel (red). On both the IC Low and current channel oscillations are seen. They are caused by high gain increase. But compared to Figure 13 the clamping of the membrane potential is much better.

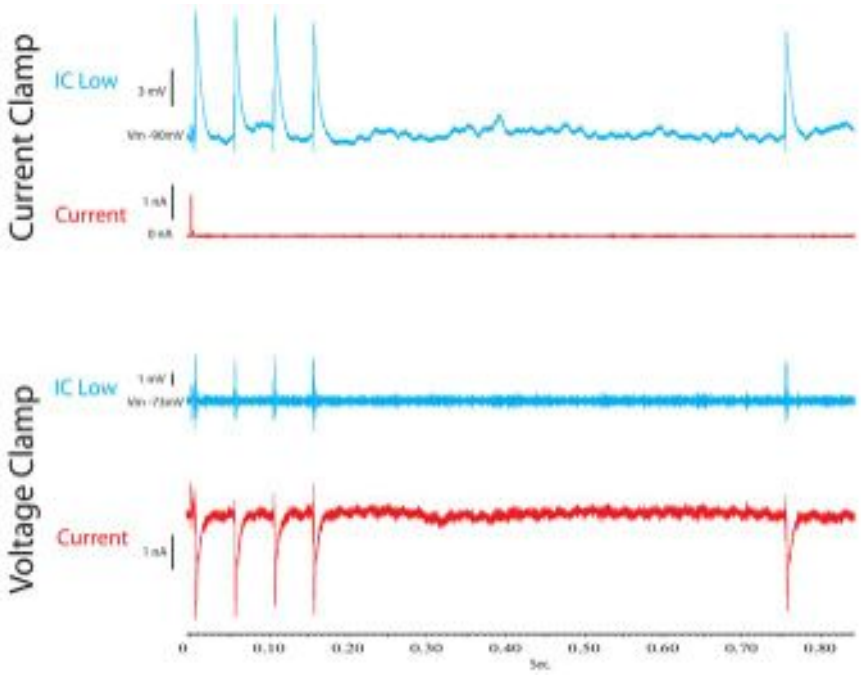

Figure 15: Here two averages are shown from before and after the switch to current clamp. PActD can be measured from both (sizes of the EPSPs are of course measured from the current channel in voltage clamp), but since the clamping is still not perfect, the results are not expected to be equal. In this cell the membrane potential changes were actually not a big issue, but it is still clear how the voltage clamp flattens out all movement.

Since a bit of potential still escapes the clamp, the measured amount of PActD in the cell shown on Figure 15 is not expected to be the identical to the value obtained in current clamp, but it is only off by about 8%, $PActD_{CurrentClamp} = 82.00\%$ and $PActD_{VoltageClamp} = 88.47\%$. These clamping settings were acceptable for first trials of voltage clamp in mice in vivo and for clamping EPSPs, but the electrode could not manage to switch fast enough for clamping events like action potentials. This can maybe be achieved by working with the parameters of the microelectrode.

4 Discussion

4.1 Summary of results

The major achievement of this study was showing that it is possible to accurately record subtle changes in events as small as EPSPs. A measure for the time course and the magnitude of PActD in adult mice in vivo was acquired first of all in control mice where factors like age, muscle group, membrane potential and conditioning EPSP size were found to be without significant influence on the PActD.

By keeping constant rigid criteria for exclusions of frames for averaging, reliable averages with small ranges can be achieved. The ranges being constant for both conditioning and test EPSP provides confidence in the validity of the comparisons. Examples of the best range obtained on an average and a standard range can be seen in Appendix C. Secondly a reduced magnitude of PActD was measured in mutant mice models of the neurodegenerative motoneurone disease ALS. These results complement findings of reduced PActD in human studies of spasticity and they contribute to pave the way for future studies using mouse models of motor disorders.

4.2 Comparison with previous literature

The results for the time course of PActD were highly comparable with previous findings in the cat model from which the method used in this study was replicated. PActD seems to be a consistent feature across species and it appears to change due to injury or motoneurone diseases, but what actually causes PActD is as yet not fully understood. Hultborn and colleagues have ruled out classic presynaptic inhibition and decreased motoneuronal excitability and concluded that it must originate from mechanisms influencing the probability of transmitter release acting on the presynapse[4].

It would be interesting to follow further investigations into this, not least because maybe the depression effect is not restricted to the motoneurones in the spinal cord; an analogous effect has been discovered in the hippocampus in rats[15][16]. They describe that synapses exhibit a so called PostBurst Depression (PBD) with a maximum magnitude of 25%, lasting a couple of hundred milliseconds to seconds and they ascribe the effect to a reduced transmitter release probability, which all indeed sounds familiar. But, they also showed that the PBD can be abolished by application of an astrocytic metabolic inhibitor.

4.3 Technical issues

There exist different strains of SOD1 mutant mice which all develop a phenotype with similar symptoms to the human counterpart[20]. Each strain is bred with an individual point mutation, and there are pros and cons to using either strain. The one chosen in this lab is the SOD1^{G127X} strain, which has the advantage that it does not cause over-expression of the malfunctioning SOD1 enzyme (a built in disadvantage in the more commonly used G93A strain in order for the disease to be expressed), which would create artifacts that are hard to control for[17].

The disadvantage lays in the pragmatic problem of not being able to genotype the mice. The best control subject for a mutant mouse is an unaffected littermate, but distinguishing between the affected and unaffected mice in a litter requires a specific genetic primer for the SOD1^{G127X} mutation. Such a primer is not yet available, and thus the mice have to be bred as homozygotes[19][17] and that implies losing the ability to control with unaffected littermates. Therefore this lab uses the inbred, very genetically stable C57BL/6J strain of healthy mice for controls, which actually is the genetic base for the SOD1^{G127X} mutant. To test whether the found reduction in PActD might be consequence of inbreeding, other SOD1 mutant strains could be tested with the PActD protocol.

[19]Which means that both parents have ALS and all pups will get the decease as well, in contrast to heterozygote breeding where some get ALS and some do not.

The main technical constraint associated with electrophysiology in mice in vivo is the membrane potential stability and ability to stay inside cells for longer, because the microelectrode can be ejected from the cell with one breath of the mouse. The ability to carry out measurements in voltage clamp could prove to be a beneficial way of controlling the changes in membrane potential, resulting in a larger number of includable frames for averaging. By experimenting with electrodes of different resistances and improving the relationship between the switching frequency and the gain, perfect clamping can hopefully be achieved, and together with the larger amount of averaged frames at the same membrane potential the accuracy would increase. Indeed, voltage clamp gives us the ability to:

- Minimize the influence of changing membrane potential on measurements, hereby improving the includable frame number and reducing the ranges on averages, which in the end will increase precision of the results.
- Perform measurements of EPSPs and IPSPs during rhythmic activity that changes the membrane potential, e.g. during respiratory drive potentials or locomotor drive potentials[18].
- Measure the actual amount of Persistent Inward Current (PIC) which is believed to partake in the increased hyperexcitability associated with ALS. At the moment it is only possible to measure the corresponding membrane potential changes when the PICs are activated, but it will be useful to be able to measure the actual current flowing into the cell precisely.

When that is said, the stability in some cells and the ranges on most cells of the present study have been remarkably good considering these measurements take place in such a small animal and the yield in terms of intracellular penetrations from each mouse has been comparable to that observed in cats (info from personal communication with C.F. Meehan).

One thing we did have great problems with was establishing the quiet stress free environment free from high frequency sounds needed when anaesthetizing the mice. Mice get easily stressed by sudden noises and sounds of certain frequencies and if stressed they will fight the anaesthesia making the basic surgery before the actual recordings impossible. Because of noisy renovation work currently being carried out near the lab in the daytime hours, we resolved to perform the experiments during the evenings and on weekends, which solved the problem entirely.

4.4 Implications and future experiments

A closer look at figure12 reveals that the lower data points up to and including the mean value for the SOD1 mice lay within the spread of the control mice data. This indicates that maybe only some motoneurones are affected by ALS, and it is in fact known that so called “fast” (referring to their higher signal conduction velocity) motoneurones are more vulnerable to the ALS disease, an elaborate description is found in this review[19]. It could be interesting to find out whether the data points above the mean in the figure do in fact belong to the class of fast motoneurones. The classification can be done by measuring the duration of after hyperpolarization resulting from an action potential initiated in the soma[20]. The equipment can easily be switched to a setting doing exactly this while the glass microelectrode is inside a cell, and we are actually in possession of just such recordings from some of the cells used for this thesis, because measurements of APs were needed for another students project.

Transgenic mouse models have many possible uses, various models in which different genes are ‘knocked out’ could in the future help reveal underlying reflex mechanisms. Mice with Cerebral Palsy, another motor disorder usually characterized to cause spasticity, exist and it could be interesting to try the PActD protocol on these mice and see whether the magnitude of depression is more like that of the control mice or the SOD1 mutants, or maybe a completely different value. What is to be expected in terms of depression is not so straight forward as one would think; because it is actually debated to what extent the ‘spasticity’ diagnosed in the humans suffering from this

[20] A fast motoneurone needs to have a short after hyperpolarization period to be able to fire APs repetitively with high firing frequency

disorder really is a phenomenon occurring in the central nervous system, i.e. hyperreflexia, or whether it might be a problem in the muscle (shortening of the muscle due to lack of early use). The mice with Cerebral Palsy are currently being created in the group and I plan to return to be involved in these experiments and use the protocols developed for this project.

Regarding the findings of the PostBurst Depression in the rat hippocampus, the discovery that the PBD was abolished if the astrocytes were blocked is very interesting. Assuming that their PBD might be the same phenomenon as our PActD but expressed in two different regions of the body, trying to block the astrocytes surrounding the motoneurones in the spinal cord and then applying the PActD protocol may unveil a possible mechanism underlying the PActD of the Ia EPSP in the spinal cord.

5 Conclusion

The mouse in vivo preparation has proven to be a viable model for measuring even subtle changes like differences in EPSP sizes, despite the stability issues associated with the experiments. Controls for features possibly influencing the magnitude of depression like age, differences in nerves innervating different muscle groups, membrane potential and size of conditioning EPSP all turned out not to be significant. Furthermore, pilot-testing of the voltage clamp technique found it to be a promising way of eliminating membrane potential changes, thus creating better circumstances for averaging which should increase the precision of the results; but the practical implementation of the technique still needs optimizing.

The results obtained in the healthy control mice appear to be consistent with what has previously been found in the cats and humans in terms of the duration. The magnitude of PActD at different time intervals are comparable between mouse and cat since the experimental method was the same, but magnitude of PActD is not comparable with the values of H-reflex depression in humans, since it shows the indirect result of the depressed EPSPs. However, the results in the mice do inspire a preliminary assumption that the PActD effect, and in particular the duration of it, is similar across species.

The experimental hypothesis concerning the transgenic SOD1 mutants, which was derived from the work on spasticity in human patients and predicted that PActD will be reduced in the mouse model of ALS, was supported by the results from the SOD1 mutant mice. In continuation of this study, it would be worthwhile to increase the number of both control and SOD1 mice and maybe include another type of transgenic mice with a motoneurone disease believed to cause spasticity. In the near future, a Cerebral Palsy model will be available to this lab.

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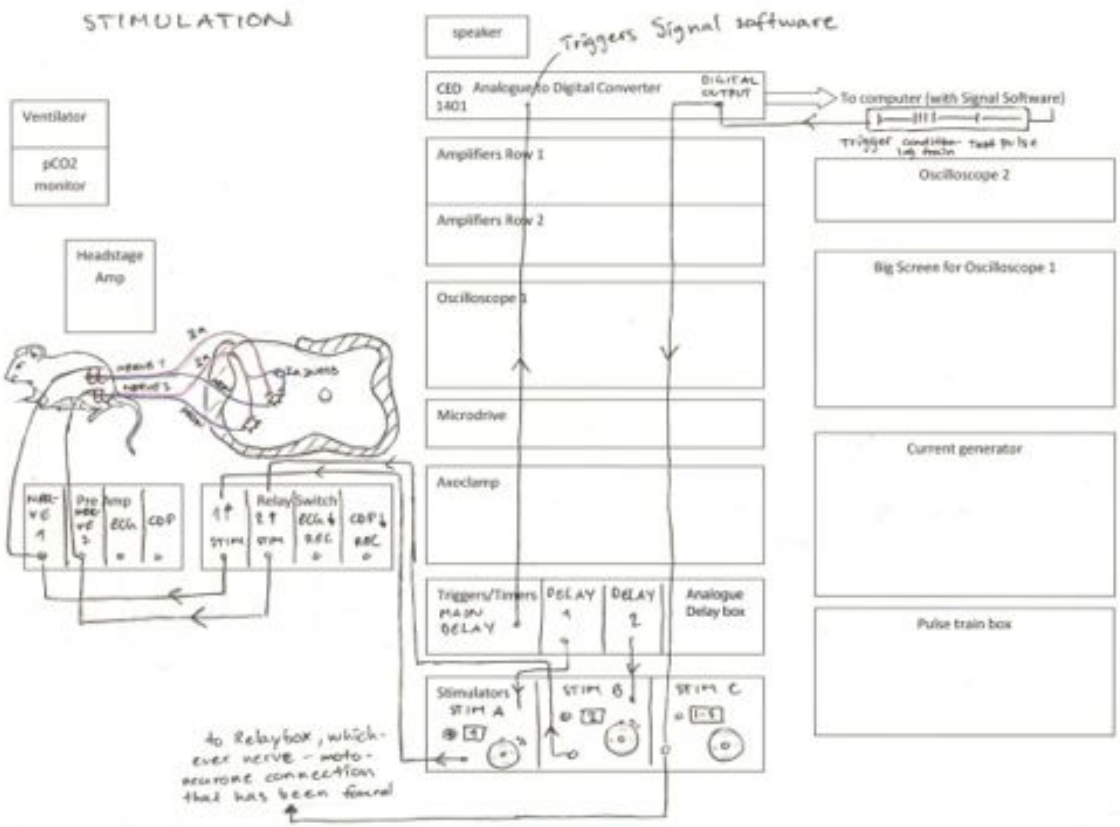
Appendix A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CED Analogue to Digital Converter 1401
DIGITAL OUTPUT
To computer (with Signal Software)
Ventilator
pCO2 monitor
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Amplifiers Row 2
Oscilloscope 2
Headstage Amp
Oscilloscope 1
Big Screen for Oscilloscope 1
Microdrive
Current generator
Axoclamp
Pre Amp
ECG
CDP
Relay Switch
STIM
Triggers/Timers
MAIN DELAY
DELAY 1
DELAY 2
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Pulse train box
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STIM A
STIM B
STIM C
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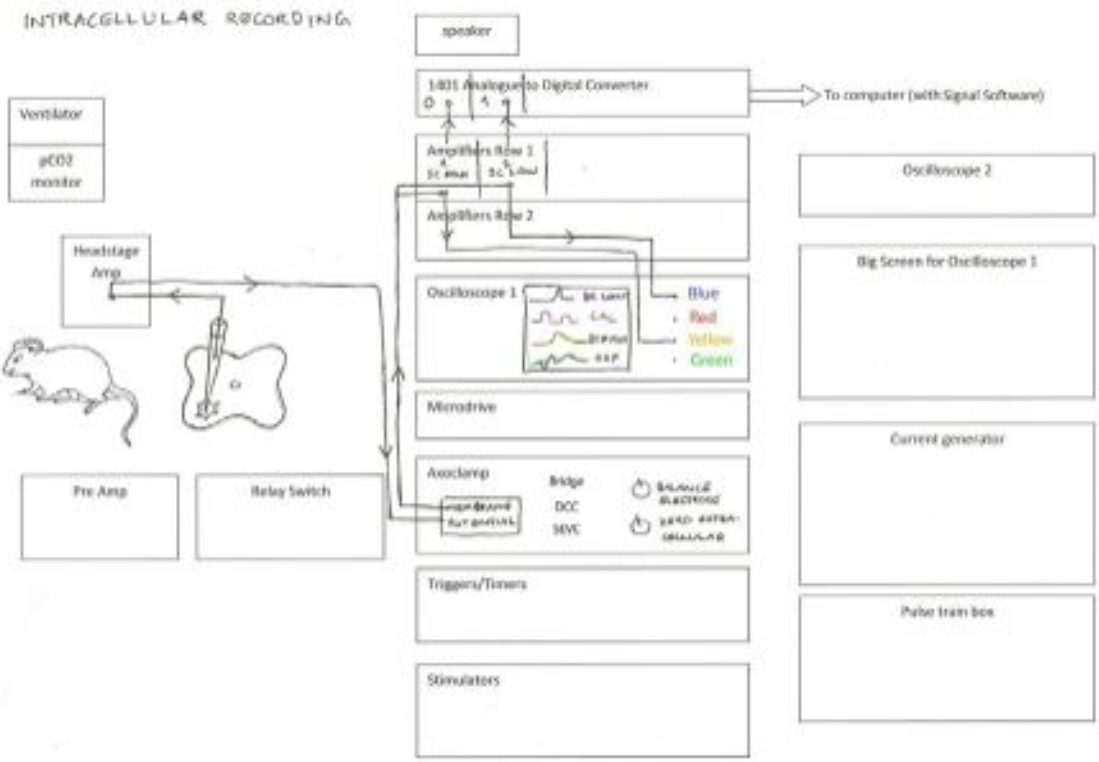
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speaker
1401 Analogue to Digital Converter
To computer (with Signal Software)
Ventilator
pCO2 monitor
Amplifiers Row 1
Amplifiers Row 2
Oscilloscope 2
Headstage Amp
Oscilloscope 1
Big Screen for Oscilloscope 1
Blue
Red
Yellow
Green
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Axoclamp
Bridge
DCC
SEVC
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Relay Switch
Triggers/Timers
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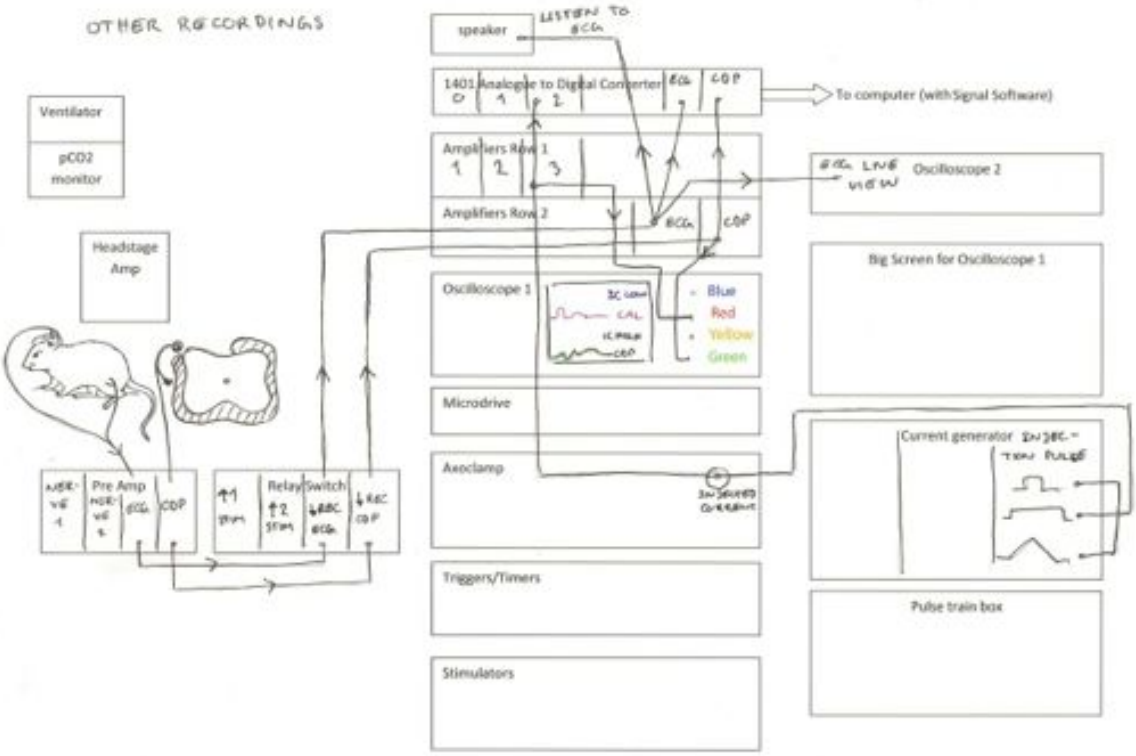
Stimulators


OTHER RECORDINGS
speaker
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Amplifiers Row 1
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Oscilloscope 1
Blue
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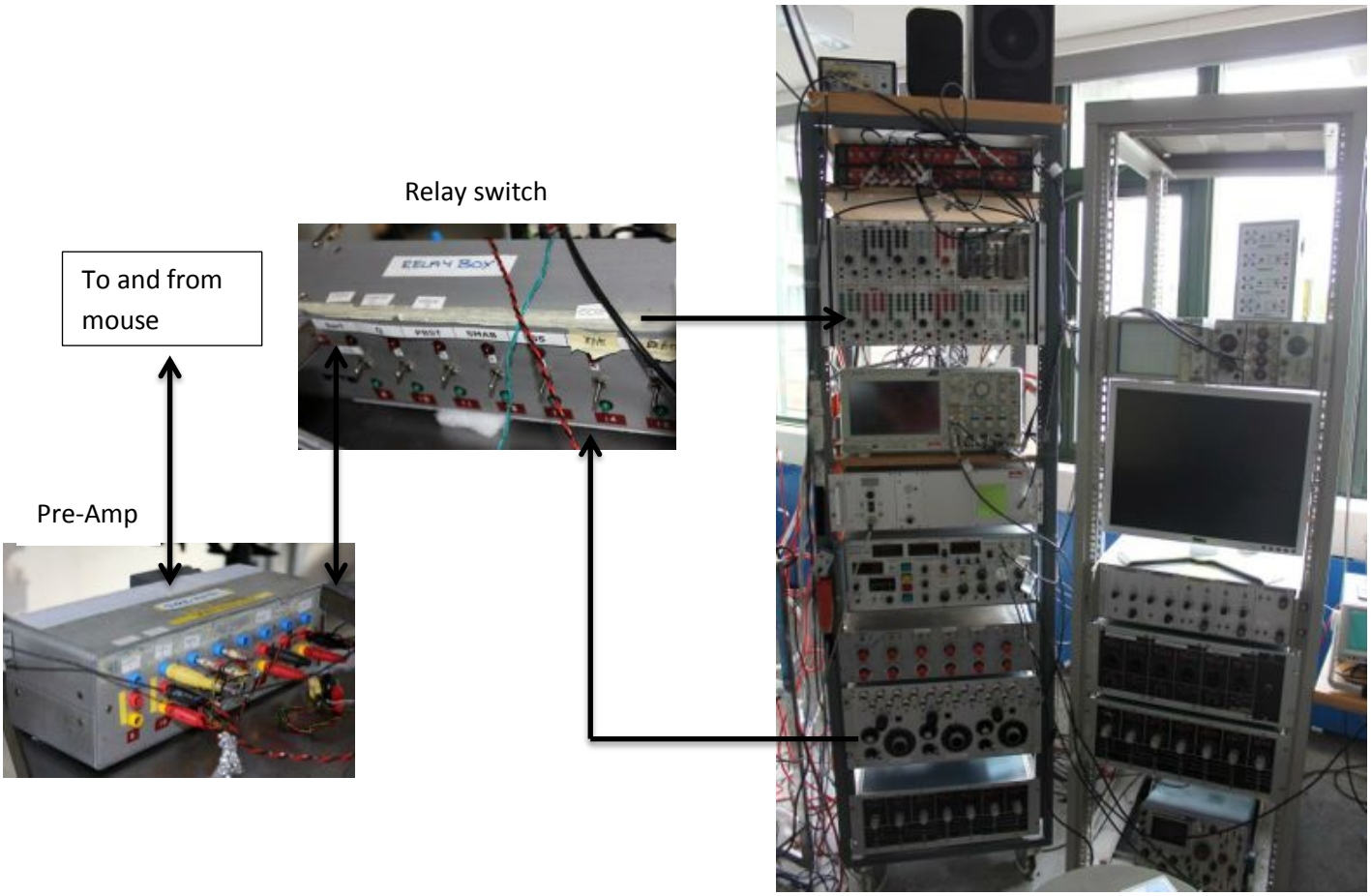
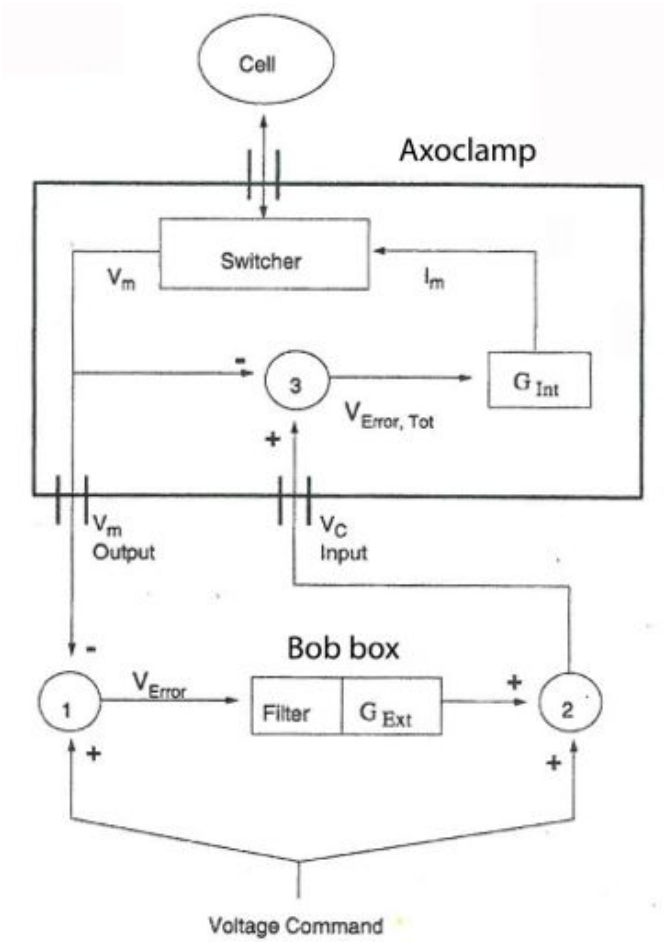
Pre Amp
Relay Switch
Triggers/Timers
Pulse train box
Stimulators


Relay switch
To and from mouse
Pre-Amp

Appendix B

Circuit of the Bob box



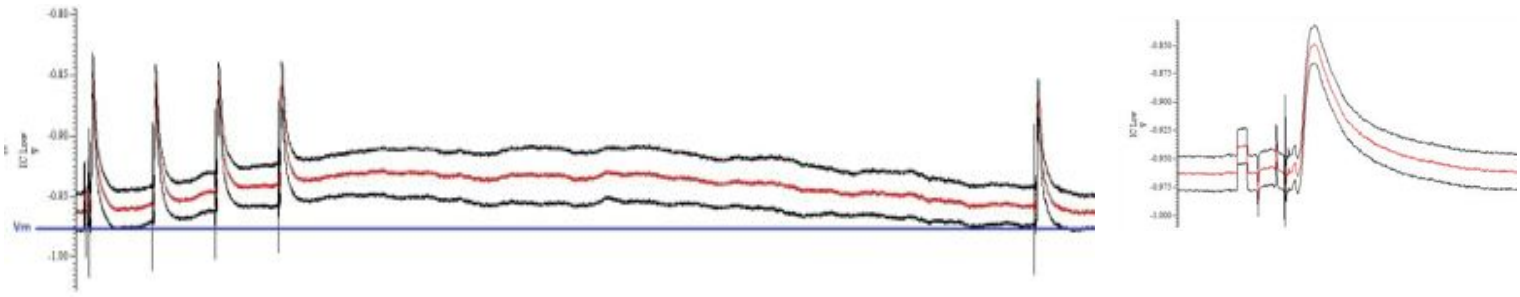
Picture of the actual Bob box

Circuit is from: Heckman C.J., Lee R.H (2001) *Advances in Measuring Active Dendritic Currents in Spinal Motoneurones In Vivo* Chapter 4 in the book: *Motor Neurobiology of the Spinal Cord*, edited by Cope T.C. pp. 97 CRC Press LLC

(I added the text: “Axoclamp” and “Bob box”)

Appendix C

An example of a standard measurement, average is shown in red and ranges in black. 17 frames were included in the average and it can be seen how random fluctuations have been smoothed out. To the left it the blue line illustrates that even though the membrane potential changes during the train, frames where it returns to the same level for the test EPSP have been chosen, which means it can be compared with the size of the conditioning EPSP. To the right a close up of the conditioning EPSP is shown.



Here an example of really good ranges in a very stable cell is shown, 16 frames were included in the average. From such averages, the presynaptic inhibition within the train and the depression of the test EPSP is visible.