An Electrophysiological Investigation of the SNAP-25 Isoforms as Possible Regulators of Short-term Synaptic Plasticity

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KTH Computer Science and Communication

Master of Science Thesis
Stockholm, Sweden 2006
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Master’s Thesis in Biomedical Engineering (20 credits)
at the School of Electrical Engineering
Royal Institute of Technology year 2006
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TRITA-CSC-E 2006:006
ISRN-KTH/CSC/E--06/006--SE
ISSN-1653-5715

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Abstract
Neurons communicate with each other primarily through chemical synapses, where electrical signals are converted into chemical signals and then back to electrical signals. In the synapse, the electrical signal is transformed to a chemical signal through fusion of neurotransmitter-containing vesicles with the presynaptic plasma membrane. This regulated transmitter release is mainly promoted by a presynaptic core protein complex comprised of vesicular VAMP-2/synaptobrevin, plasma membrane-associated syntaxin 1A and synaptosomal-associated protein of 25 kDa (SNAP-25). SNAP-25 is normally expressed in neurons as two alternative isoforms, SNAP-25a and SNAP-25b. The expression of these isoforms is different during development with SNAP-25a predominating early and SNAP-25b becoming dominant in week one and three after birth.

Although these basic molecular components that promote regulated neurotransmitter release are well established, the contribution of these proteins as regulators of synaptic plasticity is less well understood.

The aim of this thesis has been to investigate if and how short-term synaptic plasticity is altered in genetically modified mice that lack SNAP-25b in comparison to wildtype mice expressing both SNAP-25a and SNAP-25b. To examine this, Paired-Pulse Facilitation (PPF) experiments were performed in brain slices from these different mice. PPF is a form of short-term, activity-dependent synaptic plasticity common to many chemical synapses. The experiments were performed by stimulation of presynaptic axon bundles with two electrical pulses in rapid succession while neurotransmitter-induced postsynaptic responses were measured by whole-cell voltage clamp.

The obtained results indicate that SNAP-25b deficient mice show a significantly reduced facilitation. This suggests that SNAP-25b acts presynaptically as a positive regulator of short-term synaptic plasticity. The developmental shift from SNAP-25a to mainly SNAP-25b expression, mediated by alternative splicing, contributes to synaptic transmission by enhancing the facilitory process of neurotransmitter release.
En elektrofysiologisk undersökning av SNAP-25 isoformerna som möjliga regulatorer av synaptisk kort-tids plasticitet

**Sammanfattning**


Dessa proteiner är sedan tidigare kända som ansvariga för frisättningen av neurotransmitter, men deras roll som regulatorer av synaptisk plasticitet är mindre välkänd.

Målet med detta examensarbete har varit att undersöka om och hur synaptisk kort-tids plasticitet är förändrat i genetiskt modifierade möss som saknar SNAP-25b, i jämförelse med normala (wildtype) möss som uttrycker både SNAP-25a och SNAP-25b. För att undersöka detta så har två-puls faciliteringsexperiment (PPF) utförts i hjärnskikt från dessa olika möss. PPF är en typ av aktivitetsberoende synaptisk kort-tids plasticitet som förekommer vid många kemiska synapser. Experimenten genomfördes genom extracellulär stimulering av presynaptiska axonbuntar med två elektriska pulser i snabb följd och sedan mättes de neurotransmitter-inducerade postsynaptiska responserna med whole-cell voltage clamp.

Resultaten indikerar att möss som saknar SNAP-25b uppvisar en signifikant reducering av PPF. Detta antyder att SNAP-25b kan agera presynaptiskt som en positiv regulator av synaptisk kort-tids plasticitet. Skiftet som sker under utveckling från uttryck av framförallt SNAP-25a till SNAP-25b, genom alternativ splitsning, bidrar till synaptisk transmission genom att förstärka faciliteringen av frisättningsprocessen av neurotransmitter.
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1 Introduction

Neurons communicate with each other primarily through chemical synapses. At such synapses, an action potential generated near the cell body propagates down the axon to nerve terminals where calcium (Ca\(^{2+}\)) influx induces fusion of vesicles containing neurotransmitter with the presynaptic plasma membrane. This regulated transmitter release is mainly promoted by a presynaptic core protein complex comprised of vesicular VAMP-2/synaptobrevin and plasma membrane-associated syntaxin 1A and SNAP-25 (Sollner et al., 1993; Chen and Scheller, 2001). SNAP-25 (synaptosomal-associated protein of 25 kDa) is normally present as two alternative isoforms, SNAP-25a and SNAP-25b (Bark, 1993).

The effect of a signal transmitted from one neuron to another can vary depending on the history of activity at either or both sides of the synapse. Such variations at synapses can last from milliseconds to weeks (Staubli and Lynch, 1987) and is generally referred to as synaptic plasticity. The capacity of the nervous system to change is obvious during the development of neural circuits and the transition from immature to mature synaptic connections. However, the adult brain must also possess some plasticity to learn new skills, establish memories, perform synapse regrowth and remodel after neural injury. The mechanisms responsible for ongoing changes in the adult brain are not completely understood, but it appears that these alterations in neural connectivity and neurotransmission rely at least partly on regulated changes in the strength of synaptic transmission.

Although the basic molecular presynaptic core protein complex that promotes regulated synaptic transmission is well known, the contribution of these proteins as regulators of synaptic plasticity is less fully elaborated (Bark et al., 2004).

1.1 Definition of the Problem

During the period of synaptic growth and maturation in the brain, the expression of SNAP-25a and SNAP-25b is altered through alternative splicing of pre-mRNA transcripts. This developmental switch occurs at the age of week one to week three after birth, from expression of predominately SNAP-25a to SNAP-25b transcripts in adult mouse brain (Bark et al., 1995: Boschert et al., 1996: Jacobsson et al., 1999). This regulated expression is also correlated with a shift in the localization of the protein from fibers to nerve terminals (Oyler et al., 1991: Bark et al., 1995). Studies in chromaffin cells indicate that the two isoforms differ in their abilities to stabilize the pool of fully matured synaptic vesicles, where SNAP-25b appears to stabilize these vesicles more efficiently than SNAP-25a (Sørensen et al., 2003). A recent study in a mouse model where the SNAP-25a isoform was overexpressed indicates an alteration in synaptic facilitation (Bark et al., 2004). These results, together with the timing of the developmental shift in the expression of the two SNAP-25 isoforms that occurs during a period of high neural plasticity in the brain, indicates that the two isoforms acts differently in the regulation of synaptic plasticity.

The issue here is therefore: what effect does SNAP-25b have on synaptic transmission and how is it involved in short-term synaptic plasticity?

1.2 Background of the Project

The work of this thesis was performed under supervision of Dr Martin Wikström (assistant Professor) at the Nobel Institute for Neurophysiology, Department of Neuroscience, Karolinska Institute and Dr Christina Bark (associate Professor) at the Unit of Endocrinology at the Department of Molecular Medicine and Surgery, Karolinska Institute. The thesis is part of a major research project performed by Dr Bark and her research group, which aims towards a better understanding of the role and function of the two isoforms of the protein SNAP-25: SNAP-25a and SNAP25b. To examine this, Dr Bark has
generated a mouse model that only expresses one of the two isoforms: SNAP-25a. These genetically modified mice that lack SNAP-25b and only express SNAP-25a are referred to as the “SNAP-25b knockout mouse model”.

1.3 The Aim of the Thesis Project

The aim of this thesis was to investigate if and how short-term synaptic plasticity is altered in the CA1-region of the hippocampus in the SNAP-25b knockout mouse model in comparison to wildtype mice. This may help to further understand the computational behaviour of synapses and how presynaptic proteins can act as regulators of activity-dependent synaptic plasticity.

Electrophysiological techniques were used to study how the synaptic strength between neurons may be altered during short periods of time. The main form of short-term synaptic plasticity investigated is called Paired-Pulse Facilitation (PPF), which is dependent on presynaptic mechanisms. Experiments were performed with extracellular stimulation of presynaptic axon bundles in order to elicit action potentials, exocytosis and plastic mechanisms.
2 Synaptic Transmission via Chemical Synapses

Synapses are the functional connections between neurons and are made up of the end of the signal-transmitting (presynaptic) neuron: the signal-detecting machinery on the receiving (postsynaptic) neuron: and the small gap in between (the synaptic cleft). See Figure 1. Chemical synapses enable neurons to communicate via the secretion of neurotransmitters, which are chemical agents acting as messengers between the pre- and postsynaptic neuron. Secretion is triggered by $\text{Ca}^{2+}$ influx due to arrival of action potentials, which are electrochemical signals generated near the soma of a neuron that rapidly propagates down the axon until it reaches the presynaptic terminal.

Neurotransmitters are contained in synaptic vesicles, small membrane-bound organelles in the presynaptic terminal. Upon action potential arrival in the presynaptic terminal, synaptic vesicles fuse with the presynaptic membrane and neurotransmitter are released into the synaptic cleft. The neurotransmitters diffuse over the synaptic cleft and bind to receptors clustered in the postsynaptic plasma membrane, initiating a new wave of electrical signals in the postsynaptic neuron.

2.1 Structure of Chemical Synapses

The key feature of chemical synapses is the presence of synaptic vesicles that contain neurotransmitter. This is shown in Figure 1 that describes the general structure and function of a chemical synapse. It also illustrates the sequence of events that synaptic transmission at chemical synapses is based on. The process is initiated when an action potential reaches the presynaptic terminal, which depolarises the membrane in the synapse that leads to opening of voltage-gated $\text{Ca}^{2+}$ channels. This causes a rapid influx of $\text{Ca}^{2+}$ into the presynaptic terminal. This elevation of the intracellular $\text{Ca}^{2+}$ concentration stimulates synaptic vesicles to fuse with the presynaptic membrane. Fusion of vesicles with the membrane is called exocytosis, and during exocytosis the neurotransmitters in the synaptic vesicles are released into the synaptic cleft and bind to specific postsynaptic receptors in the plasma membrane. Different neurotransmitters affect the postsynaptic neuron differently although many of their effects lead to opening or closing of postsynaptic ion-channels, thus changing the ability of ions to flow into or out of the postsynaptic neurons. The resulting neurotransmitter-induced current (the flow of ions through the membrane) alters the membrane potential of the postsynaptic neuron, increasing or decreasing the probability that the postsynaptic neuron will fire an action potential. In this way, information is transmitted from one neuron to another, by converting electrical impulses into chemical signals and back again.
2.2 Molecular Mechanisms of Neuroexocytosis

Neuroexocytosis depends both on the presence of a pool of readily releasable or “primed” vesicles, formed by maturation of vesicles already docked to the plasma membrane (Parsons et al., 1995) and on the strong dependency of the fusion probability of primed vesicles on the intracellular calcium concentration (Dodge and Rahamimoff, 1967). The readily releasable pool (RRP) contains vesicles that are in a fully matured state so that they can fuse with the plasma membrane directly upon the arrival of an action potential in the presynaptic terminal.

It seems that the Ca$^{2+}$-binding function of a protein called “synaptotagmin I” is likely to form the basis for the calcium sensor of exocytosis (Mackler et al., 2002; Shin et al., 2003). The question is, however, what the molecular basis is for the formation of the primed pool of vesicles and especially how the developmentally regulated isoforms of SNAP-25 regulate vesicular fusion and thereby synaptic plasticity.
2.2.1 SNAREs

Soluble N-ethylmaleimide-sensitive factor attached protein receptors (SNAREs), are proteins implicated in many fundamental cellular processes that require membrane fusion (Weimbs et al., 1997; Bock et al., 2001). Especially the interactions of the SNARE proteins SNAP-25, syntaxin 1A and VAMP/synaptobrevin have been extensively studied (see Figure 2). These proteins can be divided into target-SNAREs (t-SNAREs), located in the cell plasma membrane, and vesicle-SNAREs (v-SNAREs) located in the vesicle membrane. The t-SNAREs are composed of the syntaxin and SNAP-25 groups, and the v-SNAREs are constituted by the VAMPs (vesicle-associated membrane proteins), that are also named synaptobrevins.

SNAP-25, syntaxins, and VAMPs present on opposing membranes can associate to form a high-stability complex in which the core consists of a bundle of four parallel alpha helices, in which SNAP-25 contributes with two out of these four helices (Brunger 2000). Formation of this SNARE complex brings opposing membranes into close proximity, which promotes vesicle fusion (Jahn and Südhof, 1999; Chen and Scheller, 2001).

Figure 2: Structure of the SNARE complex responsible for membrane fusion.
(From Neuroscience, third edition. Purves et al., 2004)

2.2.2 SNAP-25

SNAP-25 is located in the presynaptic plasma membrane and was first identified in 1989 (Oyler et al., 1989). In neonatal rodent brain there is a low level of SNAP-25 mRNA, consisting primarily of SNAP-25a. However, as the brain matures the level of SNAP-25 is increased due to a developmental switch from expression of predominately SNAP-25a to SNAP-25b transcripts that ultimately constitute over 80 % of SNAP-25 mRNA in adult mouse brain (Bark et al., 1995; Boschert et al., 1996; Jacobsson et al., 1999). Figure 3 shows that this developmental switch occurs between the first and the third week after birth.
The *Snap* gene is a single copy gene in which nine exons have been identified and localized (see Figure 4). Exon 5 exists in two similar but different sequences called exon 5a and 5b (Bark IC, 1993). The SNAP-25 protein is expressed by alternative splicing of these exons, giving rise to the two highly homologous isoforms SNAP-25a and SNAP-25b that differ by only 9 out of 206 amino acids (Bark and Wilson, 1994; Bark et al., 1995). Some differences in between the two isoforms include non-conservative changes within the N-terminal helical SNARE domain (Fasshauer et al., 1998) and clustered cysteine residues that provide sites for palmitoylation (Hess et al., 1992; Veit et al., 1996). These are involved in membrane association (Lane and Liu, 1997; Gonzalo and Linder, 1998; Gonzalo et al., 1999; Koticha et al., 1999) and disassembly of the SNARE complex after exocytosis (Washbourne et al., 2001).

**2.2.3 Vesicle Priming**

Before a vesicle fuses with the plasma membrane, it must go through several maturation steps, starting with the biogenesis of the vesicle followed by translocation and docking to the plasma membrane. In order for the neuron to be able to respond rapidly to stimulation it needs a pool of vesicles that have already passed slower maturation steps and that are ready for release directly when an action potential reaches the presynaptic terminal. The vesicles in this pool (RRP) are characterized by very low release rate constants at resting intracellular Ca$^{2+}$ levels and very high release rate constants at stimulated intracellular Ca$^{2+}$ levels (Rosenmund and Stevens, 1996). There are also pools of vesicles that are in
less mature states. These function as reserve pools to the RRP and are mobilized in a use-dependent manner. Therefore, at least three vesicle pools can be distinguished: undocked, docked but unprimed, and readily releasable vesicles (see Figure 5).

The fully primed vesicles in the RRP must be in a state which is stable with respect to other states. The relative rate constants for entry into (priming) and exit out of (depriming) this state must be adjusted so that a significant number of vesicles will occupy this state at rest. This means that the primed state of vesicles must be relatively stable in order for such a pool of vesicles to build up. It also indicates two ways in which release can be regulated: by changing the stability of vesicles in the RRP through either the priming rate or the depriming rate (Sørensen, 2004).

It seems that regulation of the priming of vesicles has important consequences for synaptic function, by regulating the amount and activity dependency of neurotransmitter release. Thus, vesicle priming might be crucial in determining the fidelity with which presynaptic terminals transfer signals to the postsynaptic neuron (Dobrunz and Garner, 2002).

Experiments done in chromaffin cells where SNAP-25a and SNAP-25b were over-expressed in a complete SNAP-25 knockout mouse model (Sørensen et al., 2003), suggests that the two isoforms confer different stability to the primed vesicles by affecting the priming or depriming rate. It seems that SNAP-25b has a higher ability to stabilize the pool of primed vesicles than SNAP-25a, primarily by decreasing the depriming rate. In these experiments, it was also seen that by overexpressing SNAP-25b the size of the RRP increased threefold compared to in wildtype chromaffin cells.

**Figure 5**: The figure shows the last stages of vesicular trafficking in the vicinity of the presynaptic membrane. (1) Vesicles are recruited to the target membrane. (2) Vesicle docking. (3) Vesicle priming. This intermediate step makes vesicles fusion competent so that they are in a fully matured state. These are the vesicles in the readily releasable pool (RRP) of vesicles. (4) Vesicle fusion. The SNARE proteins are the most likely candidates to promote membrane fusion.

In the picture, synaptobrevin is the (blue) rod on the vesicle, SNAP-25 is the (green) double rod in the plasma membrane, and syntaxin is the (red) coiled rod vertically positioned from the plasma membrane. (From Toonen and Verhage, 2003)
3 Synaptic Plasticity

Synaptic transmission is a dynamic process. That is, the strength of synapses (how effectively they relay information) is not fixed but can be modified by the activity at the synapse. This modifiability means that the brain is plastic, that it can change and adjust to different activity patterns. Indeed, the plasticity mediated by changes at synapses is the principal means by which the nervous system adapts to the external environment. Synaptic plasticity is crucial to the development of the nervous system and thereafter to the ability of an individual to learn and remember new information and to adjust its behaviour accordingly.

Synaptic plasticity can be divided into three broad categories:

1. **Long-term plasticity**, involving changes that last for hours or longer, is thought to be cellular substrates to learning and memory.

2. **Homeostatic plasticity** of both synapses and neurons allows neural circuits to maintain appropriate levels of excitability and connectivity despite changes brought about by protein turnover and experience-dependent plasticity.

3. **Short-term synaptic plasticity**, which is the main phenomena of interest in this thesis, occurs over milliseconds to minutes and allows synapses to perform critical computational functions in neural circuits. (Abbot and Regehr, 2004)

3.1 Short-term Synaptic Plasticity

Virtually all types of synapses are regulated by a variety of short-lived and long-lasting processes, some of which lead to a decrease, termed depression, in synaptic strength and others that lead to synaptic enhancement. In most cases multiple processes are present, and the result can be a combination of enhancement and depression in which synaptic strength is highly dependent on the timing and nature of synaptic activation (Tsodyks and Markram, 1997; Varela et al., 1997; Magleby, 1987; Dittman et al., 2000).

Synaptic enhancement is often attributed to effects of a residual elevation in presynaptic intracellular calcium concentration, $[\text{Ca}^{2+}]$, acting on one or more presynaptic molecular targets, while depression can be attributed to depletion of vesicles in the RRP.

The form of short-term synaptic plasticity investigated in this thesis is a form of synaptic enhancement termed facilitation that acts on the time scale of hundreds of milliseconds.
Figure 6: Short-term synaptic plasticity, here shown at the neuromuscular junction. The figure illustrates postsynaptic electrical recordings of end plate potentials (EPPs) elicited in a muscle fiber by a train of electrical stimuli applied to the presynaptic motor nerve. Facilitation of the EPPs is seen at the first few pulses as enhanced responses, which is then followed by depression of the EPPs seen as decreased responses. After the train of stimuli ends, EPPs are larger than before the train. This form of short-term synaptic plasticity is called post-tetanic potentiation. (From Neuroscience, third edition. Purves et al., 2004).

[Note: In the experiments performed during the thesis, postsynaptic responses are measured as currents and not potentials, therefore the difference in their appearance in comparison with this figure.]

3.1.1 Facilitation

Synaptic facilitation is a transient enhancement in synaptic strength and can occur when two or more action potentials invade the presynaptic terminal in close succession. This can be seen experimentally with pairs of presynaptic stimuli while recording the postsynaptic responses to these stimuli, in which the second response can be up to five times the size of the first response (see Figure 6 and 8). The main experimental method used to investigate this phenomenon is called Paired-Pulse Facilitation (PPF) and is described in detail in Ch 5.3.1.

Facilitation is the result seen by more neurotransmitters being released by each succeeding action potential, increasing the amplitude of the postsynaptic current/potential progressively.

3.1.2 The Role of Ca\(^{2+}\) Ions in Synaptic Plasticity

Synaptic facilitation is most likely the result of prolonged elevation of presynaptic Ca\(^{2+}\) levels following synaptic activity. As described earlier, the invasion of action potentials in the presynaptic terminal leads to opening of voltage-gated Ca\(^{2+}\) channels which results in a rapid influx of Ca\(^{2+}\) into the presynaptic terminal and these elevated levels of Ca\(^{2+}\) concentration trigger exocytosis (see Figure 1 and 6).
Figure 7: Ca$^{2+}$ influx in the presynaptic terminal triggers exocytosis. The figure shows microdomains with high Ca$^{2+}$ concentrations that form in the cytosol around open Ca$^{2+}$ channels and trigger exocytosis. (A) Demonstrates a model of Ca$^{2+}$ dynamics in the terminal, where open Ca$^{2+}$ channels are spaced along the x-axis. Near the mouth of the channel there is a high local concentration (>800 µM) of Ca$^{2+}$, but within just 50 nm of the channel the concentration is only about 100 µM. (B) In the active zone (shaded area), an action potential has opened a fraction of the Ca$^{2+}$ channels and synaptic vesicles in the RRP that are docked and primed may come under the influence of one or more of these microdomains and thereby be triggered to fuse with the membrane. (C) A few milliseconds after the action potential, the channels have closed and the microdomains have dispersed. The overall Ca$^{2+}$ concentration is now slightly higher than before the action potential occurred. That is, some residual Ca$^{2+}$ still remains. If another action potential arrives at the terminal in close succession to the first, there will be a build up of Ca$^{2+}$ in the terminal that may influence more vesicles than the first action potential since the Ca$^{2+}$ microdomains will then be larger. If no action potential occurs, the cell will pump the extra Ca$^{2+}$ out across the plasma membrane and restore the initial condition after several 100 ms. (From Fundamental Neuroscience, second edition, Squire et al., 2003)
According to the residual calcium hypothesis (Katz and Miledi, 1968; Miledi and Thies, 1971; Parnas et al., 1982), facilitation is caused by an action of Ca\textsuperscript{2+} remaining in the nerve terminals after the first action potential. That is, while the inflow of calcium is very rapid and occurs within a millisecond or two, the diffusion and removal of this calcium out of the presynaptic terminal is much slower. Thus, when a second action potential arrives at the presynaptic terminal in rapid succession to the first, there will still be residual calcium left within the presynaptic terminal. This leads to calcium build up inside the terminal which results in an enhancement of the probability that a vesicle will be released. Thus, more transmitter are released during this second action potential than the first, and the postsynaptic response is thereby greater, or facilitated, relative the postsynaptic response of the first action potential (see Figure 7 and 8).

Figure 8: The figure shows the two forms of short-term synaptic plasticity called facilitation and depression. To the left, electrical recordings of postsynaptic currents during presynaptic electrical stimulation is illustrated. For facilitation, the proposed role of calcium ions, represented by grey shading, is also shown. For depression, depletion of vesicles is shown. (From Xu-Friedman and Regehr, 2004)

3.1.3 Mechanisms of Enhancement

To our knowledge, in all synapses studied facilitation has been shown by quantal analysis to be presynaptic in origin and to involve specifically an increase in the number of transmitter molecules released by an action potential. The Ca\textsuperscript{2+} dependent enhancement is thought to be due to one or more of mainly three underlying mechanisms: (1) an increase of the release probability for the transmitter, (2) a change in the number of vesicles in the RRP, (3) an increase in the number of release sites or active zones, which is the site of synaptic vesicle clustering, docking and transmitter release in the presynaptic membrane that immediately oppose postsynaptic receptors. (Zucker, 1989; McLachlan, 1978; Zucker, 1973)

All these mechanisms are tightly linked to each other and alterations in the probability of neurotransmitter release might occur at several stages in the release process, such as during docking, priming or fusion of synaptic vesicles. This tight linkage makes the exact interpretation of underlying mechanisms of facilitation very complex: it seems that short-term synaptic enhancement reflects an increase in the probability of release of available synaptic vesicles, with perhaps also an increase in the number of release sites capable of releasing a vesicle. Either statistical change could be due in turn to an increase in the probability of activating exocytosis of a docked vesicle or an increase in the probability that a release site is occupied by a docked vesicle ready for release (Worden et al., 1997). The latter could occur if the pool of vesicles available to rapidly occupy release sites is increased. (Zucker and Regehr, 2002)

However, while some studies have shown that release probability is correlated with the RRP size across a population of hippocampal synapses (Dobrunz and Stevens, 1997; Murthy et al., 2001) another study indicate that the RRP size does not actually influence release probability (Hanse and Gustafsson, 2001). One hypothesis is that this relationship is causal, and that RRP size is one factor
that determines release probability. Alternatively, the correlation between release probability and RRP could be coincident to some other factor that varies across the population of synapses.

In addition to this, the size of hippocampal synapses varies by up to 10-fold (Sorra and Harris, 1993; Harris and Sultan, 1995; Schikorski and Stevens, 1997; Shepherd and Harris, 1998), and as discussed above many aspects of the presynaptic terminal scale together, including active zone size, RRP size, number of docked vesicles, and presynaptic terminal volume (Harris and Sultan, 1995; Schikorski and Stevens, 1997; Murthy et al., 2001). If the correlation observed between RRP and release probability across the population of synapses is in fact secondary to some other difference between synapses then RRP might not be a determining factor of release probability. However, within individual hippocampal synapses release probability does seem to be correlated with RRP size (Dobrunz, 2002).

According to the hypothesis that SNAP-25a and SNAP-25b differ in their ability to stabilize the pool of primed vesicles and thereby affecting the size of the RRP, such a difference should possibly be seen in the amount of facilitation between wildtype mice expressing both SNAP-25a and SNAP-25b and the SNAP-25b knockout mouse model. However, if such a difference is a direct effect of a change in RRP size could be more complex to evaluate.
4 Bioelectricity

Neurons are cells specialized for the integration and propagation of electrical events. At the cellular level, neuronal activity and, ultimately, behavior are dependent upon movement of ions through the surface membranes of neurons. However, the cell membranes are primarily composed of lipids which do not allow ionic flow. Instead, these semipermeable membranes contain large specialized protein aggregates that form ion-specific channels through the lipid membrane in which ions can flow between the inside and outside of the cell. The most abundant ions that flow through these channels are sodium (Na$^+$), potassium (K$^+$), chloride (Cl$^-$) and calcium (Ca$^{2+}$), see Table 1.

Table 1: Extracellular and intracellular ion concentrations in neurons. The concentration of ions is different for the inside and outside of a neuron which results in a concentration gradient over the cell membrane. (From Neuroscience, third edition. Purves et al., 2004).

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concertration (mM)</th>
<th>Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Squid neuron</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium (K$^+$)</td>
<td>400</td>
<td>20</td>
</tr>
<tr>
<td>Sodium (Na$^+$)</td>
<td>50</td>
<td>440</td>
</tr>
<tr>
<td>Chloride (Cl$^-$)</td>
<td>40–150</td>
<td>560</td>
</tr>
<tr>
<td>Calcium (Ca$^{2+}$)</td>
<td>0.0001</td>
<td>10</td>
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<tr>
<td><strong>Mammalian neuron</strong></td>
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<tr>
<td>Potassium (K$^+$)</td>
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<td>5</td>
</tr>
<tr>
<td>Sodium (Na$^+$)</td>
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<td>145</td>
</tr>
<tr>
<td>Chloride (Cl$^-$)</td>
<td>4–30</td>
<td>110</td>
</tr>
<tr>
<td>Calcium (Ca$^{2+}$)</td>
<td>0.0001</td>
<td>1–2</td>
</tr>
</tbody>
</table>

The ability of these channels to permit ion flow is determined by several factors, most prominently the electrical potential that exists across the membrane, the gradient of ions set up by membrane pumps, and the selective nature of the ion-channels. Different ion-channels are permeable to different ions and this is due to the channel pore that has a certain size and polarization and that therefore only permits certain ions to pass the pore.

4.1 Electrical Properties of Neurons

A neuron derives its electrical properties mostly from the electrical properties of its membrane, which in turn acquires its properties from its lipids and proteins. The membrane potential of a neuron refers to the electrical potential difference between the inside and outside of the neuron, and according to a commonly accepted convention the potential of the external solution is zero. The transmembrane potential is defined as the potential at the inner side of the cell membrane relative to the potential at the outer side of the membrane.

The resting membrane potential is the result of two opposing forces: a chemical force and an opposing electrical force (see Figure 9). The concentration of ions is different for the inside and outside of the neuron which creates concentration gradients, leading to a flow of ions proportional to the concentration gradient. When an ion leaves one side of the membrane, it leaves an unmatched charge on the side it left and it will itself become unmatched on the side it reaches, thus producing a charge separation. This charge separation introduces a non-random new electrostatic force acting on the ions. This electrostatic force is opposite to the chemical force and tends to drive ions in the opposite direction and at the same time it tends to brake the flow of ions down the concentration gradient.
final result is that the charge separation will build up a voltage across the membrane \( V = Q/C \) that will continue to increase until the flow in both directions becomes equal due to the increased electrostatic force that will tend to balance the flow produced by the concentration gradient. In this state, any ion that crosses in one direction will be counterbalanced by another crossing in the opposite direction, maintaining an equilibrium situation. The electrical potential generated across the membrane at electrochemical equilibrium, the equilibrium potential, can be predicted by a formula called the Nernst equation: 

\[
E_X = (RT/zF) \ln([X]_{\text{outside}}/[X]_{\text{inside}}),
\]

where \( E_X \) is the equilibrium potential for any ion X, \( R \) is the gas constant, \( T \) is the absolute temperature, \( z \) is the valence of the ion, and \( F \) is the Faraday constant. The brackets indicate the concentrations of ion X on each side of the membrane.

The resting membrane potential describes a steady-state condition determined by the intracellular and extracellular concentrations of ions, membrane transporters, and importantly on the relative permeability of the membrane to these ions. At rest, membranes of neurons are mainly permeable to potassium (\( K^+ \)) through so called leak channels. The resting membrane potential of hippocampal neurons is typically around -70 mV. For a detailed discussion on how the resting membrane potential is generated, see Ch 7 in “Principles of Neural Science” (Kandel et al., 2000).

In electrical terms, the membrane of a cell can be represented as a resistor of value \( R \) in parallel with a capacitance \( C \). The resistance, or conductance which is the inverse of resistance, refers to the ion channel properties of the membrane.

The current through an ion channel is described by Ohm’s law: 

\[
I_{\text{ion-channel}} = \Delta V/R = \Delta V/\gamma
\]

\( \Delta V \) represents the electrochemical driving force, which is the membrane potential minus the reversal potential. The reversal potential is the potential at which the net flow of charge through the channel is zero. The conductance of an individual ion channel is represented by \( \gamma \).

The capacitance refers to the ability of the membrane to store charge on its inner and outer surface. That is, as a consequence of the membrane’s thinness (typically less than 10 nm thick) and the
properties of its lipids to resist ion flow, the membrane serves as an excellent capacitor. The current flowing through a capacitor is proportional to the voltage change with time and is referred to as a capacitive current: 

\[ I_{\text{capacitive}} = \frac{dQ}{dt} = C \frac{dV}{dt} \]

That is, as long as the voltage across the membrane remains constant this current is zero and the effect of the membrane capacitance on the currents flowing through ion channels can be ignored. However, while the voltage across the membrane changes: there are transient capacitive currents in addition to the steady-state currents through ion channels.

A thorough description of the electrical properties of neurons can be find in chapter one of the Axon Guide (see references).
5 Electrophysiology

In order to study the role of SNAP-25b as a regulator of plasticity, different electrophysiological techniques are used. To elicit action potentials within nerves of presynaptic neurons, extracellular electrical stimulation is made with a concentric metal bipolar electrode that is placed above nerves within the hippocampus. To examine the responses of these action potentials in the postsynaptic neuron, intracellular recordings are performed with borosilicate glass microelectrodes filled with artificial intracellular solution. This way, one may elicit action potentials presynaptically and directly measure the currents or potentials that they give rise to in the postsynaptic neuron as a consequence of exocytosis and neurotransmitter activation of ionotropic receptors in the postsynaptic neuron. The recording technique used is called the patch clamp method.

5.1 The Patch Clamp Method

The patch clamp method was developed by Neher and Sakmann in the mid 70’s. With this technique it is possible to study the properties of ion channels, both those associated with neurotransmitters and/or second messengers and voltage-dependent ion channels (Neher and Sakmann, 1992).

The technique is based on the use of a glass pipette with a very small tip opening (approximately 1 micrometer in diameter) that is filled with artificial intracellular solution. The pipette is used to make tight contact with a small area, or patch, of the cell membrane (see Figure 10, 12, 16 and 18). The electrode is gently placed in close contact with the membrane of the cell body and by applying a small amount of suction to the back of the pipette, a high-resistance (giga ohm) seal is formed between the pipette and the membrane. This seal is so tight that no ions can flow between the pipette and the membrane and thus, all ions that flow through ion channels in this patch must flow into the pipette. The resulting electrical current from this ion movement, though very small, is then measured by the use of a very sensitive amplifier connected to the glass pipette.

The patch clamp method can be applied in at least four configurations (see Figure 10), giving the technique formidable adaptability for testing the molecular mechanisms of receptors and their associated ion channels and second messengers. Three of these configurations (cell-attached recording, inside-out recording, and outside-out recording) are used to study individual ion channels under different conditions.

However, to study the postsynaptic responses to presynaptic activity, the “whole-cell voltage clamp” configuration provides the most accurate and direct means for measuring the effects of neurotransmitters. This is performed by recording the intracellular postsynaptic currents, during voltage control, that result from the opening and closing of ion channels during the binding of neurotransmitters to their receptors.
5.1.1 Whole-cell Voltage Clamp

In the whole-cell configuration, a brief but strong suction is applied to the pipette after the formation of the tight seal which ruptures the membrane under the tip without disrupting the tight seal or cell viability. By doing so, the interior solution of the pipette becomes continuous with the cytoplasm of the neuron and the contents of the pipette equilibrate over time with the cytoplasm. This arrangement allows measurements of the “macroscopic” or summed currents flowing through all channels in the entire cellular membrane, rather than through a single channel. That is, recordings of electrical potentials and currents from the entire cell can be done and therefore the name whole-cell recording method is used. The voltage-clamp technique means that one controls the membrane potential and measures the transmembrane current required to maintain that voltage. That is, the membrane potential of the neuron is held constant, “clamped”, at a specific voltage.
The measured membrane current itself is not actually the focus of interest, but rather the membrane conductance because this is directly proportional to the ion channel activity. That is, in a voltage clamp experiment, when N channels, each of conductance \( \gamma \), are open, the total conductance is \( N\gamma \). The electrochemical driving force \( \Delta V \) thus produces a current \( I = N\gamma \Delta V \). When ion channels open and close, \( N \) changes and thereby also the voltage-clamp current \( I \). Hence, the voltage clamp current is proportional to the number of open channels, given that the conductance is constant. The number of open channels is in turn dependent on the amount of neurotransmitters being released from the presynaptic neuron which we are interested in. Current is measured because there is no direct way to measure the conductance.

Holding the membrane potential constant ensures that the current measured is linearly proportional to the conductance being studied. That is, if the voltage would not be held constant, the current flowing across the membrane would change the voltage by charging the membrane capacitance, and when this happens it is considerably more difficult to relate an electrical measurement to the functional state of a channel. Also, by holding the voltage constant the capacitive current is eliminated, except for a brief time following a step to a new voltage.

**5.1.2 The Patch Clamp Setup**

Figure 11 shows the standard experimental setup used to perform patch clamp experiments. The setup consists of a microscope (Olympus BX50WI) with a maximal magnification of 120 times, standing on a vibration isolation table in order to achieve a stable environment. The recording electrode is connected to a patch clamp amplifier (Axopatch 200B, Axon Instruments Inc.), which is the most important equipment since it is both clamping the voltage across the cell membrane and at the same time measuring the currents through the ion channels of the membrane. The concentric metal bipolar stimulation electrode (Fredrik Haer & Co) is connected to an isolated current source (SIU90, Cygnus Technology) which is triggered by an external pulse generator (Master-8, A.M.P.I. Instruments LTD). The recording electrode is connected to a headstage, and both electrodes are mounted on electrical micromanipulators (Luigs and Neumann SM-5) that are used to steer the electrodes. The patch clamp amplifier is connected through a digital interface (Digidata 1200, Axon Instruments) to a personal computer on which data acquisition is performed using “pClamp7” software (Axon Instruments). The setup ensures a stable experimental environment, where effects of external electrical noise have been minimalized by thorough grounding and shielding of the equipment. A detailed description of the patch clamp setup can be found in “Unit 6.1 of Current Protocols in Neurophysiology” (see references).
5.1.3 Recording Electrode

As described previously, a small glass pipette is used to measure currents from ion channels in the membrane of neurons. The pipette itself is mounted in a pipette holder that is attached to a preamplifier inside the headstage which in turn is connected to the patch clamp amplifier. A syringe is connected via a tube to the pipette holder so that suction can be applied to the pipette to enable the formation of a tight seal between the neuronal membrane and the pipette.

Pipettes are constructed by pulling glass microcapillaries on a special puller (model PP-830, Narishige) which heats the glass and at the same time pulls on both ends of the capillary. This is performed in two steps until the microcapillary divides into two pipettes with very fine tips. The diameters of the pipette tips are approximately 1 µm and have a resistance of about 5-10 MΩ when they are filled with artificial intracellular solution.

Inside the pipette holder and the pipette there is an Ag-AgCl (silver-silverchlorided) wire which is in contact with the pipette solution at one end and connected to the preamplifier inside the headstage via a copper wire in the other end. The bath solution in the recording chamber is grounded by another Ag-AgCl wire, connected to ground of the headstage amplifier. Thus, when the recording electrode is inside the bath of the recording chamber, there is a complete electrical circuit (see Figure 12).
At the electrodes, current is transformed from a flow of electrons in the silver wire to a flow of ions in solution. This liquid-metal junction is also the interface where the largest junction potential occur, along with the liquid-liquid junction formed at the tip of the micropipette. Junction potentials are unwanted potential differences that occur wherever dissimilar conductors are in contact. The sum of all junction potentials is eliminated before recordings so that they do not interfere with the measurements. This is done by applying a single DC potential of opposite polarity (see Ch 8.3.1).

5.3 Electrical Stimulation

In order to study neurotransmitter induced postsynaptic responses of presynaptic action potentials, extracellular electrical stimulation is used to evoke action potentials within presynaptic axons. By applying current pulses through a stimulating electrode positioned over bundles of presynaptic axons (see Figure 15), charges on the axon membranes are relocated. This leads to a local artificial depolarization in axons which evokes action potentials within these axons. The action potentials then travel down their axons to synapses where neurotransmitter release is triggered.

Current pulses are produced in an isolated current source, triggered by an external pulse generator. In the external pulse generator the exact stimulation protocols are programmed, such as the time in between stimulation pulses and the duration of pulses.

5.3.1 Paired-Pulse Facilitation

To examine how the lack of SNAP-25b affects synaptic facilitation, experiments with paired presynaptic stimuli are performed (see Figure 13). Paired-Pulse Facilitation (PPF) is a relatively short-term, use-dependent form of synaptic plasticity, which occurs at many chemically transmitting synapses. As described in Ch 3.1.1, PPF is defined as an increase in the size of the synaptic response to a second pulse delivered within a short time interval following the first pulse (40-300 ms). The comparison of the amplitude of the second excitatory postsynaptic current (EPSC) to the first is called the PPF ratio=amplitude of EPSC$_2$/amplitude of EPSC$_1$, and is the way of quantifying the enhancement.

The generally accepted explanation of PPF resides in the residual calcium hypothesis (see Ch 3.1.3) and a common extension of this hypothesis postulates that any plastic change that alters the probability of transmitter release should also alter the magnitude of facilitation (Santschi and Stanton, 2002). Therefore, as discussed in Ch 3.1.3, effects of SNAP-25b on short-term synaptic facilitation should possibly be seen as alterations in the magnitude of facilitation.

PPF is maximal at short interpulse intervals and declines exponentially over a period of approximately 500 ms.

It is well established that PPF is a purely presynaptic phenomenon and should ideally be studied under voltage clamp conditions with synaptic inhibition blocked pharmacologically (Bortolotto et al., 2003). If inhibition is not blocked, inhibitory responses summate together with excitatory responses and the interpretation of the results will then become more complex. The reason to examine PPF under voltage clamp conditions is that if the membrane potential is allowed to change, then nonlinear summation of
synaptic potentials needs to be taken into account. Nonlinear summation means that the second response appears smaller than it should because it occurs at a membrane potential that is closer to its reversal potential.

![Figure 13: Postsynaptic macroscopic current measured intracellularly. The figure shows a typical EPSC recording from a PPF experiment. The two upward spikes reflect artefacts from the stimulation of presynaptic fibers that is detected by the recording electrode, and the EPSCs are seen as downward responses. The currents are measured with whole-cell voltage clamp at a holding potential of -70 mV and reflect mainly a flow of positively charged ions into the postsynaptic neuron.](image-url)
6 Transgenic Mice

Advances in transgenic technologies to express cloned and modified genes in mice have made them one of the most useful animal models for biomedical research. This mammalian model is comparable to the human in respect to organ systems, tissues, physiologic systems, and even behavioural traits, which is a great advantage in comparison with other model organisms. Also, most genes are conserved between mouse and human, which results in a possibility to understand the relationships between genotype and phenotype. Mice are also easy to breed and have short generation times. (Öbrink and Waller, 1996).

6.1 The SNAP-25b Knockout Mouse Model

The design and breeding of the SNAP-25b knockout mouse model has been developed and performed over a few years by Dr Bark. This was carried out in the following manner:
The SNAP-25b knockout mouse model was designed by generating a targeting vector where exon 5b was replaced with an additional exon 5a in the Snap gene. That is, exon 5b coding for the nine amino acids in SNAP-25b that differ from SNAP-25a was replaced by a targeted vector containing exon 5a. Thus, this altered Snap gene contains two identical exon 5a in tandem and therefore only encodes for the SNAP-25a isoform of the protein.

This targeted mutation was then introduced into totipotent embryonic stem-cells (ES-cells), which are undifferentiated cells that are able to multiply and become differentiated into any sort of cell in the body. Modified ES cells that demonstrated homologous recombination and correct and expected replacement of the selected region of the Snap gene were then injected into blastocysts and implanted into a foster mother. The offspring were then tested for their ability to transmit the introduced mutation to the next generation by mating with Charles River C57BL6 mice. Males demonstrating germline transmission were then used to produce the SNAP-25b knockout mouse model that contains the mutation in the desired gene in all nucleated cells.

For detailed information on how targeted mouse mutations are constructed, see “Müller U, 1999”.

6.2 The SNAP-25b Knockout Mouse Model versus Tkneo Mouse Model

Dr Bark has previously performed experiments in a different mouse model in which targeted mutation was used to impair the shift from SNAP-25a to SNAP-25b (Bark et al., 2004). This mouse model is referred to as the “Tkneo mouse model”. The Tkneo mouse model and the SNAP-25b knockout mouse model differ in their SNAP-25 protein levels in the following manner:

Tkneo mouse model: The total SNAP-25 protein level is 50 % compared to wildtype mice. Of the total protein level, 65 % is SNAP-25a and 45 % is SNAP-25b.

SNAP-25b knockout mouse model: The total SNAP-25 protein level is 80 % compared to wildtype mice and it is all SNAP-25a. That is, of the total level there is 100 % SNAP-25a and 0 % SNAP-25b.

Experiments are performed at PN12-PN16 and at this age the protein ratios in wildtypes is roughly 30 % SNAP-25a and 70 % SNAP-25b (see Figure 3).


7 In Vitro Preparation

To physically assess and investigate neurons of interest, a special type of in vitro preparation termed brain slice preparation is used. These brain slices are segments of brain tissue that are dissected from a fresh mouse brain. The brain itself is first rapidly removed from the skull of the mouse and placed in ice-cold artificial cerebrospinal fluid (aCSF) perfused with oxygen and carbon dioxide gas (95 % O₂, 5 % CO₂). The oxygenation of cells is essential for survival and the very low temperature reduces brain activity which also improves cell survival. Thin slices that contain the region of interest are cut by the use of a “vibratome” (VT 1000S, Leica), which is a high-precision knife specialised to cut slices of brain tissue.

7.1 The Hippocampal Slice Preparation

The slice preparation used is the hippocampal slice. The hippocampus is a horseshoe-shaped sheet of neurons located within the temporal lobes of the brain. It is known that the hippocampus is highly involved in formation of memory and learning, and it is thought that activity dependent synaptic plasticity is one of the underlying neurobiological mechanisms involved.

Experiments are performed in the hippocampus first of all because SNAP-25 is known to be expressed in hippocampal neurons (Bark et al., 1995), but importantly also because this is the principal region of the brain for studying synaptic plasticity. Moreover, the hippocampus has an orderly arranged anatomy which facilitates experiments, and several pathways within the hippocampus are known to display remarkably pronounced plasticity. The brain contains two hippocampi, one in each hemisphere. This can be seen in Figure 14 and 15 that shows the location and appearance of the hippocampus, which in cross-section has a highly defined laminar structure with visible layers of pyramidal cells orderly arranged in rows.
The connections within the hippocampus form well characterized closed loops. The pathway studied here is the Schaffer Collateral/Associational Commissural (Sch/com) fibers to the CA1 pyramidal cells (see Figure 15). This pathway is derived from axons that project from the CA3 region of the hippocampus and make contact with dendrites of CA1 pyramidal neurons. The Schaffer Collateral fibers comes from CA3 neurons in the same hippocampus (ipsilateral) and the Associational Commissural fibers originates in the CA3 neurons of the hippocampus in the opposite hemisphere (contralateral).

In Figure 15, the placement of the stimulating and recording electrodes is seen. The Sch/com fibers are electrically stimulated extracellularly to evoke action potentials within the fibers, and the postsynaptic responses to the action potentials are recorded from the soma of the CA1 pyramidal neurons.
7.1.1 The CA1 Pyramidal Neuron

Activation of the Sch/com fibers leads to glutamate release from their nerve terminals. Glutamate is the major neurotransmitter in this pathway and its binding to postsynaptic receptors leads to an excitatory response. By excitatory response one means that the underlying flow of ions in the activated ionotropic channel has an equilibrium potential that is more depolarized than the threshold for generating an action potential. That is, the postsynaptic neuron is depolarized.

The major inhibitory neurotransmitter in the hippocampus is GABA (Roberts 1976) and this inhibition arises from feedforward and feedback connections via inhibitory interneurons. However, in the experiments performed during this thesis, GABA\(_A\) receptor–mediated inhibition is blocked by addition of the GABA\(_A\) receptor antagonist picrotoxin. This is done to facilitate the interpretation of PPF results as GABA interneurons projecting to the CA1 pyramidal neurons are also activated by stimulation of the Sch/com fibers.

7.2 Advantages and Disadvantages of Slice Preparations

An important advantage with the *in vitro* slice preparation compared to most *in vivo* methods is the relative ease of obtaining long-term, stable intracellular recordings without anaesthetics or immobilizing agents. An equally important advantage of the brain slice for electrophysiological studies is that the local circuits and cellular architecture of the tissue are relatively intact. This allows straightforward identification of the neuron being studied by visualization, but most importantly one can easily do stimulations and recordings as described above since structures and pathways are kept intact. Furthermore, the ethical problems are fewer in an *in vitro* preparation compared to *in vivo* methods since no experiments are performed on living animals.

The most significant disadvantage is normally that the slice is a relatively isolated preparation, which results in a lack of many normal afferent inputs and efferent targets. However, in the preparation used
in this study it is only the direct connections between the pre- and postsynaptic neurons of the investigated pathway that is of interest. Therefore, for this study the slice preparation is ideal. The slice is, of necessity, situated in an artificial environment rather than the natural and more complex milieu of the brain. Therefore, results may be somewhat biased by the particular experimental conditions and protocols used in an individual lab. This should, however, not complicate the results of this study.
8 Materials and Methods

8.1 Dissections

Mice in the age of postnatal days 12 to 16 are terminally anesthetized with carbon dioxide gas (100% CO₂) and then sacrificed by decapitation. The brain is quickly removed from the skull by cutting the scalp down the midline with a fine pair of scissors, taking care not to cut the underlying brain tissue. With the use of a fine spatula, the brain is scooped out of the brain case and placed on a wetted filter paper. The cerebellum, part of the occipital lobe and some of the frontal lobe are quickly removed by coronal incisions with a scalpel. By removing outer tissue which is not of interest, diffusion of oxygen into the hippocampus becomes more efficient.

The brain is then placed into a container with ice-cold artificial cerebrospinal fluid (aCSF) oxygenated with oxygen and carbon dioxide gas (95 % O₂, 5 % CO₂). This aCSF has a relatively low Ca²⁺ and high Mg²⁺ concentration and the total composition of the aCSF is (in mM): 124 NaCl, 5 KCl, 1.24 NaH₂PO₄, 0.5 CaCl₂, 10 MgSO₄, 26 NaHCO₃, and 10 glucose. The pH of the aCSF is adjusted to 7.40 by adding NaOH or HCl. The very low temperature (close to 0ºC) and the low Ca²⁺ high Mg²⁺ composition lowers the activity in the nervous tissue, which improves the survival of neurons (Aitken et al., 1994). The aCSF is a balanced salt solution and the exact composition can vary according to species and cell type. A balanced salt solution is an isotonic solution containing appropriate ions and buffers suitable for maintaining neurons in vitro.

The brain is then carried to the location of the electrophysiological setup and placement of the vibratome, approximately 10 min away from the animal facility. The ice-cold aCSF surrounding the brain is constantly oxygenated during this time by the use of a balloon containing oxygen and carbon dioxide gas, and the container itself is kept in ice to keep the aCSF cold.

In the vibratome (Leica VT1000S), the brain is glued caudally onto a plate, with the cerebral cortex facing the razorblade in the vibratome. 350 µm thick coronal slices are cut at very low speed from the brain. These are directly transferred to regular aCSF, same composition as above but with 2.4 mM CaCl₂ and 1.3 mM MgSO₄, and kept at room temperature (22-25ºC) for at least one hour before being transferred to the recording chamber. Because the slices are so thin, diffusion of oxygen into the slice is efficient and the health of the tissue is good even though they are kept at room temperature and at physiological concentrations of Ca²⁺ and Mg²⁺.

8.2 Setup

The recording chamber (see Figure 16) in which the slice is placed has a volume of about 1 ml and is perfused with oxygenated aCSF at a perfusion rate of approximately 2-3 ml/min. In order to block inhibitory responses, the aCSF contain 50 µM of picrotoxin which is an antagonist of GABAₐ receptors. Slices are kept still in the recording chamber by nylon threads attached to a metal ring, so that recordings can be performed with the slice kept steady. Recording pipettes are filled with artificial intracellular solution with the following composition (in mM): 135 Cs-methanesulphonate, 10 Hepes, 1 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 2-5 QX-314, and 8 NaCl. The pH is adjusted to 7.25 by adding CsOH or HCl and the osmolarity is adjusted to 270-280 mOsm by adding distilled water or glucose. The intracellular solution is made in large quantities previous to experiments and then aliquoted in 1 ml tubes kept in a freezer at -70ºC for a maximum of 3 months.

Two ion channel blockers are used in the intracellular solution to ensure that the measured current in the postsynaptic cell is the result of neurotransmitter-binding, and not by any additional voltage-dependent channels. The drugs used are Cs-methanesulphonate which is a general K⁺ channel blocker and QX-314 which is a voltage-dependent Na⁺ channel blocker.
8.3 Whole-cell Voltage Clamp

8.3.1 Positioning the Recording Electrode

The electrode is mounted in the electrode holder (partly seen in Figure 16 on the right side) and a slight internal positive pressure is applied to the recording electrode before it is immersed in the bathing solution. This pressure is applied in order to blow any contaminations in the bathing solution away from the pipette tip and must be applied before the tip is immersed in the bathing solution as contaminations often accumulate at the air-fluid interface.

As soon as the tip is immersed, the recording electrode resistance is calculated by applying a small test pulse of 2 mV for 10 ms while reading the current, and by Ohm's law calculating the resistance (R=U/I). Electrodes with a resistance of 6-10 MΩ are used. The resistance of the recording electrode is monitored constantly during the approach towards the cell (see Figure 17a-e), since increases in resistance prior to contact with a cell means the electrode tip probably has been contaminated and then the formation of a gigaseal is less likely.

The location of the electrode is monitored visually with the microscope and it is moved towards the soma of the pyramidal cells of the CA1 region in the hippocampus (see Figure 15) using a micromanipulator. As the electrode approaches the cell of interest, the positive pressure is decreased so that the cell is not blown away by the pressure. The electrode tip is gently pushed against the cell surface which is monitored both visually and by changes in the resistance. That is, when the electrode comes into close contact with the cell membrane the resistance in between the two increases because of occlusion of the pipette opening by the cell. This is seen as a reduction of the current test pulse response (see Figure 17b). At this point, the current baseline is nulled to cancel all junction potentials as this is the last stage where the recording apparatus is not influenced by the cell.
Figure 17: Test pulses produce different current responses as the electrode proceeds through the establishment of a whole-cell voltage clamp recording. The physical relationship between the recording electrode and the cell is illustrated schematically on the left. The amplitude of the current response decreases as the resistance across the recording electrode tip increases. Thus, a reduction in current indicates closer contact between the electrode tip and the cell. (A) The electrode is just above the cell, but not in direct contact, so the resistance is low (6-10 MΩ) and the current response is large. (B) The electrode touches the cell surface, the resistance increases slightly, and the current decreases. (C) A gigaseal has formed as the result of gentle suction. The resistance is high (>1 GΩ), so except for the capacitance transients, the current response is virtually flat. (D) The electrode capacitance transient is nulled. (E) Break-in is achieved by strong suction that removes the patch of membrane in the electrode tip, but leaves the seal and cell intact. The resistance decreases and large capacitance transients are seen. (From Meyer and Jackson, 2003)

8.3.2 Forming a Gigaseal
To establish a gigaseal between the electrode and the cell, the positive pressure is completely removed and instead a gentle negative pressure, suction, is applied to the pipette interior. This pulls a small patch of membrane up into the electrode tip and leads to a very tight seal and thus a very high resistance, a “giga ohm seal” is established. This high resistance results in a virtual flattening of the DC part of the test pulse current. That is, the current in between the transients (see Figure 17c). These transients reflect charging of the electrode- and membrane capacitance and are cancelled out through a negative feedback system in the amplifier.

8.3.3 Establishing the Whole-cell Voltage Clamp
Now the holding potential, -70 mV, is set on the patch clamp amplifier in order to avoid a large change in membrane potential upon the rupture of the membrane (since the expected resting membrane potential of the cell is around -70 mV). To rupture the membrane under the patch, short pulses of strong suction are applied to the pipette while checking the test pulse current response. A rupture is characterized by a broadening of the transients in the test pulse response and slightly lower resistance (see Figure 17c). Suction is immediately terminated upon their appearance. The broadening of the transients reside in that the test pulse also charges the cell interior after the membrane rupture, and not just the walls of the pipette electrode. Whole-cell voltage clamp is now established (see Figure 18), and the test pulse is turned off and the actual experiment may begin. That is, since the inside of the electrode is now in contact with the inside of the cell, currents in the postsynaptic cell that result from binding of neurotransmitters that has been released from the presynaptic cell upon stimulation can be measured (see Figure 13).
To monitor the health of the cell, the resting membrane potential of the cell is checked before and after experiments and the drift of the current required for keeping the cell at the clamped voltage is constantly monitored.

![Whole-cell patch clamp](image)

**Figure 18**: Whole-cell patch clamp. The left side of the figure shows the glass recording electrode attached to a pyramidal neuron. The membrane underneath the tip of the electrode has been ruptured and the interior solutions of the electrode and the cell are now in contact. Whole-cell mode is established. The right side of the figure demonstrates this situation schematically.

### 8.4 Electrical Stimulation

Before establishment of whole-cell voltage clamp, a concentric metal bipolar stimulation electrode is connected to the isolated current source and then mounted on the electrical micromanipulator. The electrode is moved with the micromanipulator and placed above the Sch/com fibers (see Figure 15) projecting to the neurons of the CA1 field where the recording electrode is located.

When the whole-cell voltage clamp is established, the electrical stimulation can begin. To evoke action potentials within the Sch/com fibers, DC square current pulses of 200 µs duration are delivered to the electrode from the current source. To be able to evoke action potentials, the current strength has to be high enough to induce a sufficient depolarization in the presynaptic fiber and the electrode also needs to be placed very close to a presynaptic fiber. Both the location of the stimulation electrode and the strength of the current are set individually in between experiments in order to achieve stable postsynaptic responses with a low failure rate. The strength is, however, kept as low as possible in order to activate as few presynaptic fibers as possible but yet achieve stable responses. This strength is normally in the range of 50-400 µA and as soon as desirable responses are achieved the same current strength is used throughout the entire experiment in that particular cell.

Paired stimuli are performed at six different interpulse intervals: 40 ms, 55 ms, 70 ms, 90 ms, 150 ms, and 300 ms. The reason for using so many different interpulse intervals is that SNAP-25a and SNAP-25b might affect facilitation differently at different interpulse intervals. Also, a potential difference might not be discovered if too few interpulse intervals are used.

The paired stimuli are also elicited at two different frequencies referred to as low-frequency PPF (LF-PPF) and high-frequency PPF (HF-PPF). During LF-PPF the paired stimuli are elicited every fifth second (0.2 Hz) while during HF-PPF stimuli are elicited every other second (0.5 Hz). This can be seen schematically in Figure 19.
Figure 19: Schematic illustration of the two different PPF protocols: HF-PPF and LF-PPF. The different time periods in between the paired stimuli in the two protocols can clearly be seen. During HF-PPF, the time period in between the paired stimuli is 2 seconds and during LF-PPF, the time period is 5 seconds.

Ideally, in the low-frequency stimulation scenario the frequency should be even lower in order to let the synapse fully recover before the next pair of stimuli is delivered to the synapse. However, the lower the frequency the longer the time interval in between the paired stimuli and thus the longer the entire experimental time will be. If the experimental time is too long the patched cell will either die or at least deteriorate so that the recorded responses cannot be used. Therefore there has to be a balance in between choosing a frequency which is low enough to let the synapse recover as much as possible and choosing a frequency high enough so that the cell is in good shape throughout the entire experiment.

In contrast, in the high-frequency stimulation scenario the frequency is deliberately set to be high so that the synapse probably has not fully recovered in between the delivery of the paired stimuli. This could possibly lead to different responses in the wildtype and SNAP-25b knockout mice if they differ in their ability to sustain facilitation during more intense stimulation.

8.5 Measurement Conditions

All recordings are performed at room temperature and the duration of the experimental sessions are always less than 9 hours. The slices are normally in good health for such periods of time. Individual cell recordings are performed until at least 25 PPF-responses per interpulse interval are recorded. That is, one cell is normally patched for about 10-30 minutes depending on the time it takes to find good presynaptic stimulations that result in stable postsynaptic responses.

Measurements are performed using an Axopatch 200B patch clamp amplifier (Axon Instruments) and the measured signals are digitized with a Digidata 1200 (Axon Instruments). The signals are filtered at 5 kHz using a low-pass filter and sampled at 50 kHz using the Clampex 7 software (Axon Instruments), and are stored on a personal computer.

Analyses are performed in the software program Clampfit (Axon Instruments) and Excel (Microsoft Office). Statistical analyses are performed in GraphPad Prism 4 (GraphPad Software Inc.).
9 Results

Paired-pulse facilitation was examined in both wildtype and SNAP-25b knockout mice under two different frequency conditions: low-frequency PPF (0.2 Hz) and high-frequency PPF (0.5 Hz). Both PPF protocols were performed at six different interpulse intervals (40-300 ms) in the CA1 region of hippocampal slices. Recordings were done in whole-cell voltage clamp configuration at a holding potential of -70 mV.

9.1 Low-frequency PPF

Paired-pulse facilitation is significantly reduced in the SNAP-25b knockout mouse model relative to wildtype mice (see Figure 20) during low frequency stimulation.

Figure 20: Low frequency paired-pulse facilitation elicited at 0.2 Hz at CA1 hippocampal synapses in slices from the SNAP-25b knockout mouse model (8 cells/6 mice) and wildtype mice (5 cells/5 mice). The PPF ratio=EPSC₂/EPSC₁ is calculated from EPSCs recorded in whole-cell voltage clamp configuration. Paired-pulse facilitation is significantly reduced in the SNAP-25b knockout mouse model relative to wildtype mice (p<0.05 by two-way ANOVA analysis, see table 2 and parameter “genotype”). Data points and error bars represent means ± SEM.
Figure 20 shows the PPF ratio in SNAP-25b knockout and wildtype mice for the different interpulse interval. The mean PPF ratio displayed in Figure 20 is determined from the individual mean PPF ratios per cell and interpulse interval. In every cell, the PPF ratio is based on >15 individual EPSCs per interpulse interval (typical EPSC appearance can be seen in Figure 21). The results are based on recordings from 8 cells in 6 mice of the SNAP-25b knockout mouse model and from 5 cells in 5 wildtype mice.

Figure 21: The figure shows typical individual EPSCs at 55 ms interpulse interval. (A) Representative trace from a wildtype cell. (B) Representative trace from a SNAP-25b knockout cell.

The reduction in facilitation in the SNAP-25b knockout mouse model relative to wildtype mice is seen throughout the different interpulse interval. The difference is rather small and to statistically evaluate the reduction seen in the paired-pulse facilitation in the SNAP-25b knockout mouse model relative to wildtype mice, a two-way ANOVA analysis was performed (see Table 2). This test indicates a significant reduction of paired-pulse facilitation in the SNAP-25b knockout mouse model relative to wildtype mice (table 2, parameter “genotype”, p<0.05).

Table 2: The table shows the results from the two-way ANOVA analysis of the low frequency PPF data. The most important result in the table is the significance for genotype, p<0.05.

<table>
<thead>
<tr>
<th>Low Frequency PPF</th>
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<tbody>
<tr>
<td>Two-way ANOVA</td>
</tr>
<tr>
<td>Source of Variation</td>
</tr>
<tr>
<td>Interaction</td>
</tr>
<tr>
<td>Genotype</td>
</tr>
<tr>
<td>IFI</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>P value summary</th>
<th>Significant?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
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</tr>
<tr>
<td>Genotype</td>
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<td>IFI</td>
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<th>Sum-of-squares</th>
<th>Mean square</th>
<th>F</th>
</tr>
</thead>
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9.2 High-frequency PPF

Paired-pulse facilitation is significantly reduced in the SNAP-25b knockout mouse model relative to wildtype mice (see Figure 22) during high frequency stimulation as well. This was statistically evaluated by a two-way ANOVA analysis (see Table 3) which indicates a significant reduction of the
paired-pulse facilitation in the SNAP-25b knockout mouse model relative to wildtype mice (table 3, parameter “genotype”, (p<0.05). These results are based on recordings from 7 cells in 7 mice of the SNAP-25b knockout mouse model and from 7 cells in 7 wildtype mice. However, in comparison to the low frequency stimulation the overall magnitude of the facilitation is less in both SNAP-25b knockout and wildtype mice (see Figure 22). This is probably because the deliverance of the paired stimuli is at such a high frequency (0.5 Hz) that the synapse is constantly in a suppressed state. That is, the time interval in between the paired stimuli is to short for the synapse to fully recover until the next pair of stimuli is delivered to the synapse.

Figure 22: High frequency paired-pulse facilitation elicited at 0.5 Hz at CA1 hippocampal synapses in slices from SNAP-25b knockout mouse model (7 cells/7 mice) and wildtype mice (7 cells/7 mice). The PPF ratio=EPSC}$/EPSC_1$ is calculated from EPSCs recorded in whole-cell voltage clamp configuration. Paired-pulse facilitation is significantly reduced in SNAP-25b knockout mouse model relative to wildtype mice (p<0.05 by two-way ANOVA analysis, see Table 3). Data points and error bars represent means ± SEM.

By inspection of Figure 20 and Figure 22, there is no indication that the genotype would affect facilitation differently at any particular interpulse interval in any of the two frequency protocols. That is, the interpulse interval seem to have the same effect in both the SNAP-25b knockout mouse model and wildtype mice, with increasing facilitation at decreasing interpulse intervals in both genotypes. This is also indicated by the statistical analyses that does not show any significant interaction in
neither the low frequency results (Table 2, parameter “interaction”, \( p=0.7114 \)) nor the high frequency results (Table 3, parameter “interaction”, \( p=0.592 \)).

Table 3: The table shows the results from the two-way ANOVA analysis of the high frequency PPF data. The most important result in the table is the significance for genotype, \( p<0.05 \).

<table>
<thead>
<tr>
<th>High Frequency PPF</th>
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<td>Two-way ANOVA</td>
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<th>Source of Variation</th>
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<tr>
<td>Interaction</td>
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<td>No</td>
</tr>
<tr>
<td>Genotype</td>
<td>**</td>
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</tr>
<tr>
<td>IFI</td>
<td>ns</td>
<td>No</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Df</th>
<th>Sum-of-squares</th>
<th>Mean square</th>
<th>F</th>
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</thead>
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</table>
SNAP-25 is known to be essential for evoked synaptic transmission (Washbourne et al. 2002, Molnar et al. 2002), and it appears that one difference between the two isoforms, SNAP-25a and SNAP-25b, is their ability to stabilize the pool of primed vesicles, the Readily-Releasable Pool (RRP). SNAP-25b appears to be able to stabilize a larger pool than SNAP-25a. However, the exact function of the two SNAP-25 isoforms as regulators of synaptic plasticity is currently unknown.

As a first approach to investigate this, Dr Bark and her colleagues used a different mouse model called the Tkneo mouse model in which the alternative splicing of choosing between exon 5a and 5b was impaired. This resulted in that the relative ratio of SNAP-25b was decreased and SNAP-25a increased compared to wildtype mice (Ch 6.2). Their results indicated that in this mouse model short-term facilitation was enhanced in comparison to wildtype mice.

The aim of this thesis has been to further investigate the role of the two SNAP-25 isoforms as regulators of short-term synaptic plasticity in a different mouse model. In this mouse model, the expression of SNAP-25b is completely abolished and only the SNAP-25a isoform is expressed (Ch 6.1 and 6.2). By performing experiments of paired stimuli, short-term synaptic plasticity has been evaluated in this SNAP-25b knockout mouse model.

In contrast to the results in the previous Tkneo mouse model, the results obtained during this thesis suggest that the loss of SNAP-25b may produce a reduction in synaptic facilitation in hippocampal CA1 synapses. This suggests that SNAP-25b acts presynaptically as a positive regulator of short-term synaptic plasticity. That is, the developmental shift from SNAP-25a to mainly SNAP-25b expression, mediated by alternative splicing, contributes to synaptic transmission by enhancing the facilitatory process of neurotransmitter release. However, to fully investigate this, more experiments need to be performed and the number of cells and mice increased.

The results obtained in the Tkneo mouse model where facilitation was enhanced rather than reduced, is probably due to the totally different protein levels and because that model actually contains as much as 45 % SNAP-25b (Ch 6.2). Also, recording techniques, experimental setups, protocols and the age of the mice are quite different from the SNAP-25b knockout model.

By what mechanism does SNAP-25b regulate short-term synaptic plasticity? The reduction in synaptic facilitation in the SNAP-25b knockout mouse model could result either from a decrease in the effectiveness of the facilitatory process or an increase in the depression of release after the initial EPSC in the SNAP-25b mouse model. As discussed in Ch 3.1.3, the underlying mechanism for the reduction in facilitation may be one or several.

One mechanism could involve a change in the initial release probability which may be coupled to the size of the readily releasable pool of vesicles. Although it is commonly assumed that an increase of RRP size causes an increase in initial release probability which results in a decreased paired-pulse ratio (Dobrunz and Stevens, 1997: Zucker and Regehr, 2002), experiments performed in hippocampal CA1 neurons of rats in which the RRP size was increased and thereby also the initial release probability showed an increase in the paired-pulse ratio. For example, a doubling of RRP size could cause a 50 % increase in initial probability of release which was associated with anything from no change to a 100 % increase in paired-pulse ratio (Hanse and Gustafsson, 2001b) This is in agreement with the results of this thesis, in which the SNAP-25b knockout mouse model that probably has a smaller RRP size show decreased paired-pulse facilitation compared to wildtype mice.

However, regarding the initial release probability a primary problem in the evaluation of mechanisms directly involved in short-term synaptic plasticity is that manipulations affecting the baseline level of transmission indirectly influence the magnitude of short-term synaptic plasticity (Zucker and Regehr, 2002). Therefore the interpretation of effects on short-term synaptic plasticity often requires a measure of the effects on the baseline level of transmission in order to determine whether changes in plasticity are direct effects on facilitation or depression, or if they are secondary consequences of changes in the initial probability of release.
Initial release probability can be experimentally evaluated in different ways, but in hippocampal CA1 synapses where NMDA receptors are present it is preferably performed by the use of a substance called MK-801. MK-801 is a NMDA receptor antagonist and can be used to detect changes in the initial probability of release (Rosenmund et al. 1993, Hessler et al. 1993). To interpret the results thoroughly and correctly, such experiments will be performed after the end of the thesis in order to evaluate if the initial probability of release is changed in the SNAP-25b knockout mouse model compared to wildtype mice.

If initial release probability is not changed, it is most likely that SNAP-25b enhances facilitation by increasing the probability of release after an initial conditioning stimulus. That is, when the second pulse arrives at the presynaptic terminal it seems that SNAP-25b increases the probability of releasing vesicles more than SNAP-25a. This change in the release probability at the second pulse may be coupled to size of the RRP.

Another possible mechanism for this altered synaptic facilitation could be that SNAP-25a and SNAP-25b are actually members of different SNARE complexes that contain different Ca\(^{2+}\) binding proteins that differ in their affinity for Ca\(^{2+}\). The results would then suggest that SNAP-25b is part of a SNARE complex that contain a Ca\(^{2+}\) binding protein with higher affinity for Ca\(^{2+}\) and this would then lead to an enhanced vesicular fusion during the residual Ca\(^{2+}\) build up at the arrival of the second pulse.

It seems that SNAP-25b plays an important role in the balance between positive and negative regulation of short-term synaptic plasticity. The precise molecular mechanism through which SNAP-25b functions at the synapse during short-term synaptic plasticity is of great interest and will be the subject of future investigation.

The results of this thesis may help to understand the function of proteins that mediate neuroexocytosis in presynaptic mechanisms, both in development and in response to neural injury. Also, it is of great interest to understand more about the function and regulation of synaptic plasticity and how the synapse actively processes information. Long-term changes in the transmission properties of synapses provide a physiological substrate for learning and memory, whereas short-term changes support a variety of computations. By expressing several forms of synaptic plasticity, a single neuron can convey an array of different signals to the neural circuits in which it operates.
11 Summary

In short, the work that has been performed during the thesis can be summarised as below:

- Gathering of information related to SNAP-25, synaptic plasticity, electrophysiology, electrical properties of neurons, neurophysiology etc. This was performed mainly by reading scientific articles.
- Choosing methods and learning the different techniques.
- Attending research seminars and lectures.
- Interacting with researchers working in related fields of neuroscience.
- Handling of mice, dissection, and slicing of mouse brain.
- Performing whole-cell patch clamp and stimulating axon bundles. This has been the main part of the thesis and has been very time consuming.
- Analysing the obtained data.
- Writing the thesis.

11.1 General Thoughts and Future Work

Neurophysiology is the scientific study of the functioning nervous system and is an interesting field of neuroscience. It is also a rather complex and challenging field of science and a vast amount of time has been spent to learn and understand the techniques and methods. Problems encountered during the thesis have mainly been related to the different techniques, the electrophysiological setup, optimisation of the survival of neurons etc. Also, the success of experiments varies from day to day. On a good day one may patch 10 neurons, but then there may be several days in a row without a single success during patching. However, this is also an exciting challenge with this field of science since it truly requires patience and skill to fully master the techniques and theory.

Regarding the SNAP-25b knockout mouse model, additional experiments have also been performed on a similar mouse model. However, the data from those experiments has been excluded from the thesis in order to not confuse readers with different mouse models.

The aim of the thesis project was to evaluate if, and how, short-term synaptic plasticity is altered in the CA1-region of the hippocampus in the SNAP-25b knockout mouse model in comparison to wildtype mice. This goal has, at least partly, been fulfilled and the results indicate that synaptic facilitation is reduced in the SNAP-25b knockout mouse model compared to wildtype mice. However, as discussed in Ch 10, more experiments need to be performed to more exactly understand the role of SNAP-25a and SNAP-25b as regulators of short-term synaptic plasticity.


