Chapter 1

General Introduction

Introduction

Understanding the physiological mechanisms governing learning and memory is one of the major goals in neuroscience. The importance of this topic is highlighted by many publications appearing in high ranking journals and the widespread interest of the popular media. A clearer understanding of the mechanisms of learning and memory does not only satisfy our curiosity, but may also have widespread clinical implications.

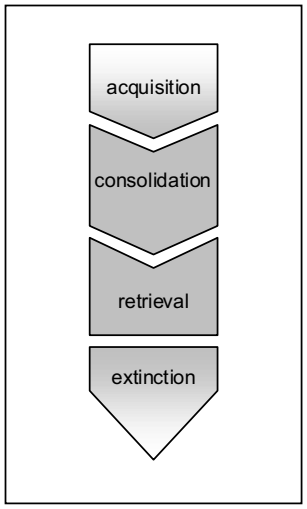

Figure 1. Learning takes place during the **acquisition** phase. Information is gathered through the sensory system and stored in the nervous system. During **consolidation**, the acquired information is maintained as memories. Some types of memory, such as working memory, are short lasting and do not have a proper consolidation phase. During **retrieval**, memories are recalled or remembered. At least in humans, this may be a purely introspective experience, but in experimental research it is measured as an adaptive change in behavior. **Extinction** is the tendency of memories to fade with time.

Most commonly, learning and memory is defined as those processes that modify behavior as a result of previous experiences (Fig.1). Learning and memory allows organisms to adapt to the ever-changing environment by enabling them to familiarize themselves with new situations and display appropriate behaviors. The ability to learn and remember helps organisms to find food and shelter, adjust to social situations, and avoid objects and situations that are harmful. Learning and memory is found throughout the animal kingdom. Even organisms as small as the 2 millimeter long nematode *C.Elegans*, which has a nervous system of only 302 cells, shows behavioral modifications as a result of previous experiences (Rankin et al., 1990). The sea slug *Aplysia* and the fruit fly *Drosophila* are capable of different types of conditioning and the analysis of these organisms has provided valuable information about the molecular cascades involved (Albright et al., 2000;Dubnau and Tully, 1998). Interestingly, the cellular mechanisms that have been implicated in learning and memory appear highly conserved in evolution, and it seems that through the course of phylogenetic history, learning and memory has evolved by exploiting old mechanism into ever-expanding nervous systems (Milner et al., 1998). Therefore, there is good reason to assume that findings in other mammals such as mice, or even from studies in invertebrates, will generalize to humans.

The synapse as a site for information storage

Somehow, experience changes the nervous system. At the end of the 19th century, Cajal suggested that these changes occurred at the connections between neurons, an idea that is still widely

supported. The connections between neurons are called synapses and changes at synapses are called synaptic plasticity. Synaptic plasticity is a mechanism that has the potential of storing information in the nervous system. Fig.2 depicts a simplified network of neurons where some synapses are stronger than others. Input from the sensory system causes a flow of neuronal activity through the different layers of neurons towards the motor output. Some synapses are stronger then others, resulting in a differential motor output for different sensory inputs. New information can be stored by increasing or decreasing the strength of particular synaptic connections. Computer models have shown that neuronal networks with such modifiable connections can store large amounts of information and have a number of attractive properties (Churchland and Sejnowski, 1992). For instance, they show resistance to noise, meaning that they are able to yield the correct output even if the input is noisy or faulty. Furthermore, they show graceful degradation, meaning that partial destruction of the network does not result in complete annihilation of memory traces. Also, they show pattern completion, meaning that they are able to give the correct output when only part of the input is presented. Finally, new information can be stored in an established neural network without destroying earlier information. These properties are strikingly similar to recognition and recollection of declarative memory in humans (Churchland and Sejnowski, 1992). Thus, there are good theoretical grounds to consider the synapse as a site for information storage. Although the idea is over a 100 years old, and has received a lot of experimental attention, it is still far from clear how this may work in the real, intact brain (Albright et al., 2000;Milner et al., 1998;Sanes and Lichtman, 1999).

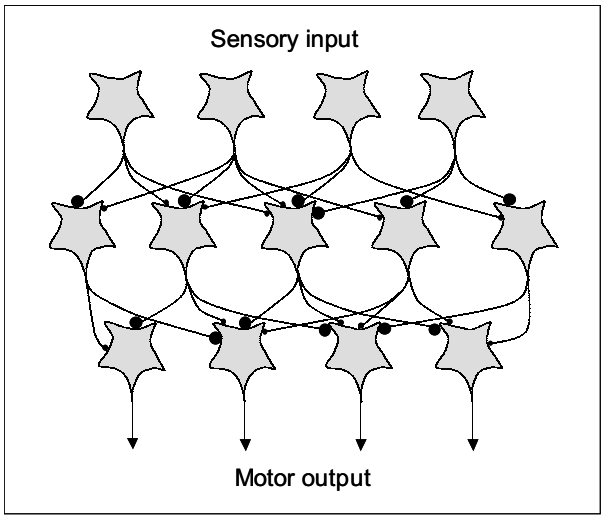

Figure 2. A simplified neuronal network capable of yielding different patterns of output upon different patterns of input. Large circles represent strong synapses (strength=2), small circles represent weak synapses (strength=1). A neuron requires at least a summed input of 3 to fire. This particular network is able to separate at least 8 different input patterns and can yield 8 unique output patterns.

Synaptic plasticity

Neurons express a wide variety of physiological mechanisms capable of changing synaptic strength (Bear and Abraham, 1996;Bliss and Collingridge, 1993;Albright et al., 2000;Zucker, 1999;Thomson, 2000a). Some forms of synaptic plasticity require simultaneous activity in both the presynaptic and the postsynaptic neuron (Bliss and Collingridge, 1993). Only those synapses that experience this association of neuronal activity are changed. This type of plasticity is called associative plasticity and

its role in learning and memory is widely studied. On the other hand, non-associative plasticity is not limited by this strict requirement and can occur as a result of only presynaptic activity or only postsynaptic activity (Zucker, 1999;Bear and Abraham, 1996). Non-associative presynaptic plasticity occurs after a period of neuronal activity and is expressed by a change in neurotransmitter release at all synaptic terminals of the active neuron. Presynaptic plasticity is a basic property of synapses, and therefore appears to be a fundamental way for a synapse to change its strength as a function of previous experiences. In this thesis, the role of presynaptic plasticity in learning and memory is studied.

Unreliability of neurotransmitter release

The synapse consists of a presynaptic bouton that releases neurotransmitter, and a postsynaptic specialization, often shaped in a spine, that contains neurotransmitter receptors (Fig.3). The

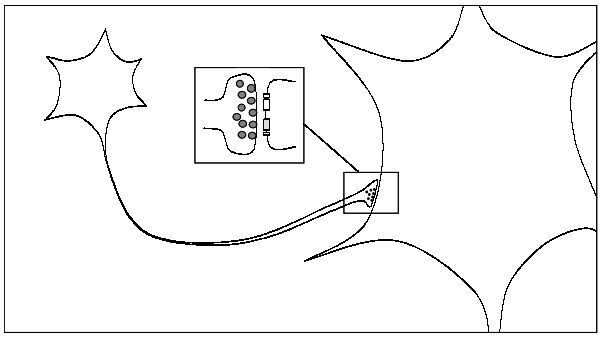

Figure 3. The presynaptic neuron on the left projects with its axon to the postsynaptic neuron. The presynaptic nerve terminal contains vesicles with neurotransmitter. When released, the postsynaptic neuron detects neurotransmitter with receptors.

presynaptic bouton has a specialized region called the active zone, where vesicles dock and await arrival of action potentials (Fig.4). These docked vesicles constitute the readily releasable pool of synaptic vesicles, whereas undocked synaptic vesicles constitute the reserve pool. Regulated secretion of neurotransmitter is characterized by an extremely high speed (under 100 µs) and strict calcium dependence, and this regulation requires sequential actions of many proteins (Sudhof, 1995;Neher, 1998;Lin and Scheller, 2000).

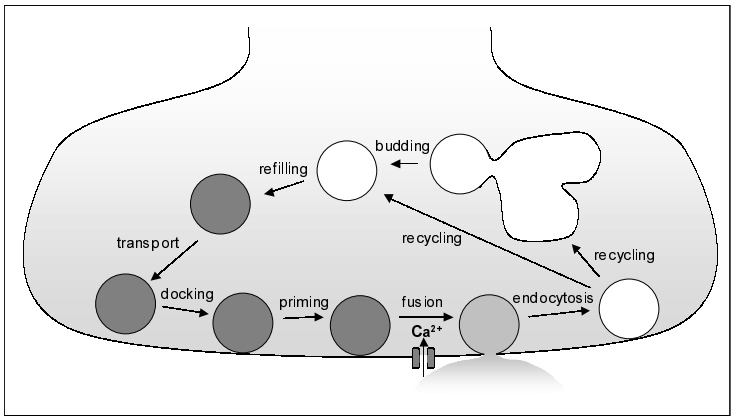

Figure 4. The synaptic vesicle cycle. A sequence of steps is necessary to enable vesicle fusion and neurotransmitter release. First, empty vesicles are filled with neurotransmitter. Consequently, they are transported to the active zone, where they dock and are primed to undergo fusion. Upon arrival of an action potential, voltage dependent calcium channels are opened and neurotransmitter is released. Afterwards, empty vesicles are recycled and refilled. Completion of this cycle takes about 60s (Sudhof, 1995).

Release probability is defined as the chance that a synapse releases neurotransmitter upon arrival of an action potential. At central synapses, there are about 5-12 docked and readily releasable vesicles at each active zone. It appears that release is usually limited to one vesicle per action potential (Schikorski and Stevens, 1997;Dobrunz and Stevens, 1997). At synapses in the hippocampus and cortex, the probability of release ranges from almost zero to about 0.9. Thus, in many cases, single action potentials do not trigger secretion of neurotransmitter at a given synapse. The probability of release varies widely between individual synapses and appears to be a function of synapse size. Larger synapses have a larger active zone, more docked vesicles and a higher release probability than small synapses (Schikorski and Stevens, 1997;Dobrunz and Stevens, 1997). Thus, due to differences in synapse size, different presynaptic terminals of the same neuron may have different release probabilities. Although related to synapse size, release probability is not static but can change dynamically as a result of previous neuronal activity.

Short-term presynaptic plasticity

Short-term presynaptic plasticity is a basic property of probably all peripheral and central synapses in invertebrates and vertebrates (Fisher et al., 1997). This type of plasticity can result in an enhancement

Presynaptic Process	Time constant	Requirements
Increased release		
Paired pulse facilitation (PPF)		
First component	50 ms	1 action potential
Second component	200 ms	1-5 action potentials
Augmentation	7 s	5-10 action potentials
Post tetanic potentiation (PTP)	20 s to min.	10-100 action potentials
Decreased release		
Depression		
Fast component	5 s	5-10 action potentials
Slow component	minutes	1000 action potentials

Table 1. Different forms of short-term presynaptic plasticity.

or a decrease in neurotransmitter release. A number of different forms of short-term presynaptic enhancement can be distinguished: fast-decaying facilitation, slow-decaying facilitation, augmentation and post-tetanic potentiation (Table 1). These processes are caused by a change in neurotransmitter release, and not by changes in the postsynaptic response (Zucker, 1999;Fisher et al., 1997;Thomson, 2000a). Facilitation is believed to be caused by a local, high rise in calcium (~50-100μM), that has persisted after an action potential. Facilitation may be caused by a simple addition of residual calcium to incoming calcium so that the local calcium concentration is heightened. Alternatively, facilitation may be caused by residual calcium binding to the exocytosis sites themselves (Zucker, 1999). The amount of facilitation is related to initial release probability. Synapses with a low release probability show stronger facilitation than synapses with a high release probability. At synapses with a very high

release probability, further increases may not be possible and in those cases paired pulses may even lead to paired pulse depression (Thomson, 2000b).

Augmentation and post-tetanic potentiation (PTP) are longer lasting forms of presynaptic plasticity. Augmentation has a duration of approximately 8-20s, while PTP can last many minutes. Both processes are believed to be caused by elevated cytosolic calcium (in the order of 1 μM) that has occurred during trains of action potentials. Artificial elevations of calcium levels are sufficient for induction of augmentation, and the strength of augmentation and PTP are directly related to cytosolic calcium concentrations (Zengel et al., 1994;Brain and Bennett, 1995;Delaney and Tank, 1994). However, PTP requires a longer train of action potentials which results in longer periods of elevated calcium levels.(Lin et al., 1998) PTP is believed to be caused by calcium buildup in mitochondria and slow extrusion of calcium from these calcium stores through the cytoplasm to the extracellular medium (Tang and Zucker, 1997). The concentrations of residual cytoplasmatic calcium during augmentation and PTP are much lower than local calcium concentrations during an action potential. Thus, augmentation and PTP do not appear to be mediated by a simple addition of cytosolic and local calcium. Instead, it seems that the residual calcium acts through binding to certain targets that facilitate release (Zucker, 1999;Thomson, 2000b). The nature of these targets is unknown. A sudden drop in calcium concentration by photo-labile calcium chelators blocks all forms of short-term plasticity, with augmentation and post-tetanic potentiation decreasing with a time constant of about 350ms, and facilitation with a time constant of 10ms (Kamiya and Zucker, 1994). This indicates that there are two different processes mediating short-term enhancement, one that is quick and predominantly active during facilitation and requires high calcium levels, and one that is slower and requires lower but possibly more prolonged calcium elevations (Kamiya and Zucker, 1994).

Depression can be distinguished in two phases: a short lasting form which recovers after 20s and a long lasting form which recovers in about 3 min (Stevens and Wesseling, 1999). Short lasting depression is believed to be dependent on the time course of refilling of the readily releasable pool of synaptic vesicles. Refilling takes about 10 to 20s (Stevens and Wesseling, 1998). After longer periods (~20s) of high-frequency activity a depression of release develops that lasts 1-3 minutes (Stevens and Wesseling, 1999). This type of depression may be mediated by depletion of the reserve pool of vesicles, but also by a temporal disruption of the active zone (Stevens and Wesseling, 1999).

At active synapses both depression and enhancement co-occur and the interaction of all determines the presynaptic response. Whether enhancement or depression dominates at a given synapse seems to depend on the initial release probability. It is believed that synapses with a low initial release probability are mostly governed by enhancements of release, whereas synapses with a high release probability are dominated by depression (Fisher et al., 1997;Zucker, 1999;Neher, 1998;Thomson, 2000b).

Does presynaptic plasticity occur at central synapses in freely moving animals?

Although it is quite clear that central synapses are capable of showing these different forms of presynaptic plasticity, this does not necessarily mean that such *in vitro* findings also generalize to the

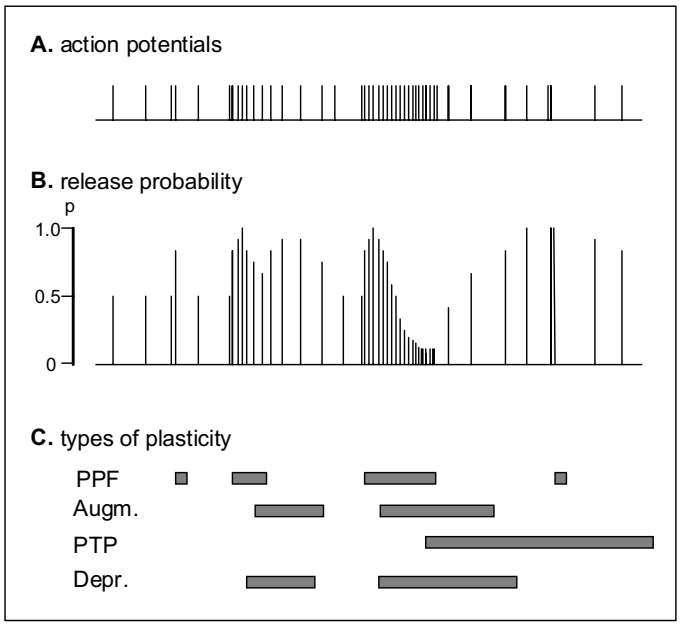

Figure 5. Interactions of presynaptic plasticity. A hypothetical example based on Dobrunz and Stevens (1999). The time scale (left to right) is not realistic and sometimes condensed for purposes of clarity.
A a dispersed pattern of action potentials arrives at the presynaptic terminal.
B the synapse has a basal release probability p of 0.5, but this changes dynamically as a result of patterned action potentials.
C different types of plasticity acting at the presynaptic nerve terminal.

intact brain. It is not possible to measure spontaneous, behaviorally induced presynaptic plasticity at central synapses, but there are indications that the circumstances required for the induction of presynaptic plasticity occur in freely moving animals.

When a rat traverses through an environment up to 50% of the pyramidal cells in the hippocampus show location specific firing (O'Keefe and Dostrovsky, 1971). Firing rates range from below 1 spike per second at baseline to about 5 to 40 (average 8) spikes per second at peak rates (Muller et al., 1987). The location at which a particular cell increases its firing rate is called the place field. These place fields generally have a mountain-like shape, with the highest firing rates at the center of the placefield (Muller et al., 1987;Thompson and Best, 1990). Although place-specific firing is a prominent feature of hippocampal pyramidal cells, they also show increased firing rates to head-direction, odors, behaviors, and even show increased firing when the stimulus is absent in a delayed matching to sample task (Wood et al., 1999;Watanabe and Niki, 1985;Hampson et al., 1999). Thus, neurons in the hippocampus fire in complex spiking patterns, which consist of short periods of activity interdispersed with periods of relative inactivity (Muller et al., 1987;O'Keefe and Dostrovsky, 1971). Dobrunz and Stevens (Dobrunz and Stevens, 1999) have stimulated synapses with such naturally occurring spiking patterns. There were dramatic fluctuations in enhancement and depression, with responses varying 2-fold or more in different parts of a pattern. Fig.5 depicts a hypothetical example of what may happen during complex spiking activity. The changes in release probability are the net result of different types of presynaptic plasticity acting simultaneously on synaptic release probability. Computer simulations have shown that inclusion of such dynamic changes in release probability in neural networks greatly increases their capabilities (Liaw and Berger, 1996). However, virtually nothing is known about the role of these types of presynaptic plasticity in learning and memory.

Associative plasticity

Associative plasticity has been widely studied and is regarded a promising candidate mechanism for synaptic changes during learning. Associative plasticity is dependent on activation of the NMDA-receptor (Fig.6). The NMDA-receptor is a postsynaptic voltage- and ligand gated calcium channel, that needs both depolarization and glutamate binding in order to be activated (Bliss and Collingridge, 1993). Thus, this receptor can detect simultaneous activity in the pre- and postsynaptic cell, and could therefore function as a coincidence detector. Long-term Potentiation (LTP) is a long-lasting increase synaptic strength and is induced by a large calcium rise (Bliss and Collingridge, 1993). The inverse of LTP is Long-term Depression (LTD), a long-lasting decrease in synaptic strength. LTD is believed to be triggered by a relatively moderate rise in postsynaptic calcium levels (Artola and Singer, 1993;Bear and Abraham, 1996).

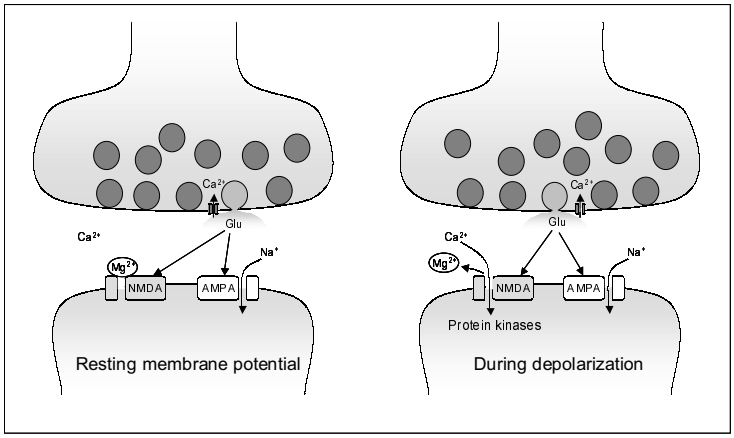

Figure 6. Mechanism of NMDA-receptor dependent synaptic plasticity. Normally, Mg^{2+} ions block the NMDA-receptor channel, thus preventing calcium influx when the receptor is activated by glutamate. After postsynaptic depolarization, Mg^{2+} ions dissociate from the channel and simultaneous glutamate binding will cause an inward flow of calcium, initiating a cascade that results in Long-Term Potentiation (LTP) or Long-Term Depression (LTD). The early stages of LTP are believed to be mediated by the protein kinases PKC, CaMKII and tyrosine kinase, while the later stages of LTP require protein synthesis (Albright et al., 2000).

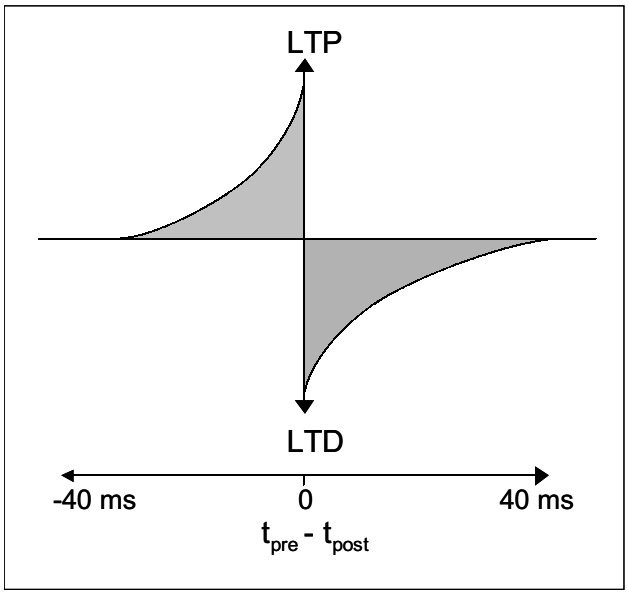

Figure 7. Coincidence detection at the temporal level. LTP is induced when the presynaptic spike precedes the postsynaptic spike. Inversely, LTD is induced when the presynaptic spike occurs after the postsynaptic spike.

Recent studies suggest that LTP and LTD induction requires strict timing (Fig.7). It is believed that the postsynaptic spike, which is generated by postsynaptic depolarization, must co-occur with presynaptic neurotransmitter release within a time-window of approximately 50ms. This is called spike-time dependent synaptic plasticity. Presynaptic activity that precedes postsynaptic firing or depolarization can induce LTP, whereas reversing this temporal order causes LTD (Levy and Steward, 1983;Gustafsson et al., 1987;Debanne et al., 1994;Markram et al., 1997;Magee and Johnston, 1997). The mechanisms are not fully understood, but seem to depend on the interplay between the dynamics of NMDA-receptor activation and the timing of the action potential backpropagating through the dendrites of the postsynaptic neuron (Linden, 1999). There are indications that LTD has a somewhat larger time window resulting in a net depression of random synaptic input which may be important in regulating total synaptic drive on a neuron and preventing excessive firing (Feldman, 2000;Abbott and Nelson, 2000).

Associative plasticity and the hippocampus

The function of associative plasticity in learning and memory is often studied in relation with the hippocampus (Fig.8). In humans, the hippocampus is involved in declarative memory, which are memories for factual information, such as places and events. Damage to the hippocampus selectively disrupts the ability to acquire and retrieve new declarative information (Vargha-Khadem et al., 1997;Milner et al., 1998). To facilitate animal research, tests were developed that were able to measure the animal equivalent of declarative memory. Consequently, it was shown that the hippocampus is essential for many complex learning tasks, including working memory in the radial maze, spatial learning in the Morris water maze and contextual fear conditioning (Olton et al., 1982;Morris et al., 1982;Phillips and LeDoux, 1992).

If associative plasticity is important for memory, then a blockade of associative plasticity would have to result in impairments in learning tasks. This is indeed the case. NMDA-receptor antagonists disrupt Morris water maze learning, contextual fear conditioning, and radial maze learning (Morris et al., 1986;Danysz et al., 1988;Fanselow et al., 1994;Kawabe et al., 1998). Genetic studies in mice, where associative long-term plasticity was disrupted by genetic deletion of mediators of associative plasticity, have provided similar results (Elgersma and Silva, 1999;Mayford and Kandel, 1999). Furthermore, overexpression of a potent subunit of the NMDA-receptor in mice heightened LTP and improved memory performance (Tang et al., 1999). Although NMDA-receptor dependent long-term plasticity appears involved in hippocampus dependent learning and memory, it cannot be the only mechanism. For instance, NMDA-receptor antagonists fail to disrupt spatial learning in pre-trained rats. Pre-training ensures acquisition of the procedure requirements of the Morris water maze task. When these pre-trained rats were consequently trained in a new room and were simultaneously given NMDA-receptor antagonists, they were still able to show spatial memory. This indicates that associative plasticity may be important for acquisition of the task requirements but not for spatial learning *per se* (Bannerman et al., 1995;Cain et al., 1996). Also, when animals were already familiar with the environment, working memory in the radial maze is not disrupted by a blockade of NMDA-receptors (Shapiro and O'Connor, 1992;Bolhuis and Reid, 1992). Finally, a disruption of associative LTP by AMPA-receptor knockout or

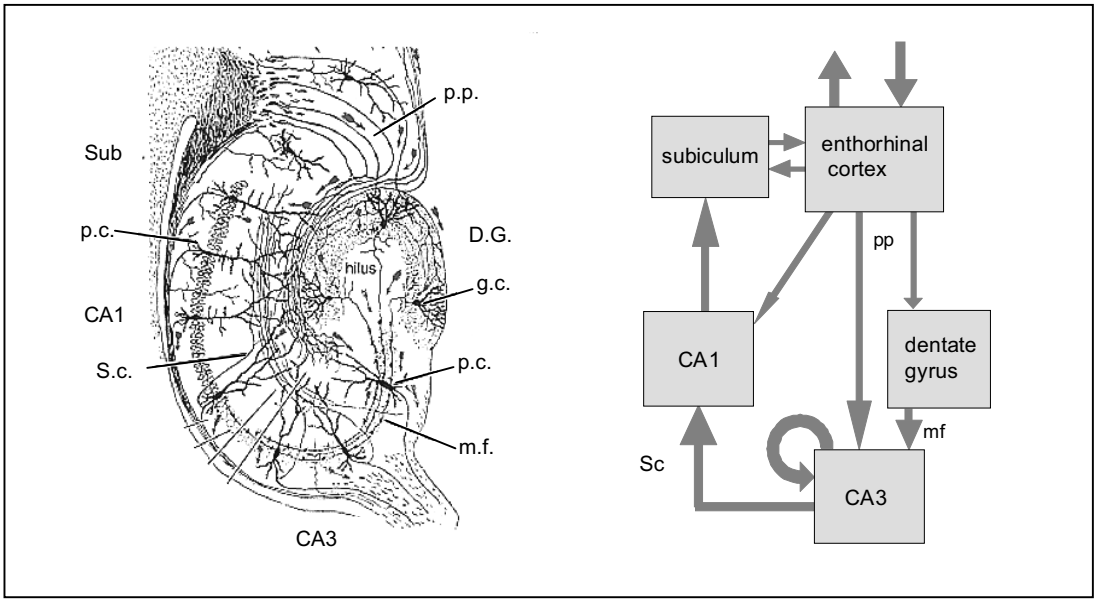

Figure 8. Anatomy of the hippocampus. Major excitatory pathways are shown on the right. The enthorhina cortex projects, by means of the perforant path (pp), to the granule cells (gc) of the dentate gyrus (DG). The dentate gyrus projects to the pyramidal cells (pc) of area CA3 through a sparse but strong connection called the mossy fibers (mf). The pyramidal cells of area CA3 also receive direct input from the enthorhinal cortex. CA3 pyramidal cells are interconnected through an extensive recurrent network, that connects each pyramidal cel with about 5% of all other pyramidal cells. Furthermore, CA3 pyramidal cells project to pyramidal cells of area CA1 by means of the Schaffer/collaterals (S.c.). Area CA1 is the output region of the hippocampus and projects back to the enthorhinal cortex through the subiculum (Treves and Rolls, 1992). Please note that the drawing by R.Cajal on the left is not correct on the latter connection.

Kv1.4 (a type of potassium channel) antisense infusion does not disrupt spatial learning (Meiri et al., 1998;Zamanillo et al., 1999). Thus, other mechanisms must be involved in these particular examples of hippocampus dependent memory. A candidate mechanism is long-term plasticity at the mossy fiber projection of the hippocampus.

Long-term plasticity at hippocampal mossy fiber synapses

The mossy fiber projection is one of the principal excitatory pathways of the hippocampus (Fig.8). Mossy fiber synapses are rather unusual synapses. They are large, have multiple active zones and are believed to be very powerful (Henze et al., 2000). Activity in only a few mossy fiber synapses may be sufficient to depolarize a CA3 pyramidal cell above threshold (Henze et al., 2000). Mossy fiber synapses show forms of non-associative LTP and LTD that are independent of the NMDA-receptor and expressed by an enhancement of neurotransmitter release (Yamamoto et al., 1980;Harris and Cotman, 1986;Staubli et al., 1990;Weisskopf and Nicoll, 1995;Derrick and Martinez, Jr., 1996;Kobayashi et al., 1996). The cellular mechanisms of mossy fiber LTP and LTD induction are not entirely clear. It seems that mossy fiber LTP can be induced through multiple pathways that can be accessed by different stimulation protocols (Jaffe and Johnston, 1990;Urban and Barrionuevo, 1996;Kapur et al., 1998;Yeckel et al., 1999). A summary of recent findings is depicted in Fig.9.

The function of mossy fiber plasticity in memory is unclear. It was shown that CA3 field potentials, evoked by mossy fiber stimulation, increased in amplitude over the course of a few days of radial

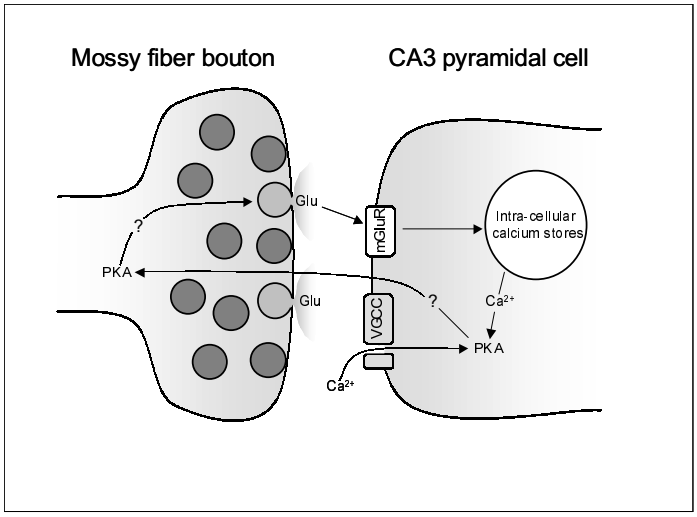

Figure 9. Induction of LTP at mossy fibers. Repeated neurotransmitter release is believed to cause a postsynaptic rise in calcium through metabotropic glutamate receptors and indirectly, by depolarization induced opening of voltage-gated calcium channels. Calcium is believed to activate PKA which, through some unknown retrograde messenger, results in enhancement of neurotransmitter release (Yeckel et al., 1999). AMPA and kainate glutamate receptors are not shown for purposes of clarity.

maze learning (Mitsuno et al., 1994;Ishihara et al., 1997). Thus, mossy fiber plasticity appears to be induced by learning, indicating that mossy fiber long-term plasticity may serve a function in memory.

Aim and outline

Learning and memory is believed to be mediated by changes at synaptic connections suggesting that activity in neurons is somehow translated in changes in synaptic strength. Many studies have focused on associative mechanisms and although partially successful, a number of issues have remained unanswered. In the previous sections, non-associative presynaptic mechanisms of synaptic plasticity were introduced. Virtually nothing is known about the function of these types of synaptic plasticity. However, non-associative presynaptic plasticity is believed to occur at all synapses and it appears to be a fundamental way for a synapse to change its strength. Therefore, in this thesis, the function of presynaptic plasticity in learning and memory is studied. In the next section this aim is outlined with specific questions.

As was shown above, neuronal representations of the environment are characterized by repeated firing. Due to the dynamic nature of release probability, these neuronal representations are subject to short-term modifications at the synaptic level. These changes in release probability may have a function during acquisition, such as in the induction of synaptic plasticity, but also during retrieval by ensuring activation of the proper synaptic patterns: **What is the function of short-term presynaptic plasticity in learning and memory?**

Working memory is the capacity to remember a limited amount of information for a short period (Glassman, 1999). Associative plasticity does not appear to be essential for working memory (Bolhuis and Reid, 1992;Shapiro and O'Connor, 1992). Given the timecourse of working memory and the fact that working memory has a low capacity, it has been suggested that post-tetanic potentiation may be

important (Churchland and Sejnowski, 1992): **Is post-tetanic potentiation involved in working memory?**

The induction of associative plasticity obeys relative strict rules and may require certain aspects of short-term presynaptic plasticity. For instance, if repetitive neurotransmitter release is required for sufficient depolarization, alterations in release probability may alter the timing of the postsynaptic spike. Also, alterations in release probability may affect the timing of presynaptic release. Such changes may even shift the sign of plasticity, resulting in LTD when LTP is required and vice-versa. Thus, given the dynamic nature of release probability, it seems likely to be an important variable for the induction of associative plasticity: **Is short-term presynaptic plasticity involved in the induction of associative plasticity?**

Mossy fiber evoked potentials increase over the course of radial maze training (Mitsuno et al., 1994), indicating that mossy fiber long-term plasticity may serve a function in memory. As mentioned, a blockade of associative plasticity by pharmacological or genetic means does not block long-term spatial learning and working memory. Thus, it appears that other mechanisms must be involved. Mossy fiber long-term plasticity is expressed in one of the principal excitatory pathways of the hippocampus. **What is the role of mossy fiber long-term plasticity in learning and memory?**

Material and methods

Presynaptic plasticity is not easily manipulated with conventional pharmacology in freely moving animals. Fortunately, the development of targeted mutagenesis in mice has provided genetic tools to manipulate the physiology of neurons. Previously, a number of different mutant mice were developed that lack certain presynaptic proteins. Some of these mutants had altered short- and long-term presynaptic plasticity. Two of these mutants were selected for this thesis: rab3A null-mutants and munc18-1 gene-dose (heterozygous) mutants.

Munc18-1

Munc18-1 is a mammalian, neuron-specific member of the Sec1/munc18-1 protein family. It is soluble and expressed throughout the brain (Garcia et al., 1994). Munc18-1 has been implicated in exocytosis due to its competition with synaptobrevin/VAMP for binding with syntaxin (Fig.10)(Hata et al., 1993;Garcia et al., 1994;Pevsner et al., 1994). This has led to the proposal that Munc18-1 is a negative regulator of secretion by controlling syntaxin availability. However, this is not supported by genetic studies in flies and mice (Harrison et al., 1994;Wu et al., 1998;Verhage et al., 2000). For instance, mice that lack munc18-1 show no regulated secretion of neurotransmitter (Verhage et al., 2000), which supports a role as a positive regulator of secretion.

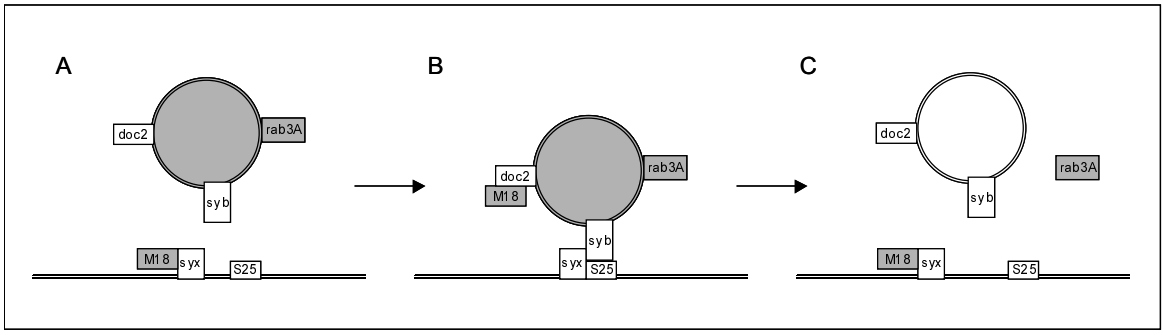

Figure 10 Simplified description of munc18-1 (M18) and rab3A in the nerve terminal. Fusion of neurotransmitter vesicles involves the SNAREs (soluble NSF attachment protein receptors) synaptobrevin/VAMP (syb), syntaxin (syx) and SNAP25 (s25) (Chen and Scheller, 2001). Together they seem to constitute the minimal requirement for membrane fusion (Weber et al., 1998). **A** munc18-1 prevents syntaxin to participate in the SNARE complex, rab3A-GTP is asociated with the vesicle **B** after docking syntaxin participates in the SNARE complex. Munc18-1 binds to doc2, but also to MINT and Munc13 (not depicted) **C** "kiss-and-run" release of neurotransmitter. After release rab3A-GDP dissociates from the vesicle. (Sudhof, 1995; Sudhof, 1997; Jahn, 2000).

Rab3A

The protein rab3A is a member of the GTP-binding Rab proteins, enriched in nerve terminals where it is associated with synaptic vesicles (Fig.10)(Sudhof, 1997). Mice that lack rab3A show normal long-term potentiation (LTP) in area CA1(Geppert et al., 1994), a form of LTP that is NMDA-receptor dependent. In contrast, rab3A mutants show enhanced tetanic depression, indicating impairments in the replenishment of synaptic vesicles (Geppert et al., 1994). Recent studies have shown that rab3A mutants lack mossy fiber LTP and LTD (Castillo et al., 1997;Tzounopoulos et al., 1998). Furthermore, rab3A mutants show enhanced paired pulse facilitation and a reduction in the limitations of neurotransmitter release, implicating rab3A in a late step of secretion process (Geppert et al., 1997).

In **chapter 2**, munc18-1 gene-dose mutant mice were studied at the biochemical and electrophysiological level. The effects of changes in presynaptic plasticity in munc18-1 gene-dose mutants for learning and memory were studied in **chapter 3**. Furthermore, munc18-1 gene-dose mutants were analyzed for other behavioral measures (**chapter 3** and **chapter 4**). The role of mossy fiber long-term plasticity in learning and memory was studied using rab3A null-mutant mice (**chapter 5**). The possibility of compensation of different types of long-term plasticity in learning and memory was studied in **chapter 6**. In **chapter 7,** the role of presynaptic plasticity in learning and memory is discussed.

The hippocampus as a model system to study memory

Virtually nothing is known about the function of presynaptic plasticity. Therefore, the effects of manipulations of presynaptic plasticity are unpredictable. In such a situation, it would be advantageous to use a model system that is well known. The hippocampus is an extensively studied brain region and a lot is known about its physiology and anatomy, and about its function in learning and memory

(Eichenbaum et al., 1999). Also, information processing in the hippocampus is of a rather complex nature, both in terms of anatomy and terms of neuronal activity, and may be especially vulnerable to manipulations of presynaptic plasticity. Therefore, in the present thesis, an emphasis was placed on hippocampal electrophysiology and hippocampal dependent learning tasks.

A number of learning tasks were used, that are all dependent on the hippocampus, but differed in many variables, such as incentives, behavioral strategies, and number of trials. Using such a variety of tasks allowed an assessment of the persistence of a phenotype in different test situations. It also allowed an assessment of the role of different kinds of presynaptic plasticity in different kinds of hippocampus dependent memory. Furthermore, behavioral changes in mutants that affect performance in ways unrelated to learning and memory may be detected with the use of multiple tasks.

Spatial learning was studied in the **Morris water maze**. Spatial learning is the ability to learn relations between particular cues in the environment, and use this information to determine ones place and heading. The water maze is a pool of water, which contains a hidden escape platform. The location of the platform is fixed and after a number of training trials, rodents learn to locate the platform based on environmental cues (Morris et al., 1982).

Cued and contextual fear-conditioning are forms of classical conditioning. During the conditioning trial, rodents are placed in a cage (context), presented with a cue (usually a sound signal), and are given a mild foot shock. Conditioning to the cue and context is measured at a later time, during which the cue and context are presented separately. Cued fear-conditioning is not dependent on the hippocampus, but contextual fear-conditioning is (Phillips and LeDoux, 1992).

Working memory is a transient type of memory that has a limited capacity but is of a flexible nature. Working memory is often referred to as scratchpad memory. In rodents, it can be tested in the **radial maze**. The radial maze contains a number of food rewards and, during a particular trial, the subject has to remember which places were already visited in order to find the food rewards efficiently. This task lasts a few minutes and after completion of a trial there is no need remember the sequence of events in to perform well at the next trial (Walker and Olton, 1979). It should be noted that working memory in humans is often defined as of a much shorter duration (~10s). It has been argued, that this may just be related to the nature of information that is remembered. Thus, spatial working memory may last much longer that verbal working memory (Glassman, 1999).

Breeding considerations

Differences between mutant mice and controls may be caused by differences in genetic background (Gerlai, 1996). The genetic background refers to all other genes but the one that is manipulated. Differences in genetic background can occur quite easily. Keeping the mutants and control lines in separate breeding colonies will almost certainly introduce differences in the genetic background. This problem is easily overcome by avoiding such a breeding strategy. More serious is the problem of genetic linkage (Fig.11).

An essential step in targeted mutagenesis in mice is embryonic stem (ES) cell manipulation. ES-cells are special, totipotent cells that are maintained in tissue culture. Totipotent cells have retained the

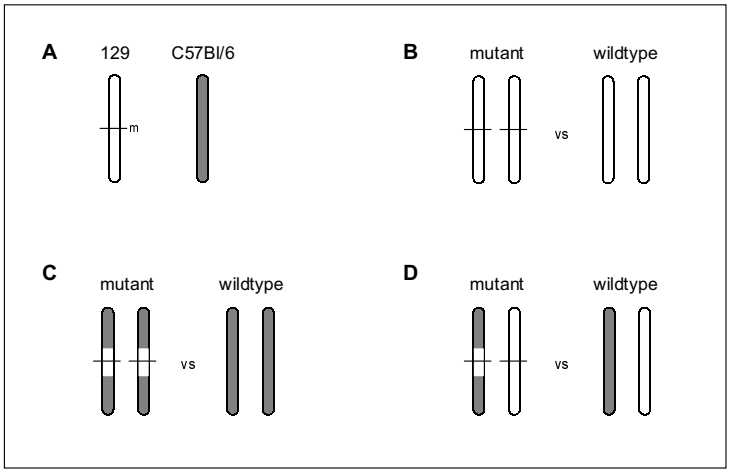

Figure 11. Genetic linkage. Only the mutated chromosome is shown. **A** The mutation (m) is induced in the 129 chromosome. The corresponding C57Bl/6 chromosome is always depicted in grey. **B** Ideal comparison with no differences in genetic linkage. This is preferred if the behavioral task allows it. **C** Backcrossing to C57Bl/6 introduces problems of genetic linkage. **D** Optimal solution for many behavioral tasks. Wildtypes perform well due to hybrid vigour and the genetic linkage problem is reduced to 1 chromosome instead of 2. Breeding strategies B and D were used in this thesis

ability to develop into a complete animal. In targeted mutagenesis, the genome of ES-cells is manipulated, and consequently reimplanted in foster mothers to produce mutant mice. This manipulation is most successful when the ES-cells are derived from 129 strains (Simpson et al., 1997). Therefore, genetic mutations are usually induced in 129 ES-cells, and consequently, in 129 chromosomes (Fig.11A). The best way to compare mutants to wildtypes is to breed the mutation in 129 mice and use these as controls (Fig.11B). Unfortunately, 129 mice are poor breeders, rather docile and unsuitable for a number of learning tasks because of poor performance (Crawley et al., 1997;Owen et al., 1997). One way to overcome these problems is to backcross the mutation into another strain, such as the C57Bl/6. However, this breeding strategy introduces the problem of genetic linkage (Fig.11C), caused by a proportion of the 129 chromosome that will always remain linked to the mutation. With 12 backcrosses (at least 2 years of breeding), the length of the 129-type chromosomal segment surrounding the manipulated gene would be, on average, about 16 centiMorgan (Gerlai, 1996). As the mouse genome covers 1600 centiMorgan, this represents at least 300 genes. If 129 and C57Bl/6 genomes were to be similar this would not present a problem. However, even a conservative estimate indicates that approximately 36% of the genes of 129 strains differ from C57Bl/6 on the protein level (Lathe, 1996;Simpson et al., 1997). Thus, even after a fairly large number of backcrosses, at least 100 genes are different and a comparison between wildtypes and mutants would also test differences in these genes. Furthermore, 129-strains and C57Bl/6 differ to large degrees in about every behavioral phenotype that has been tested, such as general activity, levels of fear and anxiety, stress responses, susceptibility to addiction, cocaine sensitivity, and learning and memory (Owen et al., 1997;Owen et al., 1997;Miner, 1997;Crawley et al., 1997;Crawley et al., 1997;Kuzmin and Johansson, 2000). Thus, this strategy introduces significant interpretation problems. Even very large numbers of backcrossing will not eliminate the problem because cross-overs between chromosomes is a stochastic process and the size of the linked chromosomal region relates in an asymptotic fashion to generation numbers. A partial solution to this problem is to use a hybrid 129-C57Bl/6 background (Fig.11D)(Banbury Conference Report, 1997). These are created by intercrossing

heterozygotes with a 129-background and heterozygotes with a C57Bl/6-background. Such hybrids outperform their parental strains on many tasks, a phenomenon known as hybrid vigor (Owen et al., 1997;Wolfer et al., 1997). Using this strategy, the genetic linkage problem is reduced by half because the linked region of only 1 chromosome differs. Furthermore, better performance in controls is advantageous for the detection of deficits in mutants.

Thus, in the present thesis, the following breeding strategy was used. Mutants were repeatedly backcrossed to 129S3 (a 129 substrain). Rab3A mutants were also backcrossed to C57Bl/6. Usually, the 129 genetic background backcross was used for behavioral experiments. The hybrid background was used in the Morris water maze task because the 129S3 is a notoriously poor swimmer and unsuitable for the Morris water maze (Wolfer et al., 1997;Banbury Conference Report, 1997).