Presynaptic plasticity and memory

A behavioral analysis of short and long-term plasticity at the nerve terminal

Presynaptische plasticiteit en geheugen

Een gedragsanalyse van korte en lange-termijn plasticiteit aan het zenuwuiteinde

(met een samenvatting in het Nederlands)

Proefschrift

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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.

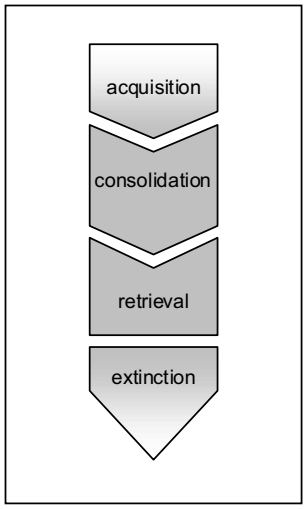

Figure 1. Learning takes place during the **acquisition** phase. Information is gathered through the sensory system and stored in the nervous system. During **consolidation**, the acquired information is maintained as memories. Some types of memory, such as working memory, are short lasting and do not 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).

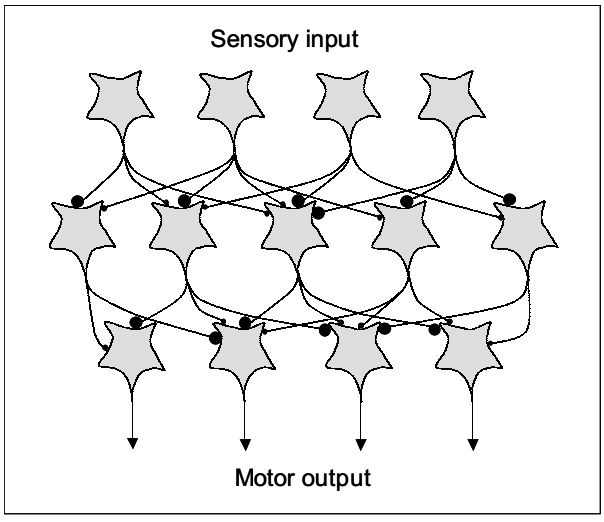

Figure 2. A simplified neuronal network capable of yielding different patterns of output upon different patterns of input. Large circles represent strong synapses (strength=2), small circles represent weak synapses (strength=1). A neuron requires at least a summed input of 3 to fire. This particular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

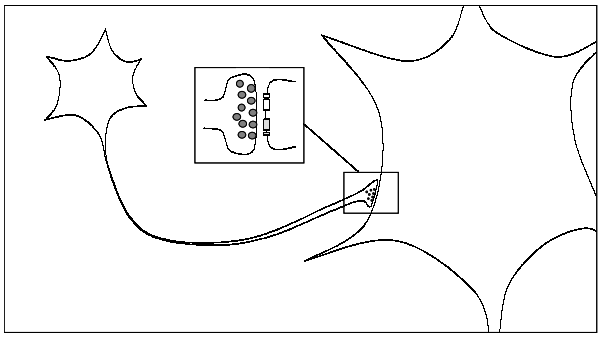

Figure 3. The presynaptic neuron on the left projects with its axon to the postsynaptic neuron. The presynaptic nerve terminal contains vesicles with neurotransmitter. When released, the postsynaptic neuron detects neurotransmitter with receptors.

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).

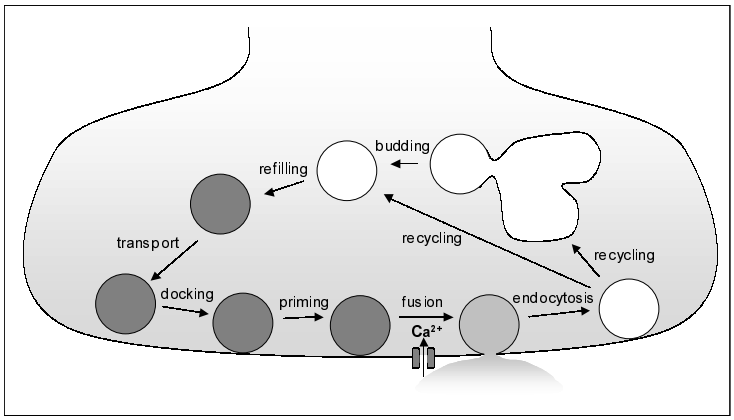

Figure 4. The synaptic vesicle cycle. A sequence of steps is necessary to enable vesicle fusion and neurotransmitter release. First, empty vesicles are filled with neurotransmitter. Consequently, they are transported to the active zone, where they dock and are primed to undergo fusion. Upon arrival of 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

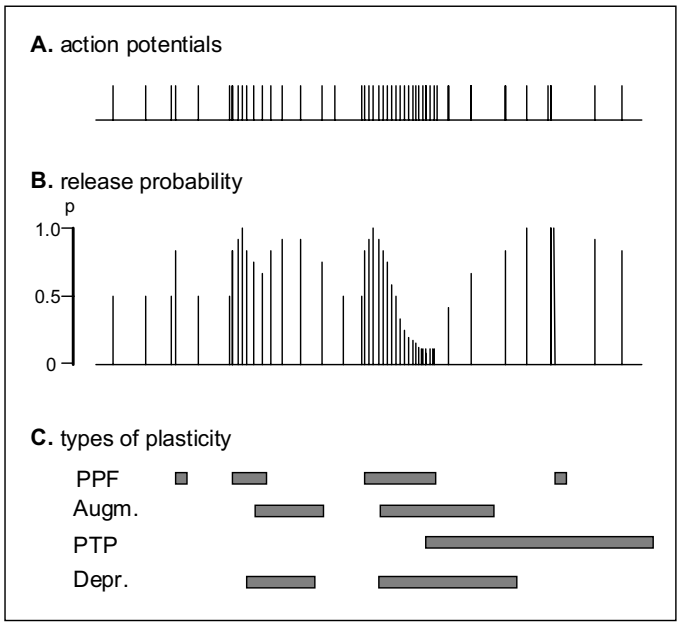

Figure 5. Interactions of presynaptic plasticity. A hypothetical example based on Dobrunz and Stevens (1999). The time scale (left to right) is not realistic and sometimes condensed for purposes of clarity.
A a dispersed pattern of action potentials arrives at the presynaptic terminal.
B the synapse 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).

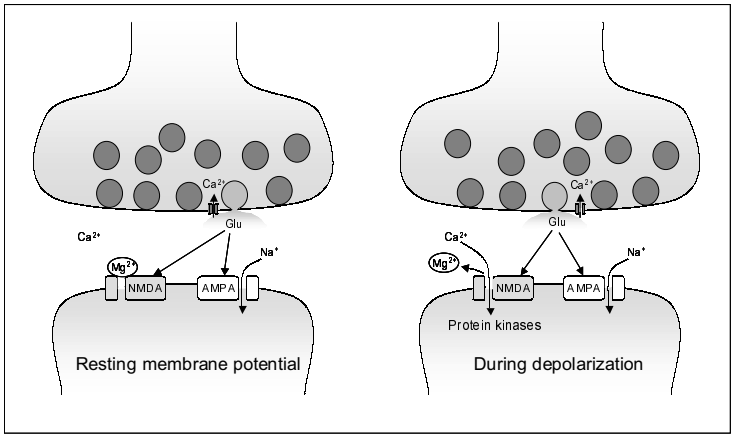

Figure 6. Mechanism of NMDA-receptor dependent synaptic plasticity. Normally, Mg^{2+} ions block the NMDA-receptor channel, thus preventing calcium influx when the receptor is activated by glutamate. After postsynaptic depolarization, Mg^{2+} ions dissociate from the channel and simultaneous glutamate 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).

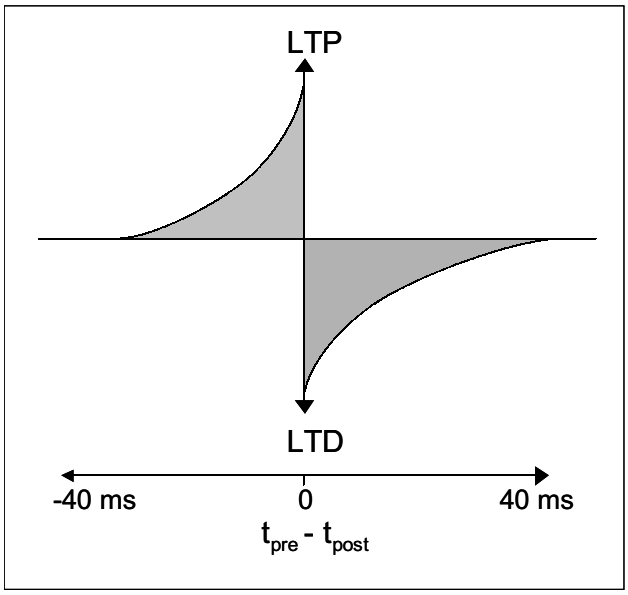

Figure 7. Coincidence detection at the temporal level. LTP is induced when the presynaptic spike precedes the postsynaptic spike. Inversely, LTD is induced when the presynaptic spike occurs after the postsynaptic spike.

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

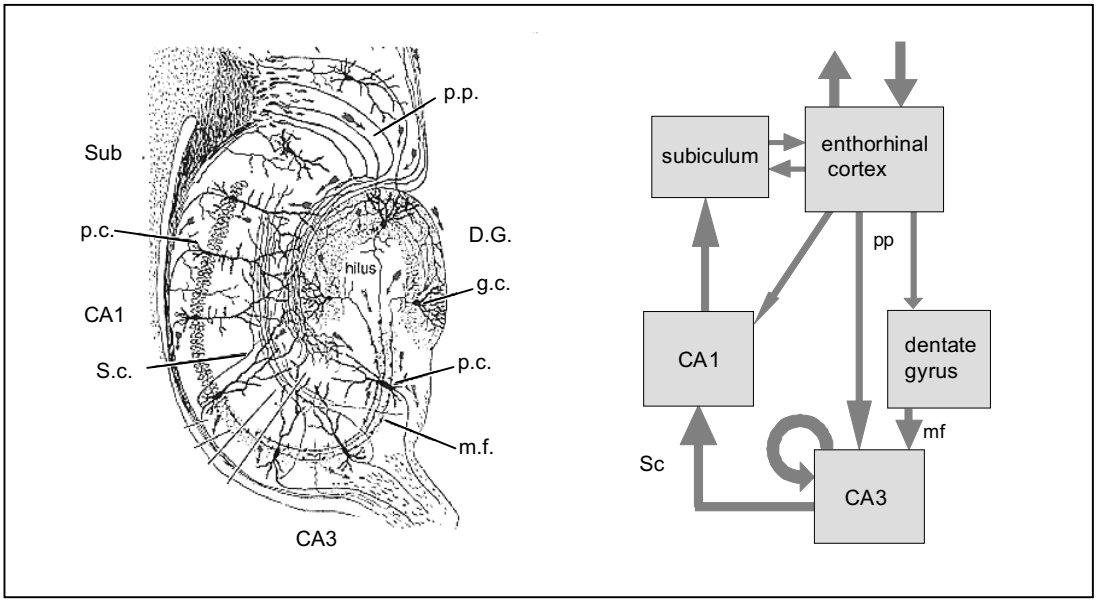

Figure 8. Anatomy of the hippocampus. Major excitatory pathways are shown on the right. The enthorhina cortex projects, by means of the perforant path (pp), to the granule cells (gc) of the dentate gyrus (DG). The dentate gyrus projects to the pyramidal cells (pc) of area CA3 through a sparse but strong 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

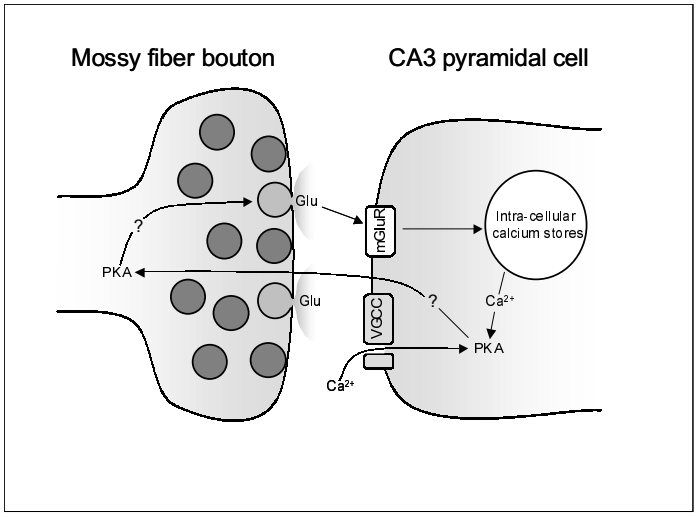

Figure 9. Induction of LTP at mossy fibers. Repeated neurotransmitter release is believed to cause a postsynaptic rise in calcium through metabotropic glutamate receptors and indirectly, by depolarization induced opening of voltage-gated calcium channels. Calcium is believed to activate PKA which, through 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.

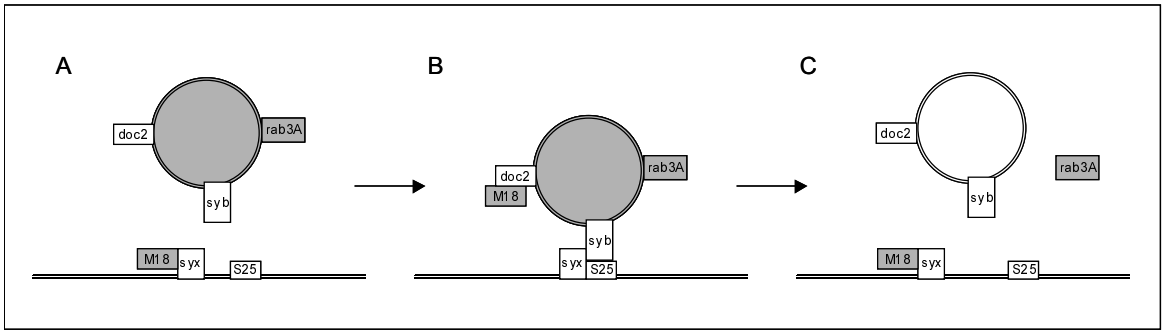

Figure 10 Simplified description of munc18-1 (M18) and rab3A in the nerve terminal. Fusion of neurotransmitter vesicles involves the SNAREs (soluble NSF attachment protein receptors) synaptobrevin/VAMP (syb), syntaxin (syx) and SNAP25 (s25) (Chen and Scheller, 2001). Together they seem to constitute 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

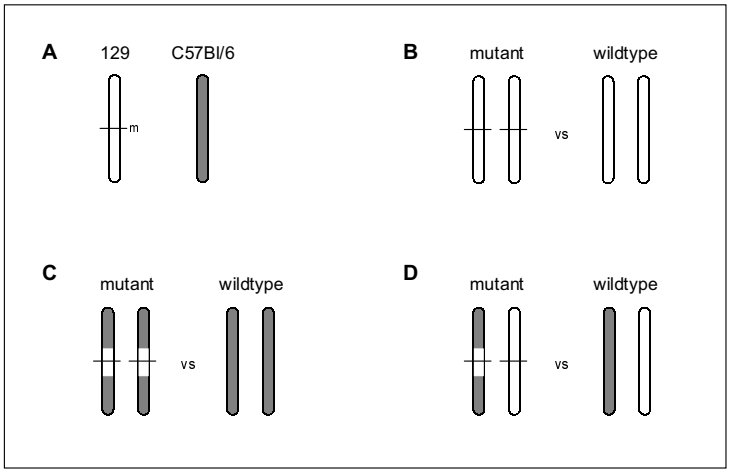

Figure 11. Genetic linkage. Only the mutated chromosome is shown. **A** The mutation (m) is induced in the 129 chromosome. The corresponding C57Bl/6 chromosome is always depicted in grey. **B** Ideal comparison with no differences in genetic linkage. This is preferred if the behavioral task allows it. **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).

Chapter 2

A reduction of munc18-1 in mice leads to deficits in presynaptic plasticity.

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Abstract

The neuronal protein munc18-1 (also known as nSec1, Munc18-a and rbSec1) is essential for secretion of neurotransmitters. Deletion of munc18-1 results in a blockade of neurotransmitter secretion in mice. The effects of reduced levels of munc18-1 were studied in the central nervous system of the mouse. For this, munc18-1 gene-dose (or heterozygous) mutant mice were used. We show that gene-dose mutants have a reduction of munc18-1 protein levels throughout the brain, including the hippocampus. Neither the input-output relationship nor paired pulse facilitation was altered in hippocampal preparations of mutants, indicating that basal transmission and the ability to show enhancement of neurotransmitter release were not affected by reduced munc18-1 levels. However, repetitive stimulation at frequencies of 5, 10 and 20 Hz caused a greater depression of field potentials in mutants, suggesting that during high activity, neurotransmitter release was reduced in mutants. Post-tetanic potentiation is a form of short-term plasticity that lasts several minutes and appears mediated by increases of presynaptic calcium levels. Surprisingly, gene-dose munc18-1 mutants failed to show post-tetanic potentiation. Long-term potentiation was studied in area CA1 and at the mossy fibers of the hippocampus. Long-term potentiation in area CA1 was marginally reduced in gene-dose mutants, but mossy fiber long-term potentiation, which is expressed by an increase in neurotransmitter release, was virtually absent in mutants. In summary, a reduction of munc18-1 in gene-dose mutants leads to deficits in neurotransmitter release that is evident during and after episodes of high neuronal activity. Furthermore, our results suggest a novel and essential role for munc18-1 in two forms of presynaptic plasticity, post tetanic potentiation and mossy fiber LTP.

Introduction

Regulated secretion of neurotransmitter is the primary form of neuronal communication in the nervous system and is characterized by an extremely high speed and strict calcium dependence (Goda and Sudhof, 1997;Neher, 1998). Secretion is maintained by a specialized membrane trafficking cycle that includes assembly of secretory vesicles, transport to the active zone, vesicle docking, maturation and, finally, fusion with the plasma membrane (Sudhof, 1995;Lin and Scheller, 2000). A large number of proteins have been implicated in these steps, but the exact role of most of them remains unclear (Sudhof, 1995;Lin and Scheller, 2000). Fusion of neurotransmitter vesicles appears to involve the so-called SNAREs (soluble NSF attachment protein receptors) synaptobrevin/VAMP, syntaxin and SNAP25 (Chen and Scheller, 2001). Cleavage of any of these proteins by clostridial neurotoxins disrupts release of neurotransmitter (Montecucco and Schiavo, 1994;Jahn and Niemann, 1994) and together they seem to constitute the minimal requirement for membrane fusion (Weber et al., 1998). Syntaxin is associated with the plasma membrane and its availability appears to be regulated by munc18-1, which competes with synaptobrevin/VAMP for binding with syntaxin (Hata et al., 1993;Pevsner et al., 1994). Munc18-1 not only binds syntaxin but is also able to modulate syntaxin function by inducing conformational shifts (Dulubova et al., 1999;Misura et al., 2000;Jahn, 2000).

Munc18-1 (also known as nSec1, Munc18-a and rbSec1) is a mammalian member of the Sec1/Munc18-related proteins that appear to be involved in all eukaryotic membrane fusion reactions (Jahn, 2000). Munc18-1 is soluble and expressed throughout the brain (Garcia et al., 1994). Munc18-1 has been proposed as a negative but also as a positive regulator for neurotransmitter release (reviewed in (Jahn, 2000)). Null-mutant mice for munc18-1 have a disruption of neurotransmitter release in the brain and muscle-endplate (Verhage et al., 2000). Similarly, null-mutant flies for rop, the Drosophila ortholog of munc18, show a blockade of neurotransmitter secretion (Harrison et al., 1994). Furthermore, rop gene-dose (or heterozygous) flies show a reduction in neurotransmission (Wu et al., 1998). In contrast, a role as a negative regulator for neurotransmission was derived from overexpression of rop in Drosophila, which show reduced secretion of neurotransmitter (Schulze et al., 1994;Dresbach et al., 1998;Wu et al., 1998). Furthermore, rop overexpression results in decreased evoked responses during repetitive stimulation indicating that rop may be involved in short-term modulation of neurotransmitter release (Schulze et al., 1994).

Here, the effects of decreased levels of munc18-1 were studied in the central nervous system of the mouse. For this, gene-dose (or heterozygous) mutant mice for munc18-1 mutants were used. First, we established that heterozygosity leads to reduced protein levels in the brain. Second, the hippocampal slice preparation was used to study the effects of reduced munc18-1 levels on neurotransmission. Our results show that reduced munc18-1 levels results in stronger tetanic depression, absence of post-tetanic potentiation, and a severe impairment of long-term potentiation in the mossy fiber projection of the hippocampus.

Materials and methods

Mice

Mutants were created by replacing exon 2-5 of the Munc18-1 gene with a Neomycin resistant gene (Verhage et al., 2000). Subsequently, mutants were repeatedly backcrossed to 129S3 (also known as 129/SvImJ; Jax code: JR2448) for at least 4 generations, resulting in a line with a standardized genetic background. Mice were bred in our laboratory under standard conditions. At three weeks, they were weaned and housed with 2-4 mice of the same sex. Mice were kept at a 12h-light/dark cycle with lights on at 7:00 PM. Food (Hope Farm) and water were freely available. Gene-dose mutant and wildtype experimental animals were obtained by crossing heterozygote males with 129S3 females. Mice were genotyped by PCR. All experiments were approved by the Ethical Committee of the Utrecht University Medical Faculty.

Western blot

Brains of adult mice were dissected and the hippocampus, the cerebellum, the neocortex and brainstem were isolated. Proteins were separated and analysed by SDS-PAGE and Western blotting using 11% gels and semi-dry blotting onto nitro-cellulose membranes. Munc18-1 was detected with a monoclonal antibody (Transduction labs, Lexington, USA) and quantified with alkaline phosphatase conjugated secondary antibodies and enhanced chemifluorescence on a Molecular Dynamics Fluor Imager (Storm, Sunnyvale, USA). GDI was detected using similar procedures and the polyclonal antibody D633 (Verhage et al., 2000).

Electrophysiology

Transversal, 400μm-thick slices were prepared and kept in oxygenated (95% O_2, 5% CO_2) medium at room temperature for at least 60min, before being used. Medium composition in distilled H_2O (mM): 124.0 NaCl, 3.3 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 10.0 glucose, 20.0 $NaHCO_3$ and 2.5 $CaCl_2$. Recordings were done at 30°C. Bipolar stainless-steel stimulation electrodes (tip electrode diameter of 50μm), insulated except for the tip, were placed at the Schaffer collaterals. Field excitatory postsynaptic potentials (fEPSPs), determined from the linear part of the trace, were recorded in the stratum radiatum of area CA1 with glass microelectrodes (tip diameter approximately 2μm). fEPSPs were quantified by determining the slope of the trace. The input/output relation was determined with 5 stimulation intensities that were evenly spaced between intensities that induced threshold and maximum field potentials. The stimulus intensity was adjusted to evoke fEPSPs of half-maximum amplitude (at least 0.125mV), and kept constant thereafter. Stimulation frequency was 0.05Hz. Paired-pulse facilitation was determined with a 50ms interval. Tetanic stimulation was at 5, 10 and 20Hz for 50 pulses. LTP was induced with 100Hz, 1s stimulation, as was PTP but with the addition of 50μM AP5 to the bath to block NMDA-receptors. Mossy fiber LTP was determined with a stimulation electrode at the afferent mossy fibers and recording electrode at the stratum radiatum of area CA3. Mossy fiber LTP was induced with a stimulation of 100Hz, 1s at half-maximum stimulation intensity.

Statistics

Results were analyzed using t-tests for independent samples, single-sample t-tests, univariate ANOVA and ANOVA for repeated measures. Huynh-Feldt correction was applied when the sphericity assumption of ANOVA for repeated measures was not met.

Results

Munc18 gene-dose mutants have reduced munc18-1 protein levels throughout the brain

Having only one copy of a gene may lead to lower expression levels of the protein. Unfortunately, there is no specific munc18-1 antibody suitable for immunocytochemistry. Therefore, we quantified munc18-1 levels using western blotting. In all brain areas tested, including the hippocampus, mutants showed a gene-dose dependent reduction in munc18-1 protein levels ($p<0.005$) (Table 1). GDI was a used as a general marker for neuronal proteins and was not reduced in munc18-1 gene-dose mutant mice (Table 1).

Table 1. Quantification of munc18-1 protein levels in brain tissue by western blot. Values (mean±sem) are measures of chemifluorescence intensities. Munc18-1 was detected using a monoclonal antibody (Transduction labs, Lexington, USA) and GDI was detected using a polyclonal antibody (D633). In all brain areas tested, including the hippocampus, mutants showed a gene-dose dependent reduction in munc18-1 protein levels. GDI was a used as a general marker for neuronal proteins and was not reduced in the mutant. Wildtypes: n=4, munc18-1 gene-dose mutants: n=4.

	Wildtype	**Munc18 +/-**	**Statistics**
Munc18-1			
Hippocampus	3.2 ± 1.0	0.71 ± 0.35	Genotype: $F_{1,6}=28.3$, $p<0.005$
Cortex	5.9 ± 0.85	2.9 ± 0.67	Area: $F_{3,18}=9.0$, $p<0.005$
Cerebellum	1.7 ± 0.63	0.56 ± 0.18	Area*Genotype: $F_{3,18}=0.62$, n.s.
Brainstem	4.3 ± 0.29	2.2 ± 0.75	
GDI			
Hippocampus	9.2 ± 3.2	6.4 ± 1.3	Genotype: $F_{1,6}=0.61$, n.s.
Cortex	7.8 ± 1.1	7.8 ± 0.9	Area: $F_{1.8,10.8}=9.4$, $p<0.01$ (Huynh-Feldt corrected $\varepsilon=0.60$)
Cerebellum	5.9 ± 0.4	5.8 ± 0.8	Area*Genotype: $F_{1.8,10.8}=0.49$, n.s. (Huynh-Feldt corrected $\varepsilon=0.60$)
Brainstem	13.0 ± 0.8	13.1 ± 0.6	

A reduction of Munc18-1 does not alter basal transmission and paired pulse facilitation

The hippocampal slice preparation was used to analyze the physiological consequences of reduced munc18-1 levels. First, the relationship between stimulus strength and field potential responses was tested. Five intensities were tested, evenly spaced between the threshold intensity and the minimal

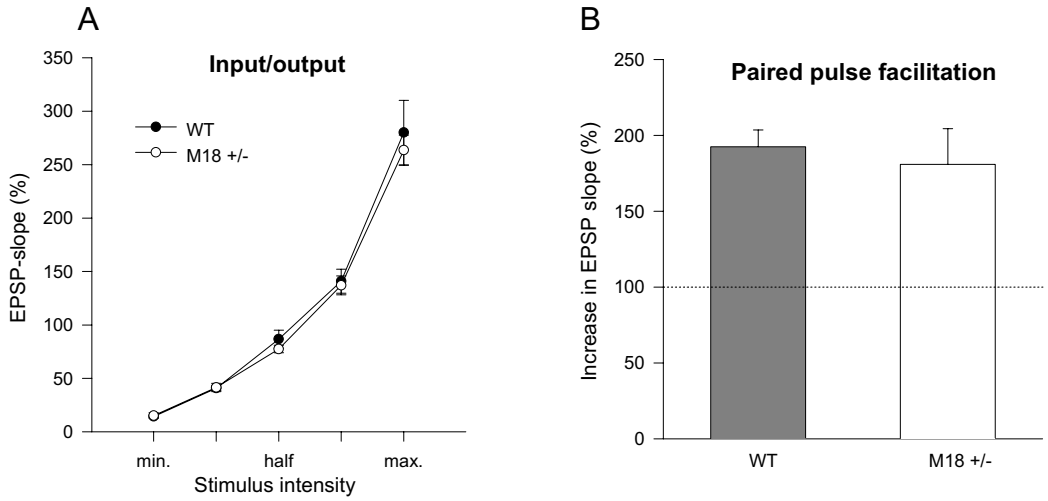

Figure 1. A. Relationship between stimulation intensity and field responses. The interval between threshold stimulation intensity and the minimal stimulation intensity that evoked a maximal response was divided in half to yield the halfmaximal stimulation intensity. A further division resulted in two further intermediate intensities. No differences were detected (genotype: $F_{1,18}$=0.58, n.s.; intensity*genotype: $F_{1.8,33.3}$=0.32, n.s., (Huynh-Feldt corrected ε=0.46)). Wildtype: 10 slices of 6 animals, munc18-1 gene-dose mutant: 10 slices of 5 animals. **B.** Paired-pulse facilitation. Halfmaximal stimulation intensity was used with a pulse interval of 50ms. No differences were detected (t(df=8)=0.45, n.s.). Wildtype: 5 slices of 5 animals, munc18-1 gene-dose mutants: 5 slices of 5 animals.

intensity that induced a maximum response. No differences were detected (Fig.1A), indicating that basal synaptic transmission was normal. Next, paired-pulse facilitation (PPF) was tested. PPF is a short-lasting enhancement of secretion believed to be caused by residual calcium (Zucker, 1999;Thomson, 2000a). PPF was tested with a 50ms interpulse interval. Both wildtypes and mutants showed PPF, and the strength of PPF was not different (Fig.1B). This shows that mutants are able to display a rapid enhancement of neurotransmitter release. Furthermore, this is an additional indication that basal transmission was not changed in mutants, because the strength of facilitation is believed to be correlated with initial release probability (Dobrunz and Stevens, 1997;Zucker, 1999;Thomson, 2000a).

A reduction of Munc18-1 results in enhanced tetanic depression

Tetanic stimulation was applied with frequencies of 5, 10 and 20Hz. Both wildtype and mutant slices showed depression of the response, but this was stronger in gene-dose mutants (Fig.2A, only 10Hz stimulation is shown). After 40-50 pulses, wildtypes showed a depression of 40%, while this was 58% in gene-dose mutants. The plateau phase that is reached after a number of pulses demonstrates the balance between replenishment and release. Mutants also showed a stronger depression at 5 and 20Hz. The relationship between the different stimulation frequencies and the amount of depression is summarized in Fig.2B. Mutants showed stronger depression than wildtypes with all stimulation frequencies ($p<0.001$). Furthermore, during 5Hz stimulation wildtypes showed only 10% depression, while this was already 40% in munc18-1 gene-dose mutants. This difference was significantly larger than the differences between mutants and wildtypes during 10 and 20Hz stimulation ($p<0.05$).

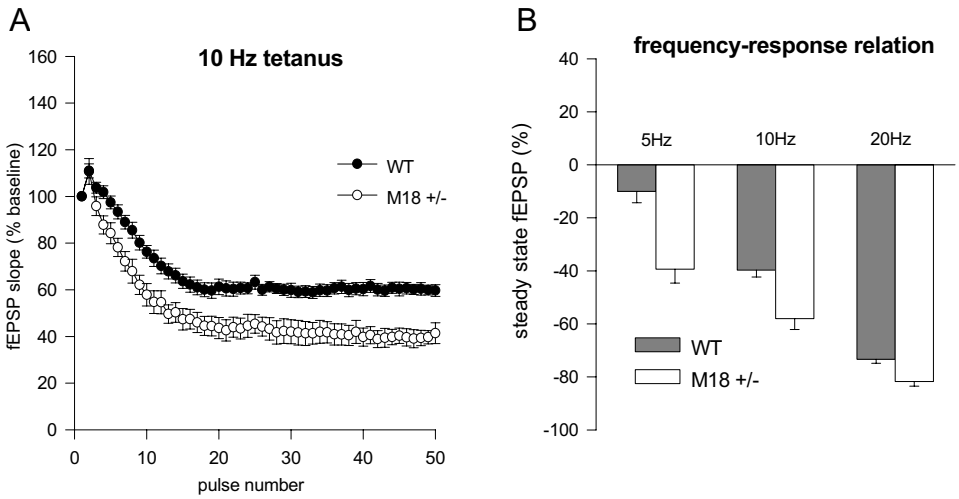

Figure 2. Tetanization experiments **A.** Field responses during 10Hz stimulation at halfmaximal intensity. Mutants showed stronger depression of field responses. Average of last 10 pulses (mean±sem): wildtype 60.3%±2.6, munc18-1 mutants 42.0%±4.1, t(df=13)=4.33, p<0.005. Wildtype 8 slices of 8 animals, mutants 7 slices of 7 animals. **B.** Summary of 5, 10 and 20Hz stimulation. Genotype*frequency: $F_{2,45}$=5.03, p<0.011; frequency: $F_{2,45}$=140.9, p<0.001; genotype: $F_{1,45}$=51.9, p<0.001. 5Hz: wildtype: 8 slices of 7 animals, mutants: 7 slices of 7 animals; 20Hz: wildtype: 12 slices of 10 animals, mutants: 9 slices of 8 animals.

Depression of neurotransmitter release is caused by vesicle depletion and the degree of depression is thought to reflect the refilling rate of the readily releasable pool (Dobrunz and Stevens, 1997;Stevens and Wesseling, 1998). Thus, a reduction of munc18-1 may have resulted in changes in the refilling rate of the readily releasable pool. However, tetanic stimulation will cause a rise in presynaptic calcium levels and induce facilitation, augmentation and post-tetanic potentiation (PTP) (Fisher et al.,

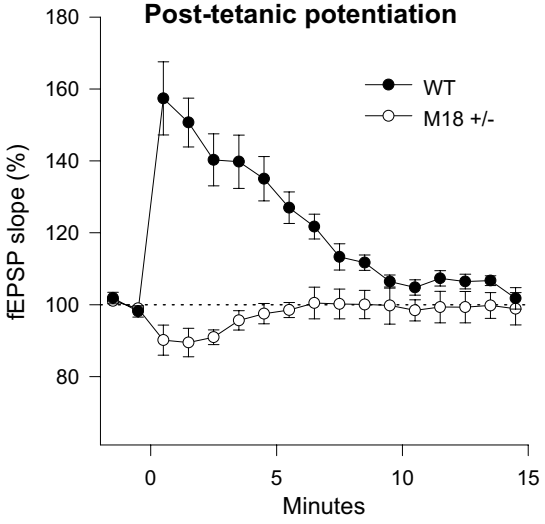

Figure 3 Post-tetanic potentiation. Slices were stimulated with a 100Hz tetanus at half-maximum stimulation intensity for 1s. AP5 (50µm) was added to the bath solution. Wildtypes showed potentiation of the response but munc18-1 mutants failed. Repeated measures ANOVA with 6 levels was used (t=1,3,5,7,9 and 11 minutes). Time*genotype: $F_{2.2,23.8}$=17.3, p<0.001, (Huynh-Feldt corrected ε=0.43). Wildtype: 7 slices of 6 animals, munc18-1 gene-dose mutant: 6 slices of 4 animals.

1997;Zucker, 1999). These enhancements of release may have decreased the degree of depression differentially in mutants and wildtypes. As was shown, facilitation was not changed in munc18 -1 gene-

dose mutants. When the readily releasable pool is emptied, refilling of the pool takes about 10 to 20s (Stevens and Wesseling, 1998). A measurement of augmentation, because of its short duration (~10s), will be contaminated by depletion of the readily releasable pool. Therefore, this hypothesis was tested by inducing PTP.

Reduced levels of munc18-1 levels abolish post-tetanic potentiation (PTP).

PTP was induced with a 100Hz tetanus that lasted 1 second. NMDA receptors were blocked by bath application of 50µM AP5. After induction, responses were measured every 20s giving ample time for refilling of the readily releasable pool. Wildtypes demonstrated PTP that lasted several minutes but munc18-1 gene-dose mutants failed and instead showed a transient decrease of the response ($p<0.001$) (Fig.3). Thus, a reduction of munc18-1 results in a blockade of post-tetanic potentiation.

A reduction of Munc18-1 causes minor deficits in CA1-LTP and severe deficits in mossy fiber LTP

Next, the effects of reduced munc18-1 levels on two different types of long-term plasticity were studied. Long-term potentiation (LTP) at the Schaffer/collateral-CA1 connection is NMDA-receptor dependent and believed to be expressed by post-synaptic changes (Malinow et al., 2000). LTP at this connection was induced with a 100Hz, 1s tetanus. Both heterozygotes and wildtypes showed LTP (Fig.4A), and it tended to be lower in heterozygotes, but the difference was not significant ($p=0.09$).

The second type of long-term plasticity that was studied was mossy fiber LTP. The mossy fibers are the axons from granule cells of the dentate gyrus and project to area CA3. Mossy fiber LTP is expressed by an enhancement of neurotransmitter release (Harris and Cotman, 1986;Staubli et al.,

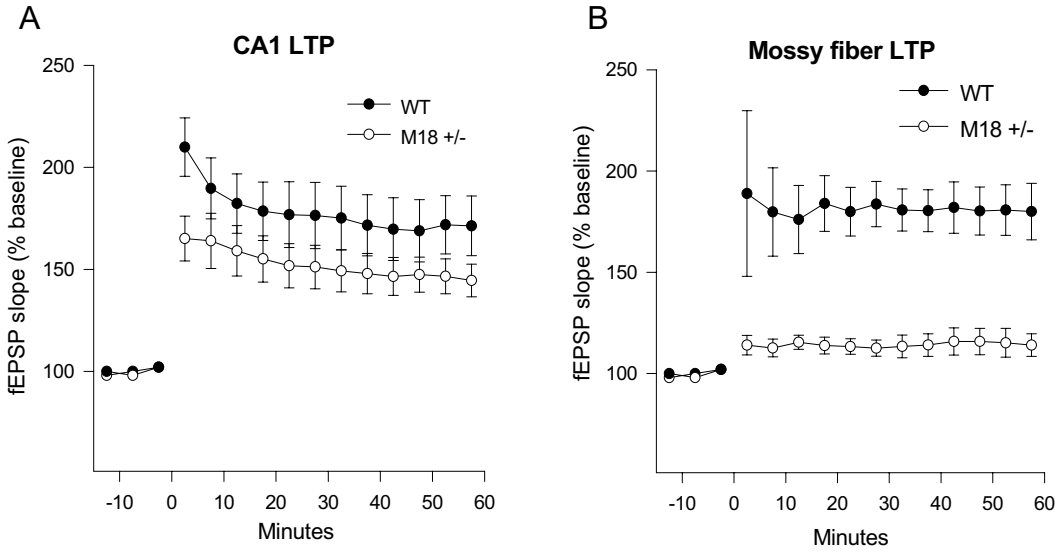

Figure 4 A. LTP in CA1. Changes in field EPSPs after tetanization (100Hz, 1s) of the Schaffer collaterals. Long lasting increases in field EPSPs are induced in wildtypes and munc18-1 gene-dose mutants (average responses 40-60 min after tetanization (mean±sem): wildtype 170%±15, mutants 146%±9, t(df=19)=1.38, p=0.092). Wildtype: 11 slices of 11 animals, munc18-1 gene-dose mutant: 10 slices of 9 animals. **B.** Mossy fiber LTP. Changes in field EPSPs after tetanization (100Hz, 1s) of the mossy fiber projection. Wildtype showed higher increases in field EPSPs than mutants (average responses 40-60 min after tetanization (mean±sem): wildtype 181%±13, munc18-1 gene-dose mutants 117%±6, t(df=12)=4.58, p<0.001). Munc18-1 mutants showed only little potentiation but it was more than baseline. One-sample t-test against 100% for munc18-1 mutants: t(df=6)=2.86, p<0.05. Wildtype: 7 slices of 7 animals, munc18-1 gene-dose mutant: 7 slices of 4 animals.

1990;Weisskopf and Nicoll, 1995). A 100Hz, 1s tetanus induced robust mossy fiber LTP in wildtypes (Fig.4B). Munc18-1 gene-dose mutants showed only a modest potentiation, which was higher than baseline ($p<0.05$), but different from wildtypes ($p<0.001$). Thus, a reduction of munc18-1 levels results in a severe impairment in a type of LTP that is expressed by enhancements of neurotransmitter release.

Discussion

The protein munc18-1 is essential for secretion of neurotransmitter from nerve terminals (Verhage et al., 2000). Presently, gene-dose mutants were analyzed at the biochemical and electrophysiological level. Mutants showed a gene-dose dependent reduction of munc18-1 protein levels throughout the brain. This reduction caused a number of electrophysiological changes. Although basal transmission and paired pulse facilitation appeared normal, mutants showed enhanced tetanic depression, absence of post-tetanic potentiation and a severe impairment in mossy fiber LTP. These results indicate that a reduction of munc18-1 results in a selective impairment in neurotransmitter secretion during and after periods of high neuronal activity.

Basal release and facilitation

The stimulus-response relationship appeared normal. This is in contrast to rop gene-dose mutant flies, which do show a decreased response to single stimuli (Wu et al., 1998). Perhaps, neurotransmitter release at the neuromuscular synapse in flies is more sensitive to decreased rop levels, as neurotransmitter release at central synapses in mice is to decreased munc18-1 levels. Alternatively, rop is involved in cellular membrane fusion reactions in non-neuronal cells (Harrison et al., 1994), and a decrease in rop levels may have led to general deficits in cell function. An additional measure for neurotransmitter release is paired pulse facilitation (PPF). PPF is a short-lasting enhancement of neurotransmitter release and, because of the strong correlation with release probability, is often used to measure changes in neurotransmitter release (Dobrunz and Stevens, 1997;Zucker, 1999;Thomson, 2000a). PPF was not changed in the munc18-1 gene-dose mutant, which is a further indication that basal release properties were not altered.

Post-tetanic potentiation

The absence of PTP in munc18-1 gene-dose mutants was unexpected and striking. PTP is dependent on moderate increases of presynaptic calcium and expressed by an enhancement of neurotransmitter release, which suggests the involvement of a high affinity calcium detector able to modulate neurotransmitter release (Kamiya and Zucker, 1994;Zucker, 1999). Little is known about the molecular mechanisms involved in PTP. Munc18-1 has been implicated in the translocation of peptide vesicles (Voets et al., 2001). Thus, translocation of new synaptic vesicles may be impaired in this mutant after high frequency stimulation of central synapses and cause a transient decrease of neurotransmitter release. However, given the normal timecourse of refilling (~20s) (Stevens and Wesseling, 1998), and the length of PTP in the present experiment (-6min), this does not appear to be able to explain the disruption of PTP. The deficit in refilling rate should be so large that tetanic stimulation at 5, 10 or

20Hz should have quickly resulted in complete extinction of secretion, and this was not observed. Therefore, our results suggest that decreases of munc18-1 disrupt some aspect of the expression of PTP and implicate munc18-1 in calcium-dependent presynaptic plasticity. Possible molecular mediators will be discussed below.

Tetanic depression

Munc18-1 gene-dose mutants showed an enhancement of tetanic depression. During tetanic stimulation a complicated interaction develops between depletion of the readily releasable pool of synaptic vesicles, enhancements of release by presynaptic calcium influx, and activation of inhibitory feedback. Any of these variables may have contributed to the changes in tetanic depression. For instance, the refilling rate of the readily releasable pool may have been altered. This hypothesis is supported by the fact that decreased munc18-1 causes impairments in the translocation of large peptide vesicles to the cell membrane (Voets et al., 2001). However, there are no indications for a similar role in trafficking of neurotransmitter vesicles in the central nervous system. For instance, null-mutants for munc18-1 have similar amounts of docked vesicles as wildtypes at birth (Verhage et al., 2000). Furthermore, impairments in recycling would probably be most evident at higher tetanic stimulation frequencies. However, the relationship between stimulation frequency and the amount of depression shows the opposite. The largest difference between wildtypes and mutants is during 5Hz stimulation and not at 10 or 20Hz (Fig2B). Another explanation for the differences in tetanic depression is increased inhibition by GABAergic interneurons. However, tetanic stimulation experiments were also performed at the neuromuscular junction by direct stimulation of motoraxons. In this preparation as well, mutants showed enhanced depression (Toonen et al., in prep). There is no inhibitory circuit in this preparation suggesting that enhanced depression in the hippocampal slice was not mediated by increases in the efficiency of inhibitory circuits. Finally, increased depression in munc18-1 mutants may have resulted from changes in the interaction between different types of presynaptic plasticity. Neurotransmitter release can be enhanced by facilitation, augmentation or post-tetanic potentiation (Fisher et al., 1997;Zucker, 1999;Thomson, 2000a). Differential expression of these types of presynaptic plasticity may have alleviated the amount of synaptic depression during tetanic stimulation differentially in wildtypes compared to mutants.

Relationship between facilitation, augmentation, post-tetanic potentiation and vesicle depletion

Facilitation is believed to be mediated by a local, high rise in calcium, while augmentation and PTP appear to be caused by a moderate, general rise in calcium (Fisher et al., 1997;Zucker, 1999). Facilitation was not changed in munc18-1 gene-dose mutants, indicating that the ability to show short-term enhancements of synaptic release is not affected by lowered levels of munc18-1. Augmentation and PTP are longer lasting types of presynaptic plasticity. There are indications that augmentation and PTP are mediated by similar molecular mechanisms. Both augmentation and PTP are directly related to presynaptic cytosolic calcium levels (Brain and Bennett, 1995), and both respond to sudden drops in calcium levels with similar time constants (Kamiya and Zucker, 1994). PTP is believed to be caused by calcium extrusion from mitochondria (Tang and Zucker, 1997), while augmentation seems to reflect a transient increase of cytoplasmatic calcium during moderate stimulation (Kamiya and Zucker, 1994). The different duration of augmentation (~10s) and PTP (~minutes) may just reflect different levels of

calcium buildup and time-course of extrusion (Fisher et al., 1997;Tang and Zucker, 1997). It is not likely that PTP was induced with the moderate stimulation frequencies that were applied during tetanic stimulation. However, as PTP was absent in munc18-1 gene-dose mutants, augmentation may also have been affected. Augmentation is readily induced with moderate tetanic stimulation, and a disruption of augmentation may explain the enhancement in tetanic depression in mutants. Clearly, further examinations are needed to elucidate this issue.

Mossy fiber LTP

Mossy fiber LTP is a form of LTP that is expressed presynaptically by an enhancement of neurotransmitter release (Yamamoto et al., 1980;Harris and Cotman, 1986;Staubli et al., 1990;Weisskopf and Nicoll, 1995). The mechanism of induction is not clear but appears to require postsynaptic calcium influx (Williams and Johnston, 1989;Yeckel et al., 1999). It may be argued that enhanced depression caused a decrease in neurotransmitter release during the induction of MF-LTP. However, MF-LTP was induced with short, high frequency stimulation (1s, 100Hz). This type of stimulation will probably result in secretion of all vesicles of the readily releasable, but appears too short to be influenced much by depression. The exact mechanisms of mossy fiber LTP are not known but synaptic vesicle proteins like rab3A and RIM have been implicated (Castillo et al., 1997;Castillo et al., 2000). Our results suggest that munc18-1 is involved in the induction or expression of mossy fiber LTP.

Molecular mechanisms

Our results show that a decrease in munc18-1 leads to deficits in the short- and long-term forms of presynaptic plasticity. The molecular mechanisms leading to enhanced neurotransmitter release are poorly understood. Our results implicate munc18-1 as an essential modulator of some of these processes. The role of munc18-1 may be mediated by munc18-1 interacting proteins such as syntaxin and Doc2 (Pevsner et al., 1994;Verhage et al., 1997;Duncan et al., 2000;Jahn, 2000). Doc2 contains high affinity calcium binding domains and has been implicated in the modulation of neurotransmitter release (Orita et al., 1995;Mochida et al., 1998;Kojima et al., 1996;Sakaguchi et al., 1999;Duncan et al., 2000). Syntaxin is essential for secretion of neurotransmitter (Bennett et al., 1992;Blasi et al., 1993). Furthermore, syntaxin is known to bind and inhibit calcium channels, and altered syntaxin function may cause changes in calcium kinetics (O'Connor et al., 1993;Leveque et al., 1994;Bezprozvanny et al., 1995;Stanley and Mirotznik, 1997;Sutton et al., 1999). The interaction between munc18-1 and syntaxin can be affected by PKC, which is able to phosphorylate munc18-1 in an activity dependent matter (Fujita et al., 1996;de Vries et al., 2000). Interestingly, activation of PKC results in an enhancement of neurotransmitter release and an increase in the size of the readily releasable pool (Carroll et al., 1998;Majewski and Iannazzo, 1998;Stevens and Sullivan, 1998;Yawo, 1999). Furthermore, PKC appears necessary for mossy fiber LTP (Son et al., 1996;Son and Carpenter, 1996). Perhaps the actions of PKC are mediated by munc18-1, and a decrease in munc18-1 levels may have altered these actions. Thus, a number of interactions exists by which munc18-1 may be involved in the modification of neurotransmitter release. However, further biochemical and electrophysiological characterization of these interactions is needed to elucidate the exact role of munc18-1.

In summary, munc18-1 gene-dose mutants were analyzed at the biochemical and electrophysiological level. Munc18-1 gene-dose mutant mice showed a gene-dose dependent reduction of munc18-1 protein levels throughout the brain which caused a number of electrophysiological changes. Mutants showed enhanced tetanic depression, absence of post-tetanic potentiation and a severe impairment in mossy fiber LTP, while basal transmission and paired pulse facilitation appeared normal. Our results suggest a novel function for munc18-1 in the induction and/or expression of short and long-term presynaptic plasticity.

Chapter 3

Disruptions of presynaptic plasticity do not affect spatial memory but cause hyperactivity and impair working memory performance

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Abstract

Learning and memory are believed to be mediated by changes in synaptic strength. Synaptic strength can be modulated by changes in neurotransmitter release, but the importance of such changes for learning and memory is not clear. Here, this relationship was tested using munc18-1 gene-dose (or heterozygous) mutant mice that have impairments in presynaptic plasticity. Previously, it was shown that munc18-1 gene-dose mutants show a reduction of munc18-1 protein levels throughout the brain. Such a decrease of munc18-1 does not affect basal transmission and paired-pulse facilitation but causes an enhancement of tetanic depression and a disruption of post-tetanic potentiation and mossy fiber LTP. Despite these severe impairments in presynaptic plasticity, we observed no deficits in the Morris water maze. The eight-arm radial maze was used to assess spatial working memory. Mutants showed no improvements in this task and remained at pre-training levels. Thus, these forms of presynaptic plasticity may be involved in working memory, but are not important for spatial memory. Munc18-1 gene-dose mutants showed a higher locomotor activity in the radial maze. Such increases in locomotor activity were also observed in the open-field. The sensitivity to psychostimulants was studied with d-amphetamine, which strongly increased locomotor activity in mutants compared to wildtypes. We conclude that tetanic depression, post-tetanic potentiation and mossy fiber LTP are not important for spatial memory, but may be necessary for working memory. Disruption of presynaptic plasticity does not lead to selective effects on learning, but also to other deficits such as hyperactivity.

Introduction

Learning and memory are believed to be mediated by modifications in synaptic strength, but the exact mechanisms are not clear. One of the mechanisms involved may be a change in neurotransmitter release. Neurotransmitter release can be altered by short-term enhancements such as facilitation, augmentation and post-tetanic potentiation (PTP), but also by different types of short-term depression (Goda and Sudhof, 1997;Fisher et al., 1997;Zucker, 1999;Thomson, 2000b). Furthermore, neurotransmitter release can be altered for longer periods, such as occurring during long-term potentiation (LTP) and long-term depression (LTD at the mossy fiber projection of the hippocampus (Yamamoto et al., 1980;Harris and Cotman, 1986;Staubli et al., 1990;Weisskopf and Nicoll, 1995;Kobayashi et al., 1996). These changes in neurotransmitter release may be important in learning and memory, but this hypothesis has not been studied extensively due to the difficulty of manipulating neurotransmitter release in freely moving animals. Recently, a number of studies have appeared that used genetically manipulated mice to study the function of presynaptic plasticity in learning and memory, but the result were inconclusive. For instance, synapsinII null-mutants and synapsinI/II doubly mutants show lowered PTP and display mild deficits in contextual fear conditioning (Silva et al., 1996). The function of mossy fiber long-term potentiation (LTP) was tested in mGluR1 and type 1 adenylyl cyclase (AC1) null-mutants, which show impaired mossy fiber LTP and impaired performance in the water maze (Conquet et al., 1994;Wu et al., 1995;Villacres et al., 1998). However, other studies failed to relate deficits in presynaptic plasticity to memory impairments. For instance, rab3A null-mutants show enhanced paired-pulse facilitation, stronger tetanic depression and an abolishment of both mossy fiber LTP and LTD (Geppert et al., 1994;Geppert et al., 1997;Castillo et al., 1997;Tzounopoulos et al., 1998). Despite these impairments of presynaptic plasticity no deficits were observed in contextual fear conditioning, water maze learning and radial maze performance (chapter 5). Also, mice that lack a catalytic subunit (Cβ1) or a regulatory subunit (RIβ) of PKA have no MF-LTP but show normal Morris maze performance and normal contextual fear-conditioning (Huang et al., 1995). Thus, although a number of efforts have been made, the role of short and long-term presynaptic plasticity in learning and memory is unclear, and further studies are needed.

Presently, this relationship was tested using munc18-1 gene-dose mutant mice. Munc18-1 is expressed in all neurons (Garcia et al., 1994), and is essential for neurotransmitter release (Verhage et al., 2000). Gene-dose mutants (or heterozygotes) lack one copy of the gene for munc18-1 and are viable. Previously, it was shown that these mutants show a reduction in munc18-1 protein levels throughout the brain, which does not affect basal transmission and paired-pulse facilitation but causes an enhancement of tetanic depression, a disruption of post-tetanic potentiation and a severe impairment in mossy fiber LTP (chapter 2). The learning capabilities of these mutants were tested in the Morris water maze and the radial maze. Our results show that spatial learning was normal but that working memory performance was severely impaired. Mutants also showed a marked increase in locomotor activity.

Materials and methods

Mice

Mutants were created by replacing exon 2-5 of the Munc18-1 gene with a Neomycin resistant gene (Verhage et al., 2000). Subsequently, mutants were repeatedly backcrossed to 129S3 (also known as 129/SvImJ; Jax code: JR2448) for at least 4 generations, resulting in a line with a standardized genetic background. Mice were bred in our laboratory under standard conditions. At three weeks, they were weaned and housed with 2-4 mice of the same sex. Mice were kept at a 12h-light/dark cycle with lights on at 7:00 PM. Food (Hope Farm) and water was freely available.

Gene-dose mutant and wildtype experimental animals were obtained by crossing heterozygote males with 129S3 females. For the Morris water maze task, heterozygote males were crossed with C57Bl/6 female to obtain a C57Bl/6-129S3 genetic background. This breeding strategy was used because 129S3 mice have a propensity to show excessive floating in the Morris water maze, while C57Bl/6-129S3 hybrids perform well in this task (Wolfer et al., 1997;Banbury Conference Report, 1997). This breeding scheme was also used to study open-field behavior on different genetic backgrounds. The experimenter was always blind with respect to genotype until the end of the experiments. Mice were genotyped by PCR. All experiments were approved by the Ethical Committee of the Utrecht University Medical Faculty.

Developmental measures

Seven sensorimotor responses and bodyweight were recorded daily from day 3 to day 21. The age of appearance of the following responses were measured. Cliff drop aversion was shown when the pup turns around and crawls away when its head is placed over the edge of a tabletop. Hindlimb placing is displayed when the pup raises its limb and places it on top of a pencil when the front of hindlimb paw is touched with that pencil. Forelimb grasp is shown when a rod is grasped when the inside of the forelimb paw is touched with that rod. Stop pivoting is displayed when the pup does not pivot around anymore when placed on a tabletop. Rightning reflex is shown when the pup turns around within 5s after being placed on its back. Straight line walking is displayed when the pup walks away in a straight line when placed on a tabletop. Eye opening is shown when both eyes are fully opened.

Morris Water Maze

The Morris maze was a circular white pool (diameter 132cm), filled with water (25°C) that was made opaque by adding small quantities of the white pigment Acoat X (Akzo Nobel, Sassenheim, The Netherlands) at a dilution of approximately 1:10.000. The platform was circular (diameter 15cm) and made of perforated Plexiglas that provided the mice with extra grip. The platform was placed 1cm below the surface of the water in the center of a quadrant. The training protocol was essentially the same as described by others (Wolfer et al., 1997). To avoid visual orientation prior to release, the mice were transported in a white bucket from which they glided in the water towards the wall of the pool. Starting positions changed every trial. Their location was sampled every 0.1s using Ethovision software (Noldus technology, The Netherlands). A trial ended when a mouse was 10s on the platform or when 120s had passed. The mice received six trials a day with a 45-60min interval between trials. At the start of the 4th day, the platform was transferred to the opposite quadrant. During the first 30s of

trial 1 at day 4 preference for the old quadrant was determined (transfer test); mice that found the relocated platform in less then 30s were excluded from the analysis. At the end of the 5th day of training, the platform was removed and a probe test of 30s was given.

Eight-arm Radial Maze

The radial maze was made of transparent Plexiglas. All arms (23cm long, 6cm wide, 5cm high) were baited with food rewards (20mg, Noyes precision food pellets, P.J. Noyes Company Inc.), which were placed behind a small barrier (1cm high, 1 cm long) that prevented visual detection. At the end of each arm, food pellets were deposited behind a perforated wall. These pellets were not retrievable by the mice but supplied the arm with food odors that prevented detection of the real food rewards by olfaction. A standard protocol was used (Schwegler et al., 1990). Twenty-four hours before training the animals were food, but not water, deprived. During training, they were kept at 85–90% of their pre-test bodyweight. Mice received 1 trial per day. The first day, they received a 10min habituation trial. On the following 8 days, between arm visits, mice were confined for 5 s in the central area by lowering guillotine doors located at the entrance of each arm. This procedure is known to disrupt chaining responses and kinesthetic strategies. Trials ended when all baits had been eaten or when 15min had past, whichever came first. During later trials, all mice routinely took all baits within 15min. An error was defined as an entry with all four paws in a previously visited arm.

Open Field

The open field task was used to study general measures of activity. The open field was a circular, moderately illuminated (80lux) arena constructed of gray PVC (diameter 78cm, walls 30cm high). The open field was divided in two parts, a central area with a diameter of 55cm and an outer ring. Mice were placed in the outer ring and allowed to freely explore the open field for the duration of 15min. The location of the mouse was recorded every 0.5s using Ethovision (Noldus technology, The Netherlands).

Amphetamine

Mice received subcutaneous injections of d-Amphetamine (OGP, Utrecht, The Netherlands), at a dose of 0, 1 and 4 mg/kg bodyweight. Immediately after injection, they were placed in an observation cage (48cm long, 24cm wide, 24cm), that was moderately illuminated (80lux). Locomotor activity was monitored for 60min thereafter, by recording the location of the mouse every 0.5s using Ethovision (Noldus technology, The Netherlands).

Statistics

Non-parametric data was analyzed with the Chi-square test and Mann-Whitney-U test. Parametric data was analyzed by t-tests for independent samples, and univariate and repeated measures of ANOVA. When the sphericity assumption was not met, Huynh-Feldt correction was applied.

Results

Munc18-1 gene-dose mutants have lower bodyweights but showed normal sensorimotor development

Mutants appeared normal upon visual inspection. At weaning, genotypes were represented according to a mendelian distribution (Table 1). However, munc18-1 gene-dose mutant pups had a lower

Table 1 Genotype frequency, bodyweight and sensorimotor development. Shown are means±sem.

	Wildtype	M18 +/-	Statistics
Genotype frequency	155	135	χ^2(df=1)=1.38, n.s.
Bodyweight			
Males at 21 days (g)	9.1±0.9 (n=4)	8.6±0.3 (n=6)	Sexe: $F_{1,20}$=0.78, n.s.
Females at 21 days (g)	10.2±0.3 (n=3)	7.8±0.2 (n=11)	Genotype: $F_{1,20}$=12.0, p<0.005
Males at 84 days (g)	28.7±2.1 (n=4)	26.2±0.5 (n=6)	Sexe: $F_{1,19}$=50.9, p<0.001
Females at 84 days (g)	23.2±0.6 (n=3)	19.4±0.3 (n=10)	Genotype: $F_{1,19}$=13.3, p<0.005
Sensorimotor development	n=7	n=17	
cliff drop aversion (day)	8.0±0.4	7.5±0.2	MWU=41.5, n.s.
hindlimb placing (day)	8.1±0.3	8.5±0.2	MWU=46.0, n.s.
forelimb grasp (day)	9.1±0.5	9.2±0.2	MWU=53.0, n.s.
stop pivoting (day)	10.7±0.8	11.7±0.3	MWU=42.0, n.s.
rightning reflex (day)	11.3±0.7	12.8±0.2	MWU=33.0, n.s.
straight line walking (day)	11.6±0.4	11.9±0.4	MWU=54.5, n.s.
eye opening (day)	13.6±0.4	13.4±0.2	MWU=50.5, n.s.

bodyweight at weaning (p<0.005). Such a decrease in bodyweight may result in retarded development, so next we determined the age of appearance of a variety of sensorimotor abilities. No differences were detected, indicating that the lowered bodyweight did not affect general development (Table 1). As adults, gene-dose mutants also had a lower bodyweight (p<0.005). General sensorimotor ability in adults was tested on a rotarod. No impairments in munc18-1 gene-dose mutants were detected (data not shown).

Spatial learning and memory in the Morris water maze are normal

The Morris water maze was used to assess long-term spatial reference memory. During the first three days of Morris maze training, both groups showed a rapid decline in search time (Fig.1A). At the first day, search time in wildtypes appeared higher compared to mutants, but this difference was not significant (t(df=22)=-1.74, p>0.1). Transfer of the platform to the opposite quadrant resulted in an increased search time on day 4, which returned to lower levels on day 5, indicating the ability of mutants and wildtypes to relearn a new platform location. Spatial memory was determined during the transfer test on day 4 and during the probe test on day 5. Wildtypes and munc18-1 gene-dose mutants showed a strong preference for the quadrant were the hidden platform used to be (Fig.1B), both during the transfer test at day 4, and during the probe test at day five. These results show that long-term

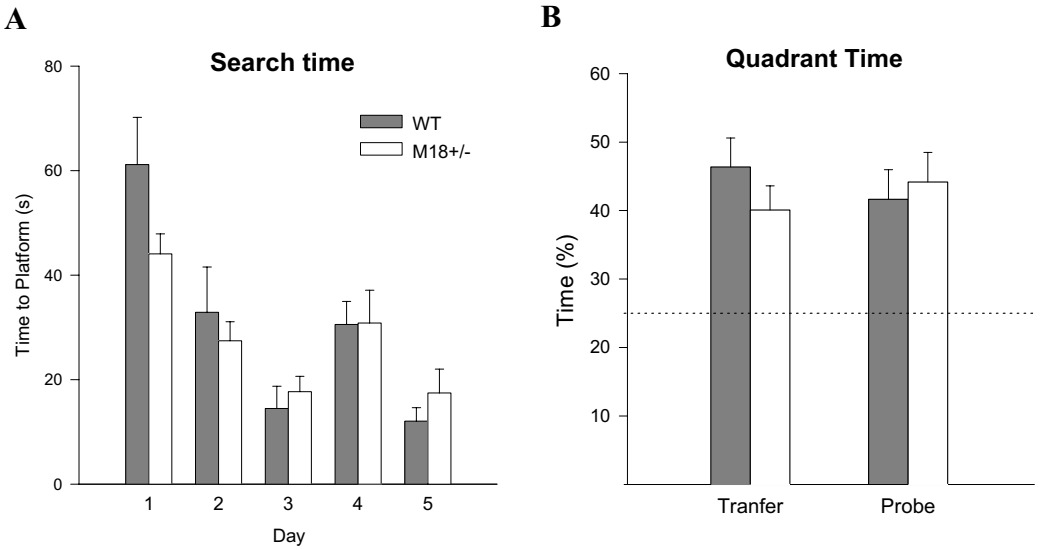

Figure 1. Morris maze performance of wildtypes (6 male and 6 female mice) and gene-dose mutants (5 male and 7 female mice). **A.** Search time to find the hidden platform. Shown is average of six daily trials. No significant main effects or interactions were detected: day*genotype*sex $F_{3.3,66.5}$=1.75, p=0.16 (Huyhn-Feldt corrected ε=0.83); day*sex $F_{3.3,66.5}$=1.71, p=0.17 (Huyhn-Feldt corrected ε=0.83); day*genotype $F_{3.3,66.5}$=2.43, p=0.067 (Huyhn-Feldt corrected ε=0.83); genotype $F_{1,20}$=0.03, n.s.; sex: $F_{1,20}$=0.77, n.s. The trend in the interaction day*genotype was attributable to search times on day 1 were wildtypes showed somewhat longer latencies than munc18-1 gene-dose mutants. **B.** Search time (in percentage of total time) in the quadrant were the hidden platform used to be. No differences were detected at day 4: wildtype n = 5 male and 3 female mice, gene-dose mutants n = 2 male and 4 female mice (genotype*sex: $F_{1,10}$=2.15, p=0.17; sex: $F_{1,10}$=0.38, n.s.; genotype: $F_{1,10}$=0.54, n.s.), and at day five: wildtype n = 6 male and 6 female, gene-dose mutants n = 5 male and 7 female. (genotype*sex: $F_{1,20}$=0.13, n.s.; sex: $F_{1,20}$=0.26, n.s.; genotype: $F_{1,20}$=0.14, n.s.).

spatial memory is not affected by lowered levels of munc18-1 and indicate that PTP and mossy fiber LTP are not essential for acquisition and retrieval of spatial information.

Performance in the radial maze is severely impaired

The eight-arm radial maze was used to assess spatial working memory i.e. the ability to remember previously visited arms within the same trial. Fig.2A shows the number of errors over the course of 8 days of training. Wildtypes showed a decline in the amount of working memory errors that reached a plateau after approximately 4 trials. In contrast, mutants failed to show improvements in their performance. A number of significant effects were detected, including a main effect for genotype ($p<0.01$) and a trial*genotype effect ($p<0.05$). Thus, munc18-1 gene-dose mutants performed worse than wildtypes. The accumulation of errors is shown in Fig.2B. Gene-dose mutants seemed to accumulate most errors during the collection of the last baits ($p<0.06$). At that point working memory load is highest, which indicates that the difference between mutants and wildtypes may have been the result of a decreased ability to maintain and use larger amounts of information in working memory.

Munc18-1 gene-dose mutants show increased activity

While conducting the radial maze task, it was noticed that mice that performed poorly seemed to have a higher locomotor activity. Post-hoc analysis revealed that these mice were mutants. Mutants spend less time in an arm that was previously visited ($p<0.01$), but were comparable to wildtypes when an arm was visited for the first time (Table 2). Swimming speeds in the Morris water maze were not changed (Table 2), and it is possible that increased activity may have selectively interfered with radial

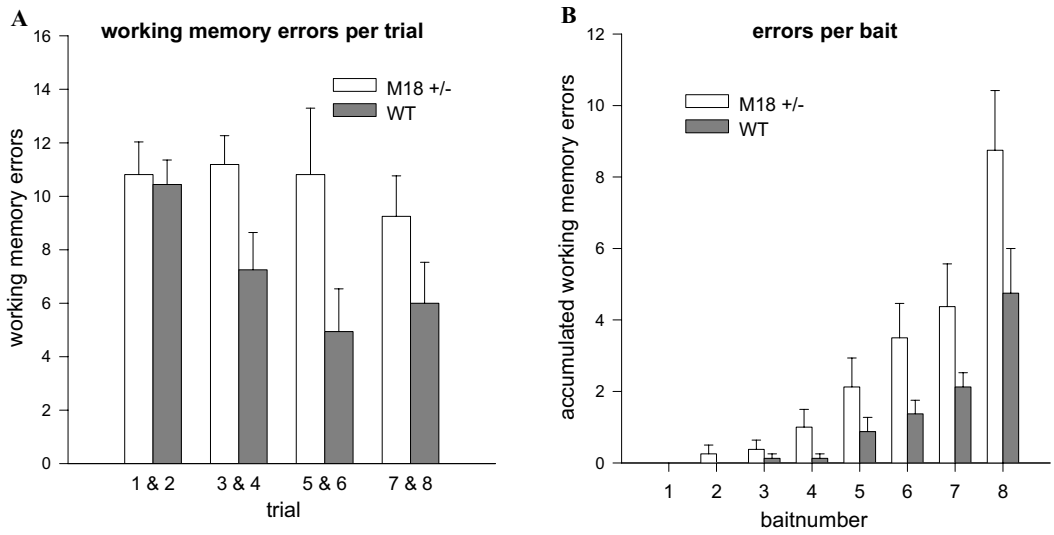

Figure 2. Radial maze performance of wildtype (5 male and 3 female mice) and mutant (3 male and 5 female mice) mice. **A.** Average errors per two trials. Statistical analysis showed significant interaction effects for: trial*genotype ($F_{3,36}$=3.88, p<0.05) and trial* sex ($F_{3,36}$=6.46, p<0.005). Also, a significant main effect was detected for genotype ($F_{1,12}$=9.56, p<0.01), whereas sex showed a trend ($F_{1,12}$=4.58, p=0.054). **B.** Accumulation of errors during the last trial. Shown are the number of accumulated errors before the next bait is found. The interaction baitnumber*genotype showed a trend ($F_{2.79,33.5}$=2.71, p=0.064 (Huynh-Feldt corrected ε=0.40)). There was a significant main effect for genotype ($F_{1,12}$=6.24, p<0.05). No interactions or main effect for sex were detected.

maze performance. In order to further analyze this increased activity, the open field task was used, which provides general measures of exploratory activity. Different genetic backgrounds were used in the radial maze (129S3) and the Morris water maze (C57Bl/6-129S3). Therefore, both genetic backgrounds were tested in the open field. In this task, gene-dose mutants walked much larger distances then wildtypes (p<0.001)(Fig.3A), which was mainly attributable to fact that mutants showed only little inactivity (p<0.001)(Fig.3B). Wildtypes 129S3 mice showed lower levels of activity than wildtype C57Bl/6-129S3 mice (wildtype only: strain effect $F_{1,24}$=27.6, p<0.001). The time in the central area was used as a measure of dispersion in the open field (Fig.3C). In the 129S3 genetic background, mutants and wildtypes differed (129S3 only: genotype*time effect: $F_{1.4,32.2}$=7.13, p<0.01 (Huyhn-Feldt corrected ε=0.70)). However, mutants and wildtypes did not differ when they had a

Table 2 Measures of activity in the radial maze and Morris water maze. Running speed in the radial maze was determined during the last trial, visits to empty arms were separated from visits to arms that still contained food rewards. Swimming speed in the Morris maze was determined during the last probe trial. Shown are means±sem.

	Wildtype	M18 +/-	Statistic
Radial maze	n=8	n=8	
Time in baited arm (s)	24.1±2.0	23.1±2.8	t(df=14)=0.47, n.s.
Time in empty arm (s)	25.3±3.7	11.7±1.2	t(df=14)=3.38, p<0.01
Morris maze	n=12	n=12	
Swim speed (cm/s)	18±1.0	17±1.0	t(df=22)=1.70, n.s.

C57Bl/6–129S3 background. Thus, munc18-1 gene-dose mutants showed enhanced activity in the

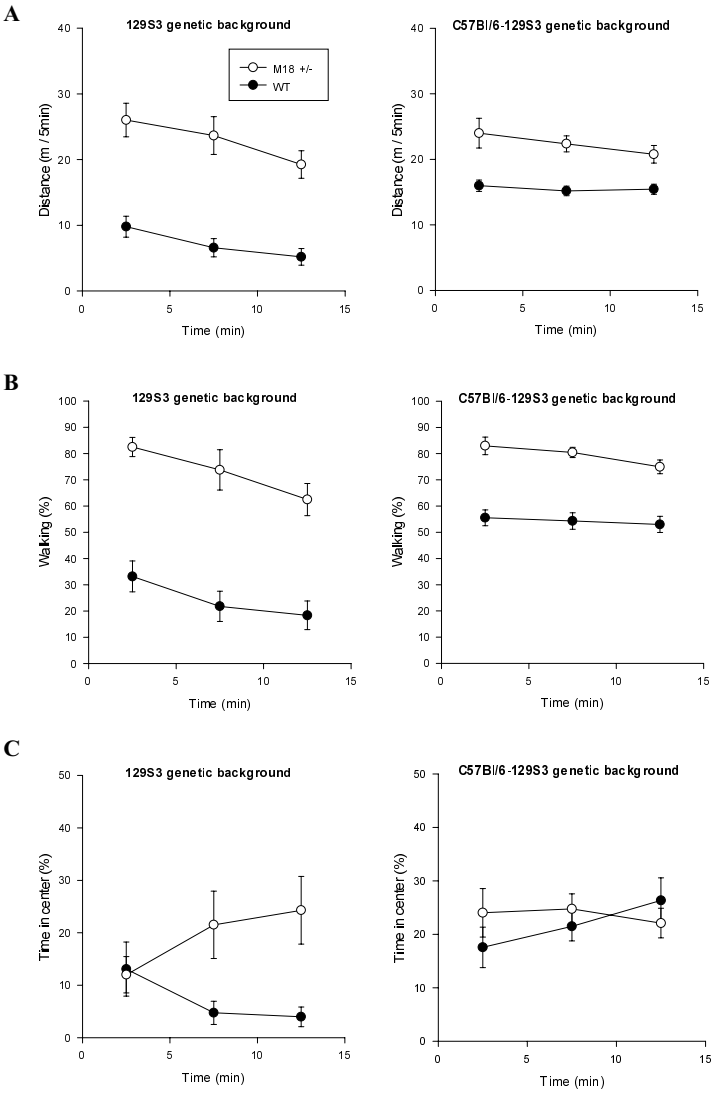

Figure 3. Open field behavior. 129S3 genetic background: gene-dose mutants (5 male and 5 female mice) and wildtypes (9 male and 8 female mice); C57Bl/6-129S3 genetic background: gene-dose mutants (5 male and 5 female mice) and wildtypes (6 male and 5 female mice). **A**. Traveled distance in 5min bins. Significant effects: time*strain ($F_{2,80}$=3.91, p<0.05), genotype*strain ($F_{1,40}$=9.54, p<0.005), strain ($F_{1,40}$=7.17, p<0.05) and genotype ($F_{1,40}$=60.9, p<0.001). **B**. Time spent moving in 5min bins. Significant effects: time*strain ($F_{2,80}$=3.79, p<0.05), genotype*strain ($F_{1,40}$=6.92, p<0.05), strain ($F_{1,40}$=16.7, p<0.001) and genotype ($F_{1,40}$=69.1, p<0.001). **C.** Time in center in 5min bins. Significant effects: time*sex*genotype*strain ($F_{1.94,77.6}$=4.58, p<0.05 (Huynh-Feldt corrected ε=0.97)), time*genotype*strain ($F_{1.94,77.6}$=8.62, p<0.001 (Huynh-Feldt corrected ε=0.97)), strain ($F_{1,40}$=8.21, p<0.01) and genotype ($F_{1,40}$=4.34, p<0.05).

open-field, which was mainly caused by incessant walking. Interestingly, the phenotype of mun c18-1 gene-dose mutants persisted in both genetic backgrounds, and genetic background had surprisingly little influence on measures of open-field behavior in mutants. In contrast, wildtype scores were much more dependent on genetic background, which shows the need for well-defined genetic backgrounds in tasks such as the open-field.

Munc18-1 gene-dose mutants are suspectible to d-amphetamine

To characterize the increased locomotor activity of munc18-1 gene-dose mutants, their response to d-amphetamine (AMPH) was studied. AMPH is a psychostimulant that is well known for its ability to increase random, non-directed locomotor activity. Paradoxically, in some animal models of hyperactivity and in patients with attention deficit hyperactivity disorder (ADHD), psychostimulants like AMPH are able to reduce symptoms of inattentiveness, hyperactivity and impulsivity (Solanto, 1998).

Mice were injected with saline, 1 and 4 mg AMPH per kg bodyweight. Injection of 4 mg/kg AMPH

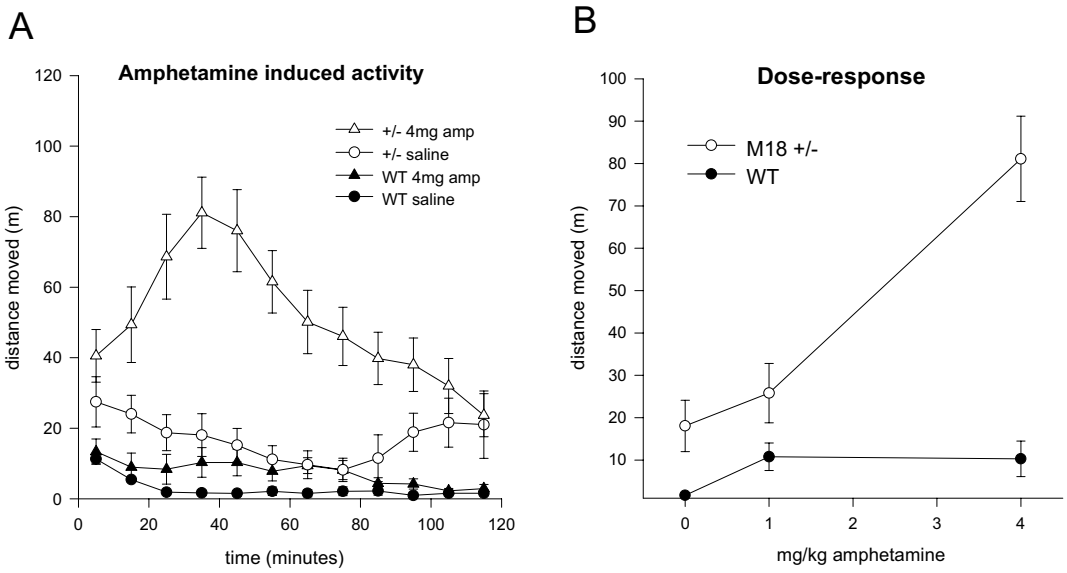

Figure 4. Response to amphetamine in wildtype (3 male and 3 female mice) and munc18-1 gene-dose mutants (3 male and 3 female mice). **A.** Response to 4 mg/kg amphetamine measured in 10min time bins. **B.** Dose response curve to 0, 1 and 4 mg/kg amphetamine. Y-axis shows moved distance in time-bin 4 (30-40min after injection). Significant effects: dose*genotype ($F_{1.74,13.9}$=16.6, p<0.001 (Huynh-Feldt corrected ε=0.87)), dose ($F_{1.74,13.9}$=22.7, p<0.001 (Huynh-Feldt corrected ε=0.87)) and genotype ($F_{1,8}$=26.5, p<0.005).

caused a strong increase of locomotor activity in gene-dose mutants and a relatively mild increase in wildtypes (Fig.4A). The dose-response relation (Fig.4B) shows that munc18-1 gene-dose mutants displayed a higher basal activity than wildtypes, which is moderately increased by 1 mg/kg AMPH but strongly by 4 mg/kg AMPH. Wildtypes showed relatively mild increases in activity when given 1 and 4 mg/kg AMPH. A significant interaction between dose and genotype was observed (p<0.001), indicating that munc18-1 gene-dose mutants respond stronger to AMPH than wildtypes. Thus, the hyperactive phenotype of munc18-1 gene-dose mutants is not alleviated by AMPH, but instead strongly increased, suggesting a dissimilarity with the mechanisms mediating hyperactivity in ADHD patients and certain animal models of hyperactivity (Solanto, 1998).

Discussion

Munc18-1 gene-dose mutants have a reduction of munc18-1 protein levels and display enhanced tetanic depression, absence of post-tetanic potentiation and a severe impairment of mossy fiber LTP. Despite all these changes no deficits in spatial learning and memory in the Morris water maze was detected. However, mutants did show severe deficits in the radial maze, a spatial working memory task. Furthermore, munc18-1 gene-dose mutants showed hyperactivity and responded strongly to d-amphetamine.

Munc18-1 gene-dose mutants performed normal in the Morris water maze. This task is widely used in learning and memory research and is sensitive to hippocampal lesions and disruptions of NMDA-receptor dependent LTP (Morris et al., 1982;Morris et al., 1986). Previously, other mutants with impairments in mossy fiber LTP were studied in this task. For instance, mGluR1 and AC1 null-mutants have impaired mossy fiber LTP and perform poorly in the water maze (Conquet et al., 1994;Wu et al., 1995;Villacres et al., 1998). However, mGluR1 mutants suffer from ataxia, which may have interfered with this task (Aiba et al., 1994;Conquet et al., 1994). Also, LTP in area CA1 is impaired in the AC1 mutant (Wu et al., 1995;Qi et al., 1996). Given the reported importance of CA1 LTP in spatial memory (Tsien et al., 1996), such impairments could also have caused deficits in this task. In contrast, rab3A null-mutants have disruption of both mossy fiber LTP and LTD and do not show impairments in spatial learning in the Morris maze (Castillo et al., 1997;Tzounopoulos et al., 1998)(chapter 5). Furthermore, mice that lack a catalytic subunit (Cβ1) or a regulatory subunit (RIβ) of PKA have no MF-LTP buts show normal Morris maze performance and normal contextual fear-conditioning (Huang et al., 1995). Our results are in agreement with the latter two studies and do not support a role for mossy fiber LTP in spatial learning and memory. Our results also show that post-tetanic potentiation (PTP) is not involved in spatial learning and memory in the Morris water maze. Previously, PTP has been implicated in spatial learning (Silva et al., 1996). αCaMKII gene-dose mutant mice show an enhancement of PTP and this mutant displays severe deficits in water maze learning (Silva et al., 1996). However, the results of the latter study appear to have been confounded by genetic background problems (Frankland et al., 2001). Thus, our results indicate that post-tetanic potentiation and mossy fiber LTP are not important for spatial learning in the Morris water maze.

Munc18-1 mice performed poorly in the eight-arm radial maze. Like the Morris water maze, performance in the radial maze is sensitive to hippocampal lesions (Olton, 1977;Olton et al., 1982). Furthermore, disruptions of NMDA-receptor dependent LTP impair performance in this working memory task (Danysz et al., 1988;Kawabe et al., 1998). However, impaired performance after NMDA-LTP blockade is only observed in a novel environment and not in a familiar one, provided that no lengthy within-trial intervals are imposed (Bolhuis and Reid, 1992;Shapiro and O'Connor, 1992). Thus, other mechanisms must be involved in the mediation of working memory *per se*. The munc18-1 mutant has impairments in tetanic depression, post-tetanic potentiation and mossy fiber LTP, which indicates that any of these different types of presynaptic plasticity may be involved in working memory. However, rab3A mutants lack mossy fiber LTP and show enhanced tetanic depression but perform normal in the eight-arm radial maze, suggesting that these types of plasticity are not involved in

working memory (Geppert et al., 1994)(chapter 5). As an alternative, post-tetanic potentiation may be involved in working memory (Churchland and Sejnowski, 1992). Like working memory, post-tetanic potentiation is short-lasting. Post-tetanic potentiation does not have properties of associativity like NMDA-receptor dependent LTP. If PTP is induced, it is likely to be induced in all synaptic connections of a neuron. However, this lack in specificity may not be necessary in working memory because the capacity of working memory is relatively low (Churchland and Sejnowski, 1992;Glassman, 1999). Furthermore, given the time frame of a few minutes before completing the radial maze task, it is hard to conceive of a mechanism for retention that does not contain some form of plasticity. Thus, post-tetanic potentiation may serve a function in working memory in the radial maze and this hypothesis is supported by our present findings.

A potential difficulty in interpreting our results is the fact that mutants showed increased activity in the radial maze. This was not observed in the Morris water maze, in which mutants performed normal. Hyperactivity is often associated with decreased attention and impulsive behavior and may result in poor cognitive performance (Solanto, 1998). Therefore, it may be suggested that the hyperactive phenotype of munc18-1 mutants interfered with radial maze performance but not with Morris water maze performance. On the other hand, variations in radial maze running speed in a variety of inbred strains do not correlate with radial maze performance, suggesting that higher locomotor activity does not affect radial maze performance to a large degree (Schwegler et al., 1990;Crusio et al., 1993;Ennaceur, 1994).

The hyperactive phenotype of munc18-1 gene-dose mutants was not alleviated by d-amphetamine, which has been reported to reduce activity in several animal models for hyperactivity (Solanto, 1998;Wilson, 2000;Zhuang et al., 2001). One of these models is the heterozygous coloboma mutant mouse. This mutant carries a chromosomal deletion of 1-2cM on chromosome 2, encompassing the gene for SNAP-25 (Wilson, 2000). This results in a reduction of SNAP-25 protein levels and also in reduced bodyweight and hyperactive locomotor behavior (Hess et al., 1996). The hyperactive phenotype of this mutant can be rescued by expression of a SNAP-25 transgene, which suggests a direct involvement of SNAP-25 (Steffensen et al., 1999). The function of SNAP-25 is related to munc18-1. Both proteins bind to syntaxin, and both are essential for neurotransmitter release (Montecucco and Schiavo, 1994;Sudhof, 1995;Verhage et al., 2000). In contrast to the munc18-1 gene-dose mutant, the coloboma mutant shows a reduction in locomotor activity after d-amphetamine injection, a response shared with ADHD patients (Hess et al., 1996;Solanto, 1998). Thus, although similar in many aspects, the coloboma and munc18-1 mutants differ markedly in amphetamine sensitivity, which suggest that different mechanisms are involved in the mediation of hyperactivity in these mutants.

In summary, mutant mice with impairments in tetanic depression, post-tetanic potentiation and mossy fiber LTP were analyzed in memory tasks. Spatial learning in the Morris water maze was not affected. Mutants showed poor performance in the radial maze, a working memory task. Furthermore, mutants showed hyperactive behavior that was not alleviated by d-amphetamine. Our results suggest a role for PTP in working memory, but interpretation is hampered by the additional hyperactive phenotype of the munc18-1 gene-dose mutant.

Chapter 4

Reduction in Munc18-1 expression leads to loss of atonia during REM sleep

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Abstract

REM sleep is associated with vivid dream mentation, desynchronous cortical EEG and paralysis of the muscles (atonia). A number of disorders of REM sleep exists, one of which is REM sleep behavior disorder (RBD). RBD is characterized by the intermittent loss of atonia during REM sleep. Here, we report that munc18-1 gene-dose mutant mice show a phenotype that resembles RBD. This mutant shows intrusions of abnormal motor activity during sleep. This behavior consists of tail twitches, limb movements, body stretching, body rocking, body shaking and jumps, after which the mice often wake up. EEG recordings showed that this abnormal motor activity coincided with cortical desynchrony that had a high power at 5-8Hz, which is a feature of REM sleep. No indications of epileptic discharges were observed. Thus, our results show that a reduction of munc18-1 protein levels leads to abnormal motor behavior during REM sleep, and suggests that this mutant may prove to be a genetic model for RBD.

Introduction

Rapid eye movement (REM) sleep, also known as paradoxal sleep, is associated with ocular saccades, vivid dreaming, desynchronous cortical EEG with a predominance of theta rhythm, and atonia (paralysis) of the postural muscles. A number of disorders of REM sleep exist, such as narcolepsy and REM sleep behavior disorder. A prominent aspect of narcolepsy is cataplexy, which is the intrusion of atonia during wakefulness. The study of narcolepsy, and of REM sleep in general, was greatly aided by genetic canine models (Foutz et al., 1979;Baker et al., 1982;Siegel et al., 1991), that turned out to have mutations in the hypocretin/orexin receptor gene (Lin et al., 1999). Soon afterwards, a deficiency of hypocretin/orexin was demonstrated in human narcoleptics (Nishino et al., 2000;Peyron et al., 2000). Apparently the opposite of cataplexy is REM sleep behavior disorder (RBD). In this disorder, all the components of REM sleep are normal except for atonia, which is frequently lost and results in complex motor behaviors that are often injurious and associated with dreaming (Schenck et al., 1986). The exact prevalence is unclear, but recent studies suggest it might not be an uncommon condition (Schenck et al., 1993;Olson et al., 2000).

Atonia appears to be regulated by the pontine-medullar system. Lesions in pontine areas or the medial medulla disrupts atonia during REM sleep (Jouvet, 1980;Schenkel and Siegel, 1989;Shouse and Siegel, 1992). Furthermore, a portion of pontine and medullar cells fire at a high rates during REM sleep (el Mansari et al., 1989;Kanamori et al., 1980;Steriade et al., 1990;Kayama et al., 1992;Schenkel and Siegel, 1989). Interestingly, in narcoleptic dogs, a number of medullar neurons increase their firing rate only during REM sleep and during cataplectic attacks, which specifically implicates these neurons in atonia but not in other aspects of REM sleep (Siegel et al., 1991). Finally, electrical stimulation of the medial medulla causes paralysis in awake cats (Hajnik et al., 2000), which appears mediated by direct inhibitory connections to motor neurons in the spinal cord (Morales et al., 1987;Fort et al., 1993). Thus, there appears to be a neuronal system in the brainstem that is activated during REM sleep and specifically involved in the maintenance of atonia.

In the present paper, we have studied sleep in munc18-1 gene-dose (heterozygous) mutant mice. Munc18-1 is present in all neurons, and has been implicated in release of neurotransmitters and hormones (Garcia et al., 1995;Verhage et al., 2000;Voets et al., 2001). Previously, it was demonstrated that gene-dose mutants show a decrease of munc18-1 protein levels throughout the brain. This decrease did not affect basal transmission and paired pulse facilitation but caused an enhancement of tetanic depression, a blockade of post-tetanic potentiation and a severe impairment of mossy fiber LTP. Thus, it appears that a reduction of munc18-1 leads to impairments in secretion specifically during and after high neuronal activity (Chapter 2). Furthermore, this mutant showed hyperactivity in an open field task and deficits in a working memory task (Chapter 3).The present study was initiated to analyze the behavior of this mutant under conditions of rest in the home-cage. We report that munc18-1 gene-dose mutant mice did show higher behavioral activity in the home cage. Furthermore, when they appeared to be asleep, mutants displayed short episodes of abnormal motor activity, which appeared to coincide with REM sleep.

Material & Methods

Mice

Mutants were created by deleting the second exon of the munc18-1 gene by homologous recombination (Verhage et al., 2000). Subsequently, mutants were repeatedly backcrossed to 129S3 (also known as 129/SvImJ; Jax code: JR2448) for at least 6 generations, resulting in a line with a standardized genetic background. Mice were bred in our laboratory under standard conditions. At 25 days, they were weaned and housed with 2-4 mice of the same sex. Mice were kept at a 12h-light/dark cycle with lights on at 7:00 PM. Food (Hope Farm) and water was freely available. Heterozygotes were used for the experiments. Wildtype littermates served as controls. The experimenter was always blind with respect to genotype until the end of the experiments. Mice were genotyped by PCR, which was occasionally confirmed by western blot. All experiments were approved by the Ethical Committee of the Utrecht Medical Center.

Behavioral recordings

At the age of 3 months, mice were individually housed and allowed to habituate to this situation for 24 hours. The next day, during the fifth hour of the light cycle (from 0:00 to 1:00 A.M.) video recordings were made, which were analyzed afterwards. Two behavioral categories were used. The category inactive behavior was used to represent quiet rest and sleeping, which were defined as the lack of body movements. The category active behavior was used to measure active wakefulness and consisted of walking, digging, rearing, climbing, scanning, eating, drinking and grooming. Also, the number of motor bursts was counted. These motor bursts consisted of uncoordinated jumps and vigorous body shakes.

Detailed video-recordings were made from the mice that were used for the EEG recordings. These recordings were used to analyze episodes of abnormal motor activity during sleep in more detail. The categories were inactivity, tail movements, chewing, stretching, walking, body rocking (slow rocking motion of the whole body), body shaking (vigorous body movements) and jumps.

Electroencephalogram (EEG) analysis

2 wildtypes and 2 heterozygous mutants were implanted with cortical electrodes. Mice were anaesthetized with a Hypnorm (Janssen Pharmaceutica Inc., Beers, Belgium), Dormicum® (Roche Netherlands Inc., Mijdrecht, The Netherlands) and demiwater mixture (1:1:2), at a dose of 2-3µl per gram bodyweight, injected subcutaneously. After surgery, post-operative care was given with a subcutaneous injection of Temgesic® (Reckitt Benckiser plc, Berkshire, United Kingdom) at a dose of 1µl / gram bodyweight and an injection of saline (20µl/gr bodyweight). The electrodes were placed on the on the surface of the cortex: one in the frontal region (coordinates with the skull surface and bregma zero-zero (A 1.5, L –2.0), and the other in the parietal region (A –3.5, L –3.0) (Franklin and Paxinos, 1997). The ground electrode was placed over the cerebellum. After surgery, the animals were housed separately and were allowed to recover for at least 2 weeks.

EEG recordings were made for 12 hours, beginning at the start of the light-phase. Mice were put in a transparent recording cage and connected to EEG leads. The EEG (in a bandwidth between 1 and 100Hz, sample frequency of 256Hz) was amplified, digitized, monitored and stored for off-line analysis

using the WINDAQ system (DATAQ Instruments, Akron, OH). Simultaneously, continuous video recordings were made. EEG traces were analyzed using Fast Fourier Transformatio n (FFT).

Statistics

The behavioral data was analyzed using univariate ANOVA for parametric data, and Kruskall Wallis and Mann Whitney U for non-parametric data.

Results

Mutants show higher levels of behavioral activity and abnormal motor bursts.

Previously, it was observed that munc18-1 gene-dose mutants showed hyperactive behavior in the open-field task (Chapter 3). The present study was started to determine whether this hyperactive behavior persisted in the home-cage. Munc18-1 gene-dose mutant mice were monitored in the home-cage for a period of 60min during the fifth hour of the light phase. Munc18-1 gene-dose mutants showed a five-fold increase in active behavior ($p<0.001$)(Table1). Surprisingly, heterozygotes showed occasional bursts of motor activity that consisted of jumps and vigorous body shakes. These bouts of abnormal activity occurred in all munc18-1 gene-dose mutants (ranging from 1 to 9 bursts), but were

Table 1. Behavior during the light phase in wildtypes (n = 5 males and 6 females) and munc18-1 gene-dose mutants (n = 3 males and 5 females). Shown are means and SEM. Mutants showed more active behavior then wildtypes ($F_{1, 15} = 25.2$, $p<0.001$). No other effects on active behavior were detected (sex: $F_{1, 15} = 0.05$, n.s., sex*genotype: $F_{1, 15} = 0.61$, n.s.). Heterozygotes showed occasional bursts of motor, which was never observed in wildtypes ($Z=-4.05$, $p<0.001$).

	wildtype	munc18-1	statistics
active behavior (min per hour)	3.4 ± 1.2	17.8 ± 2.6	$p < 0.001$
motor bursts (number per hour)	0	4.4 ± 1.0	$p < 0.001$

never observed in wildtype mice ($p<0.001$). No differences were observed between males and females. Motor bursts are preceded by periods of u ncoordinated motor activity.

The motor bursts were further analyzed using a new set of video recordings of individual mice that allowed a more detailed analysis. During the whole recording period, wildtype mice never showed conspicuous motor activity. Again, gene-dose mutants showed motor bursts, and these motor bursts were usually preceded by short episodes of motor activity that was of a less vigorous nature. Behavior during these episodes had an uncoordinated and undirected appearance, and consisted of raising and twitching of the tail, chewing, body stretching, short walks, body rocking, and were often concluded by body shakes (28%) or jumps (41%) through the cage (Fig.1). On occasion, abnormal grooming behavior was observed. During this abnormal groomi ng the paws did not touch the face. Prior to these episodes of abnormal behavior, mice always had their eyes closed, were completely inactive and seemingly asleep. Also, during the episodes of abnormal motor behavior they kept their eyes closed

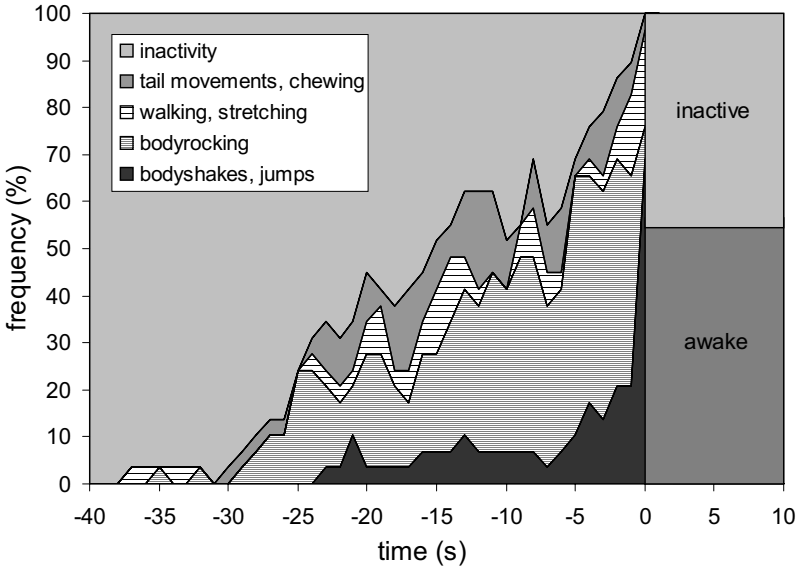

Figure 1. Description of abnormal motor behavior. 29 of these episodes from videorecordings of 2 male gene-dose mutants were collected and analyzed. These episodes were aligned at the moment these episodes of abnormal behavior ended (at t=0). Behavior was classified in a mutually exclusive fashion in the categories inactivity, tail movements and chewing, walking and stretching, body rocking, and finally vigorous bodyshakes and jumps. The graph shows that the latter categories were present to a greater extent towards the end. When these episodes ended, mice became inactive and seemingly asleep in 45% of the episodes. In 55% of the episodes they appeared to have been awakened.

and these episodes were often interrupted by short periods of complete inactivity. Thus, this abnormal motor activity appeared to occur during sleep. Jumps almost always caused awakening (92%), the other classes of behavior were usually followed by inactivity (41% resulted in awakening). In general these episodes of abnormal behavior caused awakening in approximately half (55%) of the cases, but in a large percentage of cases (45%) mice resumed inactive behavior with closed eyes. Thus, these behavioral observations suggest that this abnormal motor behavior occurred during sleep. This hypothesis was further explored using EEG recordings.

Motor activity occurred during theta rhythm

In order to determine if the motor acts occurred during sleep, and if so, to determine the phase of sleep, mice were prepared for EEG analysis. The mice were videotaped for 12 hours and continuous EEG measurements were made. During inactivity, wildtype mice showed periods of large, irregular waves and periods of small, regular waves, which are characteristics of slow-wave sleep and REM sleep, respectively (Hobson et al., 1998). Gene-dose mutants also showed episodes of inactivity with synchronous or desynchronous EEG but, as observed before, these periods of inactivity were frequently interrupted by episodes of abnormal motor activity. Corresponding EEG traces were collected and revealed that these episodes of abnormal motor activity coincided with cortical desynchrony. Furthermore, after Fast Fourier Transformation (FFT) the traces showed a predominant theta rhythm of 5-8Hz (Fig.2). As mentioned, theta rhythm is a feature of REM sleep and these results indicate that the gene-dose mutant mice show REM sleep without atonia. Representative examples of individual EEG traces and corresponding FFT of heterozygous mutants are shown in Fig.3. The first and second traces were collected when mice were inactive and had their eyes closed. Fig.3A shows an example of synchronous waves. Note the large, irregular waves and the predominance of a 1-4 Hz

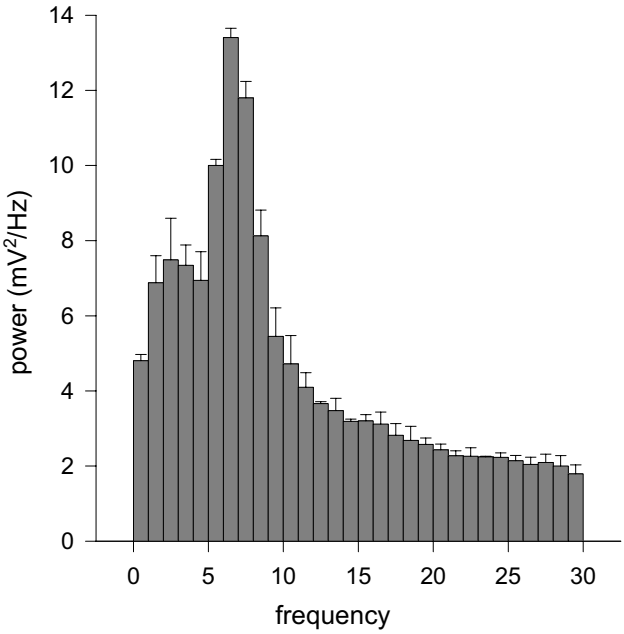

Figure 2. Fast Fourier Transformation of EEG traces that coincided with the episodes of abnormal motor behavior. 10 episodes of abnormal behavior that lasted at least 10s were collected from each mouse. FFT was applied and the 10 episodes were averaged for each mouse. Shown are means ± SEM (n = 2 males).

rhythm in the FFT figure. These are characteristics of slow-wave sleep (Hobson et al., 1998). Behavioral abnormalities never coincided with such EEG traces indicating that mutants had normal slow-wave sleep. Fig.3B shows an example of desynchronous EEG. The FFT shows a predominance of theta (5-8 Hz) rhythm. The combination of behavioral inactivity, closed eyes, and predominant theta rhythm suggests that mutants do show episodes of normal REM sleep. Furthermore, these periods of normal REM sleep appeared to outnumber the episodes with abnormal motor activity. Fig.3C shows a trace of sleep with motor activity, which in this case consisted of chewing and vigorous body shakes. Note the resemblance with Fig.3B, and the predominance of theta rhythm. Furthermore, sharp wave discharges such as those typical for epileptic attacks (Montagna et al., 1997) were not present. Fig.3D shows a trace taken from an episode of body shakes that was concluded with a violent jump. Again, this trace shows cortical desynchrony with a predominance of theta rhythm. The blow-up shows that during, and immediately before the jump, erratic waves were not present. Sharp wave discharges were never observed throughout the recording period. Thus, the abnormal motor behavior in munc18-1 gene-dose mutants was unrelated to epileptic attacks.

Discussion

Munc18-1 gene-dose mutants displayed episodes of abnormal motor activity that intruded on periods of rest. All mutants showed it, but none of the wildtypes (approximately 40 hours of behavioral recordings of wildtypes were made), and males and females were affected to a similar degree. Furthermore, this phenotype was observed in young adults of 3 months of age. Thus, this phenotype displays an autosomal dominant inheritance pattern that has full penetrance at an early age. EEG

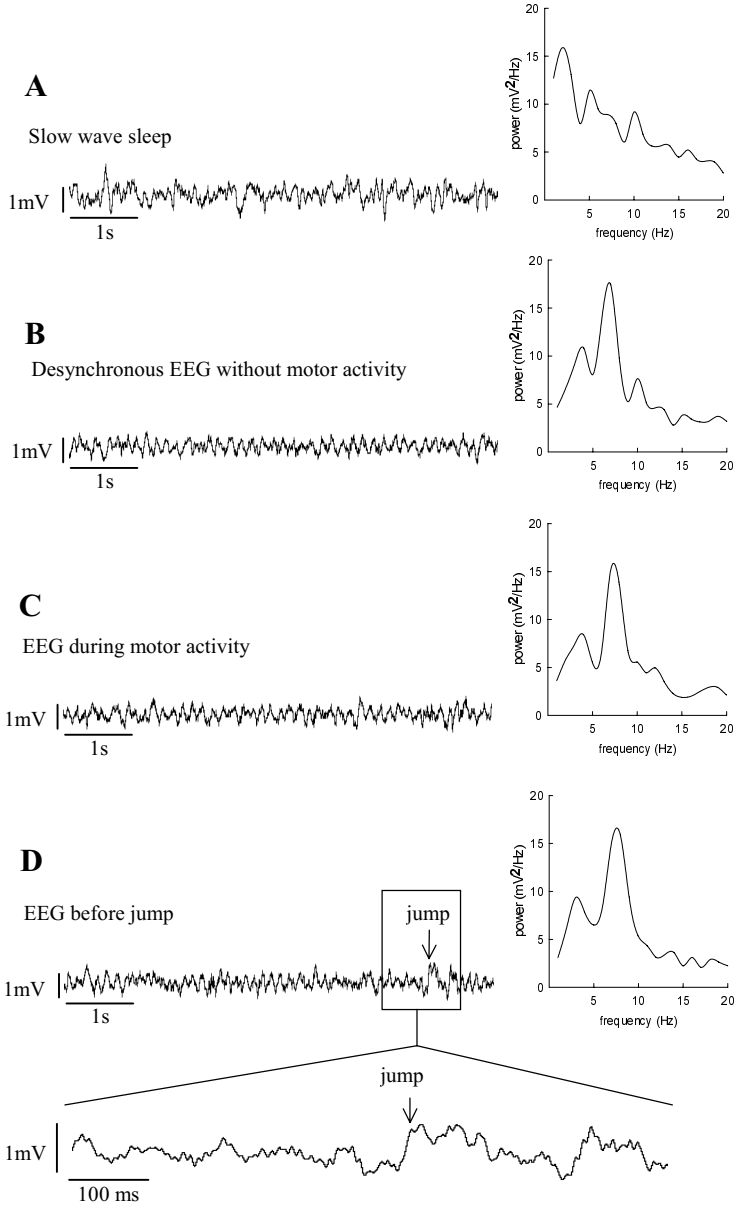

Figure 3. Individual EEG traces with FFT. **A.** example of synchronous EEG during a period of inactivity. The EEG trace on the left shows large, irregular waves and FFT on the right shows a predominance of delta waves (1-4 Hz). **B.** example of desynchronous EEG during behavioral inactivity. The FFT shows a predominance of theta waves (5-8 Hz). **C**. example of abnormal motor activity without a jump. The FFT shows cortical desynchrony and a predominance of theta rhythm. **D.** example of an episode of bodyshakes that was concluded with a violent jump. Again, this trace shows cortical desynchrony with a predominance of theta rhythm. The blow-up shows the trace immediately before and after the jump.

measurements showed that the abnormal motor activity coincided with cortical desynchrony with a predominance of theta rhythm. No signs of epileptic attacks were observed. Thus, the munc18-1 gene-dose mutant appears to have abnormal motor activity during REM sleep.

During REM sleep the brain is highly activated and a suppression of motor output is necessary to prevent uncoordinated motor activity. This suppression seems to be mediated by a specialized system in the pontine-medullar region (Jouvet, 1980;Schenkel and Siegel, 1989;Shouse and Siegel, 1992). A proportion of medullar neurons fire at high discharge rates only during atonia (Siegel et al., 1991) and it appears that these neurons suppress motor activity by direct glycinergic inhibitory connections to motor neurons in the spinal cord (Morales et al., 1987;Fort et al., 1993). Munc18-1 gene-dose mutant mice show reductions in munc18-1 protein levels in the brainstem (Chapter 2). These mutants had normal slow-wave sleep, indicating that their phenotype was specific for REM sleep. Also, REM sleep

was often normal, indicating that the mechanisms that enable atonia were still present. However, longer periods of REM sleep require longer periods of atonia, and consequently longer periods of high neuronal activity (Siegel et al., 1991). Thus, the appearance of abnormal motor activity during REM sleep may be caused by impairments in maintaining secretion during high neuronal activity resulting in the gradual loss of motor inhibition during REM sleep.

REM sleep behavior disorder (RBD) is characterized by a frequent loss of atonia during REM sleep, and results in complex motor behaviors that are often injurious and associated with dreaming (Schenck et al., 1986). The munc18-1 gene-dose mutant displays a phenotype that resembles RBD and may prove to be a valuable genetic model for this disorder. The phenotype that we have described is fully penetrable in both males and females, and already present at an early age. This, however, is not the case in human patients with RBD. Onset of this disorder is at approximately 60 years of age, there is a strong male preponderance and familial cases have not been observed (Schenck et al., 1993;Olson et al., 2000). Thus, there is no sign of a dominant inheritance pattern as that observed in the gene-dose mutant mice.

In summary, a reduction of munc18-1 in munc18-1 gene-dose mutants leads to abnormal motor activity during REM sleep. This phenotype may have been caused by the loss of atonia as a result of impairments in secretion during high neuronal activity. The munc18-1 gene-dose mutant may prove to be a mouse model for RBD on the physiological level. Further research is required to unravel more of the molecular mechanisms that regulate atonia during REM sleep.

Chapter 5

Spatial, contextual and working memory are not affected by the absence of mossy fiber long-term potentiation and depression

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Abstract

The mossy fibers of the hippocampus display NMDA-receptor independent long-term plasticity. A number of studies addressed the role of mossy fiber long-term plasticity in memory, but have provided contrasting results. Here, we have exploited a genetic model, the rab3A null-mutant, which is characterized by the absence of both mossy fiber long-term potentiation and long-term depression. This mutant was backcrossed to 129S3/SvImJ and C57Bl/6 to obtain standardized genetic backgrounds. Spatial working memory, assessed in the eight-arm radial maze, was unchanged in rab3A null-mutants. Moreover, one-trial cued and contextual fear conditioning was normal. Long-term spatial memory was tested in the Morris water maze. Two different versions of this task were used, an "easy" version and a "difficult" one. On both versions, no differences in search time and quadrant preferences were observed. Thus, despite the elimination of mossy fiber long-term plasticity, these tests revealed no impairments in mnemonic capabilities. We conclude that spatial, contextual and working memory do not depend on mossy fiber plasticity.

Introduction

Granule cells of the dentate gyrus are a principal relay station of the hippocampus, receiving excitatory input from the enthorhinal cortex and projecting, by means of the mossy fibers, to area CA3. A number of findings have implicated granule cells in memory. For instance, granule cell lesions impair spatial memory (Walsh et al., 1986;Czeh et al., 1998), and the acquisition of spatial information is blocked by a reversible inactivation of mossy fiber neurotransmission (Lassalle et al., 2000). In addition, the size of the mossy fiber projection is positively correlated with performance in the eight-arm radial maze (Crusio et al., 1987). Finally, new granule cells are continuously generated in adults through neurogenesis, and not only are these new neurons important in trace-conditioning (Shors et al., 2001), but neurogenesis itself is enhanced by training in this hippocampus dependent learning task (Gould et al., 1999).

Much research has focused on the involvement of long-term potentiation (LTP) and long-term depression (LTD) in learning and memory. LTP and LTD are rapid and persistent changes in synaptic strength. NMDA-receptor dependent LTP and LTD are particularly well studied. A blockade of NMDA-receptor dependent plasticity by pharmacological (Morris et al., 1986) or genetic (Tsien et al., 1996) means impairs learning in the Morris water-maze and suggest an essential role for this type of synaptic plasticity in memory. Mossy fibers also display LTP (MF-LTP) and LTD (MF-LTD) but they are rather unusual. MF-LTP and MF-LTD are independent of the NMDA-receptor and expressed by changes in neurotransmitter release (Yamamoto et al., 1980;Zalutsky and Nicoll, 1990;Harris and Cotman, 1986;Staubli et al., 1990;Weisskopf and Nicoll, 1995;Kobayashi et al., 1996;Derrick and Martinez, Jr., 1996).

If MF-LTP and MF-LTD have a role in memory then changes in synaptic strength should occur during learning. Such measurements were done in rats. It was shown that CA3 field potentials, evoked by mossy fiber stimulation, increased in amplitude over the course of a few days of radial maze learning (Mitsuno et al., 1994;Ishihara et al., 1997). A number of genetic studies addressed the role of mossy fiber long-term plasticity in memory, but the results were not conclusive. Mice lacking type 1 metabotropic glutamate receptors (mGluR1) or type 1 adenylyl cyclase (AC1) have deficits in MF-LTP and are impaired in the Morris water maze (Conquet et al., 1994;Wu et al., 1995;Villacres et al., 1998). Other genetic models showed different results. Mice that lack the catalytic subunit Cβ1 or the regulatory subunit R1β of PKA also have no MF-LTP, but show normal Morris maze performance and normal contextual fear-conditioning (Huang et al., 1995). Furthermore, the mGluR2 null-mutant has normal MF-LTP but impaired MF-LTD and shows no deficits in the Morris maze (Yokoi et al., 1996).

Resolving this apparent contradiction is problematic for a number of reasons. Firstly, the genetic background of the mice in the studies was poorly defined. This will result in different genetic backgrounds of mutant and wildtype mice (Gerlai, 1996), and may have contributed to the apparently contrasting results. Secondly, the presence of MF-LTD was not tested in the mGluR1, AC1, Cβ1 and R1β mutants. It has been suggested that mossy fiber plasticity may be involved in reducing signal-to-noise ratios in granule cell signaling (Treves and Rolls, 1994). This might be achieved, at least to a certain degree, by using only MF-LTD and may be adequate to allow relatively normal hippocampal

functioning and normal cognitive performance. Thirdly, the mGluR1 mutant was also ataxic (Conquet et al., 1994), and the AC1 mutant also had impairments in NMDA-LTP in area CA1 (Wu et al., 1995). These deficiencies may have influenced performance in the learning tasks. Thus, the role of mossy fiber plasticity in learning and memory is unclear and new experiments are needed for a better understanding of this relationship.

In the present study, we analyzed a mutant that lacks both MF-LTP and MF-LTD, the rab3A null-mutant (Geppert et al., 1994). Rab3A is a neuron-specific protein, enriched in nerve terminals and implicated in the regulation of vesicular secretion (Sudhof, 1997). Rab3A deletion in mice does not affect basal transmission or short-term plasticity in the mossy fiber projection but eliminates both MF-LTP and MF-LTD (Castillo et al., 1997;Tzounopoulos et al., 1998). As far as we know, this is the only model with a simultaneous blockade of MF-LTP and MF-LTD. Mutants were tested in a variety of hippocampus-dependent learning tasks in order to measure different aspects of memory. Spatial working memory was tested in the eight-arm radial maze. Long-term spatial memory and spatial reversal learning were tested in the Morris water-maze. Contextual memory was tested in the fear-conditioning task. We report that rab3A null-mutants, although lacking mossy fiber long-term plasticity, show normal performance in all these tasks.

Materials and methods

Mice

Mutants were created by deleting the promoter and first two exons of the rab3A gene by homologous recombination (Geppert et al., 1994). Subsequently, mutants were repeatedly backcrossed to 129S3 (also known as 129/SvImJ; Jax code: JR2448) for at least 4 generations, resulting in a line with a standardized genetic background. A second line was obtained by backcrossing to C57Bl/6 for 4 generations. Mice were bred in our laboratory under standard conditions. At 25 days, they were weaned and housed with 2-4 mice of the same sex. Mice were kept at a 12h-light/dark cycle with lights on at 7:00 PM. Food (Hope Farm) and water were freely available. Null mutants were obtained by crossing heterozygotes. Wildtype littermates served as controls. The experimenter was always blind with respect to genotype until the end of the experiments. Mice were genotyped by PCR, which was occasionally confirmed by western blot. In all experiments, except for the Morris water maze, the backcross to 129S3 was used. The 129S3 strain is unsuitable for the Morris water maze because of its propensity to float (Wolfer et al., 1997). As recommended (Banbury Conference Report, 1997) we used the F1 between the 129S3 backcross and C57Bl/6 backcross. All experiments were approved by the Ethical Committee of the Utrecht Medical Center.

Electrophysiology

Transversal, 400μm-thick slices were prepared and kept in oxygenated (95% O_2, 5% CO_2) medium at room temperature for at least 60min, before being used. Medium composition in distilled H_2O (mM): 124.0 NaCl, 3.3 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 10.0 glucose, 20.0 $NaHCO_3$ and 2.5 $CaCl_2$. Recordings were done at 30°C. Bipolar stainless-steel stimulation electrodes (tip electrode diameter of 50μm),

insulated except for the tip, were placed at the afferent mossy fibers. Field excitatory postsynaptic potentials (fEPSPs), determined from the linear part of the trace, were recorded in the stratum radiatum of area CA3 with glass microelectrodes (tip diameter approximately 2μm). fEPSPs were quantified by determining the slope of the trace. The stimulus intensity was adjusted to evoke fEPSPs of half-maximum amplitude (at least 0.125mV), and kept constant thereafter. Stimulation frequency was 0.05Hz. After 15min of baseline recording, MF-LTP was induced with high-frequency stimulation (200Hz) for 1s, after which the responses were recorded for 60min.

Open field

The open field was a circular, moderately illuminated (80lux) arena constructed of gray PVC (diameter 78cm, walls 30cm high). The open field was divided in two parts, a circular central area with a diameter of 55cm and an edge area. Mice were placed in the edge area and allowed to freely explore the open field for the duration of 30min. The location of the mouse was recorded every 0.5s using Ethovision (Noldus technology, The Netherlands).

Eight-arm radial maze

The radial maze was made of transparent Plexiglas. All arms (23cm long, 6cm wide, 5cm high) were baited with 20mg food rewards (Noyes precision food pellets, P.J. Noyes Company Inc., Lancaster, New Hampshire, United States), which were placed behind a small barrier that prevented visual detection. At the end of each arm, food pellets were deposited behind a perforated wall. These pellets were not retrievable by the mice but supplied the arm with food odors that prevented detection of the real food rewards by olfaction. We followed the protocol of others (Schwegler et al., 1990). Twenty-four hours before training the animals were food, but not water, deprived. During training, they were kept at 85–90% of their pre-test bodyweight. Mice received 1 trial per day. The first day, they received a 10min habituation-trial in which they were allowed to freely explore the maze and gather 16 scattered food rewards. During the following four trials, only the ends of the arms were baited. Between successive arm visits, mice were confined in the central area for 5s by lowering guillotine doors located at the entrance of each arm. This procedure is known to disrupt chaining responses and kinesthetic strategies (Schwegler et al., 1990). Trials ended when all baits had been collected or when 15min had past, whichever came first. During later trials, all mice routinely took all baits within 15min. An error was defined as an entry with all four paws in a previously visited arm.

Morris water maze

The Morris maze was a circular white pool (diameter 132cm), filled with water (25°C) that was made opaque by adding small quantities of the white pigment Acoat X (Akzo Nobel, Sassenheim, The Netherlands) at a dilution of approximately 1:10.000. The platform was circular (diameter 15cm or 8cm) and made of perforated Plexiglas that provided the mice with extra grip. The platform was placed 1cm below the surface of the water in the center of a quadrant. To avoid visual orientation prior to release, the mice were transported in a white bucket from which they glided in the water towards the wall of the pool. Starting positions changed every trial in a pseudo-random fashion. A trial ended when a mouse was 10s on the platform or when 120s had passed. Mice that failed to find the platform within 120 s were lured to the platform with a wire mesh shovel, so that they climbed the platform by themselves and were left there for 10s. The shovel was also used as a means to retrieve the mice

from the platform in a stress-free manner. Their location was sampled every 0.1s using Ethovision (Noldus technology, The Netherlands). The first experiment used the 15cm platform. In this version of the task, mice received six trials a day with a 45-60 min interval between trials. At the start of the 4th day, the platform was transferred to the opposite quadrant. During the first 30s of trial 1 at day 4 preference for the old quadrant was determined (transfer test); mice that found the relocated platform in less then 30s were excluded from the analysis. At the end of the 5th day of training, the platform was removed and a probe test of 30s was given (7th trial).

In the second version of the Morris water maze, an 8cm platform was used. Mice received four training trials per day with an inter-trial interval of 30min, except for days 6 and 8 were they received three training trials. Probe tests, in which the platform was removed, were conducted 30min prior to training on day 1, 30min after training on day four, and 30min prior to training on days 6 and 8. Probe trials lasted 60s. The mice in the last study were used as a control group in a pharmacological experiment and received subcutaneous injections of saline (volume 10-15µl) 60min prior to training.

Contextual and cued fear conditioning

A transparent Plexiglas shock chamber (24*24 cm) with a stainless-steel rod floor (1 rod per 9mm, diameter rod 4mm) was used for training. The training protocol was as described by others (Bourtchuladze et al., 1994). Mice were placed in the chamber and allowed to habituate for 2 min before onset of the conditioned stimulus (CS: 30s, 85dB tone). During the last two seconds of the CS, the unconditioned stimulus (US: 0.75mA scrambled foot shock) was applied. After another 30s, the mice were returned to their home cage. The next day, mice were tested for contextual and cued fear conditioning. Mice were placed in the shock chamber (without CS) for a period of 5min in order to determine contextual fear conditioning. After a 2h interval, cued fear conditioning was assessed. Mice were placed in a novel cage for a period of 6min; during the last 3min, the tone CS was continuously applied. The strength of conditioning was determined by measuring the time spent freezing, which was defined as immobility with only an occasional head movement.

Statistics

The data were analyzed by t-tests for independent samples, and univariate and repeated measures of ANOVA. When the sphericity assumption was not met, Huynh-Feldt correction was applied.

Results

Rab3A mutant pups develop sensory motor skills normally, but have a lower bodyweight

All genotypes were represented according to a Mendelian distribution (wt:+/-: ko = 51:106:50). Null-mutants had a slightly lower bodyweight at weaning (at 21 days (mean±sem): mutants 9.1±0.3gr (n=11), wildtypes 10.1±0.4gr (n=12), $t(df=21)=1.90$, $p<0.01$). Mutants and wildtypes acquired sensomotoric skills at the same age (fore- and hind limb placing response, fore- and hind limb grasping response, stopping of pivoting, cliff drop aversion, straight-line walking, righting reflex, opening of the eyelids (data not shown)). This indicated that sensomotoric development was similar and that the reduced bodyweight did not represent a slower development of the rab3A mutants. In adult mice, bodyweight was not different (data not shown).

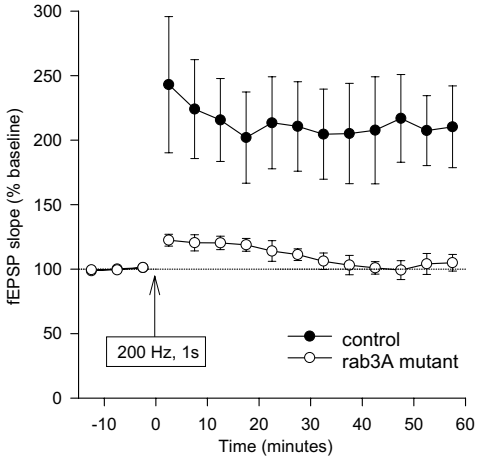

Figure 1. Changes in field EPSPs after tetanization (200Hz, 1s) of the mossy fiber projection. Mutant slices (n = 4) did not show potentiation, whereas control slices (n = 4) showed long-lasting increases in field EPSPs (average responses 30-60 min after tetanization (mean±sem): controls (heterozygotes) 209%±36, mutants 103%±5, t(df=6)=3.02, p<0.02).

Mossy fiber LTP is absent

The rab3A mutant was backcrossed to 129S3, resulting in a different genetic background than used in earlier studies. Therefore, MF-LTP was tested in these genetically standardized mice (Fig.1). Wildtype mice showed robust MF-LTP while Rab3A mutants failed (p<0.02). Thus, we confirmed earlier findings that showed the absence of MF-LTP in the rab3A mutant (Castillo et al., 1997).

Mutants lacking mossy fiber plasticity spend less time in the central area of the open field The open field test was used to study the mutant's response to a novel environment. The total test period was divided in three intervals. Mutants and wildtypes did not differ in traveled distances in the open field, and both groups showed a decline in traveled distance (Fig.2A). The time that mice spent in the

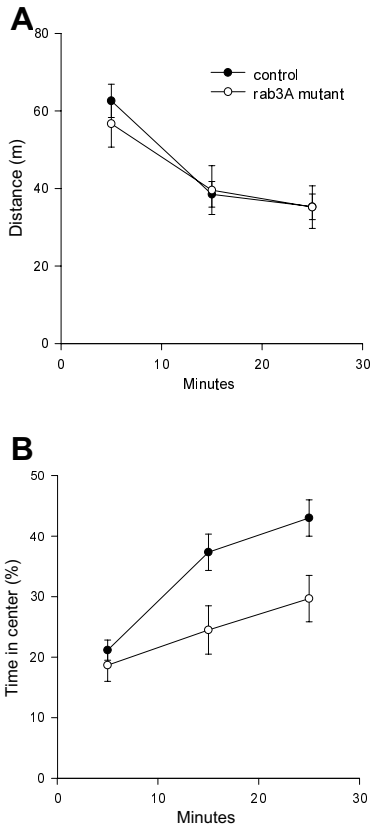

Figure 2. Open field behavior of wildtype (10 males) and mutant (10 males) mice. **A.** Traveled distance in 10min bins. Mutants and wildtypes did not differ (time*genotype: $F_{1.5,\ 27.1}$=1.63, n.s. (Huynh-Feldt corrected ε=0.75)), but both groups showed a decline in traveled distance over time (time: $F_{1.5,27.1}$=79.4, p<0.001, (Huynh-Feldt corrected ε=0.75)). **B.** Time in center in 10min bins. Wildtypes showed a stronger preference for the center at later intervals (genotype*time: $F_{2,36}$=5.41, p<0.01).

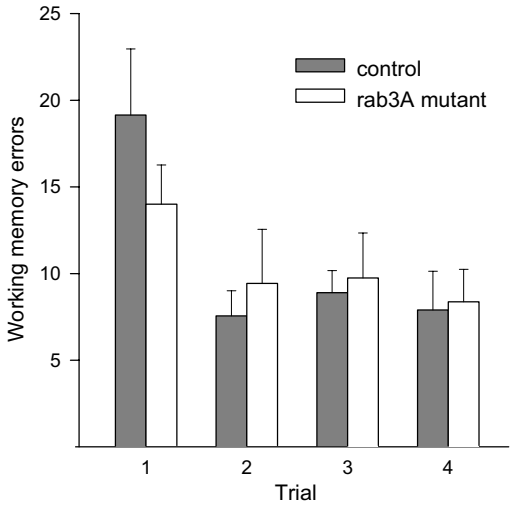

Figure 3. Radial maze performance of wildtype (10 males) and mutant (8 males) mice. The last four trials are shown. Mutants and wildtypes did not differ. Working memory errors in trial one: wildtypes 19.1±3.8, mutants 14.0±2.3, t(df=12)=1.16, n.s. Working memory errors at the last trial: wildtypes 7.9±2.2, mutants 8.4±1.9, t(df=16)=0.16, n.s.

central area increased at later intervals, but this was less in rab3A mutants ($p<0.01$) (Fig.2B). Thus, mutants showed a different response during habituation to a novel environment.

Working memory in the radial maze was not changed by the absence of mossy fiber plasticity

As a first aspect of memory formation we tested spatial working memory in the eight-arm radial maze. In this maze the ability to remember previously visited arms within the same trial is tested. The two groups showed a similar decline in working memory errors on consecutive trials (Fig.3). At the first day of training, wildtypes tended to make more working memory errors but this difference was not significant. Performance reached a plateau at the second trial in both groups and no further changes were detected on the third and fourth trial. Thus, mutants do not show impairments in this task indicating that MF-LTP and MF-LTD are not involved in working memory.

Spatial memory in the Morris water maze performance was not changed by the absence of mossy fiber plasticity

As a second aspect of memory formation we tested long-term memory for a specific spatial location using the Morris water maze. Two experiments were performed. In the first experiment a platform with a diameter of 15cm was used, and mice received 6 trials per day. During the first three days of training, the two groups showed a similar, rapid decline in search time (Fig.4A). Quadrant preference was determined during the first trial of day 4 (transfer test: Fig.4B). Controls and rab3A mutants showed a similar preference for the quadrant where the hidden platform used to be. Transfer of the platform resulted in increased search time on day 4, which was similar for both groups. At day 5, search time declined again. At the end of the fifth day, the platform was removed and quadrant preference was tested (probe test: Fig.4B). No differences were detected between controls and rab3A mutants. Furthermore, swimming speeds were not different between groups [during probe test at day 5: wildtypes 19±1 cm/s, mutants 21±1 cm/s, t(df=14)=1.6, n.s.]. In a second version of the Morris maze, task difficulty was increased by decreasing the platform size to 8cm and reducing the number of training trials to 4 per day. This version proved to be more difficult as evidenced by the longer search times (Fig.5A). Also in this difficult version of the Morris water maze, mutants performed comparably to

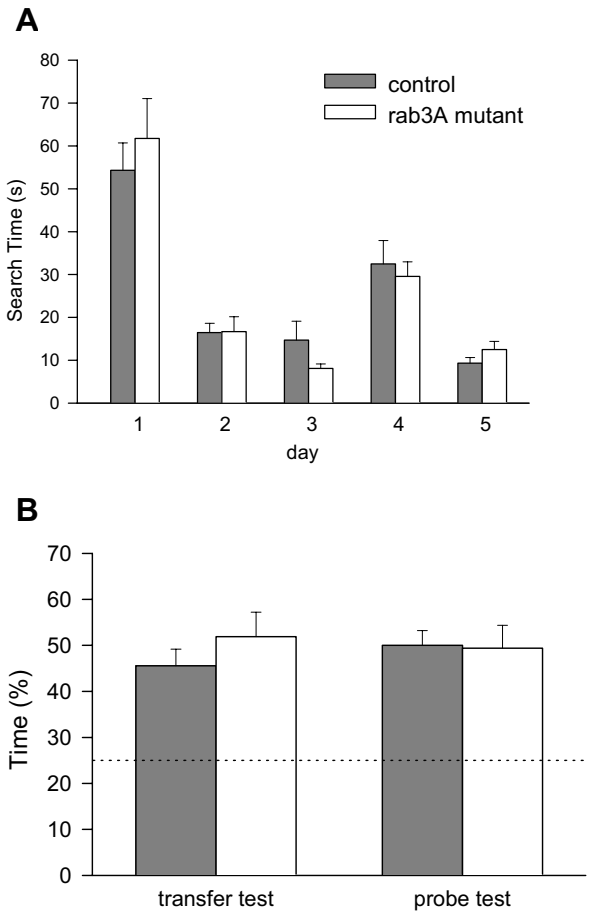

Figure 4. Morris maze "easy" version. Performance of control (wildtypes (4 males, 5 females) heterozygotes(3 males, 3 females) and null-mutant (3 males, 4 females) mice. **A.** Search time to find the hidden platform. Shown is the averaged search time of six daily trials. No main effects of sex or genotype nor interactions were observed (day*genotype: $F_{7.5, 59.6}$=0.67, n.s. (Huynh-Feldt corrected ε=0.93)). Heterozygotes and wildtypes were pooled, as were male and females. **B.** Search time (in percentage of total time) in the quadrant were the hidden platform used to be. No main effects or interactions were detected.

controls both in search times and quadrant preferences (Fig.5B). Thus, rab3A mutants performed normal in two versions of the Morris water maze, indicating that MF-LTP and MF-LTD are not involved in spatial learning and memory.

Contextual and cued fear conditioning was not changed by the absence of mossy fiber plasticity

As a third aspect of memory formation we tested contextual and cued fear conditioning using the association of a specific location (context) or a specific sound (cue) and an aversive foot-shock. It is believed that the strength of this association can be determined by measuring freezing behavior. The mice did not freeze during habituation in the first 2min or during the first presentation of tone CS (Fig.6A). This indicates that both the shock chamber and the tone CS had no *a priori* aversive properties. After the foot-shock was applied, both groups showed freezing responses. Contextual fear conditioning was assessed 24h later. During re-exposure to the conditioning chamber (Fig.6B) both groups showed similar amounts of freezing. Two hours later the mice were tested for cued fear conditioning (Fig.6C). When placed in the novel cage, the mice did not freeze, indicating that neither handling of the mice nor exposure to a novel cage elicited fear responses. During application of the tone CS, rab3A mutants and controls showed similar amounts of freezing. Again, rab3A mutants performed comparably to controls indicating that MF-LTP and MF-LTD are not involved in contextual and cued fear conditioning.

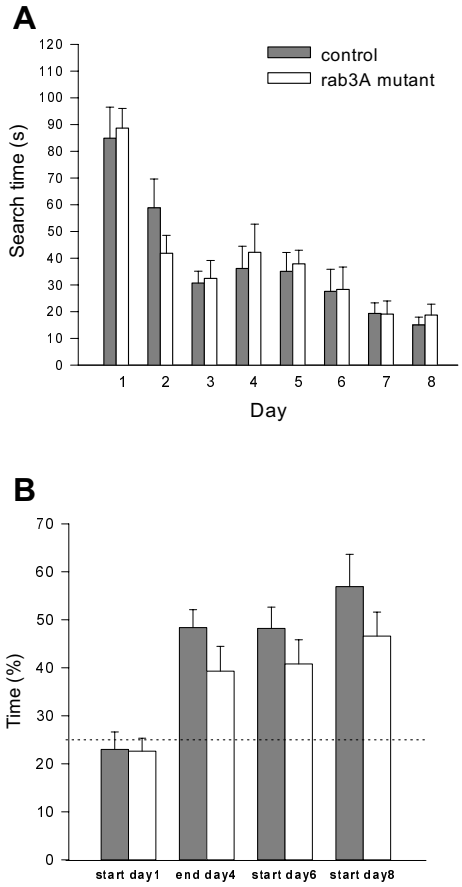

Figure 5. Morris maze "difficult" version. Performance of control (8 heterozygotes males) and null-mutant (10 males) mice. **A.** Search time to find the hidden platform. Shown is average of four daily trials, except for day 6 and 8, were only three training trials were given. Both groups showed a decline in search time (day: $F_{7,122}$ = 24.4, p<0.001), which did not differ between groups (day*genotype: $F_{7, 122}$ = 0.64, n.s). **B.** Search time (in percentage of total time) in the quadrant were the hidden platform used to be. The first probe test was before training, the second after training on day 4, the third and fourth were conducted before training on day 6 and 8. An increase in quadrant preference was observed for both groups (day: $F_{3,48}$=17.6, p<0.001), but no differences between mutants and controls were observed (day*genotype: $F_{3,48}$=0.56, n.s.).

Discussion

The aim of this study was to investigate the role of mossy fiber long-term plasticity in spatial and contextual memory. For this purpose we used rab3A null-mutant mice that have a deficit in both MF-LTP and MF-LTD. These mice were backcrossed in order to obtain a standardized genetic background and avoid the flanking region problem (Gerlai, 1996). The rab3A mutant performed normally in the radial maze, two versions of the Morris water maze and in the cued- and contextual fear-conditioning task. The only differences detected were a decrease in bodyweight in rab3A mutant pups and a minor change in open field behavior at later stages of that task.

This flanking region problem occurs when heterozygotes with a mixed genetic background (129/Sv and C57Bl/6) are used for breeding, which results in co-segregation of different regions flanking either the wildtype allele (derived from C57Bl/6) or the mutant allele (from 129/Sv). Mice that are hybrids of 129/Sv and C57Bl/6 outperform their parental strains in the Morris water maze (Owen et al., 1997), indicating that both inbred strains carry genetic polymorphisms that impair Morris water maze performance. Thus, differences in flanking regions may cause reduced or enhanced performance independent of the mutated gene. In the present study the backcross to 129S3 was used, which resulted in identical genetic backgrounds for mutant and control mice. The 129S3 inbred strain is

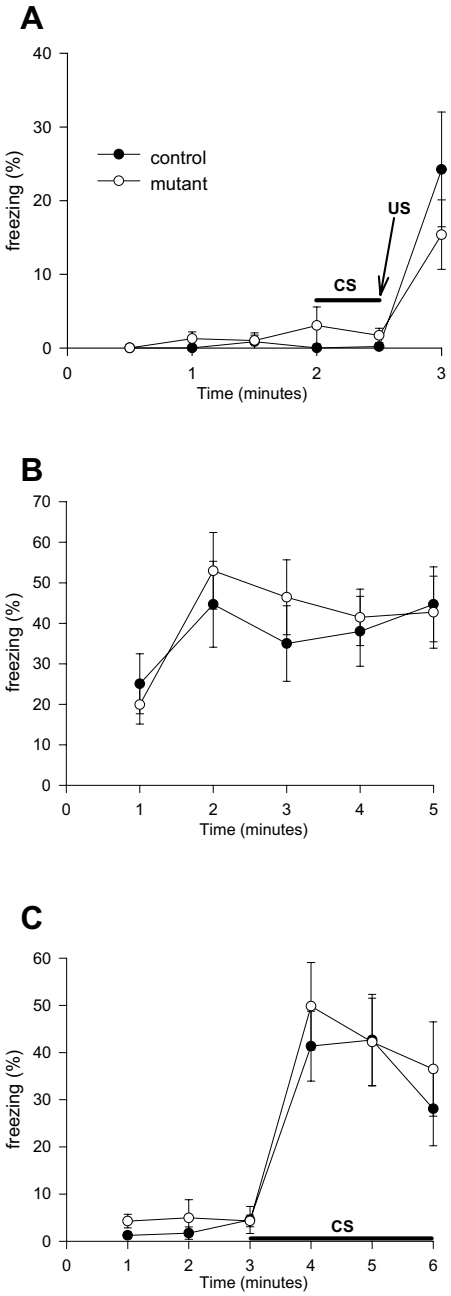

Figure 6. Cued and contextual fear-conditioning in wildtype (11 females) and mutant (12 females) mice. **A.** Freezing time in 30s bins during the training trial. Mutants and wildtypes did not show freezing during their first exposure to the shock chamber and the tone CS. After application of the foot shocks, both groups showed freezing responses that were similar (time: $F_{1.3,27.3}$=16.5, p<0.001 (Huynh-Feldt corrected ε=0.26)), (time*genotype: $F_{1.3,27.3}$=1.23, n.s. (Huynh-Feldt corrected ε=0.26)). **B.** Contextual fear-conditioning. Shown is freezing in 1min bins. Mutants and wildtypes freezed when placed in the shock chamber and showed similar levels of freezing (time: $F_{4,84}$=3.76, p<0.01), (time*genoype: $F_{4,84}$=0.45, n.s.), (genotype: $F_{1,21}$=0.16, n.s.). **C.** Cued fear-conditioning. Mice were placed in a novel environment were they were exposed to the tone CS after a 3min habituation period. During habituation, both groups showed virtually no freezing. After onset of the tone CS, both groups showed similar amounts of freezing responses (time: $F_{3.4,70.7}$=27.4, p<0.001 (Huynh-Feldt corrected ε=0.67)), responses (time*genotype: $F_{3.4,70.7}$=0.25, n.s. (Huynh-Feldt corrected ε=0.67)).

unsuitable for the Morris water maze because of its propensity to float (Wolfer et al., 1997), and the F1 between both backcrosses was used, as was recommended by others (Banbury Conference Report, 1997). In this way, the flanking region of only one chromosome differ and is also smaller in size, so that the genetic background problem is greatly reduced.

The results show that a complete elimination of long-term synaptic plasticity in the mossy fiber pathway does not impair learning and memory in a variety of tasks. This is surprising for a number of reasons. Firstly, the mossy fibers are an integral pathway in hippocampal information processing and granule cells share many firing characteristics with pyramidal cells of areas CA3 and CA1. Examples of such characteristics are spatially selective firing, theta precession, and reactivation of firing sequences during sleep (Jung and McNaughton, 1993;Skaggs et al., 1996;Shen et al., 1998).

Secondly, mossy fiber synapses are very large and believed to be powerful (Henze et al., 2000). Activity of only a few mossy fiber synapses could be sufficient to trigger a CA3 pyramidal cell (Henze et al., 2000). Thus, this pathway seems an ideal target to alter hippocampal information processing. Finally, mossy fibers show naturally occurring plasticity during radial maze learning, suggesting a role for mossy fiber plasticity in learning and memory (Mitsuno et al., 1994;Ishihara et al., 1997).

Our results do not exclude a subtle role for mossy fiber plasticity. The tasks that were used could all be learned within a limited number of trials and may not have been demanding enough to require mossy fiber plasticity. This issue was addressed using a difficult version of the Morris water maze. Even in this difficult version, mutants were not impaired. Apparently, other types of plasticity, such as those that are dependent on the NMDA-receptor are sufficient to ensure normal performance. Alternatively, synaptic plasticity in the mossy fiber pathway may not be important for the present task, but for future cognitive function. The dentate gyrus is one of the few regions in the brain that shows neurogenesis in adulthood. Interestingly, *in vivo* high-frequency mossy fiber stimulation, sufficient to induce MF-LTP, enhances neurogenesis (Derrick et al., 2000). Neurogenesis is also enhanced after a learning task (Gould et al., 1999) and in enriched environments (Kempermann et al., 1997) and these new neurons are necessary for trace conditioning (Shors et al., 2001). However, these new neurons need to be older than 6 days in order to be utilized in this learning task (Shors et al., 2001). Thus, mossy fiber plasticity may be involved in enhancement of neurogenesis, and consequently in learning and memory, but such effects will remain undetected in shorter learning tasks, such as those of the present study.

In summary, we tested the involvement of mossy fiber plasticity in learning and memory using a genetic model lacking both MF-LTP and MF-LTD. We circumvented the flanking allele problem by using appropriate breeding strategies and a selection of genetic backgrounds suitable for the different tasks. A small behavioral change was detected in the open field where mutants spent less time in the center. No differences were detected using the eight-arm radial maze, the Morris water maze and cued and contextual fear conditioning. Although we cannot exclude a subtle role for mossy fiber plasticity in memory, we can conclude that it is not essential for spatial, contextual and working memory.

Chapter 6

Complementary actions of two forms of long-term plasticity in spatial memory

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Abstract

The hippocampus is essential for the acquisition of spatial memory and changes in synaptic strength are believed to be important for this process. The leading candidate mechanisms are NMDA-receptor dependent long-term potentiation and depression. NMDA-receptor dependent plasticity is displayed by most hippocampal pathways and its disruption by pharmacological or genetic means abolishes water-maze learning in naïve rodents. However, NMDA-receptor blockade fails to disrupt spatial memory in rats that have received drug-free pre-training in another room. Pre-training ensures familiarization with only the procedural requirements of the water maze. Thus, NMDA-receptor dependent plasticity does not appear essential for spatial learning. To resolve this issue, we blocked NMDA-receptor dependent LTP and LTD and hippocampal mossy fiber long-term plasticity. The latter types of plasticity are NMDA-receptor independent and absent in mice that lack rab3A. These mutants showed normal spatial memory during water-maze pre-training. Subsequently, they were trained in a novel room and simultaneously received the competitive NMDA antagonist (±)CPP at 10 mg/kg. Spatial memory was retained in wildtype mice, thus confirming earlier findings in rats. In contrast, mutants failed to show long-term spatial memory. These results suggest that NMDA-receptor dependent long-term plasticity and mossy fiber long-term plasticity can compensate for each other in a mutually exclusive fashion, and indicate that long-term synaptic plasticity in the hippocampus is both necessary and sufficient for long-term spatial memory.

Introduction

Spatial memory tasks such as the Morris water maze are widely used to assess mnemonic abilities in rodents. Spatial memory is believed to be an easily accessible analog for declarative memory in humans. Both declarative memory in humans and spatial memory in rodents are disrupted by lesions of the hippocampus (Milner et al., 1998;Morris et al., 1982;Bannerman et al., 1995). Much interest has focused on the physiological mechanisms involved. The foremost candidates are long-term potentiation (LTP) and long-term depression (LTD), rapid and persistent forms of synaptic plasticity. There are at least two kinds of long-term plasticity in the hippocampus, which can be distinguished on the necessity for NMDA-receptor activation. Many studies have emphasized the importance of NMDA-receptor dependent LTP and LTD (NMDA-LTP/LTD) in spatial memory. Disruption of NMDA-LTP/LTD by pharmacological (Morris et al., 1986) or genetic (Tsien et al., 1996) means abolishes water-maze learning in naïve rodents. However, in order to perform well in this task, animals must become familiar with the procedural requirements and suppress inappropriate behavior. Inadvertently, such aspects are included when studying naïve rats or mice. The problem is solved by water maze pre-training, which ensures the acquisition of the task requirements, and allows a refined assessment of spatial memory (Bannerman et al., 1995; Saucier and Cain, 1995). Using this procedure, it was shown that NMDA-receptor blockade fails to disrupt spatial memory in rats (Bannerman et al., 1995;Saucier and Cain, 1995). Thus, it seems that other mechanisms, independent of the NMDA-receptor, support spatial memory.

Most hippocampal pathways display NMDA-receptor dependent LTP and LTD, but there is an exception for the mossy fiber pathway from the dentate gyrus to area CA3. The mossy fibers display long-term plasticity (MF-LTP/LTD) that is not blocked by NMDA-receptor antagonists (Harris and Cotman, 1986;Kobayashi et al., 1996). The function of MF-LTP and LTD is unclear. They can be disrupted by gene manipulation, but that does not affect performance in a variety of learning tasks, including the water maze (chapter 5). Previously, we suggested that the loss of MF-LTP/LTD may be compensated by other types of plasticity (chapter 5).

Presently, this hypothesis was tested. To this end, we used rab3A null-mutant mice (Geppert et al., 1994). Rab3A is a presynaptic protein that is essential for MF-LTP and MF-LTD (Castillo et al., 1997;Tzounopoulos et al., 1998)(chapter 5). In combination with the NMDA-receptor antagonist (±)CPP, we were able to disrupt MF-LTP/LTD and NMDA-LTP/LTD and study the behavioral consequences. Our results show that spatial memory is preserved with a blockade of either NMDA-receptor dependent long-term plasticity or mossy fiber long-term plasticity. However, a simultaneous blockade of all these forms of long-term plasticity disrupts long-term spatial memory.

Material and Methods

Mice

Rab3A null-mutant mice were created by deleting the first two exons of the rab3A gene (Geppert et al., 1994). Mutants were repeatedly backcrossed to 129S3 (also known as 129/SvImJ; Jax code: JR2448) for at least 6 generations, resulting in a line with a standardized genetic background. A second line was obtained by backcrossing to C57Bl/6 for 6 generations. Heterozygotes for the rab3A gene from both lines were intercrossed resulting in hybrids which are known for their proficiency in the water maze (Wolfer et al., 1997;Banbury Conference Report, 1997). Wildtype and heterozygous littermates served as controls. 15 rab3A null mutant mice (5 males and 10 females) and 15 control mice (9 males and 6 females) were used for this experiment. All experiments were approved by the Ethical Committee of the Utrecht University Medical Faculty.

Morris water maze protocol

The Morris mazes were circular white pools (diameter 132 cm), filled with water (25°C) that was made opaque by adding small quantities of the white pigment Acoat X (Akzo Nobel, Sassenheim, The Netherlands) at a dilution of approximately 1:10.000. The platform was circular (15cm diameter), and made of perforated Plexiglas. The platform was placed 1 cm below the surface of the water in the center of a quadrant. To avoid visual orientation prior to release, mice were transported in a white bucket from which they glided in the water towards the wall of the pool. Starting positions changed every trial in a pseudo-random fashion. Their location was sampled every 0.1 s using Ethovision software (Noldus technology, The Netherlands). A trial ended when a mouse was 10 s on the platform or when 120 s had passed. Mice that failed to find the platform within 120 s were lured to the platform with a wire mesh shovel, so that they climbed the platform by themselves and left there for 10 s. The shovel was also used as a means to retrieve the mice from the platform in a stress-free manner. Probe trials, in which the platform was removed, lasted 60 seconds. The training protocol is shown in table 1. The experimenter was always blind with respect to genotype until the end of the experiments. The two experimental rooms differed in size, orientation and dimensions. Animal cages were placed in different types of racks. The location of the door and the experimenter with computer setup was different. Finally, lightning of the room and spatial cues were different.

Table 1. Training protocol. Columns represent consecutive days, rows represent time after injection of (±)CPP (in minutes).

time \ day	1	2	3	4	5	6	7	8	9	10	11	12	13
0	-	-	-	-	-	-		c	c	c	c	c	-
60	p					p		p					p
90	t	t	t	t	t		room change	t	t	t	t	t	
120	t	t	t	t	t			t	t	t	t	t	
150	t	t	t	t	t			t	t	t	t	t	
180			p		p					p		p	

- no injection
c (±)CPP injection
t training trial
p probe trial

Treatment

In the second half of the experiment, mice received daily subcutaneous injections of the competitive NMDA-receptor antagonist (±)CPP [(±)–3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; Sigma RBI, Saint Louis, Missouri 63103, USA], at a dose of 10mg/kg. (±)CPP was dissolved in saline. The dose of 10 mg/kg disrupts *in vivo* dentate gyrus LTP in mice, until at least 150 minutes after injection (Davis et al., 1997), and primed-burst potentiation, an NMDA receptor dependent form of synaptic enhancement, in rats from 90-180 minutes after injection (Kentros et al., 1998). Mice received training trials from 90 to 150 minutes after (±)CPP injection. Thus, *in vivo* LTP is blocked by (±)CPP during the daily training sessions.

Statistics

Univariate ANOVA was used to analyze the data. In cases where it was inappropriate to use ANOVA, the Mann-Whitney U test was used. Spatial preference during probe tests was tested with one-sample T-tests against a test value of 25%.

Results

In the present study, experimental procedures were adapted from rats to mice. Rats are more adept in the Morris water maze so extra training is needed for mice. To assure thorough acquisition, mice received three training trails per day. Probe trials were used to measure spatial preference as these are considered the best measure for spatial memory (Lipp and Wolfer, 1998). The experimental protocol is shown in table 1. In short, mice received 5 days of drug-free pre-training. Subsequently, they were transferred to a new room and trained for another 5 days with NMDA-receptor blockade.

During drug-free pre-training, which was a standard Morris water maze procedure, all mice performed normal. Escape latencies declined during training (Fig.1A). Controls did not differ from rab3A mutants. The first probe test was performed at the start of the experiment. Mutants and controls did not show a spatial preference (Fig.1B), indicating that there was no bias towards the goal quadrant prior to training. The second and third probe tests were conducted 30 minutes after the last training trials on days 3 and 5. Little spatial preference was shown at day 3, but at day 5 both groups showed strong spatial preference (mutants $t(df=14)=4.8$, $p<0.001$; controls $t(df=14)=4.1$, $p=0.001$). The last probe test was performed one day after the last training in which both groups showed strong spatial preference (mutants $t(df=14)=4.0$, $p=0.001$; controls $t(df=14)=5.1$, $p<0.001$). Mutants never differed from controls during these probe tests. Thus, during pretraining, both groups showed short-term spatial memory as measured during the third probe trial and long-term spatial memory as measured in the fourth probe trial, and demonstrated their ability to solve the maze using spatial information.

After pretraining, mice were transferred to a new room, which had different dimensions and cues. Each day, prior to training, mice received an injection of the competitive NMDA antagonist (±)CPP (10 mg/kg). The protocol of the task did not differ from pre-training, hence mice were already familiar with the task requirements. This procedure should allow a specific assessment of spatial memory with as little interference of other aspects of Morris water maze learning as possible. Mice showed no signs of behavioral abnormalities under NMDA blockade, such as repeated deflection from the platform or

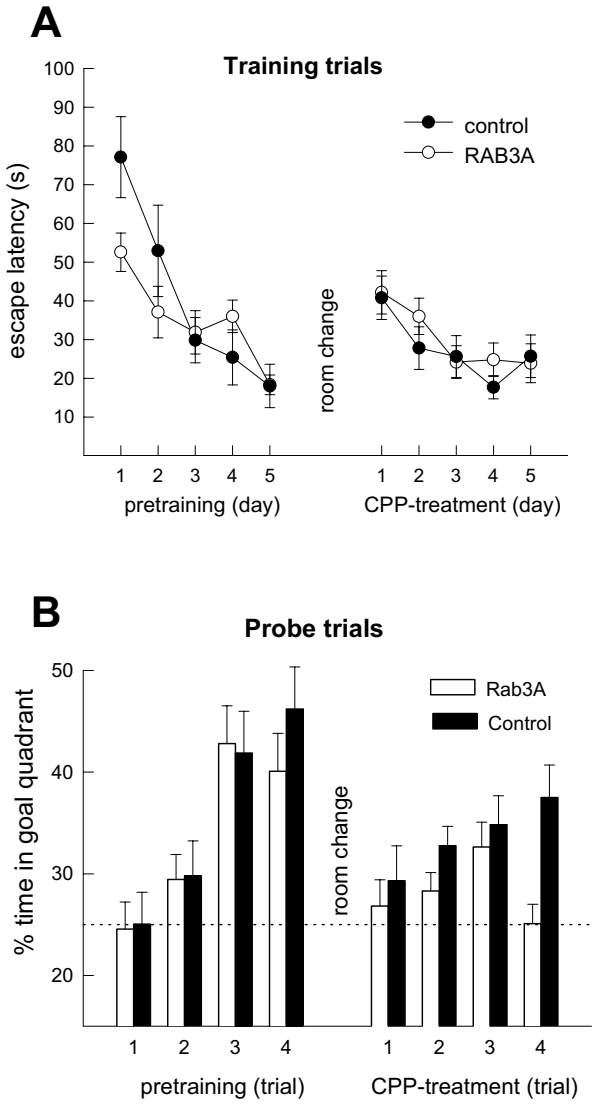

Figure 1. Morris maze performance of wildtype (n = 15) and mutant (n = 15) mice. **A.** Averaged escape latencies of 3 daily training trials expressed as mean±SEM. Shown at the left are the 5 pre-training days. At the right the 5 training days in the new room with simultaneous (±)CPP injections. No differences were detected **B**. Spatial memory was tested in probe tests during which the platform was removed. Preference for the goal quadrant was measured during 60 s free-swim trials. Preference was expressed as percentage of time in goal quadrant. Expressed as mean±SEM. Shown at the left are the 4 probe trials during pre-training. At the right the 4 probe trials in the new room with simultaneous (±)CPP injections. During both parts of the experiment, the patterns of probe trials were similar. Probe trial 1 was before training at day 1, probe trial 2 was 30 minutes after the last training trial of day 3, probe trial 3 was 30 minutes after the last training trial of day 5 and probe trial 4 was 1 day after the last training trial.

failures to climb the platform, a phenomenon that has been reported to occur in naïve rats, but not in pretrained rats (Li et al., 1997). At the first day of training, latencies were already short as compared to the first day of pre-training (Fig.1A). This shows that pre-training was effective in familiarizing the mice with the task. At later training days, latencies declined further, but controls were not different from mutants. The first probe trial (Fig.1B), given at the start of training in the new room, showed that there was no *a priori* preference for the goal quadrant. The second and third probe trial, taken 30 minutes after training on days 3 and 5 respectively, showed an increasing preference of control mice for the goal quadrant (2nd probe trial($df=14$)=4.1, $p=0.001$; 3th probe trial ($df=14$)=3.5, $p=0.004$). Mutants show only little spatial preference in the second probe trial, but demonstrated significant spatial preference in the third probe trial (2nd probe trial $t(df=14)=1.8$, $p=0.086$; 3rd probe trial $t(df=14)=3.1$, $p=0.007$). Mutants differed from controls at the 2nd probe trial ($F_{1,26}=5.9$, $p=0.023$), but not at the 3rd probe trial. Thus, both controls and mutants showed a preference for the goal quadrant immediately after training, thereby demonstrating short-term spatial memory, but mutants perform slightly worse

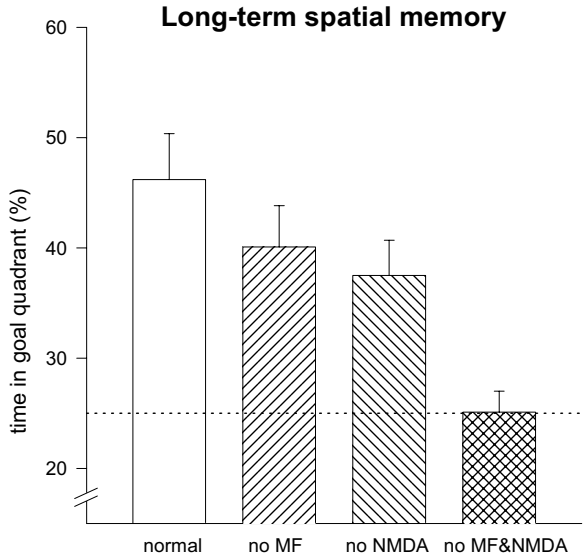

Figure 2. Summary of probe tests. Shown are results for long-term spatial memory during the last probe tests of each training phase.

then controls. The fourth probe trial, was taken 1 day after the last training trial. This probe trial measures long-term spatial memory. Control mice showed a strong preference for the goal quadrant ($t(df=14)=3.9$, $p=0.002$). Thus, control mice showed long-term spatial memory despite a blockade of NMDA receptors. Mutants, however, failed to show any spatial preference and differed from controls ($F_{1,26}=10.2$, $p=0.004$). Thus, when either mossy fiber long-term plasticity or NMDA-receptor dependent long-term plasticity is blocked, mice still display spatial memory, but under conditions where both forms of long-term plasticity are disrupted, mice fail to show long-term spatial memory in the Morris water maze (Fig.2).

Discussion

In the present study, we tested whether mossy fiber long-term plasticity and NMDA-dependent long-term plasticity can compensate for each other in spatial memory. To this end, we used rab3A null-mutant mice which lack both MF-LTP and MF-LTD. Mutants and controls were pre-trained under drug-free conditions and showed normal spatial memory. In the second phase of the experiment, mice were trained in a new room and were given the competitive NMDA antagonist (±)CPP at a dose that blocks *in vivo* NMDA-LTP. Controls and MF-LTP/LTD deficient mice did not differ in escape latencies nor did they differ on the first three probe trials. Both groups showed evidence of short-term spatial memory. The final probe trial, showed a strong spatial preference in controls, but mutants failed. Thus, mice, like rats (Bannerman et al., 1995;Saucier and Cain, 1995), are able to acquire and use spatial information with NMDA-receptor blockade. Our results suggest that in the absence of NMDA-receptor depedent plasticity, mossy fiber long-term plasticity is able to compensate.

In the present study, we used a platform of 15 cm in diameter and a pool size of 135 cm in diameter. Thus, compared to pool size, the platform size was relatively large and enabled mice to find the platform by random swim patterns. Indeed, only 3 out of 30 mice failed to find the platform during the

first training trial of the second phase. An advantage of such a setup is that potential problems such hypothermia and learned helplessness are kept minimal, and that mice are able to locate and climb the platform even when they are not aware of its spatial location. A disadvantage is that it may be hard to detect differences in escape latencies, such as in the present study. However, the only reliable way to measure spatial memory is with probe tests, because a probe test can distinguish between a spatial search strategy and a non-spatial search strategy (Lipp and Wolfer, 1998). These probe tests showed that mutants treated with (±)CPP do show a spatial preference 30 minutes after training. Hence, under conditions where both NMDA-LTP/LTD and MF-LTP/LTD are blocked, spatial memory is still supported, but this is only transient, and lost within one day.

The hippocampus has been specifically implicated in spatial learning because neurons of the hippocampus show spatially selective firing. This finding has led to the proposal that the hippocampus can support a spatial map of the environment that animals can use to navigate (O'Keefe and Dostrovsky, 1971). Spatially selective firing develops rapidly in a new environment and remains stable for many days (O'Keefe and Dostrovsky, 1971;Muller et al., 1987). Spatially selective firing is also observed in the water maze (Hollup et al., 2001). The long-term stability of these “place cells” can be affected by NMDA-receptor blockade (Kentros et al., 1998). However, spatial navigation is not blocked by NMDA-receptor blockade after pre-training (Bannerman et al., 1995;Saucier and Cain, 1995), which suggests a dissociation between the long-term stability of place cells and long-term spatial memory. However, place cells are usually measured in a pellet chasing tasks where spatial cues have little behavioral significance. Even under these circumstances, place cells tend to cluster around objects and along walls (Eichenbaum et al., 1999), which indicates that the “spatial map” concentrates around cues that may have some significant behavioral value. Thus, brain systems that are implicated in emotion and arousal may be involved in creating the spatial map. A target for such modulation of the spatial map could be provided by the mossy fibers. The terminal region of the mossy fibers also receives an extensive noradrenergic projection from the locus coeruleus (Loy et al., 1980). The induction of MF-LTP requires activation of β-adrenergic receptors (Huang and Kandel, 1996). Thus, simultaneous activity of hippocampal granule cells and activity in noradrenergic fibers appear necessary to induce long-term plasticity at the mossy fiber terminals (Bailey et al., 2000). As the locus coeruleus becomes active in situations of behavioral arousal (Huang and Kandel, 1996), it appears likely that noradrenergic activity is heightened in the life-threatening water maze. This way, mossy fibers may be able to support a spatial map in behaviorally challenging situations such as the water maze, but not in spatially undemanding situations such as a pellet chasing task.

The mossy fibers are suitable for such a role in spatial memory because mossy fiber synapses are large, have multiple active zones and believed to be very powerful. Activity in only a few mossy fiber synapses may be sufficient to depolarize a CA3 pyramidal cell above threshold (Henze et al., 2000). Also, granule cells show spatially selective firing (Jung and McNaughton, 1993). Thus, expression of MF-LTP and MF-LTD could strongly influence the spatial firing characteristics of CA3 pyramidal cells and consequently influence hippocampal function in spatial navigation.

In summary, our results suggest that short-term spatial memory is supported by transient mechanisms independent of mossy fiber long-term plasticity and NMDA-dependent long-term plasticity. The

absence of either NMDA-plasticity or mossy fiber long-term plasticity does not prevent long-term spatial memory. However, a blockade of both forms of long-term plasticity disrupts long-term spatial memory, which indicates that NMDA-plasticity and mossy fiber long-term plasticity are able to compensate for each other.

Chapter 7

General Discussion

Brief summary

The aim of this thesis was to analyze the role of presynaptic plasticity in learning and memory. In **chapter 2**, munc18-1 gene-dose mutant mice were studied at the biochemical and electrophysiological level. A decrease of munc18-1 resulted in a blockade of post-tetanic potentiation and a severe impairment of mossy fiber LTP. Furthermore, enhanced depression was observed, while basal transmission and paired pulse facilitation appeared normal. The effects of these changes on learning and memory were studied in **chapter 3**. No impairments in spatial memory in the Morris water maze were detected. However, munc18-1 mutants were severely impaired in the radial maze. Unfortunately, they also showed hyperactive behavior, which may have interfered with performance and complicates interpretation. Furthermore, locomotor activity in mutants, compared to wildtypes, was markedly enhanced by d-amphetamine. In **chapter 4**, it was shown that the hyperactive phenotype of munc18-1 gene-dose mutants persisted in the home cage. Surprisingly, gene-dose mutants displayed abnormal motor activity during sleep. Using EEG measurements, it appeared that this vigorous motor activity occurred during REM sleep. The role of mossy fiber long-term plasticity in learning and memory was studied in **chapter 5.** The rab3A null-mutant mouse is the only genetic model with a simultaneous blockade of LTP and LTD at the mossy fiber projection. Despite these impairments in mossy fiber plasticity no deficits in spatial memory, working memory and contextual fear-conditioning were detected. The possibility of compensatory mechanisms by other types of synaptic plasticity was studied in **chapter 6**. Neither a blockade of NMDA-receptor dependent plasticity nor a blockade of mossy fiber long-term plasticity impaired spatial memory. However, a simultaneous blockade of all these types of long-term plasticity blocked long-term spatial memory. This suggests that the loss of mossy fiber long-term plasticity can be compensated by NMDA-receptor dependent plasticity and vice versa.

In the opening chapter a number of general questions were put forward. In the next section, these questions are evaluated.

What is the function of short-term presynaptic plasticity in learning and memory?

Dynamic 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. Rab3A mutants showed an enhancement of PPF (Geppert et al., 1997) and an enhancement of tetanic depression (Geppert et al., 1994). These changes are likely to result in a wider dynamic range of release probability during natural spike patterns (introduction: Fig.5), which may affect acquisition and retrieval of information. However, Rab3A null-mutants do not show deficits in spatial, working and contextual memory (chapter 5). Thus, it appears that increasing the dynamic range of release probability does not affect either acquisition or retrieval of these types of memory.

Further indications were derived from studies of munc18-1 gene-dose mutants. These mutants showed enhanced tetanic depression and a blockade of post-tetanic potentiation (chapter 2). These changes are likely to result in a downward shift, i.e. more depression than facilitation, of the dynamic range of release probability. Munc18-1 gene-dose mutants did not show deficits in the Morris water maze. Thus, these changes do not appear to impair acquisition and retrieval of spatial information.

Munc18-1 gene-dose mutants did show severe impairments in the radial maze. This indicates that a downward shift in release probability may have caused working memory impairments. The relationship between working memory and post-tetanic potentiation will be discussed in the next section. In summary, increases in facilitation and enhancements of tetanic depression do not appear to affect learning and memory in a number of tasks. As release probability is believed to change rapidly at active synapses, it seems that alterations in the dynamics of release do not affect the quality of information processing to a large degree.

Is post-tetanic potentiation involved in working memory?

Working memory is the capacity to remember a limited amount of information (6-8 items) for a short-period of time (Glassman, 1999). Working memory in the radial maze appears independent of NMDA-receptor dependent associative plasticity (Shapiro and O'Connor, 1992). As completion of the radial maze takes a few minutes, it appears unlikely that continued recurrent neuronal activity could be maintained, given the inherent unreliable nature of synaptic transmission. This has led to the suggestion that post-tetanic potentiation may be involved (Churchland and Sejnowski, 1992). Munc18-1 gene-dose mutants lack post-tetanic potentiation (chapter 2) and are severely impaired in the radial maze (chapter 3). These results suggest that working memory may be mediated by post-tetanic potentiation. Munc18-1 gene-dose mutants also display enhanced tetanic depression and impaired mossy fiber LTP but, given the normal performance of rab3A mutants, these types of deficits do not seem to impair radial maze learning. Thus, the blockade of post-tetanic potentiation may have severely impaired working memory in the radial maze. Unfortunately, mutants also showed hyperactive behavior. This complicates interpretation, because hyperactivity may have interfered with radial maze performance.

Is presynaptic plasticity involved in the induction of associative plasticity?

It is widely believed that associative plasticity is involved in learning and memory. The induction of associative plasticity may require certain aspects of short-term presynaptic plasticity. For instance, presynaptic terminals may need to release neurotransmitter at high frequencies to sufficiently depolarize the postsynaptic neuron. Furthermore, precise timing between pre- and postsynaptic spikes seems required for proper induction of associative plasticity, and may require certain levels of neurotransmitter release probability. Altering the properties of presynaptic plasticity may change the effectiveness of inducing associative plasticity, and hence learning and memory. However, Rab3A mutants did not show deficits in learning and memory tasks, and munc18-1 gene-dose mutants showed normal Morris maze performance (chapter 3 and chapter 5). These learning tasks are sensitive to manipulations that block associative long-term plasticity (Morris et al., 1986;Danysz et al., 1988;Fanselow et al., 1994;Kawabe et al., 1998). Furthermore, a blockade of NMDA-receptors in rab3A mutants abolished long-term spatial memory (chapter 6). Therefore, if the characteristics of presynaptic plasticity were important for the induction of associative plasticity, deficits in spatial memory would be expected even without treatment with the NMDA-receptor antagonist CPP.

What is the role of mossy fiber plasticity in learning and memory?

The function of mossy fiber long-term plasticity was studied using rab3A null mutants. Despite the lack of mossy fiber plasticity (Castillo et al., 1997;Castillo et al., 1997), no impairments in a variety of

learning tasks were detected. This suggests that mossy fiber long-term plasticity is not important for spatial memory, working memory and contextual fear conditioning. However, compensation by NMDA-receptor dependent plasticity may have occurred. As was shown in chapter 6, a simultaneous blockade of mossy fiber plasticity and NMDA receptor dependent plasticity does impair spatial memory. This finding appears to resolve the long standing problem of incomplete correlations between LTP blockade and memory impairments. Therefore, mossy fiber long-term plasticity may contribute to learning and memory, but this is not easily detected in learning tasks due to compensation by other forms of synaptic plasticity. Possibly, learning tasks such as the Morris water maze are just not challenging enough and suffer from a ceiling effect in this respect. Mossy fiber long-term plasticity may be important in more complex and demanding cognitive situations, such as the natural environment of mice, and function alongside other types of synaptic plasticity. In the present thesis it was attempted to test this hypothesis by increasing the difficulty of the Morris water maze, but no deficits were detected. Further increases in the demands on cognitive function may require other kinds of learning tasks, probably with a higher ethological relevance than the rather unnatural water maze.

Information may be reliably processed by bursts of action potentials

It seems that changes in the characteristics of short-term plasticity do not impair cognitive abilities. Somehow, the information that is transmitted by the altered synapses of rab3A and munc18-1 mutants remains relatively intact (chapter 3, chapter 5). How is this possible? A possible explanation lies in the fact that neurons often fire in bursts. Bursts are complex spike patterns that last at most 25ms and consist of 2-6 action potentials occurring at ~200Hz (Ranck, Jr., 1973;Fox and Ranck, Jr., 1975). These burst have been recorded at the pyramidal cells of the hippocampus (Muller et al., 1987;Otto et al., 1991), but also at other regions in the brain (Lisman, 1997). It is conceivable that synapses may respond reliably to every burst, in a manner that is relatively independent of initial release probability and the exact number of action potentials within individual bursts. This is because two processes interact. First, during a burst the release probability is heightened due to facilitation. Second, after release of neurotransmitter, presynaptic terminals in the hippocampus display a short refractory period that lasts about 15ms, during which release probability is dramatically decreased (Foster and McNaughton, 1991;Stevens and Wang, 1995;Debanne et al., 1996). Thus, during a burst of action potentials, synapses are not likely to release neurotransmitter more than once, but also facilitate strong enough to ensure neurotransmitter release even at synapses with altered presynaptic plasticity or low initial release probability (Stevens and Wang, 1995;Dobrunz and Stevens, 1997). Thus, within the time window of a burst, synaptic release may actually be highly reliable. It appears that the information content of bursts is higher then the information content of the rest of the spiking pattern. For instance, spatial location is better predicted by analysis of bursts only, then by analyzing all activity of place cells in the hippocampus (Muller et al., 1987;Otto et al., 1991). Similar findings were obtained in analysis of the relation between visual information and spiking characteristics of neurons in the visual cortex (Lisman, 1997). Thus, there are indications that short bursts of action potentials are an important form of neuronal communication. Neurotransmitter release during a burst is relatively independent from differences in short-term plasticity. Therefore, the increased dynamics of rab3A

deficient synapses or the downward shift at munc18-1 gene-dose synapses may not have had a drastic impact on neuronal communication of information.

Additional findings

As might be expected from manipulations that affect basic properties of synapses throughout the brain, additional changes in behavior have occurred. Munc18-1 gene-dose mutants showed increased locomotor activity in the open field (chapter 3). This increased locomotor activity was persistent on two genetic backgrounds. Furthermore, the psychostimulant d-amphetamine greatly increased locomotor activity in munc18-1 mutants, while it did not in wildtypes. Thus, it appears that mutants show a different sensitivity to psychostimulants.

Another behavioral change in munc18-1 gene-dose mutants were the sleep disturbances (chapter 4). During REM sleep, periods of vigorous motor activity occur that resembled a loss of atonia. Such sleep problems may have been caused by an inability to maintain sufficient inhibition of motorneurons.

Although further research is needed, it appears that these different aspects of the phenotype are caused by physiological changes in different parts of the brain. As opposed to munc18-1 mutants, rab3A null-mutants appear relatively normal. They do show small decreases in bodyweight and a minor alteration in open field behavior, but these changes are mild compared to the phenotypic changes displayed by munc18-1 gene-dose mutants.

The electrophysiological phenotypes of these mutants resemble each other, so why do their phenotypes differ in these respects? This may be explained by differences in expression patterns and the presence of isoforms. For instance, the expression of rab3A is not uniform throughout the brain but restricted to specific neurons.(Moya et al., 1992;Stettler et al., 1994;Stettler et al., 1995). Furthermore, the isoforms rab3B and rab3C are expressed in the CNS, preferentially in those neurons with low rab3A expression, and it appears that they are able to compensate for the loss of rab3A (Stettler et al., 1995;Li et al., 1994). At the granule cells of the hippocampus, rab3A is the only isoform that is expressed, which may explain the severe phenotype of these neurons (Castillo et al., 1997). On the other hand, munc18-1 is expressed in all neurons, and the loss of munc18-1 does not appear to be compensated by other members of the munc18/sec1 protein family (Garcia et al., 1995;Verhage et al., 2000). Therefore, the phenotype of rab3A null-mutants was likely to be less severe than that of the munc18-1 gene-dose mutant.

The use of gene manipulation in the study of brain and behavior

Ever since the first studies of Grant. (Grant et al., 1992) and Silva (Silva et al., 1992), the use of genetic manipulation in the study of brain and behavior has been met with resistance. Over the years, a number of problems have been identified and discussed. However, the exponential increase in the numbers of publication using gene-manipulation in the study of brain and behavior, shows that these problems have not deterred neuroscientists. Evidently, the advantages outweigh the problems. In the next section some of the advantages and disadvantages of the use of gene-manipulation will be discussed.

There are a number of obvious advantages to genetic manipulation as a tool to study brain function. First, genetic manipulation is very selective. A deletion of 1 in 30.000 genes is a very precise and "clean" ablation. Second, it is a permanent manipulation. Once a genetic mutation is induced, breeding the mice will give identical copies of the same animal model that can be easily transferred between laboratories. Third, it is non-invasive and there is no need for surgery or treatment with pharmacological substances. Thus, the effects of the gene-product can be abolished without the side-effects of drugs or inconvenience for the animal.

Most importantly, this technique allows a study of new mechanisms. Genetic manipulation may be the only way to determine the precise role of many endogenous factors on behavior. Thus, genetic manipulation in mice opens up new fields of research that were previously inaccessible to scientific manipulation. Examples are the manipulations of presynaptic plasticity described in this thesis. Because short-term presynaptic plasticity is not accessible with pharmaca in freely moving animals, virtually nothing was known about the role of these processes on cognitive function in mammals.

However, there are also a number of disadvantages to use of gene-knockouts. The gene is altered in all cells of the body and at all stages of development. Any behavioral deficit may be due to the missing gene-product at the moment of testing but also due to lack of the protein during developmental processes. In addition, the absence of a single gene may alter expression of other genes and unexpected compensatory or redundancy mechanisms may be activated that obscure interpretation. Also, because the gene is lacking in all brain regions, a complex phenotype may emerge that is difficult to interpret. In the present thesis, the latter problem is illustrated by the munc18-1 mutant. Although there is a deficit in radial maze performance, claims about the specificity on working memory are hampered by the presence of hyperactivity. As all learning tasks require motoric acts, it is virtually impossible to exclude the possibility that changes in locomotor activity do not influence measures of memory. In summary, genetic manipulation is a new technique with its own strengths and shortcomings. It has the unique capability of manipulating virtually every aspect of the physiology of an animal, but it lacks temporal specificity and may result in complex phenotypes. As with other techniques, the shortcomings can be overcome by collecting converging evidence from other genetic models as well as from classical methods.

Future directions

In this final section, recommendations for future research are offered. The munc18-1 gene-dose mutants showed a number of behavioral changes that require additional experimentation to be fully understood. First, munc18-1 gene-dose mutants showed a marked increase in locomotor activity after d-amphetamine injection. D-amphetamine is a wideacting psychostimulant and inhibits re-uptake of a variety of monoaminergic neurotransmitters. The specificity of psychostimulant action in munc18-1 mutants may be studied with selective agonists and antagonist for different monoaminergic receptors. Possibly, the hyperactive phenotype of munc18-1 mutants is caused by disregulation of a monoaminegic system, such as the dopaminergic system. Further analysis of this mutant may provide valuable information about its regulation.

Second, munc18-1 gene-dose mutants showed sleep disturbances during REM sleep. Further analysis of sleep patterns, with simultaneous EMG and eye movement recordings are necessary to analyze the conditions for appearance of these abnormal motor activities. Electrophysiological analysis at the brainstem level during sleep may provide information about the induction and loss of atonia in munc18-1 mutants. Such recordings are not possible in freely moving mice. However, an artificial preparation exists that uses local infusion of carbachol in the pons to induce REM sleep. This model shows a striking resemblance with normal REM and allows single cell recordings in the pons and medial medulla during atonia (Fenik et al., 1998). Such studies may provide insights in the regulation of REM sleep and the causes of REM sleep behavior disorder.

An interesting finding in the present thesis was the observation that mossy fiber long-term plasticity and NMDA-receptor dependent long-term plasticity appear to compensate for each other in spatial memory (chapter 6). For reasons mentioned above, it is not certain whether these results were not caused by some unknown peculiarity in the rab3A mutant. Therefore, this finding would be greatly supported by a replication using another mouse mutant without mossy fiber plasticity.

It appeared that a deficit in mossy fiber long-term plasticity could only be detected after a blockade of NMDA-dependent long-term plasticity (chapter 6). This implies that mossy fiber long-term plasticity does contribute to learning and memory, but that its role is not easily detected using conventional learning tasks. Mossy fiber long-term plasticity may prove to be important in more complex and demanding cognitive situations. Thus, new memory tasks should be developed that are better able to test the full potential of mnemonic capabilities of mice. Such tasks should make good use of the ethological repertoire of the mouse, because under natural circumstances animals usually show much better cognitive performance (Tinbergen, 1989). The natural habitat of mice is dry land, and foraging for food and water in a large, aversive maze may be an ethologically relevant approach to assess memory.

Further studies on the role of post-tetanic potentiation and working memory (chapter 3) need a sophistication in genetic techniques. The problem of confounding effects within complex phenotypes can be overcome by restricted expression or deletion of certain genes. Using a combination of genetic techniques it is possible to manipulate a gene at a specific anatomical location or at a specific point in time (Mayford and Kandel, 1999). This way, undesired side effects are restricted to certain ages or anatomical locations, thereby facilitating analysis. The munc18-1 gene-dose mutant appears to be the only mutant with a complete blockade of PTP. Therefore, this specific electrophysiological phenotype should be exploited. Studies on the role of PTP in working memory would be greatly facilitated by a restricted deletion of munc18-1 in the hippocampus and/or frontal cortex. This way, PTP would be disrupted only at those regions that have been specifically implicated in working memory (Olton et al., 1982;Glassman, 1999), and this may avoid confounding factors such as hyperactivity. These novel genetic techniques may also allow an anatomical analysis of the mechanisms of working memory by restricting the deletion to subregions of the hippocampus.

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Nederlandse samenvatting

Het vermogen om te leren is een van de belangrijkste eigenschappen van onze hersenen. Er wordt al vele jaren intensief onderzoek naar gedaan. Gek genoeg wordt nog steeds niet goed begrepen hoe de hersenen nu eigenlijk informatie opslaan. De meeste wetenschappers zijn het er over eens dat informatie wordt opgeslagen door middel van veranderingen in de verbindingen tussen zenuwcellen; de zogenaamde synapsen (zie ook figuren 2 en 3 in hoofdstuk 1). Neuronen hebben een aantal mechanismen tot hun beschikking om de eigenschappen van synapsen te veranderen en deze mechanismen worden gezamenlijk synaptische plasticiteit genoemd. Zo kunnen er veranderingen optreden in het postsynaptische gedeelte door het aantal of de aard van neurotransmitterreceptoren te veranderen. Dit type synaptische plasticiteit is vaak associatief van aard: er is een gezamenlijke activiteit in zowel het presynaptische als het postsynaptische neuron nodig. Neuronen kunnen ook de secretie van neurotransmitter aan het presynaptische zenuwuiteinde veranderen. De secretie van neurotransmitter kan zowel vergroot als verkleind worden. Er zijn een aantal typen presynaptische plasticiteit die in tijdsspanne kunnen variëren van enkele honderdsten van een seconde tot vele uren. Presynaptische plasticiteit is niet-associatief van aard: er is alleen activiteit van het presynaptische neuron nodig. Er zijn aanwijzingen dat associatieve plasticiteit belangrijk is in leren en geheugen. Dit kan echter zeker niet het enige mechanisme zijn, want als associatieve plasticiteit geblokkeerd wordt is leren nog steeds mogelijk. De rol van presynaptische plasticiteit in leren en geheugen is onduidelijk. Dit komt met name omdat het voorheen niet mogelijk was om presynaptische plasticiteit in levende dieren te manipuleren. Daardoor kon de rol van presynaptische plasticiteit in geheugen nooit goed onderzocht wordt. Door de ontwikkeling van genetische manipulatie in muizen zijn er echter de wetenschappelijke gereedschappen ontwikkeld om dit wel te doen. In dit proefschrift is van deze techniek gebruik gemaakt om de rol van presynaptische plasticiteit in leren en geheugen te onderzoeken.

Er wordt in dit proefschrift gebruik gemaakt van twee verschillende mutante muizen. Als eerste wordt de munc18-1 heterozygote mutant behandeld. Munc18-1 is een eiwit dat essentieel is voor de secretie van neurotransmitter. De munc18-1 heterzygoot heeft nog maar 1 intact munc18-1 gen (normaal zijn er 2 kopieën van elk gen). In hoofdstuk 2 wordt beschreven dat dit er voor zorgt dat munc18-1, in de hersenen van deze mutant, met ongeveer 50% gereduceerd is. Tevens laat deze mutant een aantal veranderingen in presynaptische plasticiteit zien, terwijl associatieve plasticiteit vrijwel normaal lijkt te zijn. De veranderingen die deze mutant laat zien zijn in de middellange tot langdurende vormen van presynaptische plasticiteit. Zo laat deze mutant een versterkte tetanische depressie zien. Dit betekent dat als de synaps gedurende langere tijd sterk actief is, de secretie van neurotransmitter sterker afneemt dan in controle muizen. Een van de interessantste veranderingen in de munc18-1 mutant is een complete blokkade van post-synaptische potentiatie, een vorm van presynaptische plasticiteit die enkele minuten duurt. Aangezien dit de enige mutant is die een blokkade van posttetanische potentiatie heeft, zou dit geschikt model kunnen zijn om de rol van posttetanische potentiatie in geheugen te onderzoeken. Ook laat deze mutant een sterke vermindering zien van de langdurige potentiatie aan de mosvezels. Waarom deze mutant deze veranderingen in presynaptische plasticiteit

laat zien is niet geheel duidelijk en wijst op nieuwe rollen voor het eiwit munc18-1 in deze vormen van presynaptische plasticiteit. In hoofdstuk 3 is onderzocht of deze veranderingen gevolgen hebben voor het leervermogen van deze mutant. Het lange-termijn geheugen is gemeten in het water doolhof. In dit doolhof zit een ontsnappingsplatform verstopt onder het melkachtig gekleurde wateroppervlak. De muis kan het ontsnappingsplatform dus niet zien maar zal de ruimtelijke locatie na enkele ervaringen leren en er dan snel naar toe zwemmen. Deze taak meet voornamelijk het lange-termijn geheugen. Verassend genoeg blijkt de munc18-1 muis normaal te presteren in deze taak. Het blijkt dus dat het gemis van een aantal vormen van presynaptische plasticiteit geen gevolgen heeft voor lange-termijn geheugen. Werkgeheugen is een flexibele, kortdurende (seconden tot minuten) vorm van geheugen en wordt ook wel kladblok geheugen. Het werkgeheugen van deze mutant is gemeten in het 8-armige radiaal doolhof. In dit doolhof zijn een aantal voedselbeloningen verstopt. Het doel van de taak is al deze beloningen op te halen. Daardoor moet de muis gedurende het verzamelen onthouden waar al gezocht is en waar nog niet. Gedurende de training bleek de munc18-1 mutant veel fouten te maken, en nagenoeg op kansniveau te presteren. Het werkgeheugen van deze mutant lijkt dus zwaar verstoord te zijn. Er zijn aanwijzingen dat versterkte synaptische depressie en afwezigheid van mosvezel potentiatie geen effect hebben op werkgeheugen. Het is dus mogelijk dat het gemis van posttetanische potentiatie het defect in werkgeheugen veroorzaakt. Dit is een aantrekkelijke gedacht omdat posttetanische potentiatie en werkgeheugen een zelfde tijdspanne hebben. Jammer genoeg wordt de interpretatie van deze resultaten vermoeilijkt doordat de munc18-1 mutant ook een sterk verhoogde locomotorische activiteit laat zien. Het zou kunnen dat deze verhoogde activiteit de prestatie in het radiaal doolhof heeft verstoord, maar de prestatie in het water doolhof niet. Aangezien vrijwel elke leertaak in muizen sterk afhangt van locomotoriek is het uitermate lastig dit probleem te ontwijken. In hoofdstuk 4 wordt een andere gedragsverandering in de munc18-1 mutant beschreven. Deze mutant laat namelijk gedurende de slaap zo nu en dan abnormale motorische activiteit zien. Deze abnormale activiteit bestaat uit slaapwandelen en mondt dan vaak uit in ongecoördineerde sprongen waarna de muis vaak wakker wordt. In hoofdstuk 4 is onderzocht in welke fase van de slaap dit gebeurt. Dit is gedaan met een EEG registratie van de hersenactiviteit. Het bleek dat deze motorische activiteit samenvalt met REM slaap. REM slaap is die fase van slaap waar gedroomd wordt en waarbij de hersenen erg actief zijn. Zonder een speciaal mechanisme waardoor de motorische signalen van de hersenen onderdrukt worden, zou men uitvoeren waarover men droomt. Normale mensen zijn door die inhibitie nagenoeg verlamt tijdens REM slaap maar er zijn patiënten bekend die de inhibitie missen. Deze patiënten voeren dan ook hun dromen uit, wat natuurlijk gevaarlijk is voor hen zelf en hun (slaap)partners. De munc18-1 heterozygoot lijkt hetzelfde te laten zien en zou een nieuwe genetisch model voor deze ziekte kunnen zijn.

De functie van lange-termijn potentiatie en depressie in de mosvezels van de hippocampus werden onderzocht met behulp van de rab3A knock-out muis. Deze mutant mist namelijk deze vormen van presynaptische plasticiteit. In hoofdstuk 5 wordt beschreven dat deze mutant, verassend genoeg, normaal leert in alle leertaken (water doolhof, radiaal doolhof en angstconditionering) die hem worden voorgeschoteld. Het lijkt dus dat lange-termijn potentiatie en depressie aan de mosvezels geen rol speelt in werkgeheugen en lange-termijn geheugen. In hoofdstuk 6 is onderzocht of er in het laatste

geval sprake is van compensatie door andere vormen van lange-termijn plasticiteit. Er was namelijk al eerder gevonden, in ratten, dat een blokkade van associatieve postsynaptische plasticiteit het lange-termijn geheugen ook niet blokkeert. Ook muizen blijken een normaal lange-termijn geheugen te hebben wanneer associatieve plasticiteit geblokkeerd wordt. Echter, wanneer beide vormen van lange-termijn plasticiteit tegerlijkertijd geblokkeerd worden is het lange-termijn geheugen verdwenen. Dit laatste experiment laat zien dat zowel associatieve plasticiteit als mosvezel plasticiteit betrokken zijn bij lange-termijn geheugen, maar dat de een voor de ander kan compenseren.

Samenvattend kan worden gezegd dat presynaptische plasticiteit een rol lijkt te spelen in leren en geheugen. Posttetanische potentiatie zou belangrijk kunnen zijn bij werkgeheugen, maar er is nieuw onderzoek nodig om dit te bevestigen. Lange-termijn veranderingen aan de mosvezels lijken belangrijk bij lange-termijn geheugen. Aangezien er compensatie kan optreden door associatieve plasticiteit is dit echter lastig aan te tonen. Er zijn waarschijnlijk moeilijkere leertaken nodig om de functie van mosvezelplasticiteit direct aan te tonen.

Publications

Hensbroek RA, Sluyter F, Guillot PV, Van Oortmerssen GA, Crusio WE (1995) Y chromosomal effects on hippocampal mossy fiber distributions in mice selected for aggression. Brain Res 682: 203-206.

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Hensbroek, RA, Verhage M, and Spruijt BM (2001) Disruptions of presynaptic plasticity do not affect spatial memory but causes hyperactivity and impairs working memory performance. Submitted for publication

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Hensbroek RA, Kamal A, Spruijt BM, and Verhage M. A reduction of munc18-1 in mice leads to enhanced tetanic depression and absence of post-tetanic potentiation and mossy fiber long-term potentiation. In preparation.

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Nu zijn promotoren er voornamelijk voor de theorie. Op het praktische vlak zijn ze echter vaak nogal roestig. Gelukkig zijn er dan analisten om bij te springen bij echte experimenten. Ik heb in het begin, bij het moleculaire werk, veel hulp gehad van Anita. Ik had (bijna) nog nooit een pipet gehanteerd en nu moest jij me maar eventjes een van de lastigste en meest precieze experimenten aanleren. Zonder jouw hulp, engelengeduld en optimisme, was het me nooit gelukt het PCR-en onder de knie te krijgen. Bij het gedragswerk heb ik veel hulp gehad van Anne-marie. Anne-marie, je wist altijd allerlei opstellingen bij elkaar te verzamelen en in elkaar te zetten. Ik vond het erg leuk om de continue intra-cerebrale infusie in muizen met jouw op te zetten. Jammer dat het in het uiteindelijke experiment niet goed uitwerkte, maar dat heeft niet aan de techniek gelegen en we hebben er wel mooi een posterprijs aan over gehouden! Naast praktische hulp op het werk was het ook altijd gezellig ouwehoeren op je kamer en ben ik uiteindelijk niet bij Berry maar bij jouw matroos geweest. Beste Robbert, je bent niet voor niets paranimf. Toen je op het RMI aankwam wist je je binnen de kortste keren op te werken tot

een topanalist. Je kennis van zaken en je hulp bij het regelen en uitvoeren van experimenten waren voor mij van groot belang. Natuurlijk is werk belangrijk maar er moet ook zo nu en dan ontspannen worden. Dat deden we niet alleen in diverse vage kroegen, maar ook aan het water waar jij me de beginselen van het vliegvissen hebt geleerd, en natuurlijk met de gitaar, lekker raggen met de versterker op 10.

Ik heb ook nog met diverse onderzoekers samengewerkt. Op het RMI was dat onder andere met Amer. Toen ik voor het eerst wat muizen bij je langs kwam brengen, dacht ik dat je wel een paar probeermuizen nodig zou hebben om aan het veel kleinere formaat te wennen. Echter, vanaf de allereerste muis liep het perfect en lukte je het om mooie resultaten te behalen. Zonder jouw essentiële metingen zou mijn andere werk geen poot hebben om op te staan.
Een deel van de experimenten zijn uitgevoerd door Natasja de Bruin van het NICI (Katholieke Universiteit Nijmegen). Het experiment hebben we toendertijd onder het genot van enkele biertjes besproken en dat zal wel hebben bijgedragen aan het speelse gemak waarmee jij de data verzameld hebt. Het was erg prettig samenwerken en ondanks het geringe aantal muizen ben ik best wel trots op het EEG hoofdstuk.

Ik heb ook nog een student van de HLO mogen begeleiden. Beste Tjeerd, je hebt me een boel geleerd over het begeleiden van studenten. Jammer genoeg zijn je resultaten niet meer in een hoofdstuk terechtgekomen, maar dat lag zeker niet aan de kwaliteit van je werk. Wetenschap zit soms gewoon even tegen. Succes aan de operatietafel

Het welzijn van de muizen was in de handen van de jongens van de dierstallen. Wout, Joost, Bert en alle anderen; niet alleen bedankt voor het perfect verzorgen van mijn muisjes, maar ook voor de koffie en de melige gesprekken op jullie kantoortje.

Ton, bedankt voor je vele hilarische verhalen en de fantastische opstellingen die je voor me gemaakt hebt. Iedereen was jaloers op mijn radiaal doolhof!

Tijdens het onderzoek zat ik in twee werkgroepen die uitersten van de neurowetenschappen vertegenwoordigen. Aan de ene was de groep Verhage, gevuld met DNA en eiwit onderzoekers, en aan de andere kant was daar de groep Spruijt gevuld met gedragsonderzoekers.
De onstuimige Verhage clan bestond onder andere Klaas-Jan (bedankt voor de eiwit-ondersteuning), Niki, Maja, Jildau en Elisabeth. Speciaal wil ik noemen: Ex-buurman en paranimf Ruud: in den beginne, toen ik als ontheemde Groninger door Utrecht zwierf, heb jij me de betere kanten van Utrecht laten zien. Onze tripjes naar Orleans (nooit meer met aangeschoten francaises in de auto…) en Miami (maar wel weer in een convertible…) waren onvergetelijk. Ex-huisgenoot Joost: zonde dat ons intra-netwerkje niet meer bestaat, maar wanneer gaan we dat bewustzijnsprobleem nou eindelijk eens aanpakken? En toekomstig buurvrouw Danielle; wat hebben wij allemaal wel niet besproken bij een sigaretje?

De gezellige Spruijt groep bestond onder andere uit Caroline, Jacob, Bart, Claudia en Ruud. En ex-kamergenoot Leon: schaaktegenstander en discussieopponent. Nu werkzaam in San Diego als surfleraar (o ja, en nog iets met wetenschap). Doe je de groeten aan Shelby?

Voor de gezelligheid had ik geen beter lab kunnen treffen. Vanaf het begin werd ik opgenomen in “de RMI-familie” en waren er vaak borrels en stapavonden. Vaste klanten (in goede zin van het woord) waren onder andere Ruud, Niki, Julia, Marten, Ceriel&Diane, Leon, Wouter&Dorien, Martijn, Robbert, Patrick&Marjan, Joost, Danielle, Bart, Elisabeth, Josien en vele anderen. Bedankt voor alle katers...

Een hele reeks kamergenoten zijn aan me voorbijgetrokken. Ik ben op een zijkamertje begonnen met Alex, en daarna verhuist naar een grotere kamer met Jacob, Leon, Lisette, wiens bureaus later werden ingenomen door Heidi, Leontien en Hans. Soms hadden we wat strubbelingen door mijn neiging papieren bouwwerken te maken, maar dat ging gelukkig altijd met een knipoog.

De RMI-band is een publiek geheim, maar alleen de bewakers weten hoe goed ze werkelijk zijn. Peter (lead guitar), Sander (keyboards), Robbert, (rhythm guitar&vocals), Wouter (guitar&bass&drums&vocals) en diverse wisselende bandleden: bedankt voor de leuke sessies.

Gelukkig ken ik ook nog mensen die niks met het RMI te maken en die je laten zien dat er meer is dan de wetenschap. Met “ouwe seunen” Eddie, Hans, Joldert en Paul heb ik niet alleen oude herinneringen opgehaald, maar ook nieuwe herinneringen gemaakt in allerlei exotische oorden. Zulke tripjes houden een mens gaande (of laten hem uitgeput neervallen). Waar gaan we de volgende keer naartoe? Mijn fototoestel jeukt. Doctor Mike, onze e-mailtjes zouden eigenlijk eens gepubliceerd moeten worden.

Begrip en liefde van je familie zijn onmisbaar. Al wonen jullie in het verre noorden, ik heb altijd het gevoel dat jullie vlak bij zijn. Mama, het onderzoek was allemaal echt niet zo eng als je denkt, en nu is het gelukkig eindelijk af. Papa en Rob, onze inspannende en ontspannende vistripjes had ik echt nodig om goed uit te blazen. Wonderlijk, wat de belofte van de zee met je doen. Marijn, de enige echte kapitein, volgende keer ga ik weer mee zeilen. Carina en Jeannette, mijn lieve zusjes, bedankt voor alles; gelukkig hebben jullie wat beters gedaan met je tijd dan ik. Jari en Robin, ik ben de trotste oom die er is.

Lieve Johanneke, je hebt wat met me te stellen gehad in de laatste maanden. Ik beloof dat ik van nu af aan op tijd naar bed zal gaan.

Robert

Curriculum vitae

The author of this thesis was born in Harlingen on 23 July 1969. After the elementary schools, Jan Lightart and Prof.Kohnstamm, he attended the Rijksscholengemeenschap Simon Vestdijk in Harlingen (VWO, 1979-1988). He started his study Biology at the University of Groningen in 1988 and graduated in 1994. During this period two research projects were conducted. The first was at the Department of Animal Physiology under supervision of Dr.E.A.van der Zee. His second research project was conducted partly at the Department of Animal Physiology under supervision of Dr.F.Sluyter and partly at Génétique, Neuogénétique et Comportement at the University V Rene Descartes in Paris under the supervision of Prof.dr.W.E.Crusio. After graduation, he performed his military duties at the Royal Army as a “medic”. In august 1996 he started the PhD study presented in this thesis at the Rudolf Magnus Institute for Neurosciences of the Utrecht University under the supervision of Prof.dr.W.H. Gispen, Prof.dr.B.M.Spruijt and Prof.dr.M.Verhage. In September 2001, he started as a post-doc at the Department of Neurosciences of the Erasmus University in Rotterdam under supervision of Prof.dr.C.I.de Zeeuw and dr.Y.Elgersma.