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.