Chapter 7

General Discussion

Summary and scope of the discussion

Learning and memory are processes essential to animal survival. At the cellular and molecular level, memory is mediated by modifications of synapse strength and structure [1]. Molecular mechanisms involved in learning and memory have been extensively investigated for plasticity in hippocampus, focusing on postsynaptic sites. However, hippocampal function is also shaped by presynaptic forms of short- and long-term plasticity that affect neurotransmitter release [82,293]. Two key mechanisms regulated by presynaptic plasticity are the vesicular release machinery and the presynaptic calcium concentration [294]. The main objective of this thesis was to examine synaptic molecular mechanisms at the basis of learning and memory in the hippocampus. In the first part of the thesis, we investigated molecular mechanisms underlying learning and memory in two inbred strains of mice known to differ in cognitive performance (**chapter 2**) and in FHM1 transgenic mice that exhibit increased neurotransmitter release due to gain-of-function mutations in the $Ca_V2.1$ (P/Q-type) calcium channel (**chapters 3-5**). In the second part of the thesis, we investigated transcriptional changes in the hippocampus during memory reconsolidation (**chapter 6**). Knowledge derived from the studies performed in this thesis showed that memory formation and adaptation may be affected by basal neurotransmission strength, dependent on protein expression, activity, or localization. In the following paragraphs the implications of these findings and future research perspectives will be discussed.

Importance of synaptic molecular composition for function and plasticity

Pre- and postsynaptic function are impaired DBA mice

Inbred mouse strains show diverse behavioral and cognitive phenotypes [95,96,135], with C57BL/6J (C57) mice performing well in hippocampus-dependent cognitive tasks compared with (memory impaired) strains, such as DBA/2J (DBA). These memory impairments have been associated with reduced capacity for the maintenance of LTP in hippocampus [95,147]. Altered presynaptic short-term plasticity has been observed in DBA mice [95]. Genetic background in inbred mouse strains has been shown to affect gene expression levels in different tissues [250] and hippocampal micro-RNA expression [295]. The differences between inbred strains at the transcript level further imply an effect of genetic background on the hippocampal proteome, affecting synaptic function. In **chapter 2** [296], we confirmed reduced hippocampal CA1 short-term plasticity in DBA compared with C57 mice,

linked to a reduced number of vesicles and reduced expression of exocytosis-related proteins. Decreased short-term plasticity has been associated with impaired memory in mice that exhibit normal LTP [58]. Although the presence of a presynaptic phenotype in DBA mice suggests a role in learning and memory, its relative contribution compared with postsynaptic proteins would need further testing. Memory impairment in DBA has been predominantly attributed to different levels of protein kinase (e.g., type C: PKC) activity in postsynaptic plasticity pathways [97-100]. We detected a reduced expression of PKC beta1 in DBA mice. PKCbeta1 knock-out mice display impaired memory in the fear conditioning task, while synaptic transmission, short-term plasticity and long-term potentiation are all normal [59]. Furthermore, treatment with piracetam-like drugs enhances PKC function and improves cognitive functioning in DBA mice [100,297]. Thus, it would be interesting to test how such treatments affect short-term plasticity in DBA mice, as PKC(beta) was recently reported to function as a presynaptic calcium sensor, important for presynaptic short-term plasticity [298].

Is glial function affected in DBA mice?

As part of tripartite synapses, astrocytes have a major function in regulating neurotransmitter metabolism and related processes [159,299]. One of the aspects not discussed in chapter 2, is the observed regulation (up: n = 2, down: n = 9) of mitochondrial/metabolic proteins, most (n = 7) of which were related to metabolic function. Low activities of malic enzyme have been reported in DBA mice compared with other strains [300], a key component of the TCA cycle that is linked to astrocyte glutamate metabolism [299]. As a possible compensatory mechanism we observed an upregulation of mitochondrial malic enzyme (*Me3*). The lower expression of glutamine synthetase (*Glul*) and reduction in enzymes for pyruvate (*Dlat*, *Pkm*) and glycolytic (*Gpi1*, *Pgk1*) metabolism and a shift from cytosolic to mitochondrial creatine kinase expression (*Ckb*, *Ckmt1b*) seem to provide further support for the notion that metabolic function may also be affected in DBA mice. In combination with the finding that almost a quarter of the regulated proteins (n = 10/41) was of glial origin, suggests metabolic dysfunction of this cell type in DBA mice. Deficits in brain astrocytic function have not been described, apart from an impairment in potassium and glutamate buffering related to seizure susceptibility in DBA mice [301].

The role of presynaptic enhanced calcium channels in plasticity and memory

Like the vesicle release machinery, voltage-gated calcium channels are another essential molecular component in regulation neurotransmitter release, and involved in short-term plasticity [51,52]. The class of $Ca_V2.1$ (P/Q-type) voltage-gated calcium channels play a dominant role in neurotransmitter release [52,106]. We therefore expected a role for these channels in learning and memory. Cognitive consequences of decreased expression and function of $Ca_V2.1$ channels have been described. Naturally occurring heterozygous mouse $Ca_V2.1$ mutants such as *leaner* and *Rolling Nagoya*, have progressive cerebellar ataxia and age-related spatial learning deficits (homozygosity for these mutations is lethal) [302–304]. Specific ablation of Cav2.1 channels from forebrain also impairs memory without affecting motor function [305]. However, a role of gain-of-function FHM1 mutations in the $Ca_V2.1$ channel in learning and memory had not been demonstrated.

Gain of $Ca_V2.1$ function enhances neurotransmission in the hippocampus

The effects of gain-of-function FHM1 R192Q and S218L missense mutations in the presynaptic $Ca_V2.1$ calcium channel have been investigated in the context of migraine, i.e., their effects on brain regions important for FHM1 symptoms, such as the cortex and cerebellum [109]. This line of research has shown that FHM1 mutations affect $Ca_V2.1$ channel kinetics, by increasing channel opening upon depolarization [110,111]. In the cortex, these changed kinetics enhance excitatory neurotransmission, whilst inhibitory neurotransmission is left intact [112,113,222]. Whether FHM1 mutations affect neurotransmission in a similar manner in the hippocampus, where a high expression of $Ca_V2.1$ channels has been observed [119], had not been described. **Chapter 3** [227] provides the first account of enhanced neurotransmission in the hippocampus due to FHM1 mutations. We observed enhanced responses (evoked field potentials) in the CA1 areas of the hippocampus, to stimulation of the anterior commissural pathway. Although we cannot conclude whether the enhanced responses result from a selective effect on excitatory neurons, as has been described for the cortex, the results seem to fit the notion that the FHM1 mutations increase glutamate release [109]. In the cortical synaptic proteome of R192Q mice, a compensatory upregulation of glutamate transporters has been observed [217]. However, in naïve FHM1 animals, expression changes in the cortex are minimal in the synaptic proteome (19 differentially expressed proteins, 10–28% change) and transcriptome (9 differentially expressed genes, 6–34% change) [217,248]. In

accordance, our analysis of the hippocampal transcriptome in **chapter 5** revealed minimal basal changes in expression.

Gain of $Ca_V2.1$ function affects synaptic plasticity and memory in the hippocampus

As enhanced glutamatergic signaling is thought to augment long-term plasticity and memory [61,306], we expected mice with increased $Ca_V2.1$ function to show enhanced plasticity and memory. Instead, we found that enhanced LTP was accompanied by impaired spatial and contextual fear memory in the FHM1 R192Q mutants, with LTD being unaffected (**chapters 3, 4**, [227]). Although hippocampal plasticity could not be measured in S218L mice, because any conditioning stimulus triggered a spreading depression, one may speculate that this severe mutation further augments LTP as a more severe impairment in the same memory tasks was found. Since its discovery, LTP in the hippocampus has been the leading model for the synaptic mechanisms underlying memory formation [40]. Whereas substantial evidence exists in literature for a positive association between hippocampus-dependent memory, LTP and molecular mechanisms involved (reviewed in [307]), an inverse association between LTP and memory has also been reported for a number of mutants [306]. Interestingly, this inverse association has also been reported in a mouse model of 22q11 microdeletion (DiGeorge) syndrome, which shows a progressive presynaptic phenotype with enhanced neurotransmitter release and slower Ca^{2+} decay times [308]. Our findings in FHM1 mutants suggest that the enhanced early-LTP, a phase that lasts for 4–6 hours [309], leads to impaired short-term memory (2-hour contextual fear). Additional experiments would be required to determine whether other forms of plasticity are affected in the hippocampus of FHM1 mice. Working memory deficits in S218L animals seemed to point to changes in hippocampal short-term presynaptic plasticity, which may be measured by paired-pulse facilitation. The observed long-term memory impairments indicate that the early-LTP effect may have an impact on late-LTP (24 hours).

How is impaired memory related to enhanced LTP in FHM1 mice?

A possible interpretation of the memory impairment FHM1 mutants is that the enhanced LTP in the hippocampus indicates reduced quality of memory consolidation. This may be linked to a shift in LTP induction, resulting in lower a signal-to-noise ratio (Figure 1A). In our experiments, high-frequency stimulation (100 Hz) induced LTP to a different extent in wild-type and FHM1 mice. This could be specific to high-frequency stimulation (Figure 1B, dashed grey line), or it is

possible that the enhanced neurotransmission induces LTP in FHM1 mice at frequencies that do not produce strengthening in wild-type mice (Figure 1B, solid grey line). This may be assessed using LTP-stimulation protocols covering a wider range of stimulations, including those that induce physiologically relevant forms of plasticity. The LTP in field potentials measured in our experiments indicate an enhanced strengthening response of the (local) hippocampal network. This suggests that the FHM1 mutants potentially obtain activation in a higher number of synapses and/or cells during memory consolidation, compared with wild-type mice. A gain in synaptic efficacy (LTP) may not necessarily exert beneficial effects on memory when a high number of cells are activated [310,311] in view of the sparse coding for associative memories. During normal learning a small (2–4%) subset of connected hippocampal neurons is thought to be involved in memory storage [312-314]. A putative mechanism for the disruption by increased LTP in FHM1 mutants, would be that such a set of cells that is memory-specific, is less easily obtained when strengthening (LTP) is more widely spread, rather than limited to a few connections. This specific connection strengthening appears critical for the hippocampus to function as an effective memory system, requiring bidirectional modification of strength by both LTP and LTD [315]. In other words, it is likely that if LTP (signal-plus-noise) is increased, this would need to be matched by an increase in LTD (decreasing the weaker, "noisy" connections) to maintain a proper signal-to-noise ratio to finally achieve memory storage. The spatial aspect of memory is represented by organized, specific activity in hippocampal place cells [13,316], and may be sensitive to imbalances in the signal-to-noise ratio. Disruption of place cell activity, with increased noise in and reduced stability of spatial representations has been observed in nociceptin-receptor knockout mice that show enhanced LTP [209]. The enhanced LTP in R192Q mice may indicate a disruption in signal-to-noise ratio that disturbs, rather than increases, the capacity for learning and memory.

Behavioral abnormalities associated with gain of $Ca_V2.1$ function: to what extent is hippocampal dysfunction involved?

In **chapters 3, 4** we demonstrated cognitive deficits in both FHM1 mutant strains, with additional behavioral abnormalities in homozygous S218L mutants, such as decreased anxiety-like behavior. The cognitive deficits appeared in tasks that rely on intact hippocampal function, i.e., the Morris water maze and contextual fear conditioning. However, memory in these tasks may also be affected by interaction with other brain regions.

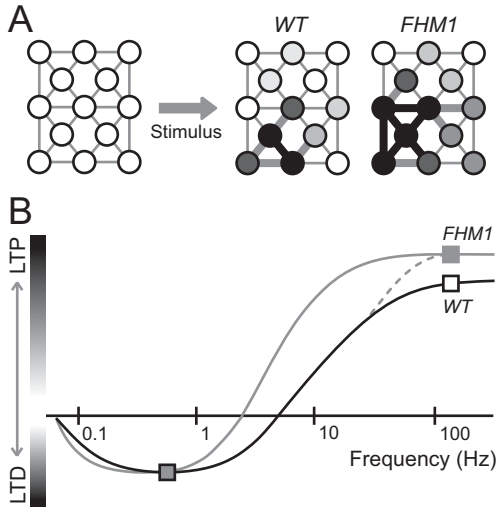

Figure 1: Degree of activation augments LTP in FHM1 mutants. (**A**) Hypothetical network in the hippocampus. Circles indicate neurons, lines the connections between cells. Responses to a stimulus, e.g., a learning task, are putatively stronger in FHM1 compared with wild-type animals (strength of the response indicated by fill colour). More connections may become potentiated (thick lines). (**B**) As a result of the enhanced neurotransmission in FHM1 mutants, LTP induction at different frequencies might be shifted. The boxes indicate the LTP and LTD measured for FHM1 mutants [227]. Black line indicates induction of plasticity in wild-type (WT) animals. In FHM1 mutants the plasticity may either be specifically shifted in the highest frequencies (dashed grey line) or may show an additional left-shift throughout the frequency range (solid grey line).

Processing of fear memory and its contextual component depends on interaction of the hippocampus with a circuit of brain structures, such as the amygdala, prefrontal cortex and thalamus [32]. Disturbed function of these structures in FHM1 mutant mice may also affect cognitive performance. The behavioral phenotype (reduced anxiety-like behavior) in S218L mutants suggests that pathways of fear expression, e.g., the interaction of hippocampus and amygdala or thalamus, may be affected. Furthermore, migraine research has shown enhanced cortical transmission and modulation of (trigeminal) signals in the thalamus of FHM1 mutants [109]. These brain structures are important for processing the sensory information during learning, with an additional role for the cortex as a site for systems consolidation, i.e., remote memory storage [35,317]. Altogether, these data suggest that dysfunction in multiple brain regions in FHM1 mutant mice may aggravate the cognitive phenotype. The relative contribution of dysfunction in hippocampal versus other brain regions in these mutants to memory deficits may be elucidated using targeted testing strategies. This could be obtained by testing fear memory deficits in mice with expression of the FHM1 mutations restricted to the hippocampus. Or reversely, by examining whether local intra-hippocampal delivery of drugs or blockers that specifically modulate $Ca_V2.1$ function [318,319] may rescue memory deficits in FHM1 mutants.

What is the link between migraine and cognitive dysfunction?

The finding of cognitive dysfunction in FHM1 mutants raises the question how this relates back to migraine. A few studies report cognitive dysfunction in FHM1 patients [191–194], with two types of mutations (T666M and V518L), both associated with progressive cognitive dysfunction in some cases [192,194]. However, definite evidence in support of cognitive deficits in "common" migraine outside attack periods is lacking. It seems that the dysfunction is more clearly linked to FHM mutations, possibly as a result of a more profound increase in excitatory neurotransmission. In FHM2 that is caused by loss-of-function mutations in the *ATP1A2* gene seem to affect glutamate clearance [109]. In heterozygous *Atp1a2* knockout mice spatial learning is impaired; in addition these mice show increased anxiety-related behavior and reduced locomotor activity [320]. Comorbid cognitive deficits have also been established in other disorders with a disruption of the inhibitory/excitatory balance in neurotransmission, such as epilepsy [321].

Another shared factor between migraine and cognition seems to be stress, which is the most frequent reported trigger for migraine [237,322]. The FHM1 mutants showed a more impaired performance in memory tests that involve a higher degree of stress. As discussed above, this may reflect additional dysfunction in other brain areas. However, the memory deficits may also result from a direct effect of glucocorticoid stress hormones such as corticosterone on hippocampal function. In wild-type animals, glucocorticoids and excitatory neurotransmitters exert a biphasic effect on synaptic function and certain types of memory, i.e., low to moderate levels of both mediators may enhance, whereas higher levels or chronic, moderate increases of both may impair memory [239]. In the FHM1 mutants the enhanced hippocampal excitatory transmission [227] may cause a shift in this biphasic curve, negatively affecting performance in stressful cognitive tasks. In the cortex of FHM1 mutants a higher sensitivity to disruption of neurotransmission by corticosterone has been reported, although an effect of natural (restraint) stress is not apparent [323].

What mechanisms are involved in regulation of memory modification during reconsolidation?

The importance of regulation of synaptic function becomes apparent from changes in molecular mechanisms during memory reconsolidation. Synaptic strength and content of memories are modified during retrieval and subsequent reconsolidation. Retrieval renders recently consolidated memories labile by inducing protein degradation [26], requiring reconsolidation to restabilize them [256]. The molecular, and in particular the transcriptional, mechanisms underlying reconsolidation have not been comprehensively described. In **chapter 6**, we reported a temporal profile of differential transcription during reconsolidation in the hippocampus. Reconsolidation of fear memory follows a pattern of initial induction (at 30 minutes) of expression of transcription factor genes. The observed changes in ion channel expression that follow (6 hours), likely affect synaptic strength.

In addition to regulation of transcription and protein expression during the initial phase of reconsolidation, AMPA receptor endocytosis is required for modulation of memory strength and content [29,30]. Blockade of AMPA receptor endocytosis using an inhibitory peptide enhances fear memory and reduces the plasticity in synaptic strength during reconsolidation [29]. This subtle perturbation may disrupt molecular mechanisms important for reconsolidation, but may also stimulate feedback mechanisms for receptor endocytosis that are normally not involved (e.g., micro-RNA 207).

Our data reflect global transcriptomal changes in the hippocampus, as a next step it would be interesting to focus on subregions of the hippocampus. Moreover, changes that would truly be at the basis of consolidation and reconsolidation can only be found when specifically selecting neurons involved in the memory engram. This may be helped by combining techniques such as histology or laser dissection of cells, with emerging genetic strategies to label activated cells [313]. Studies on the effect of changed neuronal transmission on plasticity in connected neuronal subpopulations, such as the memory engram cells, may further our understanding of the origin of memory deficits in disorders with imbalanced inhibitory/excitatory transmission.

Conclusions

Historically the postsynaptic compartment has been the main focus of attention as a locus for synaptic plasticity associated with persistent forms of memory, involving synaptic strengthening by LTP, whereas the presynaptic compartment has been mostly associated with short-term memory. The view that presynaptic plasticity mechanisms and the interaction with the postsynaptic compartment may be as important, has only started to gain more ground in recent years [78,82,324,325].

In this thesis I have demonstrated that genetic alterations in molecular function, that in turn affect the degree of neurotransmission, result in altered associative learning and memory. This demonstrates the importance of the (pre)synaptic molecular machinery in synaptic plasticity. Genetic background may affect pre- and postsynaptic protein expression important for short-term and long term plasticity (DBA inbred mice). Furthermore increased activity-dependent neurotransmitter release and plasticity may be associated with memory deficits (FHM1 mutant mice). Although a memory deficit in combination with enhanced LTP may seem paradoxical, it emphasizes that the relation between neurotransmission and memory does not simply follow a linear relationship. The changed LTP may indicate alteration of the signal-to-noise ratio and number of cells participating in a memory engram, both important for memory formation. The functional and molecular studies on inbred and mutant mouse strains described in this thesis were performed with naïve animals. It has been suggested that studies focusing on synaptic changes during or after a cognitive challenge, may provide better characterization of functional and molecular mechanisms supporting memory formation [326]. For example, by investigating differences in molecular mechanisms engaged by fear conditioning, in either the DBA strain or FHM1 mutants. In the latter case, we detected minimal molecular (transcriptome) changes in a naïve state, emphasizing that a hippocampus-dependent learning challenge may further elucidate molecular and/or functional mechanisms affected by this mutation. Furthermore, molecularly increasing postsynaptic signaling by blockade of AMPA receptor endocytosis disrupts plasticity necessary for reconsolidation of aversive memory. Although this signaling is essential to modify the memory strength and content, only minimal effects on transcription were detected in comparison with regular aversive memory reconsolidation. Changes related to protein synthesis (e.g., local translation at the synapse) and modification (e.g., phosphorylation) may be more important for memory reconsolidation.