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I. Summary of results

The objective of this thesis was to study the cellular processes that underlie the adaptation to changes in a familiar environment or, from a wider perspective, the processes that underlie the flexibility of our memory capacity) and compare them with the cellular processes involved in the formation of new memories. This question was approached at the behavioral as well as molecular level. To study this, behavioral as well as genetic approaches were used with emphasis on the balance between protein kinases and protein phosphatases that have opposite catalytic activities.

In chapter 2, we compared changes in hippocampal CaN enzyme activity, protein levels and region specific expression induced by training and reversal training in the Y maze. We found that training in the Y maze induced decreases in hippocampal CaN activity. These activity levels were restored during reversal training. This restoration was reversal-training-specific, because it did not reflect normal restoration of basal levels unrelated to subsequent learning. Hippocampal protein levels for the catalytic subunit of CaN were reduced during the early phase of training, but returned to the original level during the early phase of reversal training. CaN expression for the catalytic subunit was enhanced in area CA1 and CA3 specifically after reversal training. These findings show that memory formation is accompanied by reduced CaN activity, whereas adapting to changes in a familiar environment is accompanied by restored CaN activity with a specific function of CaN in the hippocampal subregions CA1 and CA3.

In chapter 3A, we used a transgenic approach to investigate whether reducing CaN activity in the forebrain affected the rate of acquisition during training or reversal training. Training in the Y maze was not affected by reduced CaN activity levels. In contrast, reducing forebrain CaN activity facilitated reversal training. These findings suggest that in case of Y-maze learning, the impact of reduced forebrain CaN activity is reversal learning specific.

In chapter 3B, the effect of reduced forebrain CaN activity on memory formation and memory adaptation to changes in a familiar environment was studied using a contextual fear conditioning paradigm. Reducing CaN activity facilitated consolidation of contextual fear. However, the extinction rate of previously established contextual fear memory was attenuated. These data imply that in case of contextual fear conditioning, CaN plays an opposite role in fear conditioning and extinction of previously formed fear memories.

Chapter 4 describes a study in which the role of PKA in learning and reversal learning was assessed. Elevated PKA expression in areas CA3 and DG during the acquisition phase of training and reversal training correlated positively with behavioral performance. PKA immunoreactivity was similarly enhanced in area CA1 during the acquisition phase of reversal training, but did not correlate with behavioral performance. No changes were found in the subiculum. In addition, we found that AMPA receptor phosphorylation was enhanced at the GluR1-S845 site during the acquisition phase of reversal training. These findings provide evidence that training and reversal training induce region-specific changes in hippocampal PKA expression. Moreover, they suggest a specific involvement of area CA1 in the detection of changes in a familiar environment and a specific function of the GluR1-S845 site in the detection of these changes.
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In **chapter 5**, we explored whether training and reversal training changed CaN, PKA and GluR1 protein levels as well as AMPA receptor phosphorylation at the S845 site in the striatum. We did not find any training-induced changes in PKA and CaN protein levels in the striatum. Likewise, no changes were found in the phosphorylation levels of GluR1-S845 site, which indicates that PKA and CaN signaling in the striatum is not affected by learning and reversal learning in the Y maze.

In **chapter 6**, experiments were conducted to examine the effect of sleep loss on AMPA receptor functioning in the hippocampus. Sleep deprivation (SD) gradually decreased AMPA receptor phosphorylation at the S845 site. PKA and CaN protein levels were not affected by sleep loss, however protein levels of the anchoring protein AKAP150 were decreased due to SD. The SD-mediated decrease in AKAP150 protein levels could therefore diminish PKA-dependent S845 phosphorylation. Since plasma levels of the stress hormone corticosterone were not elevated after SD, the decrease in AMPA receptor phosphorylation is due to sleep loss rather than a consequence of stress.

In **chapter 7**, we applied 5h of SD directly after each training session and / or reversal training session and investigated whether the performance was affected by loss of sleep. While SD did not directly have an effect on training or reversal training, SD applied during training decreased the performance during reversal training (regardless of SD during reversal training). Next, we assessed if SD applied during training affected the expression of the immediate early genes c-Fos and Zif268, as well as the phosphorylation of P44/42 MAPK in the hippocampus. Training enhanced the expression of Zif268 in area CA1 and DG of the dorsal hippocampus. Likewise, c-fos expression and P44/42 MAPK phosphorylation was enhanced in the DG of the dorsal hippocampus. Remarkably, SD deteriorated the training-induced increase in P44/42 MAPK activation, but not the expression of hippocampal c-fos or Zif268. Together, these data show that loss of sleep affects behavioral flexibility. Furthermore, these data show that the MAPK pathway in the hippocampus is affected by SD.

The experiments in **chapter 8** were applied to further explore the role of sleep and circadian systems in memory processing. Mice lacking the Cry1 and Cry2 genes (known to have a disturbed sleeping pattern) were tested in the Y-maze. Behavioral performance during training and reversal training was not affected by the loss of both Cry genes (e.g. see figure 3 chapter 8). These data show that disturbed sleeping patterns, as a consequence of the loss of both Cry genes does not deteriorate the consolidation during learning and reversal learning.
2. Calcineurin and memory formation

Our Y-maze experiments revealed that training induces a decrease in CaN activity that could facilitate actions of protein kinases, while at the end of reversal training, CaN activity was restored to baseline levels (Chapter 2). To examine whether the changes in hippocampal CaN activity induced by training and reversal training were Y-maze specific, an additional experiment was conducted using the Morris water maze as behavioral paradigm. Mice were trained to locate a submerged (hidden) platform that placed at a fixed location. After several days of training, the platform was relocated to a novel position (reversal training). Learning curves are shown in figure 1A and 1B. At the end of the training, a trend for a decrease in CaN activity levels in the cytosol fraction was found ($P = 0.1$). After the reversal training, activity levels were significantly increased compared to the levels found after training ($P < 0.01$) and slightly but not significantly enhanced compared to the control group (Fig. 1C). These results suggest that spatial learning and reversal learning in the water maze induce similar effects on hippocampal CaN activity as Y-maze learning, although the effects are less prominent. In line with the hypothesis that training in a hippocampus-dependent learning paradigm reduces CaN function, Monti and co-workers (2005) showed that training in a contextual fear conditioning paradigm also decreased hippocampal CaN protein levels. Yet, no studies have been conducted to examine hippocampal CaN after extinction training in a contextual fear conditioning test. Overall these studies indicate that the formation of new memories is accompanied by reduced hippocampal CaN function, while the adaptation to changes in a familiar environment is paralleled by restored CaN function (see table 1A).

It should be noted that a further reduction of CaN activity levels in the hippocampus by means of the expression of a CaN inhibitor facilitates learning the location of a submerged platform in the Morris water maze as well as the formation of an association between a context and an electrical shock in a contextual fear conditioning paradigm. In contrast, this further reduction in CaN activity in the hippocampus does not enhance learning the location of a food reward during Y-maze training (see table 1B). Apparently, the remaining fraction of CaN that is not suppressed in wildtype animals during Y-maze training does not seem to function as a constraint on the formation of a memory for the location of the food reward during Y-maze training.

3. Reversal learning enhances the expression of the regulatory subunit of CaN

As described in the previous paragraph, reversal learning was accompanied by restored CaN activity levels in the hippocampus. In addition to this, CaN immunoreactivity (CaN-ir) for the catalytic subunit was enhanced in area CA1 and CA3 at the end of reversal training (chapter 2), suggesting a specific function of CaN in relation to reversal learning in these two regions. Since CaN consists of both catalytic and regulatory subunits, the question remained whether we could also detect these enhancements in immunoreactivity for the regulatory subunit. We quantified CaN immunoreactivity for the regulatory subunit (CaNB-ir) in area CA1...
and CA3 of the dorsal hippocampus. As depicted in figure 2, similar to the catalytic subunit of CaN, expression for the regulatory subunit was enhanced in the CA3 and CA1 cell bodies, but not in dendrites after reversal training in the Y-maze. Together, these data lend evidence to the notion that reversal training specifically enhances immunoreactivity for both CaN subunits, but with differential subcellular effects on the catalytic and regulatory subunit. Future studies, examining CaN activity in these hippocampal subregions after training and reversal training should clarify whether the enhanced immunoreactivity levels indeed represent an increase in CaN enzyme activity. Such an increase might be needed to restore the AMPA receptor phosphorylation levels that were enhanced during the acquisition phase of reversal learning (Chapter 4, see also figure 3).

Figure 1. Hippocampal CaN activity is differentially affected by training and reversal training in the Morris water maze. Two groups of mice were trained consisting of three trials per day in the Morris water maze. The T-group and RT-group received 6 days of training. With ongoing training both groups gradually decreased their latency times indicating they learned to location of the platform (ANOVA $F_{17.221} = 2.966$ $P < 0.001$) (A). After training, the RT-group received 2 days of reversal training with the platform positioned in the opposite quadrant. After an initial increase latency times were reduced again, indicating that mice learned the new location of the platform (ANOVA $F_{5.35} = P < 0.05$) (B). A trend for a decrease in CaN activity was found after training ($P = 0.1$), however after reversal training, levels were restored to baseline levels and significantly enhanced compared to the levels found after training ($P < 0.01$ compared to T-group) (C). # $P = 0.01$, ** $P <0.01$.

4. Other brain areas involved in behavioral flexibility

In chapter 3A and 3B, we showed that reducing CaN activity in forebrain neurons of transgenic mice via the expression of a CaN-inhibitor had opposing effects on reversal learning in the Y maze and extinction learning in the contextual fear conditioning paradigm (see
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Table 1A. Behaviorally-induced changes hippocampal CaN

<table>
<thead>
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<th></th>
<th>Y maze</th>
<th>water maze</th>
<th>fear conditioning</th>
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<tbody>
<tr>
<td>Training</td>
<td>↓1</td>
<td>↓2</td>
<td>↓3</td>
</tr>
<tr>
<td>Reversal training / Extinction training</td>
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Table 1B. Effect of reduced forebrain CaN activity on behavioral performance

<table>
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<th>water maze</th>
<th>fear conditioning</th>
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</thead>
<tbody>
<tr>
<td>Training</td>
<td>↓4</td>
<td>↑5</td>
<td>↑6</td>
</tr>
<tr>
<td>Reversal training / Extinction training</td>
<td>↑4</td>
<td>↑5</td>
<td>↓6</td>
</tr>
</tbody>
</table>

1: Havekes et al. (chapter 2)
2: Havekes et al. (chapter 9)
3: Monti et al. 2001, Hippocampus
4: Havekes et al. (chapter 3A)
5: Malleret et al. 2001, Cell
6: Havekes et al. (chapter 3B)

In this section, we discuss the involvement of two additional brain regions that could explain the opposing results found in both learning paradigms.

4.1. The amygdala
The amygdala is a complex set of interconnected nuclei that are essential for various functions including negative emotions such as fear (LeDoux, 2000) as well as positive emotions (Baxter and Murray, 2002). It has been shown that lesions of the amygdala affect appetitive conditioning (Balleine and Killcross, 2006) and can cause reversal learning impairments in reward-motivated behavioral paradigms (Eleftheriou et al., 1972; Elias et al., 1973; McDonald et al., 2004). Furthermore, stimulating PKA activity in the amygdala has shown to facilitate reward-related learning (Jentsch et al., 2002). Based on these findings, reducing CaN activity in the amygdala could favor PKA activity and as a consequence facilitate reversal learning in the Y-maze as was shown in Chapter 3A.

Contextual fear conditioning relies on communication between hippocampus and several nuclei of the amygdala. The amygdala generates the output leading to an emotional response. In case of tone-cued fear conditioning, pharmacological inhibition of CaN activity in the amygdala was shown to impair fear extinction (Lin et al., 2003). Since the amygdala (although through different nuclei) plays a crucial role in both tone-cued and contextual fear extinction, the loss of previously established contextual fear (chapter 3B) might require CaN activity in the amygdala.
4.2. The medial prefrontal cortex

The prefrontal cortex is generally seen as one of the brain structures crucial for learning new behavioral responses, while inhibiting the execution of previously learned responses (Owen et al., 1990, 1993; Dias et al., 1996, 1997). Based on neuronal activity recordings Milad and Quirk, (2002) showed that extinction of tone-cued fear memories potentiated neuronal activity in structures that participate in the inhibition of a previously acquired response. Furthermore, facilitation of neuronal activity in the medial prefrontal cortex (mPFC) strengthens extinction (for review, Quirk et al., 2006). In line with these studies, it can be expected that facilitation of neuronal activity through the reduction of CaN activity would enhance extinction of contextual fear. We found however that reducing CaN inhibited the extinction of contextual fear (Chapter 3B), making it unlikely that the attenuated rate of extinction was due reduced CaN activity levels in the medial prefrontal cortex.

In case of reversal learning in the Y-maze, the medial prefrontal cortex could have played an important role. Lesions of the mPFC were shown to impair reversal learning in the spatial version of the water maze (de Bruin et al., 1994) and in a spatial discrimination task (Salazar et al., 2004). Therefore, facilitating prefrontal cortex activity (by reducing CaN activity) could as a consequence result in an increased performance during reversal training in the Y maze (chapter 3A).
Figure 3. Training and reversal training have subregion specific effects on hippocampal PKA and CaN function as well as AMPA receptor phosphorylation. An schematic overview of the results from chapter 2 and chapter 4. 1) Hippocampal CaN activity was reduced after training, but restored after reversal training. 2) CaN expression was facilitated at the end of reversal training. 3) PKA expression in area CA3 and the dentate gyrus was enhanced during the acquisition phase of both training and reversal learning. 4 + 5 ) PKA expression in area CA1 as well as AMPA receptor phosphorylation at the S845 site was enhanced selectively during the acquisition phase of reversal learning. Note that the results from chapter 7 are not included in the overview since Zif268 expression, c-Fos expression and MAPK phosphorylation was only assessed at the end of training.

5. Hippocampus and striatum-dependent strategies in the Y maze

The processing of information, encoded as neuronal signals goes via parallel brain systems that all have access to the same information about situations in which learning occurs. Two of these major systems are essential for spatially organized behavior (although in different ways): the hippocampal system and (dorsal) striatal system. The hippocampal system provides a map-like representation of the environment that is flexibly applicable and can be used to quickly provide a behavioral adaptive response to environmental changes. It is important for spatial learning through locating a particular goal in space based on distal cues). In contrast, the striatal system generates a more route-following or cue-approach behaviors (e.g. making responses based on their own body orientation in space also known as egocentric learning), that are rather inflexible (O’Keefe and Nadel, 1978).

Packard and McGaugh (1996) trained rats to find food in a modified T maze (Greek cross maze, for details see introduction chapter 5), by making a consistently reinforced right turn at the choice point (e.g. rats always started from the same position, and were rewarded when they choose the right arm but not the left arm). They showed that during the initial phase of learning, rats tended to use a hippocampus-dependent strategy (spatial learning), rather than a striatum-dependent strategy (response learning). However, with extended training, rats switched from using a strategy requiring the hippocampus to a strategy requiring the striatum. Similar results (e.g. switches from a hippocampus- to a striatum-dependent strategy with prolonged training) have been obtained in humans (Hartley et al., 2003; Orban et al., 2006).
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Several findings described in this thesis suggest that in our Y-maze paradigm, mice used a hippocampal strategy rather than a striatum-dependent strategy to locate the baited arm. We showed that Y-maze training: 1) reduced hippocampal CaN activity and protein levels (chapter 2), 2) enhanced hippocampal PKA expression (chapter 4), 3) increased the phosphorylation of P44/42 MAPK in the hippocampus (chapter 7), and 4) enhanced the protein expression of the immediate early genes c-Fos and Zif268 in the hippocampus (chapter 7). In contrast, training in the Y maze did not induce any changes in CaN, PKA and GluR1 protein expression or alterations in S845 phosphorylation in the striatum (chapter 5). In line with our findings, Packard and McGaugh (1996) showed that after 8 days of training in a T-maze reference task, rats still tended to use a hippocampus-dependent strategy, rather than a striatum-dependent strategy. Only in case of extended training (in their case 16 days, which is far more than the number of training days that we used in our case) rats tended to switch to a striatum dependent strategy.

Both systems can be cooperative in the sense that loss of one system can be compensated by the other. Neural inactivation of the hippocampus facilitated the use of a response strategy, while inactivation of the striatum facilitated the use of a spatial strategy (Packard and McGaugh, 1996). Likewise, in case of Huntington's disease that is characterized by the atrophy of the striatum (Huntington study group, 1996), it has been suggested that the hippocampus could partly compensate for loss of striatal function (Voermans et al., 2004). Also non-pathological factors can affect the learning strategy used. Recently, Korol and colleagues (2004) showed that depending on the phase of the reproductive cycle, female rats tend to use a spatial or response strategy, leading to the same performance in a reference T-maze task.

Loss of sleep is known to specifically affect hippocampal functioning (Campbell et al., 2002; Davis et al., 2003; McDermott et al., 2003; McDermott et al., 2006; Kopp et al., 2007; chapter 6). SD impairs performance in learning paradigms that require the intact hippocampus, while leaving hippocampus-independent memory formation undisturbed (Graves et al., 2003; McDermott et al., 2003). Hippocampal MAPK-signaling was enhanced by Y-maze training. However, hippocampal MAPK-signaling remained at baseline levels when training was combined with SD (Guan et al., 2004; chapter 7). Although hippocampal MAPK activity was impaired by SD, performance during training was not decreased due to SD (Chapter 7). One explanation for the lack of decreased performance as a consequence of SD may be that other brain systems, like the striatal system could have compensated for the dysfunctional hippocampus, leading to the same behavioral response during training. A good indication that mice receiving SD indeed used a strategy requiring the striatum, rather than a spatial strategy, came from the notion that mice receiving SD during training performed worse during reversal training (although they were not sleep deprived during reversal training). In other words, mice that received SD during training were less flexible and had more difficulties with adapting their behavioral response to the novel situation compared with non-sleep deprived controls. This is in line with the general notion that striatal system generates more route-like or cue-approach behaviors, that are rather inflexible (O'Keefe and Nadel, 1978). Analysis of the striatal system of mice that received training with and without SD should clarify whether SD indeed facilitates the use of the striatum system.
6. Disturbed sleeping patterns in contrast to loss of sleep does not affect behavioral performance in the Y-maze

Sleep is important for consolidation (formation) of memory by processing information acquired while awake (Stickgold, 2005). As reported in chapter 7, and discussed in the paragraph above, SD affected behavioral performance in the Y maze. In chapter 8 of this thesis, we tested knockout mice that have disturbed sleeping patterns due to the loss of the genes Cry1 and Cry2 (Wisor et al., 2002) in our Y-maze learning paradigm. In contrast to sleep deprivation, disturbed sleeping patterns (in case of Cry1, Cry2 double knockout mice: spending more time in non-REM sleep with higher EEG delta power; Wisor et al., 2002) did not affect behavioral performance in the Y-maze. Thus, in contrast to sleep deprivation, disturbed sleeping patterns do not affect behavioral performance in the Y-maze learning task.

7. Re-reversal learning

In various chapters in this thesis we examined the mechanisms underlying reversal learning. The question remaining unanswered was whether reversal learning reflected new learning, or whether it was just a temporary inhibition of the originally learned behavioral response. If reversal learning reflected a temporal inhibition of the originally learned response, than one would expect that the rate of acquisition during a second reversal training (in which the food reward was relocated back to the originally baited arm) would be higher.

In figure 4, the behavioral performance during training, reversal training and re-reversal training is shown. Strikingly, rates of acquisition during reversal training and re-reversal training were similar (repeated measures ANOVA for acquisition rates: \( F_{1,6} = 0.001 \) \( P > 0.9 \), Fig 4). These data show that although the food reward is relocated to the original arm during the re-reversal training, mice cannot easily re-adapt to the original situation. Moreover, these findings suggest that reversal learning is not simply a temporary inhibition of the originally learned behavioral response that can easily be overcome.

![Figure 4. Performance of young in the Y maze during training, reversal training and re-reversal training. The percentage of correct trials per session is shown.](image-url)
8. Aging-related impairments in reversal learning and future perspectives

Clinically it is widely acknowledged that loss of flexibility (e.g. the inability to adapt to changes) is one of the first hallmarks of non-pathological ageing and the development of major dementing illnesses like Alzheimer’s disease (Lafleche and Albert, 1995; Marschner et al., 1995). Also in rodents, the mechanisms underlying the ability to adapt to changes is the first to be affected by ageing (Means and Holsten, 1992; McMonagle-Strucko and Fanelli, 1993; Rahner-Welsch et al., 1995). To test whether we could assess ageing-related deficits in reversal learning in our paradigm, we trained young (3 months old) and aged C57Bl mice (24 months old) in our paradigm. As shown in figure 5, performance of the young and aged mice was similar during training. However, performance during reversal training was clearly affected in the aged mice. The question is at which organizational level the aging-induced problems occur. For instance, do aged mice tend to use a less flexible striatum-dependent strategy since their hippocampal system is impaired? Or do problems occur at specific regions within for instance the hippocampus: is the hippocampal comparator function deteriorated? Problems might also occur at the cellular level: is the balance in activity between protein kinases and phosphatases disturbed due to ageing facilitating routine-like behaviour? Future studies will have to try to answer these aging-related questions. In addition, studies with emphasis on the amygdala and prefrontal cortex function should give further insight into the importance of these two brain regions in memory flexibility. One of the most important starting points is to have a learning paradigm that is suitable to study the memory flexibility. As shown in this thesis, the symmetrical Y-maze proved to be a sensitive learning test. It would be worthwhile to use the Y-maze paradigm for these studies, since it is a robust and simple learning task ideal to study the mechanisms that are involved in the adaptation memories to match with changes in a familiar environment or situation.

Figure 5. Performance of young (n=7) and aged mice (n=8) in the Y maze during training and reversal training. Both groups gradually learned to locate the baited arm (ANOVA $F_{6,78} = 8.880 \, P < 0.001$). Ageing did not affect the rate of acquisition during training (ANOVA $F < 1$). Although both young and aged mice both improved their performance during reversal training (ANOVA $F_{6,78} = 22.564 \, P < 0.001$), rate of acquisition during reversal training was reduced in aged mice (ANOVA $F_{1,13} = 8.208 \, P < 0.05$).
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