A time to remember

Biemans, Barbara Agatha Maria

Chapter 9

General Discussion

This thesis has described experiments in rats and mice, carried out to elucidate the role of the circadian system in learning and memory processes. The most important results and conclusions are recapitulated here, and discussed in a more general framework.

Contents

I. SUMMARY

The impressive work of Holloway and Wansley in the 1970's, however convincing in demonstrating periodic memory deficits, has left two important questions unanswered, both concerning the endogenous nature of the periodic memory deficits. Circadian rhythms in behavioural processes are considered endogenous when they persist in continuous environmental conditions. The Holloway - Wansley work was all done in the presence of a light dark cycle, and it never became clear whether the periodicity in retention was based on an endogenous clock or contingent on external light cues. In the study described in chapter 2, we showed that memory deficits are present at 18 and 30 hrs after active shock avoidance (ASA) training, in rats trained and tested under constant dim red light (DD). This demonstrates that the phenomenon does not depend on light-dark information, and suggests that it is controlled or driven directly or indirectly by the circadian system. The other issue relates to the notion that true periodicity should be demonstrable within one individual, and at least for a few cycles. This particular point was proven in rats (again trained and tested under DD) repeatedly tested for their retention in a passive shock avoidance task (chapter 6). Individual rats displayed greater inhibition to enter a dark compartment (where they had previously been shocked) at 24 h intervals after the footshock than they did at non-24 h intervals.

Ascribing the periodic memory deficits -at least partly- to the circadian system, raises the question which neuropeptide or neurotransmitter systems in the suprachiasmatic nucleus (SCN), the main circadian pacemaker, may play a role. Our focus was directed to SCN vasopressin (AVP), because this is a major output system, and on the cholinergic system for its intimate involvement in learning and memory. AVP is possibly involved in the generation of memory fluctuations, since Brattleboro rats lacking the neuropeptide, did not display a memory deficit 18 hrs after ASA training in contrast to the Long Evans control strain (chapter 2). The impact of this stressful learning task on SCN-AVP was studied in a line of house mice with high basal AVP levels (chapter 3). An immediate drop in the number of stainable AVP neurones and content (measured by optical density) was found 20 minutes after an ASA session. Twenty-four hrs and another ASA session later, AVP immunoreactivity was still low compared to naïve controls. Examination of the time course of changes in SCN-AVP levels after a single ASA session revealed that AVP is apparently replenished between the moment of the acute effect and the 24 h time point, at 12 hrs post-training. This is again the case at 36 hrs post-training, between the 24 h and 48 h time point at which AVP was also decreased compared to control mice. In rats, however, no significant reduction in AVP levels was found 24 hrs after ASA (chapter 8). Although it is reasonable to assume a role for AVP in orchestrating

memory oscillations in rats and mice, more experiments need to be carried out to conclusively couple neurochemical changes to behavioural output.

Most, if not all, SCN neurones are innervated by cholinergic fibres. Muscarinic acetylcholine receptors (mAChRs) which mediate acetylcholine (ACh) signal transduction are abundantly present on SCN neurones. The cholinergic system is highly involved in attention and arousal and therefore important for (associative) learning. Twenty-four hrs after ASA and passive shock avoidance (PSA) training, mAChR immuno-reactivity (ir) in the rat SCN is considerably increased, most likely reflecting increased internalisation of the receptor peptide (chapters 4 and 8). In a follow-up experiment, the habituation phase of the behavioural tasks (the novelty element) was found sufficient to trigger this effect. The most striking aspect of this phenomenon is the finding that mAChR immuno-responsiveness waits a full circadian cycle (24 hrs) to reach full-blown proportions, while a significant increase in the number of mAChR-positive glial cells occurs as soon as 2 hrs after training. Thirteen days after the ASA training session, mAChR-ir in the SCN returned to basal levels in half of the rats, whereas the other half had levels remaining as high as after 24 hrs. This suggests an all-or-nothing event (chapter 4).

Taken together, both the AVP and the mAChR system in the SCN are affected by high-impact learning tasks, but associative learning is not required for the alterations in mAChRs. AVP might be the direct driving force behind the oscillations in memory retention (chapters 2 and 3). The decreased sensitivity for cholinergic input (reflected by enhanced mAChR-ir) in a subset SCN neurones (about 30 %) after conditioning may suggests that these cells form a substrate for a specific action. Evidently, this calls for further investigation.

The impact of ageing on the circadian system is reviewed in chapter 1, and our own findings in rats and mice are summarised in chapter 5. Age-related changes in circadian parameters were consistently found. For both rats and mice, α (activity period) lengthened with age. Locomotor activity levels and rhythm strength decreased, whereas the day to day variability in centre of gravity of activity increased. In aged Wistar and F×BN rats, τ (freerunning period) was shorter than in the young rats. In contrast, a lengthening of τ was observed in older mice. The magnitude of the alterations seemed to depend on strain, light conditions, and previous experience, such as having a running wheel. Furthermore, one has to keep in mind that both central and peripheral aspects contribute to behavioural alterations. The effect of age on SCN neuropeptides was studied by means of immunocytochemical stainings. Contrary to what has been found for other peptides in the SCN, such as AVP and vasoactive intestinal peptide (VIP) (chapter 1, 7 and 8), somatostatin (SS) is significantly increased in the SCN of aged rats (chapter 7). Because SS can reset the phase of the clock, and has an inhibitory modulating role on VIP rhythmicity, we speculated that SS may have a specific age-related role in circadian behaviour.

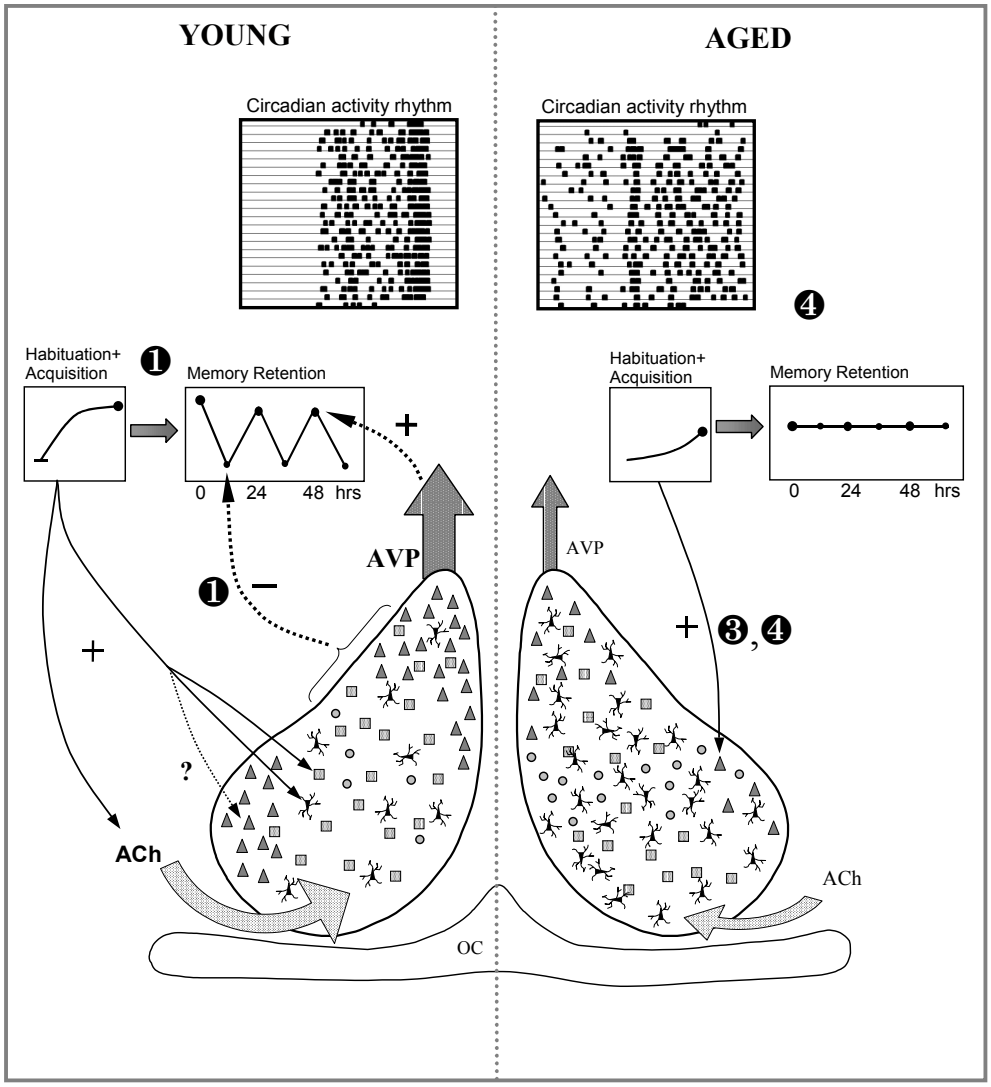
Severe cognitive disturbances and disorganised circadian behaviour occur together with ageing, especially in demented humans (chapter 1). To specifically address the question whether some of the memory deficits may be attributable to a decrease in circadian organisation, we studied behaviour in learning paradigms and the relation with SCN neurochemistry in aged rats as a model species. First, if the circadian system generates these memory oscillations, they are predicted to be absent or less manifest in individuals with a less functional pacemaker. This was indeed found in aged rats trained for PSA in DD (chapter 6). Old rats did not have any modulation in memory retention, but high entering latencies at all testing times. A positive correlation was found between the strength of rhythmicity (in general activity) and the degree of memory modulation (difference between 24 h and non-24 h values). This suggests that a more robust circadian system has a stronger suppressing effect on memory retention at non-24 h intervals after training.

Is this behavioural difference between young and aged rats reflected at the neuro-chemical level in the pacemaker, for example in neuropeptidergic response? This question was addressed in the experiment described in chapter 8. The increase in mAChR-ir, previously found in young rats 24 hrs after ASA, is indeed absent in aged rats, suggesting that the SCN neurones are no longer able to respond to cholinergic input. Contrary to this, AVP levels (neuronal counts and peptide content) were found to be increased in aged, but not in young rats. Several explanations are conceivable (see chapter 8). Possibly, young rats release their (extra) AVP, like the house mice (chapter 3), whereas the release of AVP in aged rats may be inhibited. In any case, 24 hrs after a learning task the two SCN neurochemical systems are affected differentially with age.

II. GENERAL DISCUSSION

A graphical representation, summarising the results described in this thesis, is given in Fig. 1. This section discusses some facets depicted in the figure in more detail, and ideas for future research are given where relevant. The different aspects are indicated in the figure by numbers corresponding to the paragraphs in which they are considered. First, ❶ the interaction of the circadian system with learning and memory is more closely inspected. Which aspect of the conditioning process is actually under the control of the pacemaker: acquisition, retrieval, or yet another component involved in learning and memory, such as fear? We have concluded that the SCN seems to have a suppressing action on retrieval at non-24 h intervals, but how strong is the evidence for this? ❷ Parallels and discrepancies between our own and previous studies are examined, focusing on species and paradigm differences. ❸ The next issue is the putative mechanism underlying periodic deficits: what could be the relevance of the observed alterations in the SCN muscarinic receptor and vasopressin system, and how could they interact? ❹ Finally, the current state of affairs regarding our main research question is discussed. Does a reduced organisation of behaviour contribute to the age-related decline in memory?

Figure 1. Schematic drawing of rodent SCN, summarising the results in this thesis and proposing a speculative integrating model. The left panel represents a young, and the right panel an aged SCN, and its related behavioural output. Circadian rhythms are flattened and more fragmented in aged rodents, and the circadian pattern in memory retention is absent. Input (ACh) and output (AVP) systems of the SCN are indicated in block arrows. Neural elements (putatively) involved in the regulation of memory retention: ▲ = vasopressin (AVP) producing neurone; ▨ = muscarinic acetylcholine receptor (mAChR) positive neurone; ○ = somatostatin (SS) producing neurone;  = mAChR positive astroglial cell; ACh = acetylcholine. In the aged SCN, vasopressin immunoreactivity (-ir) is severely decreased, and neuronal mAChRs to some extent. The number of mAChR positive astrocytes, and SS-ir is increased. The conditioning event affects SCN neurochemical systems and triggers the oscillation in memory retention. Arrows pointing towards the SCN indicate alterations in neurochemistry as a consequence of conditioning 24 hrs later. Arrows leaving the SCN indicate its modulation of memory retention over time. Inhibitory systems (SS, GABA) could suppress memory, whereas others (AVP, mAChR+ cells) may act to temporarily overrule inhibition at the 24 h-intervals. Note that the SCN does not determine absolute levels of performance, but only controls the degree of circadian variation. In aged rodents, effects of conditioning on neurochemistry are minimal, and the aged SCN does not influence retention. Digits (❶) refer to paragraph numbers in the General Discussion where topic is discussed in detail. OC = optic chiasm. This scheme is based on rat and mice ❷ data.


YOUNG
AGED
Circadian activity rhythm
Circadian activity rhythm
4
Habituation+
Acquisition
1
Memory Retention
0 24 48 hrs
+
AVP
1
−
+
?
ACh
Habituation+
Acquisition
Memory Retention
0 24 48 hrs
AVP
+ 3,4
ACh
OC

1. The circadian system and conditioning

Disentangling components of conditioning

The studies performed in this thesis have focused on the circadian system in relation to memory retrieval, and not to learning. This was in accordance with the compelling evidence for a role for the circadian clock from studies in which memory retention for a learned task turned out to be periodically impaired in a circadian fashion. Indeed, we found a circadian pattern in memory retention for PSA and ASA learning task. Many of the experiments combined learning tasks and SCN immunochemistry, enabling determination of relationships between them. Significant correlations between retention levels and markers in the SCN (e.g. number of stained AVP neurones, optical density measure-ments of AVP and mAChRs) were consistently absent. From this result, and from the results in chapter 2 and chapter 6 we infer that the SCN probably does not regulate performance levels per se, but merely the regulation of memory retention over time.

Initial learning was related to the neurochemical state of the SCN afterwards, in the young Wistar rats from chapter 8 (Fig. 2). The percentage correct avoidances during ASA training strongly correlated with the number of mAChR+ astrocytes in the SCN 24 hrs later (r=0.7, p=0.014). The number of mAChR+ astrocytes was found to increase after PSA and ASA in young rats (chapter 4 and 8), indicating SCN plasticity at this level. Possibly, the impact of a task, varying between individuals with their ability to acquire the response, leaves a trace in the SCN as to how important this event has been to the animal, and how much attention it should receive in the future. The habituation phase of the PSA and ASA task was sufficient to enhance the number of mAChR+ astrocytes. This could indicate that the impact of the habituation is greater for rats that learn faster, possibly because their attention and responsiveness is higher through an enhanced cholinergic drive. The staining intensity of mAChR in the SCN as a whole did not correlate with avoidances during acquisition. The specific role of astrocytes in this

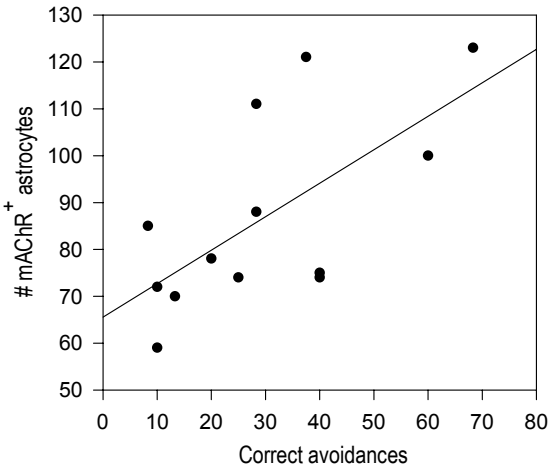


Figure 2. mAChR positive astrocytes in the SCN 24 h after active shock avoidance against number of correct avoidances during acquisition

matter remains to be elucidated.

The most plausible explanation for periodic memory deficits is that memory is temporarily "out of reach", i.e. that retrieval is suppressed. We do not know at this point, whether the interfering factor acts on the retrieval process itself, or that it somehow inhibits the behaviour we use to measure memory. The animal may also remember well that the shock was given in the dark compartment, but for some reason have lower expectancy of the shock (risk assessment), or have increased fear of staying outside at non-circadian intervals (motivational aspect). Such processes would have nothing to do with retrieval, but are involved in the action that follows. Bintz *et al.* (1970) specifically addressed this question, and concluded that the retention decrement of the original Kamin effect cannot be explained by a simple generalised fear response. Klein and Spear (1970) reached a similar conclusion in their studies. It is difficult to disentangle these aspects of learning and memory, that include motivational and anxiety factors. They all interact, resulting in the observation of "the retention" (Blokland, 1996). The work of Holloway and Wansley has shown that a simple (pre-existing) factor (like a fluctuating hormone) cannot be the direct source of the oscillations, since the pattern is independent of time of day of training (Holloway and Wansley, 1973b). It is possible that the shock triggers a separate oscillatory process in stress hormones or in cortical functions, that indirectly influences memory or what is measured as such: retention, by altering fear levels.

Suppression or stimulation?

There are two possible ways in which the SCN could cause the memory oscillation pattern, observed in young rats in the active and passive shock avoidance (chapter 2 and 6; Holloway and Wansley studies):

1. the pacemaker stimulates memory retrieval at 24 h intervals;
2. the pacemaker suppresses memory retrieval at non-24 h intervals.

The results presented in this study do not provide solid evidence that the SCN suppresses memory retention, but this is strongly implied by the data sets in chapter 2 and 6. Brattleboro rats, lacking AVP, perform well both at 18 and at 24 hrs after training. Their performance is as good as in the control Wistar strain, and even better than in the control Long Evans strain. This makes it unlikely that memory is enhanced (by AVP) at the 24 h intervals, instead of suppressed at 18 h. The other endorsement comes from the aged rats in the passive shock test (chapter 6). If a healthy circadian system would periodically enhance memory retrieval, then aged rats with a declined pacemaker amplitude would perform worse, unable to stimulate memory at the 24 h time. Aged rats were not impaired at this task, instead they had enhanced latencies. The possibility that aged rats would perform better is implausible. The most compelling evidence seems to come from the study of

Stephan and Kovacevic (1978). Their SCN lesioned rats performed as well as the non-lesioned rats at 24 hrs after learning. If the SCN would have a stimulatory action, retention levels would have dropped to the 18 or 30 h level of the intact rats. Even in the lesioning study, there is an alternative explanation. Some of the rats had extended damage to the optic chiasm as a result of the SCN lesion. This may have affected light sensitivity, a crucial factor in passive shock avoidance. Rats might have been less compelled to enter the dark compartment, had they been less sensitive to light.

Apart from the behavioural data, general SCN physiology also makes a suppressing action the more likely option. Somatostatin (SS) is an inhibitory peptide. SS neurones are tightly interconnected, and innervate other neurones (e.g. ones containing VIP) within the SCN. The increase in SS-ir with ageing (chapter 7) could reflect peptide accumulation inside the neurones (release deficit) resulting in a diminution of inhibitory actions. In addition, GABA is the principal neurotransmitter of the SCN, as it is present in (nearly) all neurones (Moore and Speh, 1993). The SCN therefore largely maintains inhibitory control over the areas it innervates. Although no evidence is at hand for a SCN- specific role in cognition for GABA or SS, the suppressing effect on retention could simply be a common feature of the SCN. The combined action of transmitters such as AVP, and glutamate from (mAChR+) astrocytes, could temporarily overrule this inhibition, via a release peak (see below). A crucial role for AVP seems at odds with the Brattleboro data, because AVP is not present to overcome this inhibition. But we have to keep in mind that the SCN does not control how well animals learn or remember. It merely modulates performance in a circadian fashion, and AVP could play a role in this action. AVP administration in the SCN could clarify this issue.

2. Species and paradigm differences: comparison with other studies

The behavioural studies presented in this thesis closely followed the Holloway and Wansley paradigms (active and passive shock avoidance) and training testing intervals (6 hrs) on purpose, as these laid the basis for our studies. The results are indeed in agreement with the data of Holloway and Wansley; memory retention deficits are present at the same intervals (6 hrs after training and 12 h multiples), in the same learning tasks (active and passive shock avoidance). Periodic memory deficits are thus reported rather consistently across time, learning task, and laboratory (see chapter 1). Together the findings support a "time-stamping" role for the SCN in memory processing. It postulates that the available information is used only if the internal circadian phase coincides with the circadian phase of training, and not at other times. As pointed out before, it makes adaptive sense to use the

experience of today as a temporal template for tomorrow (Daan and Koene, 1981; Daan, 2000). Anticipation to high-impact events in the future would increase chances for survival, and the circadian clock is the system exceptionally suited to regulate such behaviour.

A few studies have arrived at other conclusions. Ralph and colleagues (Devan *et al.*, 2001; Ralph *et al.*, 2002) conducted experiments in hamsters and rats with the goal to establish the influence of circadian rhythms on the different stages of learning and memory (acquisition, consolidation, and retrieval), using a range of different tests in rats and hamsters. A common factor in most of their studies is that they trained animals at two circadian time points, and then tested them at either the same, or another time (i.e. training and testing at the same Zeitgeber time (ZT 4 or ZT 12), or training at ZT 4, and testing at the different ZT 12, and vice versa). The acquisition phase of their learning tasks always consisted of multiple consecutive days of training. Hamsters only showed a preference for the paired condition during testing, if they had been trained at the same circadian time. Even when tested during the night (when the reward of running for nocturnal mammals is expected to be high) they did not show a preference for that room if they had been trained during the day (Ralph *et al.*, 2002). These results are in agreement with the Holloway and Wansley data. Other studies carried out in rats using a similar set-up (water maze, aversive context memory, radial maze-stimulus response), have failed to reproduce memory deficits at non-circadian intervals, even after using 6 h intervals cf. the Holloway and Wansley protocol (Ralph *et al.*, 2002). McDonald *et al.* (2002) proposed two explanations for the discrepancies between their results and the Holloway and Wansley data: the first is based on that shock sensitisation, instead of memory retrieval could be the process under circadian control. In their studies, this is not a discriminating factor, since it would affect the paired and the unpaired context equally. The second is that the difference could be due to the use of multiple versus single-session acquisition procedures, but the authors argue that these multiple sessions were the minimum amount needed for obtaining a discriminative degree of memory to manipulate (Ralph *et al.*, 2002).

To examine the generality of our findings in rats across species, CBA/ca mice were subjected to the PSA protocol used for rats (cf. chapter 6). Here, I report on the (unpublished) results of this experiment in mice. In short, we gave mice three habituations and a single training in the PSA box, and subsequently tested individual mice 24 hrs later, and then repeatedly every 6 hrs for another two days (three days in total). We trained the mice during the subjective day, but they were kept on a skeleton-photoperiod (0.5:11.5 hrs LD) to maintain entrainment. By training and testing in darkness, a direct influence of light can be ruled out. Figure 3 shows the individual patterns for young and aged CBA mice in PSA. Young mice showed more fluctuations in step-through latencies than aged mice. This is consistent with rats

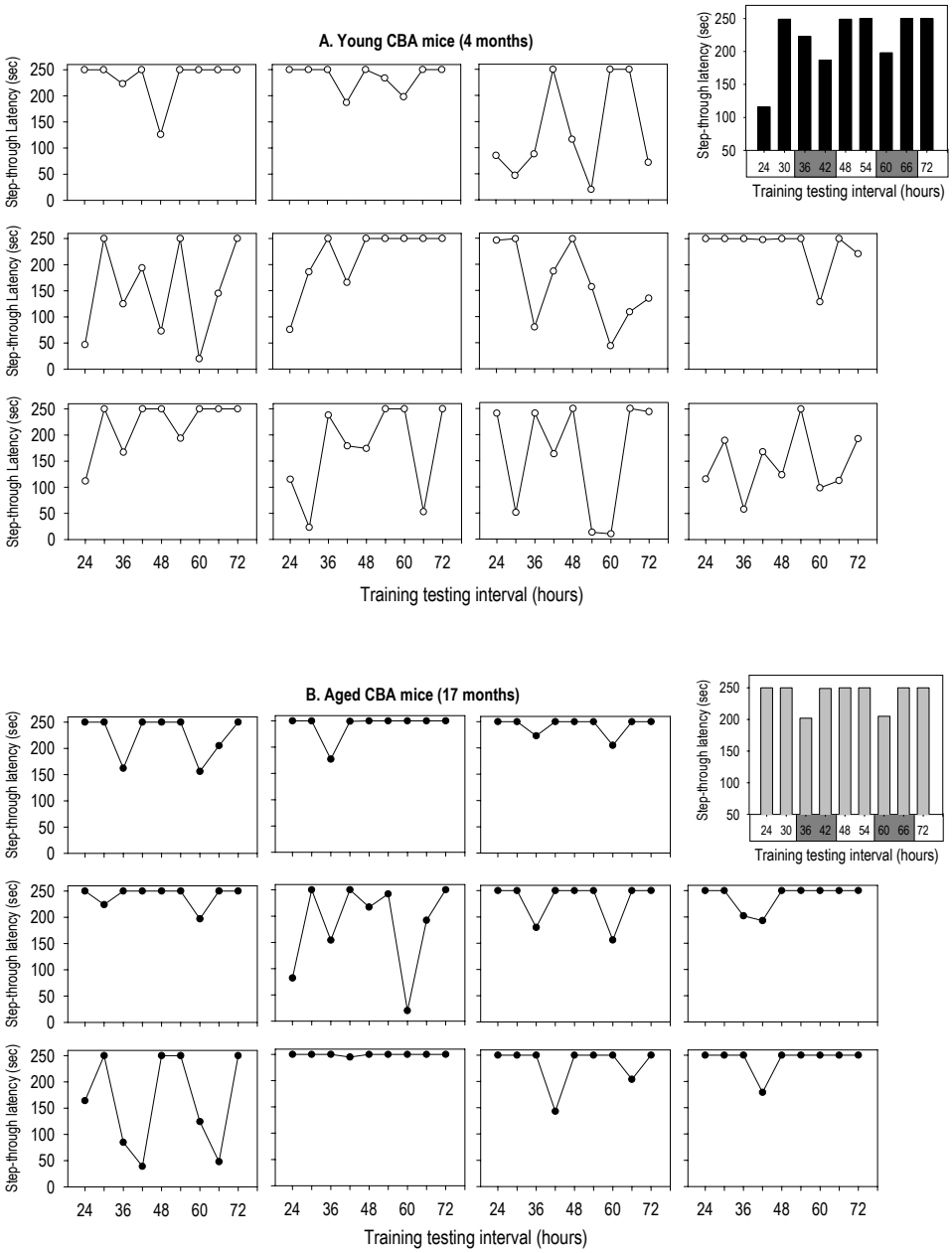


Figure 3. Individual memory retention at multiple training testing intervals after passive shock avoidance training in young (upper graphs, A) and aged (lower graphs,B) CBA/ca house mice. Median step-through latencies are depicted in the upper right corner of the individual graphs.

(chapter 6). Latencies to enter were generally lower during the subjective night, showing more passive behaviour during the subjective day. There was no clear circadian pattern in retention. Possibly, environmental factors influence, or interfere with a 24 h oscillation, if the phenomenon of periodic memory deficits exists in mice. Chaudhury and Colwell (2002) investigated circadian modulation of fear conditioning in a single training paradigm in house mice. Their results were also not fully consistent with an induced 24 h oscillation. Innate (and pre-existing) high daytime freezing seemed to interfere with a time-stamping mechanism, of which clear indications were present. Species comparison

Except for the experiments by McDonald *et al.* (2002), data from rat studies in general consistently agree with the Holloway and Wansley data, and with a time-stamping role for the circadian system in memory retention. Some hamster studies substantiate this concept as well (Ralph *et al.*, 2002), while others do not (Oklejewicz *et al.*, 2001; Ko *et al.*, 2003, *in press*). For mice, the picture is not clear yet either. Perhaps the natural behaviour of mice includes cautious responses to any stimulus during the light phase, which is potentially the more dangerous time of day. This could lead to a conflict situation in the described tasks, and hamper the detection of a time-stamping concept in mice. In any case, it would explain the discrepancy between the mice study of Chaudhury and Colwell (2002) and the rat studies. During the acquisition session, freezing as a direct result of the tone-shock pairings was much higher (in both light protocols) in light-phase trained animals than in dark-trained animals. Mice are more strictly nocturnal than rats, and in rats, the circadian phase is in most cases reported as irrelevant for training in diverse paradigms (see chapter 1). The PSA task (chapter 6) is quite comparable to the freezing to context/tone paradigm, as it requires passive behaviour as an expression of memory. In the small nest-building mice (from chapter 3), we found much better acquisition of ASA during the dark phase than during the light phase. ASA requires an active response (which is expected during the dark phase) in contrast to passive-like behaviour, which could be expected during the light. Obviously, these matters need further clarification, especially since mice are becoming popular models to study behavioural processes relating to circadian rhythms and cognition.

Paradigm problem

Apart from the choice for a particular species, the issue of which task is the "best" to study memory processes in a circadian context is also complicated. All learning paradigms entail either strongly aversive or appetitive components, as motivation is crucial in any attempt to teach an animal a (conditioned) response. In addition, fear or anxiety is an inescapability, at least in the initial stages of learning (novelty

aspect). A water maze paradigm is quite different from the context memory tasks. The former is a truly spatial task that requires using extra-maze cues, in contrast with a simple association test (e.g. unique chamber with reward/shock, or light with food). Comparing the results is therefore problematic. In the context memory task (Ralph *et al.*, 2002), rats are not inclined in any way to choose the shock-paired chamber. There is no trade off between the natural urge to enter this chamber, as there was no initial preference for it. In Holloway and Wansley's and our own studies a conflict (PSA), or an urge to perform (ASA) is present. In that view, the water maze is a "better" task, because the animal is "forced" to undertake some action. In a water maze task, Devan *et al.* (2001) found impaired memory in rats after 13 days of training, as a consequence of 5 consecutive days of 3-h phase shifts.

It could be argued that tests in which no conflict is present are preferable, for the very reason that they do not involve interfering factors. The appetitive tasks (Wansley and Holloway, 1975; Hunsicker and Mellgren, 1977) demonstrate that the periodic deficits are observed also in the absence of such a strong fear factor. In any food-motivated task, we have to bear in mind that responses could be driven by the food entrainable oscillator (FEO), which is independent of the SCN (Stephan, 1989; Mistlberger *et al.*, 1996; Walcott and Tate, 1996; Means *et al.*, 2000; Aragona *et al.*, 2002). This is also true for time-place association studies (TPA)(Boulos and Logothetis, 1990; Daan *et al.*, 1994; Carr and Wilkie, 1997). In general, one could say that the best tasks to study are those closest to the natural behaviour of the animal.

In conclusion, the grounds for the discrepancies between studies remain open for discussion. Simply ascribing them to strain or paradigm differences does not bring us any further in our understanding of periodic memory deficits if they represent a general phenomenon. It has been suggested before that "overtraining" may abolish the Kamin effect (Gabriel, 1968), and this was also reported in the study by Chaudhury and Colwell (2002). Another possible and associated explanation lies in the single trainings -as used by Holloway and Wansley and in our studies- versus multiple trainings -as used by McDonald *et al.* (2002). It is possible that a single "shocking" event has more impact on the circadian system, and with the same event recurring every day at the same time (which is the case during the training phase), it may loose its significance due to adaptation to the applied stimulus. The SCN may then no longer exert its influence on, or interfere with cognitive processing by higher cortical areas. The difficulties and pitfalls in choosing the proper test for studying cognition in a circadian context were demonstrated here: one has to carefully consider how natural behaviour can confound results, by spontaneous variation in general activity levels. Mice may well be more affected by such variation than rats.

3. Neurochemical substrate of periodic memory deficits

Our neurochemical analyses have focused on vasopressin and muscarinic acetylcholine receptors (mAChRs) in the SCN. In chapter 8, we have found indications that the two systems are not coupled in the SCN, as they respond differently to a stressor in aged compared to young rats. Studies on the interaction of vasopressinergic and cholinergic systems are indeed scarce. In the rat SCN, the second messenger system cGMP-dependent protein kinase (PKG) is co-localised with AVPir, and is involved in processing of cholinergic stimuli (Revermann *et al.*, 2002). Outside the SCN, the AVP4-9 fragment stimulated ACh release from the hippocampus in a microdialysis study in rats (Maegawa *et al.*, 1992). In BALB/c mice, the same was found in vitro in hippocampal slices, and this action is probably mediated through the V1 receptor (Tanabe *et al.*, 1999). At the behavioural level, AVP4-9 (a potent memory booster) was able to (partly) overcome the scopolamine (a selective ACh receptor blocker) induced memory impairment in an eight-arm radial maze task (Fujiwara *et al.*, 1997), and in a passive avoidance task (Tanabe *et al.*, 1999). We have not specifically addressed the issue of a cholinergic-vasopressinergic interaction, and the systems are therefore first discussed separately with respect to their role in circadian learning and memory.

Vasopressin system

In the learning and memory context of the circadian system, vasopressin (AVP) is an obvious peptide to study. Not only is AVP an important output pathway of the timekeeping system, but this peptide has a long history of being implicated in learning and memory function. CSF and blood AVP are functionally and anatomically compartmentalised, suggesting a distinct function of CSF-AVP in the central nervous system (Reppert *et al.*, 1981, 1987), and CSF-AVP most likely originates from the SCN (Schwartz *et al.*, 1983). It was never established whether endogenous AVP from the SCN could act on V1/V2 receptors in the dorsal hippocampus and neocortical brain regions. This is an intruiging possibility since evidence for a direct innervation from any AVP region is lacking. The role of SCN AVP (a clock-controlled gene), is still under debate, although several studies have aimed at elucidating part of its function (Bult *et al.*, 1993; Gerkema *et al.*, 1994; Ingram *et al.*, 1998; Reghunandanan *et al.*, 1998; Landgraf *et al.*, 1998; van Esseveldt *et al.*, 1999). AVP was proposed to be the signal from the SCN to other areas in the brain. It could serve to warn the individual that that time of day has arrived when, on a previous day, a significant event happened. Several lines of evidence now suggest that the SCN-AVP system is involved in the signalling to brain areas involved in learning and memory, as consequence of a (significant) learning event. To begin with, studies in this thesis showed that:

1) an ASA induced drop in AVPir was observed in the SCN of four separate lines/ strains of house mice, indicating the universal character: selected lines of big and small nest-builders and C57Bl/6 (chapter 3), and CBA/ca mice (Fig. 5; unpublished observations);

2) the AVP time profile in small nest-builders, presented in chapter 3, points in the direction of a stress-induced oscillation in AVP-ir in the SCN of house mice.

Adding more substance to the concept are previous studies from literature showing that:

3) within the SCN, AVP is released directly after a stressful event (forced swimming) as was shown in a microdialysis study (Engelmann *et al.*, 1998);

4) 24 hrs after PSA the SCN was found to be depleted of AVP (as demonstrated by immunohistochemistry by (Laczi *et al.*, 1983). The finding that directly after passive avoidance learning, as well as directly after the 24 h and 120 h retention, AVP is significantly enhanced in the CSF of rats (Laczi *et al.*, 1984) corresponds with this since CSF-AVP most likely originates from the SCN.

5) injections of AVP must be given within an hour after the learning task to prevent extinction at 24 and 48 hrs compared to controls (de Wied, 1971).

An AVP oscillation superimposed on the normal circadian AVP release, running independently yet phase-locked to the master clock, is an interesting concept. This could explain the finding that retention is optimal 24 h after learning, independent of the time of training. For a full answer, we need to know whether periodic memory deficits, parallel to the observed pattern in AVPir in the SCN, are demonstrable in mice in a different set-up. It would also be important to determine whether AVP release is observed in rats, where memory fluctuations have been observed. This hypothesis could be tested ideally by microdialysis, enabling "online" AVP release monitoring during (or in any case directly before and after) retention testing in the SCN (and surroundings) after a single stressful event. Such a study has yet to be conducted.

mAChR system

Acetylcholine (ACh) is an important neurotransmitter in the brain, and has attracted a great deal of attention over the years in cognitive research (Ingram *et al.*, 1994; van der Staay and Blokland, 1996; Tago *et al.*, 1987). Information on the function of the SCN-cholinergic system is mostly confined to a role in phase shifting (Zatz and Herkenham, 1981), an action mediated by mAChRs in vivo (Bina and Rusak, 1996)

and in hypothalamic slice preparations (Liu and Gillette, 1996). In our studies, we have found indications that the mAChR system is involved in associative learning, and more specifically in the novelty aspect of a learning task (chapter 4). An SCN specific time profile of increased mAChR staining was found, starting 22 h after the first event, whereas other brain areas (hippocampus and amygdala) display these changes much sooner (Van de Zee and Luiten, 1999). This suggests that mAChRs have a distinct role in the SCN, relating to its temporal properties, rather than directly responding to cholinergic stimulation.

Enhanced mAChR staining by the monoclonal antibody M35, a good tool to study these mAChR dynamics, probably reflects reduced sensitivity for cholinergic input at the mAChR level through receptor internalisation following cholinergic stimulation (Van der Zee and Luiten, 1999). Increased ACh innervation from distant brain areas thus possibly results in shutting down of a subset of SCN neurones (about 30%) to more cholinergic input. The absence of an oscillation in the mAChR staining profile suggests that cells expressing mAChRs (stained with M35) do not use cholinergic signal transduction to mediate periodic memory fluctuations. One could speculate that these neurones represent a subset of cells that have become partly independent from, or out of phase with the SCN, thus creating a cellular substrate for an independent oscillation of another output transmitter, such as AVP. Moreover, the habituation phase of the learning task is sufficient to induce the largest changes in mAChR staining (chapter 4).

Although this speculation is very appealing, it evidently calls for clarification of the interaction between cholinergic and AVP systems in the SCN. MAChR labelling does occur in the AVP-region of the SCN, making it likely that at least some AVP cells could carry these receptors, but double-labelling studies should be conducted to determine whether AVP containing cells in the SCN in fact express mAChRs. This would support the idea that the subset of mAChR positive cells acts together with AVP to generate memory oscillations. For AVP, we do not know if novelty, or associative learning is required to exert its effect on the mouse SCN-AVP system (chapter 3) and on aged Wistar rat SCN-AVP (chapter 8). The lack of correlation between AVP staining and performance parameters in ASA suggests that also SCN-AVP is not concerned with the degree of learning. The associative learning aspect may still be required to induce these changes. These aspects remain to be disentangled, as they were for mAChRs.

Finally, a more prominent role than so far presumed may be granted to astroglial cells. A significant correlation between the number of CARs during acquisition and $mAChR^{+}$ astrocytes in the SCN 24 hrs later was found. The role of astrocytes in the SCN has not been well-studied to date, although some studies exist (Botchkina and Morin, 1995; Lavialle and Serviere, 1995; Li *et al.*, 2002). Possibly undeserved, since they express receptors for a number of neuropeptides and

neurotransmitters, including serotonin, NPY, glutamate, vasoactive intestinal peptide, and vasopressin (Hosli and Hosli, 1993), all present in the SCN. Furthermore, it has been shown by Van der Zee *et al.* (1991), and again in chapter 8 that with age, the number of mAChR+ positive astrocytes significantly increases.

4. Ageing: decline in circadian organisation and memory

The central topic in this thesis was to determine to what extent the circadian system contributes to the age-related decline in memory function. Circadian organisation and cognition are both severely impaired with ageing, and especially in dementia. In contrast to the findings in chapter 6, in which we found no evidence for a age-related cognitive decline in the PSA task in F×BN rats, the Wistar rats from the study in chapter 8 did show a dramatic inability to acquire the ASA task. The temporal pattern of memory modulation, however, was not studied in these rats. So, in the described experiments, we did not demonstrate impaired cognition together with the absence of a circadian modulation in the same rats, since the aged F×BN rats lacking circadian modulation in the retention of the PSA tasks (chapter 6) performed well.

The lack of circadian modulation in aged rats offers a strong indication that a healthy circadian system suppresses memory at non-24 h intervals. Moreover, a correlation was found between the individual degree of circadian modulation and the robustness of the circadian system (chapter 6). A lack of correlations of SCN markers with performance levels per se does not dispute SCN involvement in the temporal aspect of memory retention. Antoniadis *et al.* (2000) did find that within a group of aged golden hamsters, the ones with fragmented rhythms had lower preference scores than those with more consolidated rhythms. It awaits further investigation if this discrepancy arises from species or paradigm differences. In addition, other structures beside the SCN may be involved in the temporal modulation of memory (Ko *et al.* 2003, *in press*).

At this point I therefore conclude that in rats, the aged circadian system is not responsible for impaired cognition in a narrower sense. A good way to settle the issue would be to temporarily inhibit SCN function pharmacologically in young rats, thereby mimicking the age-related decrease in circadian organisation. If the circadian system involvement in memory retention is restricted to a temporal modulation, these rats would display higher values than before at the non-24 h intervals, whereas 24 h values should remain the same.

III. CONCLUDING REMARKS

The circadian pacemaker modulates memory retention in rodents to incorporate the information in their behaviour in a time-of-day dependent manner. This is an important conclusion in itself, as a confounding factor to be taken into account by those studying learning and memory processes. We have come a little closer to characterising the pathway through which the pacemaker interacts with memory retention. The SCN is most probably not involved in learning or memory performance levels per se. In fact, a not fully intact or compromised circadian system (like with ageing, following SCN lesions, and in the Brattleboro rat) has positive effects on mnemonic capacity at testing times otherwise showing (actively) suppressed retention. Essentially, the SCN seems to modulate when to retrieve what has been learnt. This is suggested by the fact that the time-dependent fluctuations disappear in rats with a less organised circadian system (aged rats) and with a different SCN make up (Brattleboro). Like many other rhythmic functions controlled by the SCN, temporal organisation of cognitive behaviour becomes weaker or is lost with a deteriorating circadian system.

It may seem that having an intact circadian system is disadvantageous for memory function. The reverse is true: allocating specific actions to specific "time slots" is the way to cope with a (predictable) fluctuating environment, like the one we live in. A "time-stamping" concept is supported by our results. The SCN "marks" the time in the circadian cycle when an impact-full event occurs, for future reference. The SCN is not designed for determining performance levels in learning tasks, but is doing the job it is supposed to do: timing the appropriate action. It may seem trivial that information is unavailable at certain times, why not always remember maximally? It is reasonable to assume that in a young, healthy rat the system works optimally, and that this has some relevance to its well-being and survival, rather that it just being an "artefact" of a well-organised circadian system. In the animal kingdom, daily routines are common, and most events occur periodically. Suppressing fearful information, that is only relevant at certain times of day, can be advantageous as it might inhibit other relevant behaviour (such as foraging).

Using rats as a model to study effects of ageing in humans, is valuable as long as a certain degree of similarity is present in the way ageing is expressed. The decline of circadian organisation and cognition were both demonstrated in rats, as they are in humans. Comparable experiments are feasible in humans, and would be interesting to recognise points of difference and resemblance. It is very well possible that in humans the significance of this specific role for the circadian pacemaker is less pronounced. However, the deterioration of the circadian system was not found to contribute, or be detrimental to cognitive performance. Only the temporal

organisation is lost. Therefore, presuming that the human circadian system does regulate memory retention in a similar way, therapeutics aimed at re-establishing circadian organisation may not act as pure cognitive enhancers, but enhance general well-being on the whole.