CHAPTER 8

Summary
Circadian Time-Place Learning

Natural environments are constantly changing. The availability of food, mates and predators varies across both space and time. If these stimuli vary predictably, it is advantageous for animals to learn this spatiotemporal variability. The ability to encode spatiotemporal reoccurring events, and to exploit this information by efficiently organized daily activities, is believed to constitute a significant fitness advantage which has likely shaped the architecture of cognitive and circadian systems over the course of evolution. Indeed, the ability to learn spatiotemporal variability has been demonstrated in many species and has become known as Time-Place Learning (TPL).

Investigating TPL behavior has an obvious ecological relevance, as it can help to gain a better understanding of the foraging dynamics in prey as well as in predators. This thesis however focuses on what we can learn from this behavior in a neurobiological perspective. Interestingly, animals can use their circadian system for TPL, in which case we refer to circadian TPL (cTPL). However, not much is known about this specific role of the circadian system in memory formation. As a circadian system dependent learning and memory task, cTPL can be used to study the role of circadian system components on a functional behavioral level. Moreover, cTPL implies that distinct phases of an internal circadian clock can be incorporated in associative ‘what-where-when’ memory, which resembles the content of human episodic memory. This type of memory is particularly susceptible to perturbations of aging and neurodegenerative disease, yet animal models to study episodic memory are scarce.

Getting animals to show cTPL in laboratory settings proofed to be difficult. Without the proper motivation, animals will make use of external time cues (zeitgebers) in TPL tasks, and apply alternative strategies like ordinal- or interval timing. This suggests that the incorporation of circadian phase into memory, i.e. using the circadian strategy (cTPL), may be more demanding on the brain. It may therefore only be engaged in situations in which encoding the time-place contingency is essential for well-being or survival. In this light, a major step to studying TPL behavior in laboratory settings has been the development of a
suitable paradigm, in which animals consistently show cTPL. The key has been to apply a balanced approach between a reward, to motivate animals to choose correct locations (finding food while hungry), in combination with a punishment (response cost) for choosing incorrect locations. This way, the paradigm emulates the natural situation in which hungry animals seek food while different feeding locations can be predictably unsafe to visit, depending on the time of day.

Investigating the role of circadian system components in cTPL

Clock gene deficient mice
The circadian system synchronizes internal physiology and behavior to the external circadian environment. It is composed of a network of hierarchically interconnected circadian oscillators (tissues and neuronal assemblies) which drive the circadian expression of many physiological parameters. In mammals, the suprachiasmatic nucleus (SCN) is recognized as the “master clock” in the brain. This oscillator synchronizes to the environmental light/dark (LD) cycle and in turn synchronizes many subordinate oscillators in the brain and periphery. In external LD cycles, the circadian system provides entrained daily rhythms in the body. In constant external conditions, these rhythms show a free running pattern of about (circa-) one day (-dian). On a cellular level, circadian rhythms are predominantly controlled by clock genes and their protein products, which are expressed in virtually all cells in the body. Clock genes have been identified based on mutation or deletion of these genes and resulting arrhythmicity of SCN governed activity in constant conditions. In short, CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle ARNT-like protein 1) form a heterodimeric complex which acts as a transcription activator for PER (Period) and CRY (Cryptochrome) proteins. PER and CRY dimerize and translocate back into the nucleus to inhibit the CLOCK-BMAL1 transcription factor, forming a closed transcriptional-translational feedback loop.

Previously, we found that Cry1/Cry2 double knockout mice were unable to acquire TPL. Here, we investigated whether cTPL is Cry-specific or also depends on Per clock genes. Interestingly, we found that Per1/Per2 double mutant mice,
despite their arrhythmic phenotype, showed no cTPL deficiencies compared to wild-type mice. These results indicate internal timekeeping functional to cTPL that is *Cry*, but not *Per* dependent. They add to the discussion of whether PER proteins are essential in all circadian oscillators in the brain and periphery. While PER proteins are critical to maintain rhythmicity in the light-driven SCN, they may not be critical in non-photic oscillators potentially involved in cTPL. Indeed, it has become clear over recent years that clock genes can have tissue-specific functions. The discrepancy in TPL ability between *Cry* and *Per* deficient mice indicates that TPL can be a discriminating paradigm to investigate the behavioral functionality of known clock genes and validates the investigation of mice deficient in other molecular clock components.

**Involvement of a light-sensitive or a food-sensitive oscillator in cTPL**

The SCN master clock in the brain entrains to photic environmental cues (zeitgebers), and is therefore classified as a light-entrainable oscillator (LEO). In addition, brief periods of food availability form a second major zeitgeber known to entrain circadian rhythms. SCN lesions abolish light-entrainable rhythms, but do not affect the circadian properties of feeding-entrainable rhythms. Therefore, a separate (anatomically and functionally distinct) Food Entrainable Oscillator (FEO) is distinguished, although the locus and neural substrates of the FEO have not been conclusively identified. Whether cTPL is driven by the SCN LEO or a FEO has been an ongoing debate and it has also been proposed that either the SCN or the FEO may be conditionally used for cTPL.

We investigated cTPL sensitivity to abrupt light and food induced phase-shifts, with the rationale that cTPL performance should be affected if the underlying oscillator system is abruptly phase-shifted. In line with the characteristics of an underlying circadian oscillator (-system), both the light pulse and food advance negatively affected TPL performance for multiple days while performance gradually recovered. Notably, the FEO phase-shifts affected TPL performance in specific test sessions, while the LEO phase-shift more severely affected performance in all three TPL test sessions. These results may have important implications. First, the finding of session-specific disturbances in cTPL performance suggests that the cTPL underlying timing mechanism is more complex than a single underlying oscillator only. Instead, these results suggest
that cTPL may involve local timekeeping at the level of session-specific memory traces, which can thus be differentially affected and thereby result in structural session-specific disturbances of cTPL performance. Furthermore, these results suggest that the SCN LEO has a more general and prominent influence on cTPL behavior.

**The role of the SCN in cTPL**

Given the results of the light-pulse on cTPL performance, and its role as the master circadian pacemaker in the brain, we next investigated whether the SCN is critically involved in cTPL by testing SCN lesioned mice. Interestingly, none of the SCN lesioned mice showed cTPL deficiencies. This finding indicates that cTPL must be primarily driven by non-SCN (non-photic) oscillators. Moreover, instead of showing TPL deficiencies, both the Per mutant mice, and the SCN lesioned mice rather showed improved TPL performance compared to control mice. These observations may be explained by the notion that non-SCN rhythms often interact or compete with SCN-driven rhythms to influence behavior. Thus, when SCN photic-driven rhythms are perturbed, as in SCN lesioned and Per mutant mice, interference of SCN photic-driven rhythms should be absent, explaining increased performance in such animals. In line with this, it has been proposed that SCN photic-driven rhythms are attenuated during cognitive training, permitting non-SCN (non-photic) rhythms to modulate behavior. This may explain why a light pulse had such a large effect on cTPL performance of SCN intact mice, even though the SCN is not critically involved in cTPL. The light pulse was given outside of TPL testing times (thus when SCN photic-driven rhythms are not attenuated), allowing cTPL involved timing mechanisms to be phase-shifted by the photic SCN rhythms. Alternatively, the light pulse may have directly affected the cTPL involved timing mechanism.

**The role of the adrenals in cTPL**

The adrenal glands harbor an important peripheral oscillator to consider in relation to cTPL. The SCN interconnects with the adrenal cortex through SCN governed ACTH (Adrenocorticotropic hormone) secretion from the anterior pituitary gland, but also via automatic nervous system pathways, which can directly modulate adrenal ACTH sensitivity. In response to ACTH, the adrenal cortex produces glucocorticoids, while this production is gated by the local
adrenal clock. Glucocorticoids regulate a wide variety of functions, including arousal, stress response, energy metabolism and cognition. Glucocorticoid receptors are widely expressed in the hippocampus and corticosterone is known to modulate processes underlying learning and memory. Importantly, with intact behavior rhythms present (e.g. induced through masking via the light cycle or daily testing), the adrenal clock can sustain corticosterone rhythmicity in absence of a functional SCN pacemaker. Likewise, food anticipatory activity is preceded by a corticosterone (CORT) peak, which persists in SCN-lesioned animals. Adrenal outputs may therefore serve as an internal time-signal used in cTPL even in the absence of a functional SCN.

We first measured CORT in intact TPL-trained mice, who expected to be tested in their first TPL session, and home cage control mice. In line with potential involvement of adrenal rhythms in cTPL, we found a statistical trend for higher CORT levels in TPL-trained mice compared to home cage control mice. We next performed bilateral adrenalectomy on TPL-trained SCN lesioned and SCN intact mice and re-tested these mice in the TPL paradigm. None of the adrenalectomized mice showed cTPL deficiencies, indicating that neither the SCN nor the adrenals are required for cTPL. One point of discussion is that the ADX mice were TPL trained before the adrenalectomy. Therefore adrenal corticosterone signaling may still play an initial (enhancing) role in driving cTPL underlying oscillators that may become independent with training. Whether naïve adrenalectomized mice can acquire cTPL remains to be investigated. The TPL paradigm may not be sensitive enough to detect minor learning/memory enhancing effects of corticosterone signaling. Nevertheless the current results do not support an essential role for the adrenals in cTPL.

**Immunohistochemical markers and target areas for cTPL**
Finding neurobiological correlates of cTPL will shed light on the underlying mechanism. We investigated the brains of young home cage control mice and TPL trained mice, sacrificed on a TPL test-session time point at the end of their behavioral experiment. Each TPL-trained mouse thus expected to be TPL tested, but was instead brought to the perfusion room, together (pairwise) with a home cage control (HCC) mouse. Using this setup, any IHC differences between TPL-trained mice relative to home cage control mice can be attributed to the
anticipated TPL testing. We investigated the expression of vasopressin (AVP, the main circadian output of the SCN), CRY2, and a neuronal activity marker (C-Fos) in the SCN, but we found no significant differences compared to HCC mice. This corroborates with our SCN lesion results which have indicated that the SCN is not critically involved in cTPL. The most pronounced difference between TPL trained and HCC mice was found in c-Fos expression in the paraventricular thalamic nucleus (PV), which has been referred to as a circadian system relay station. Tracing studies have revealed that the PV receives input from all major components of the circadian timing system, including the SCN, subparaventricular zone, the intergeniculate leaflet, and the retina. In addition, the PV is connected to brain areas involved in learning and memory, including the ventral striatum, amygdala, entorhinal cortex, hippocampus, and cortex. The PV may thus be an interesting target area for future lesion studies in the context of cTPL. Interestingly, we also found a 26% increase in CRY2 positive cells in the hippocampal dentate gyrus of TPL-trained mice relative to HCC mice. This corroborates the finding that Cry knockout mice were unable to acquire TPL and indicates that the hippocampus may harbor a timekeeping mechanism functional to cTPL.

**A distributed memory integrated clock system**

Based on our findings, and in light of the current literature, we propose a hypothetical model for the clock system underlying cTPL. We propose that the hippocampal memory system likely holds a central place in cTPL behavior, providing essential associative memory input of previous experience to brain areas of executive function, as well as encoding specific representations of encountered biological significant events in the spatial environment. Indeed, clock genes are expressed in all subregions of the hippocampus. Recent findings suggest that hippocampal “time cells” in the CA1 region take part in episodic memory networks and include a code that can be used to distinguish time intervals on an extended scale of hours to days. In line with this we found a 26% upregulation of CRY2 in the dentate gyrus of TPL trained mice relative to home cage control mice (unfortunately, unspecific staining in the CA1 prevented us from quantifying CRY2 expression in this area). The results from the light pulse and food shift experiments indicated that performance in specific test sessions can be
differentially disrupted. These results suggest session specific, i.e. memory trace specific, local hippocampal clocks.

It is likely that such memory integrated clocks still require the setting and/or entrainment by a reference oscillator. This clock may be localized in a single brain region, or emerge from a network of interconnected brain structures, as hypothesized for the FEO. Although the first option is not excluded by our findings that cTPL is independent of the SCN and adrenals, the latter option has gained likeliness. At the systems level, the elements participating in a distributed clock may be variable. For instance, the FEO clock network may conditionally take part in the cTPL clock network depending on whether a TPL task involves restricted feeding. Moreover, a distributed clock network can be complex in the sense that it may involve widespread brain regions, different types of oscillators (self-sustained, partially self-sustained, or hourglass mechanisms, sensitive to various zeitgebers), and an intricate coupling architecture. Although not critical for cTPL, the SCN likely has a general modulatory effect on this circadian system, as advocated by the effects of a light pulse. Taken together, experience-related cues may act as zeitgebers to a distributed network of cTPL involved brain regions, including the hippocampus, where local timekeeping mechanisms may be entrained. Whether Cry, but not Per genes are essential for temporal coding in the hippocampus remains to be further investigated.

**Investigating TPL in the context of aging**

Numerous clinical studies have established a direct correlation between abnormal circadian clock functions and the severity of neurodegenerative disorders, suggesting a functional link between the circadian clock and age-associated decline of brain functions. cTPL demonstrates that animals can form so-called ‘tripartite memory codes’ consisting of associated what, where, and when information, resembling the content of human episodic memory. This type of hippocampus-dependent memory is particularly susceptible to the pathologies of aging and neurodegenerative disease. Aging is characterized by cognitive decline, as well as circadian system deterioration. We therefore predicted that cTPL is
specifically age sensitive, and investigated TPL for the first time in the context of aging.

We found that at middle-age (17 months), most untrained mice were unable to acquire TPL. Surprisingly, some mice did master the task by adapting an alternative (ordinal) TPL strategy. We hypothesize that age-related hippocampal dysfunction, together with age-related circadian system decline caused these untrained mice to adapt this ordinal TPL strategy, which is presumably less cognitively demanding than cTPL. In contrast, mice trained over their lifespan successfully maintained the circadian strategy (cTPL, learned from young age) until old age (22 months). At this age however, mice showed signs of behavioral rigidity and a lack to update time-of-day information.

The striatum and hippocampus are widely held to be components of distinct memory systems that can guide competing behavioral strategies. While hippocampus-dependent episodic memory is particularly age sensitive, the striatal system is more age-resistant. We suggest that cTPL requires the plasticity of an intact hippocampus, while ordinal TPL, as used by the untrained (naïve) mice, may instead rely more on the aging-resistant striatal (procedural) memory system. Future studies may provide more insight on the conditional use of different TPL strategies and the putative involvement of dissociable memory systems.

Together, these data show the positive effects of repeated cognitive training and shed new light on aging-related loss of behavioral flexibility to update time of day information. Most importantly, these data show the age-sensitivity of TPL, and indicate its potential as an animal model for episodic memory and aging. Such functional behavioral models are scarce, yet essential to test interventions that potentially improve detrimental effects of aging and (episodic) memory related diseases like Alzheimer's disease.