CHAPTER 1

General Introduction

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Time-Place Learning

Time–place learning (TPL) refers to the ability to secure resources when they are available under specific temporal and spatial contingencies (Crystal 2009). Many environmental aspects show circadian variation. Predators often establish hunting routes and many resources, like food and mates only become available on certain times of the day (Daan and Koene 1981; Rijnsdorp et al. 1981; Silver and Bittman 1984). This given, TPL is believed to be functional in optimizing resource localization and exploitation as well as predator avoidance in the circadian environment, decreasing energy expenditure and increasing survival chances. Although this is mainly a hypothetical explanation as to why animals possess TPL ability, an evolutionary relevance is strengthened by the fact that TPL has been shown in many species including bees (Gould 1987), ants (Harrison and Breed 1987), fish (Reebs 1996), birds (Krebs and Biebach 1989), rats (Boulos and Logothetis 1990) and recently mice (Van der Zee et al. 2008). Evidence suggests that TPL can depend on the circadian system. This circadian TPL suggests an unexplored link between the circadian system and associative memory.

The notion that time of day can be relevant in cognitive functions arose long before the concept of circadian clock systems was established. Beling published her first study on “Zeitgedachtnis”, time memory, studying sun compass orientation in honey bees (Beling 1929). In 1950, Kramer did rather similar experiments in starlings. He showed that these birds also use an internal time of day mechanism to select the appropriate orientation relative to the position of an artificial sun (Kramer 1950). These findings, of a functional internal timing in vertebrates, helped in the breakthrough of biological clock concepts (Aschoff 1954; Pittendrigh et al. 1958). Since then we gained much insight into the physiology and molecular determinants of the circadian system itself and on the manifold behavioral and physiological processes under circadian control.

In mammals, a central nervous system based pacemaker, located in the suprachiasmatic nuclei (SCN), tracks light and dark information and is connected
with many peripheral, organ based oscillators (Dibner et al. 2010). 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. 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 (Ko & Takahashi, 2006).

In external 24h light-dark (LD) cycles the circadian system results in entrained daily rhythms in the body. In constant external conditions, these rhythms show a free running pattern of about 24h (circadian). The circadian sleep-wake cycle, regulation of hormonal, body temperature and feeding rhythms are well-known examples. General cognitive- and memory performances have also been shown to vary over the circadian cycle (for review see Carrier and Monk 2000). In line with this, disruption of circadian rhythms due to age, shift work and shifts of the LD cycle (jet lag) have been associated with impairments of cognitive function (Antoniadis et al. 2000; Biemans et al., 2003; Cain et al. 2004b; Craig and McDonald 2008; Devan et al. 2001; Fekete et al. 1985; Folkard et al. 1985a,b).

Besides governing innate rhythms in physiology and behavior, it is believed that circadian oscillators can provide phase information to brain systems involved in cognition, like memory, which allows time to be used in adaptive mechanisms (Enright 1970; Enright 1975; Gallistel 1990; Mistlberger et al. 1996). It is believed that time of day information derived from an internal oscillator can be ‘stamped’ in memory as a contextual feature to form associations with other contextual features and to be used in decision making processes. Such a mechanism can only function when a clock can be consulted continuously to record time-stamps, and to check whether coded time-stamps match the actual time of the day. This ‘continues consulted clock’ function is thought to underlie cTPL as well as the adaptive time of day compensation in sun compass navigation demonstrated in several insect and bird species (Budzynski et al. 2000; Frisch 1950; Hoffmann 1960; Keeton 1974; Kramer 1950; Merlin et al. 2009).
Terminology
In order to avoid confusion, it is necessary to explain/define some terminology regarding TPL. TPL is also referred to in the literature as time-place discrimination or time-place association. Two types of TPL paradigms can be found in the literature: Interval- and daily TPL paradigms. Interval TPL paradigms are specifically designed to study interval timing (the ability to keep track of elapsed time). In such paradigms, animals typically receive a single test session each day (with multiple trials/location switches), and these sessions can occur at varying times of the day. For example, animals can learn to anticipate the switch when first food is provided only at location ‘A’, while after ‘X’ amount of time food is provided only at location ‘B’. Rats can learn an interval time-place task with at least four different feeding locations and with unequal intervals between rewarding locations (Thorpe and Wilkie 2002). In daily TPL paradigms, typically the location of a resource depends on the time of day, and animals are trained over multiple days with multiple sessions per day on fixed time-points, so that they learn to visit or avoid specific locations on specific times of the day.

In this thesis we focus on daily TPL. Thus when we refer to TPL, more specifically we refer to daily TPL. In the literature, daily TPL is sometimes referred to as circadian TPL, however, when we refer to circadian TPL (cTPL), we refer to daily TPL with the use of a circadian strategy. This distinction is necessary because animals may use multiple strategies to solve a TPL paradigm, as will be explained in the next paragraph.

Multiple strategies for TPL
We mentioned that TPL can depend on the circadian system (in this case we refer to cTPL). This is only true if animals use a so called circadian strategy to master the paradigm. Alternatively, three non-circadian TPL strategies have been identified (Carr and Wilkie 1997). These strategies will be explained here along with methods to identify these non-circadian strategies. First, animals may use the so called contextual cue strategy. This is a non-timing strategy by which animals simply learn to visit or avoid location A in the presence of one (set of-) contextual cue(s), while they learn to visit/avoid location B in the presence of another (set of-) contextual cue(s). To exclude the possibility for animals to use this strategy, any discriminating contextual cues (differences between sessions) should be excluded by a proper research setup and practice.
Second, animals may use an ordinal strategy. In this case animals remember the sequence of (daily) events, e.g. first visit location A, then B. This strategy is also referred to as an alternation strategy and can also be viewed as the establishment of a (daily) route. Two variants of the ordinal strategy are identified: A timing- and a non-timing variant. In case of the timing variant, the sequence is reset daily, while this is not the case in the non-timing variant. The use of an ordinal strategy can be identified by skipping sessions. Note however that skipping the last session of a day will not identify an ordinal strategy when the timing variant is utilized. Animals will show normal location visits in the first session of the next day because the sequence has been reset. Thus, to identify the use of an ordinal strategy, without further distinction between the two variants, the first session(s) of a day need to be skipped. Instead of visiting the second session location, animals will visit the wrong (skipped) first session location in case an ordinal strategy is used.

Third, animals may use an interval timing strategy. In this case specific delay periods relative to one or more external cues are encoded as discriminating cues to know which locations to visit or avoid. For instance, animals may learn that short (or concrete after X amount of time) after lights-on, they should visit location A, while longer after lights on (or concrete after Y amount of time) they should visit location B. Because external cues can start/stop/reset timing, interval timing is also referred to as a stopwatch like mechanism. Interval timing is thought to be utilized to track intervals in a second-to-minutes range (Buhusi and Meck 2005; Crystal 2009; Wilkie 1995), but some studies have suggested that intervals of several hours can be tracked (Pizzo and Crystal 2006). According to the classical pacemaker accumulator theory, the circadian system may be implicated in interval timing as well. However, recent advances have challenged this view (Buhusi and Meck 2005; Staddon and Higa 1999; Wearden 2004; Yin and Troger 2011). Interval timing has been shown to be independent of the SCN and Cry1,2 clock genes (Lewis et al. 2003; Papachristos et al. 2011) and thus seems to be independent of the circadian system. To rule out the possibility that animals use this strategy for TPL, any distinctive external cues should be ruled out from experimentation, for instance by testing under constant light conditions to eliminate light-dark transitions. Previous test sessions may also start/stop/reset interval timing (intersession interval timing). Similar to the ordinal strategy, this possibility can easily be ruled out by performing session skips.
Only when the above non-circadian strategies have been experimentally ruled out, one can conclude that animals must use a circadian timing strategy. In this case TPL is based on an internal clock, independent of external cues. The endogenous nature of cTPL can further be shown by identifying known oscillator characteristics, like persistence in constant conditions, and known limitations imposed by the circadian system (Crystal 2009).

**Time memory: Circadian retention paradigms vs. cTPL paradigms**

Evidence that animals remember time of day is provided by behavioral paradigms that involve a training (stimulus encounter) followed by a retention test. In such experiments, animals show optimal retention when training and testing times match, indicating memory for the time of training. A basis for this knowledge was provided by Kamin who reported retention in a passive avoidance paradigm to be minimal one hour after training compared to the retention after 24 hours, or even 19 days after training (Kamin 1957). Why retention would be enhanced after a long interval compared to after a short interval was difficult to explain and the phenomenon, still known as the Kamin effect, has long been misinterpreted as a weak transition point in the processing of short-term memory into long-term memory.

In 1973, Holloway and Wansley discovered that the Kamin effect was actually the result of a circadian periodicity in memory retention. They showed that, independent of the time of day at which training occurs, retention is always optimal 24 hours later, or multiples thereof (Holloway and Wansley 1973b; Holloway and Wansley 1973a; Wansley and Holloway 1976). Next to passive avoidance, such periodic retention effects were also shown for active avoidance (Holloway and Wansley 1973b; Cain et al. 2008), appetitive motivated learning (Wansley and Holloway 1975), fear conditioning (Chaudhury and Colwell 2002), conditioned place preference (Ralph et al. 2002; Valentinuzzi et al. 2008), and conditioned place avoidance (Cain et al. 2004a). However, it should be noted that not all mammals show this pattern (Oklejewicz et al. 2001).

Note that the retention paradigms described above do not require animals to remember the time of training. Apparently animals remember time of day automatically. Therefore, Gallistel proposed that animals automatically encode time, together with place and the nature of biological significant events (Gallistel...
Although periodic retention deficits suggest time-memory, they do not necessarily implicate that animals learn an association between a stimulus and circadian phase. An alternative interpretation is that behavioral output (e.g. freezing) has been entrained by the stimulus pulse in the same way circadian rhythms can be entrained by other time-cues (zeitgebers), such as LD transitions. However, when an animal is trained to go to different places at different times of day, as in TPL paradigms, more than entrainment of an oscillator must be involved: an association between time and place must have been learned (Biebach 1989). In cTPL paradigms, memory of time is displayed directly by active choices. Animals are stimulated to remember the time because time discriminates between correct and incorrect choices. The correctness of a choice depends on correct memory and retention of time, based on previous encounters.

In summary, animals may use different strategies for TPL. The use of a circadian strategy is of special interest. cTPL presumes that an internal clock, and time derived from it, can be used by higher cognitive brain systems in adaptive experience based behavior. Correct location choices in cTPL implicate knowledge of current time of day (internal clock consultation), training times being stored as a contextual cue in memory (time-stamping, time-memory) and an association of these contextual time-points with spatial features and the nature of the event to guide behavior. These features make cTPL a unique circadian system dependent learning and memory ability. However, much remains to be discovered regarding the origin of the underlying circadian clock mechanism and its connection with the memory system. cTPL provides the means to investigate this connection. This will be the focus of the final section of this chapter.

Putative mechanism underlying cTPL

The internal circadian process underlying cTPL currently remains elusive. The SCN (central pacemaker) would be a first educated guess. Via neuuropeptidergic, nonsynaptic pathways the SCN is connected to brain regions involved in learning and memory (Van der Zee et al. 2009 and references therein). One of the major output systems of the SCN is the neuuropeptidergic Vasopressin (AVP) system. AVP released in the third ventricle reaches numerous brain regions involved in learning
and memory, including the hippocampus. AVP receptors are expressed in the hippocampus, and AVP is able to induce LTP in various subregions of the hippocampus (Dubrovsky et al. 2003). The AVP neurons of the SCN and the formation of time memory are regulated by cholinergic input (Hut and Van der Zee 2011), by which the SCN may provide a time cue to support the formation of time-place associations in the hippocampus. Both the AVP system (Van der Zee et al. 1999) and cholinergic signaling in the SCN (Van der Zee et al. 1991) are strikingly age-sensitive. Aged rats show age-specific alteration in the AVP and cholinoreceptive systems in the SCN (Biemans et al. 2003), predicting age-related impairments in cTPL performance. Taken together, the SCN seems a good candidate as the circadian clock underlying cTPL.

An alternative candidate for circadian regulation of TPL is the FEO (Stephan 2002). Despite many lesion studies this oscillator has not yet been fully localized (Davidson 2006). Currently the FEO is thought to be comprised of a network of interconnected brain structures (Carneiro and Araujo 2009). cTPL has also been shown in experiments that did not involve food (Widman et al. 2004), arguing against the FEO as the timing mechanism behind cTPL. Another identified oscillator is the methamphetamine sensitive circadian oscillator (MASCO) (Hiroshige et al. 2009), but it has been hypothesized that both the FEO and the MASCO may be a manifestation of the same oscillator induced by arousal (Cain and Ralph 2009). Together, these known oscillators may drive the circadian expression of many potential time cues for cTPL.

Several criteria may narrow the search: 1. Potential candidate time cues should obey the characteristic of being expressed in a circadian pattern. 2. As the hippocampus is the key region for learning and spatial memory, the cue should be able to reach the hippocampus through either synaptic or hormonal routes. 3. In turn the hippocampus should be receptive for the time cue. Given these criteria, next to the already discussed SCN derived AVP, potential candidates may be hormones like leptin, ghrelin, corticosteron, glucagon and insulin (Carneiro and Araujo 2009; Lukoyanov et al. 2002), or neurotransmitters like dopamine (Aragona et al. 2002). In addition, non-hormonal metabolic signals, like free fatty acids, ketone bodies and glucose could function as a food driven hourglass timer, for instance, through depletion of glycogen stores or through SIRT1, an NAD(+) dependent deacetylase which is known to modulate CLOCK-BMAL1 activity (Asher
et al. 2008). In addition, multiple time signals and underlying oscillators and/or metabolic hourglass mechanisms may influence subjective timing, as for instance seen in the regulation of sleep (Daan et al. 1984).

Recent studies have shown that the hippocampus harbors its own clockwork. Clock genes are expressed in most regions of the hippocampus (CA1, CA3, DG) and peak expression of some clock genes seems to coincide with times at which memory is consolidated (Feillet et al. 2008; Jilg et al. 2010). Moreover, memory deficits have been shown in clock gene knockout animals (Feillet et al. 2008; Jilg et al. 2010; Lyons et al. 2006; Sakai et al. 2004). Recently, memory formation and consolidation were shown to depend on the circadian reactivation of the cAMP/MAPK/CREB pathway in hippocampal neurons (Eckel-Mahan et al. 2008). Although the circadian expression of clock genes (Per2) has been shown in isolated hippocampal slices (Wang et al. 2009), MAPK cycling seems to be driven by the SCN (Phan et al. 2011). In the SCN, activation of the MAPK pathway is light responsive (Butcher et al. 2002; Obrietan et al. 1998). It has been hypothesized that training might function similarly in hippocampal neurons as light activation and clock resetting functions in SCN neurons; inducing a unique, temporally specific molecular profile in memory-specific neuronal ensembles (Gerstner et al. 2009). Indeed, BMAL1 is negatively regulated by MAPK (Sanada et al. 2002), providing a molecular clock resetting mechanism by training. Interestingly, next to ‘place’ cells in the hippocampal CA1 and CA3 region, ‘time’ cells have recently been identified and shown to track the elapsing of an interval (Macdonald et al. 2011; Shapiro 2011). It is likely that this hippocampal clock may not only be entrained by training (event encounters), but is also modulated by internal cues like SCN and FEO outputs, thus integrating multiple time-signals.

Taken together, the SCN (LEO), the FEO, and the hippocampus, as well as rhythmically expressed hormones like corticosterone are major candidates to be involved in cTPL. Therefore the studies described in this thesis have focused on these candidates and the results may provide a first step to clarify the neurobiological mechanisms underlying cTPL.
Outline of the thesis

Chapter 2 adds to this first introductory chapter, as both chapters were part of a single published review. Chapter 2 provides a more in-depth literature overview describing the long road towards a functional laboratory TPL paradigm for mice and the first results using this setup on an earlier investigation of Cry1 and Cry2 clock gene deficient mice. The finding that cTPL depends on Cry1 and/or Cry2 clock genes led to the question whether cTPL specifically depends on Cry genes, or on other clock genes as well. In Chapter 3, we therefore studied TPL in Per1 and Per2 double mutant mice.

The circadian system as a whole is known to be particularly sensitive to time-cues of light and time-restricted food availability. There is considerable evidence suggesting that these different zeitgebers entrain dissociable circadian systems. Therefore, in chapter 4 we studied the sensitivity of cTPL to timing manipulations with light as well as food. Furthermore, in our search to locate the main internal clock used in cTPL, we investigated the role of two major clocks, namely the SCN master pacemaker in the brain, and the peripheral adrenal clock. We lesioned the SCN and removed the adrenals to investigate their requirement for cTPL in mice.

Since both hippocampal dependent associative (what-where-when) memory, as well as circadian rhythms show a markedly decline with age, cTPL is predicted to be particularly age-sensitive. TPL may therefore be a suitable model for aging and neurodegenerative disease. In chapter 5, we investigated TPL for the first time in the context of aging, using a longitudinal setup.

Finding the neurobiological correlates of cTPL will shed light on the underlying mechanism. In chapter 6 we investigate the SCN, hippocampus, cortex, and the paraventricular thalamic nucleus for potential markers involved in cTPL, by comparing TPL trained mice (sacrificed at a TPL test-session time-point) with home cage control mice. Finally, in chapter 7 we discuss the main results and conclusions in relation to each other and in the framework of existing literature. Based on what we have learned so far, we suggest targets for future studies to further unravel the mechanisms behind cTPL.

The reference list of this chapter is grouped with the references list of the next chapter and can thus be found at the end of chapter 2.