CHAPTER 6

Investigating Immunohistochemical markers for Time-Place Learning

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Abstract

With time-place learning (TPL), animals link a stimulus with the location and the time of day. Animals can use their circadian system for TPL, in which case we refer to circadian TPL (cTPL). Previously we found that cTPL does not require the SCN, but that cTPL is dependent on the core molecular clock genes *Cry1* and/or *Cry2*. However, much remains to be discovered about the specific connection between the circadian system and memory formation. Finding neurobiological correlates of cTPL will shed light on the underlying mechanism. We applied immunohistochemistry (IHC) on the brains of young mice that had successfully mastered cTPL. These mice were sacrificed the day after their last TPL test day, at the time of a daily TPL test session, together with home cage control (HCC) mice. We investigated c-Fos, *CRY2*, vasopressin (AVP), and pCREB as potential markers in the SCN, the main circadian clock, and the hippocampus, the main locus for declarative (episodic) memory formation.

In line with our previous finding that the SCN is not critically involved in cTPL, we found no differences at the level of the SCN for the investigated markers (*CRY2*, c-Fos and AVP). Moreover, in line with our previous finding that cTPL depends on *Cry* genes, we found a 26% increase of *CRY2* expression in the hippocampal dentate gyrus. These results further indicate a key role of CRY proteins in cTPL and designate involvement of the hippocampus. Interestingly, 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. The hippocampus and PV may thus be interesting targets for future (lesion) studies to further unravel the mechanism behind cTPL.
Introduction

cTPL presumes a functional connection between the circadian system and memory system(s), but not much is known about this connection regarding the origin of the underlying circadian oscillator and the molecular/neuronal signaling to the memory system. Neurobiological correlates of circadian system dependent Time-Place Learning (cTPL) potentially shed light on the underlying mechanism. We applied immunohistochemistry (IHC) on the brains of young mice that had successfully mastered cTPL. These mice were sacrificed the day after their last TPL test day, at the time of their first (or second) daily test session, together with homecage control (HCC) mice. All mice had been similarly food deprived (see the materials and methods section for more details). We used IHC (protein detection) instead of In situ hybridization (mRNA detection) because we aimed at identifying protein markers that are functional to cTPL behavior. In trained mice, such functional proteins should be expressed in anticipation of TPL testing time-points, and show higher expression levels compared to HCC mice sacrificed at the same TOD. We investigated c-Fos, CRY2, vasopressin (AVP), and pCREB as potential markers, each of which will be shortly introduced in the following paragraph. We hypothesized that a) the suprachiasmatic nucleus (SCN), the main circadian clock, would not reveal significant changes given the results of chapter 4 (SCN lesions did not affect TPL), and b) that the hippocampus, as the main locus for declarative (episodic) memory formation, would show significant changes.

The markers we chose were c-Fos, CRY2, AVP, and pCREB. C-Fos belongs to the immediate early gene (IEG) family of transcription factors. Because IEGs are rapidly induced by neuronal activity, c-Fos is widely used as a marker for activated circuits at cellular scale (Kawashima et al. 2014; Morgan et al. 1987; Sagar et al. 1988). CRY2 is the transcription product of the core molecular clock gene Cry2 (Cryptochrome 2). On a cellular level, circadian rhythms are predominantly controlled by clock genes and their protein products. 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 and Takahashi 2006). In absence of a light-dark (LD) cycle, mice lacking the Cry1 and Cry2 genes lose periodicity (i.e. become arrhythmic) in wheel-running behavior (van der Horst et al. 1999), electrophysiological activity in the SCN (Bonnefont et al. 2003) and the rhythm in core body temperature (Nagashima et al. 2005). The Cry genes are specifically interesting to investigate as neuronal markers for TPL, because we showed that TPL depends on Cry1 and/or Cry2 (Van der Zee et al. 2008), but not Per1 and Per2 clock genes (Mulder et al. 2013). We also attempted to investigate CRY1, the paralog of CRY2. Unfortunately, we could only get a specific signal with the antibody for CRY2. AVP (arginine vasopressin) is seen as the major output signal of the SCN master clock. Approximately 10-30% of the neurons within the SCN contain AVP. These neurons show a daily rhythm in AVP synthesis as well as in the number of AVP-expressing SCN neurons and release (Kalamatianos et al. 2004). AVP release is found to peak in the light phase and trough in the dark phase for nocturnal as well as diurnal animals (Kalamatianos et al. 2004; Kalsbeek et al. 2010). Released AVP is known to be active in- and outside the SCN. Vasopressin is indicated as the (humoral) output of the SCN because AVP producing SCN neurons project to distal targets areas, such as to the paraventricular nucleus (PVN). But AVP also has excitatory action within the SCN acting on the V1-type receptors (Kalamatianos et al. 2004). Interestingly, salient events have been shown to induce a circadian rhythm in the expression of muscarinic acetylcholine receptors in the SCN, with peak expression levels coinciding with the event-specific time of day (TOD) (Van der Zee et al. 2004). It has therefore been proposed that the SCN may function as a programmable “alarm clock”, using the neuropeptide AVP as an output to transfer the specific TOD information to other brain regions (Biemans et al. 2003; Hut and Van der Zee 2011; van der Veen et al. 2008; Van der Zee et al. 2004). pCREB is a widely used marker for neuronal plasticity. CREB (cAMP response element-binding protein) is a cellular transcription factor which binds to certain DNA sequences called cAMP response elements (CRE), thereby increasing or decreasing the transcription of downstream genes. The phosphorylated form of CREB (pCREB) has been shown to be integral in the formation of spatial memory. Moreover, pCREB has a well-documented role in neuronal plasticity and protein synthesis-dependent long-term memory formation in diverse behavioral paradigms among many species (Bernabeu et al. 1997; Colombo et al. 2003; Countryman et al. 2005; Guzowski
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and McGaugh 1997; Llamprecht et al. 1997). pCREB stimulates the expression of several immediate-early genes (IEGs). One of those genes is the proto-oncogene transcription factor c-Fos (Sheng and Greenberg 1990).

In the SCN we analyzed c-Fos, CRY2 and AVP, and in the hippocampus we analyzed CRY2, c-Fos and pCREB in those subregions where specific and clear immunostaining was present. pCREB was also analyzed in the cortex for its function in the storage of long-term memory. C-Fos was additionally analyzed in the (anterior) paraventricular thalamic nucleus (PV, or PVT), because a clear signal (specific staining of neurons) was observed. The PV receives input from all major components of the circadian timing system, including the suprachiasmatic nucleus (SCN), the intergeniculate leaflet and the retina. Tracing studies have shown that the PV is ideally situated to relay circadian timing information from the SCN to brain areas involved in visceral and motivational aspects of behavior and to provide feedback regulation of the SCN (Moga et al. 1995).
Materials and methods

Animals

Brain material from three different cohorts of young to middle-aged male C57BL6 mice was used. Each cohort consisted of TPL-trained mice and homecage control (HCC) mice. HCC mice had been similarly food deprived as TPL-tested mice. Details are shown in Table 1. Cohorts 1 and 2 were of similar age and specifically TPL trained for later immunohistochemical analysis (DEC 5583F). Animals from cohort 3 were SCN lesioned mice described in chapter 4 (DEC 5583D). No differences were found between SCN lesioned mice and SHAM SCN lesioned mice, indicating that the SCN lesions had no effect on the expression of the investigated markers (c-Fos and pCREB). Therefore, these mice were grouped as TPL-trained mice.

Table 1 Details of mice used for immunohistochemistry. The age is the age at which the mice were sacrificed. TOD indicates the TPL test session at which the mice were sacrificed. C-Fos in the DG was measured in both cohorts 2 and 3. These results were grouped because results were similar for both cohorts.

<table>
<thead>
<tr>
<th>marker</th>
<th>brain area</th>
<th>cohort</th>
<th>age</th>
<th>N (HCC)</th>
<th>N (TPL)</th>
<th>days trained</th>
<th>TOD (session)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRY2</td>
<td>SCN</td>
<td>1</td>
<td>4.6</td>
<td>7</td>
<td>6</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>DG</td>
<td>1</td>
<td>4.6</td>
<td>9</td>
<td>8</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CA3</td>
<td>1</td>
<td>4.6</td>
<td>8</td>
<td>7</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td>AVP</td>
<td>SCN</td>
<td>1</td>
<td>4.6</td>
<td>6</td>
<td>5</td>
<td>36</td>
<td>2</td>
</tr>
<tr>
<td>c-Fos</td>
<td>SCN</td>
<td>2</td>
<td>4.8</td>
<td>6</td>
<td>4</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DG</td>
<td>2+3</td>
<td>4.8 / 8.3</td>
<td>13</td>
<td>18</td>
<td>47/44</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>PV</td>
<td>3</td>
<td>8.3</td>
<td>4</td>
<td>9</td>
<td>44</td>
<td>1</td>
</tr>
<tr>
<td>pCREB</td>
<td>DG</td>
<td>3</td>
<td>8.3</td>
<td>5</td>
<td>9</td>
<td>44</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CA3</td>
<td>3</td>
<td>8.3</td>
<td>4</td>
<td>9</td>
<td>44</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CA1</td>
<td>3</td>
<td>8.3</td>
<td>4</td>
<td>9</td>
<td>44</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>cortex</td>
<td>3</td>
<td>8.3</td>
<td>5</td>
<td>9</td>
<td>44</td>
<td>1</td>
</tr>
</tbody>
</table>

Mice from cohort 1 were sacrificed at the time of their second daily TPL test-session, while mice from cohorts 2 and 3 were sacrificed at their first daily TPL test-session (deviation maximally 10 minutes). We did select these time points for practical reasons, but avoided selecting the third daily TPL test-session because expression of most clock genes is lower at the beginning of the light-phase. Hence, by choosing the earlier time points we increased the detectability of potentially upregulated markers (compared to HCC mice) in anticipation of TPL testing.
Collecting and processing of brain material
Under deep pentobarbital anaesthesia, mice were perfused transcardially for 1 minute with 0.9% NaCl + 0.5% heparin (400U) in H2O (15ml/min), followed by 150 ml 4% paraformaldehyde (PF) in 0.1M phosphate buffer (PB) for fixation. Brains were collected and further processed in Greiner cups (Greiner Bio-One, Container, PS, 15 ml, 40 x 24.5 mm snapdeks, cat # 203170). Brains were postfixated for 24h in 4% PF in 0.1M PB, rinsed for one day in 0.01M phosphate buffered saline (PBS, pH 7.4) and then kept overnight in 30% sucrose in PBS cryoprotectant at 4⁰C. Brains were frozen the next day using liquid nitrogen and stored at -80⁰C until further processing. Brains were cut in coronal sections of 25 μm thick using a cryotome and stored at 4⁰C. Target areas were the SCN (these sections also containing the anterior PV) which was cut from -0.34 to -0.70 relative to bregma, and the hippocampus (these sections also containing PV and cortex) which was cut from -1.82 to -2.06 relative to bregma according to the mouse brain stereotaxic atlas (Keith B.J. Franklin and George Paximos 1997, Academic press, CA, USA). Sections were equally distributed over several Greiner cups containing 0,01M PBS, to create multiple equal series that could be used for different immuno-stainings.

Immunohistochemistry
Three to five brain sections per mouse were used for each staining. Because similar protocols were used for each staining, only the procedures for the pCREB staining will be described as an example. Brain sections were rinsed three times for 5 minutes in TBS (0.01 M Tris-HCL + 0.9% NaCl, pH=7.4), and were then placed in 0.3% H2O2 in TBS for 30 minutes. After rinsing the sections in TBS four times, 5 minutes each time, the primary antibody solution was added (Rabbit α-pCREB Millipore 1:1000 with 5% Normal Goat Serum and 0.1% Triton-X 100 in TBS). Sections were incubated overnight at room temperature on a shaker. After being rinsed with TBS eight times, for 10 minutes each time, the sections were incubated at room temperature for 2 hours with the secondary antibody (biotinylated Goat anti-rabbit IgG Jackson 1:500 with 1% Normal Goat Serum and 0.1% Triton-X 100 in TBS). Next, sections were rinsed eight times with TBS for 10 minutes each time. After that, the sections were put in ABC complex (1:500 in TBS) for 2 hours and then, they were rinsed again eight times with TBS for 10 minutes each time. Finally, the labeled cells were visualized with
diaminobenzidine (DAB, 0.7mg/mL in H₂O; Sigma-Aldrich, Steinheim, Germany) with 0.1% H₂O₂ as a reaction initiator. The reaction was stopped by rinsing three times with TBS for 5 minutes each time, and stored overnight in TBS at 4°C. The following day, the slices were mounted from a 1% gelatin in aquadest solution onto microscopic glasses using a brush. The sections were placed with the posterior side faced up (during cutting the left hemisphere was marked so it could be distinguished during mounting) and ordered from anterior to posterior. Sections were left to dry for one day after which they were gently mounted (from 1% gelatin solution with 0.001% aluin) on glass slides, and left to dry overnight. Next, sections were put through a alcohol-xylol concentration series, covered with a cover glass using DPX mountant. A similar protocol was used for the other immuno-stainings, using different primary, and matching secondary antibodies. For CRY2, the primary antibody used was rabbit polyclonal anti-mCRY2 (1:200, from Alpha Diagnostic, USA). For c-Fos: rabbit polyclonal anti-c-Fos AB-5 (1:8000, vector), and for AVP: monoclonal anti-AVP (1:1000, PS41, kindly supplied by Dr. H. Gainer, NIH, Maryland).

Quantification
For each staining, the most appropriate quantification method was determined. When only few specifically labelled cells were present in the area of interest or when a variable background was present, cells were manually counted through a microscope. This applies for CRY2 in the SCN and DG, and for c-Fos in the DG and PV. For the other stainings, optical densities (OD) were measured at 50x magnification using a computerized image analysis system (Quantimet 550, Leica, Cambridge, UK). The OD is expressed in arbitrary units corresponding to grey levels. To correct for variability in background staining among sections, background labeling was measured in the corpus callosum and extracted from the OD of the area of interest. Bilateral measurements were averaged. The experimenter was blind to the treatment of individual animals during all cell counting and OD measurements. Because of the different quantification methods used, all results are expressed as percentage relative to the HCC group. Differences between TPL and HCC groups were tested by two-tailed unpaired t-tests using Microsoft Excel.
## Results and discussion

Results of the different immuno-stainings are summarized in Figure 1. Because different quantification methods were used, all results are expressed as percentage relative to the HCC group (set at 100% expression), for optimal comparison. Representative pictures of the performed immuno-stainings are shown in Figure 2.

**Figure 1** Summary of results of all performed immuno-stainings in investigated brain regions. Expression levels are relative to home cage control (HCC) mice, which are depicted by the horizontal grey line at 100% (including error bars). The Dentate Gyrus (DG), Cornu Ammonis areas 1 and 3 (CA1; CA3) are subregions of the hippocampus. Same markers are indicated by same greyscale colors. All error bars represent SEM. Statistical evaluations (two-tailed unpaired t-tests) of expression in HCC vs. TPL trained mice are included: * p<0.05, ** p<0.01, *** p<0.001. CRY2 expression in the DG showed a statistical trend toward significance (p=0.09).

A first observation from these data is that no differences between TPL trained and HCC mice were found at the level of the SCN for the investigated markers (CRY2, c-Fos and AVP). This is in line with our findings reported in chapter 4, that the SCN is not essential for TPL (Mulder et al. 2014). Notably, a significant decrease in c-Fos positive cell-count in the Dentate Gyrus (DG) and the optical density of pCREB-positive cells located in DG, CA1, CA3 and Somatosensory Barrel Cortex was found in TPL trained mice compared to HCC mice.
One explanation for these decreased expression levels is that mice were extensively trained. It has been shown that, with extensive training, c-Fos is attenuated in most brain regions (Bertaina-Anglade et al. 2000). Moreover, c-Fos and pCREB are related in the way that pCREB also stimulates the expression of c-Fos (Sheng and Greenberg 1990). In extensively trained animals, the hippocampus may be devoted to the learned task (retention rather than acquisition) activating
only the cells devoted to this task. From another perspective, training may increase synchronization of hippocampal neurons, causing less cells to be active at one given time point. It would therefore be interesting to also investigate these markers during the learning (acquisition) phase of TPL. In 1990, Kononen and his colleagues showed that in the rat brain, c-Fos levels show a circadian rhythmicity, with peak expression in the active (dark) phase (Kononen et al. 1990). Therefore, another explanation for the decreased expression levels may be that TPL testing had induced a phase shift (advance) in c-Fos (and pCREB) circadian expression relative to the ‘normal’ expression pattern in HCC mice.

The most pronounced difference between TPL trained and HCC mice was found in c-Fos expression in the paraventricular thalamic nucleus (PV). The PV has been referred to as a relay station transmitting SCN circadian rhythms throughout the brain (Moga et al. 1995). Indeed, the PV receives input from all major components of the circadian timing system, including the SCN (Pickard 1982), subparaventricular zone (Watts et al. 1987), the intergeniculate leaflet, and the retina (Moga et al. 1995). In addition, the PV is connected to brain areas involved in learning and memory, including the ventral striatum, amygdala, entorhinal cortex, hippocampus, and cortex (See Moga et al. 1995, and referenced therein). The PV may thus be an interesting target area for future lesion studies in the context of TPL.

The 26% upregulation of CRY2 in the DG of TPL trained mice compared to HCC mice is an interesting finding. cTPL likely involves the hippocampus, which is known to be involved in spatial navigation and associative (episodic) memory. The DG is one of the few brain areas where adult neurogenesis occurs, and thought to be particularly involved in the formation of new episodic memories (Amaral et al. 2007; Treves et al. 2008). It has been proposed that experience-related cues (cognitive training) may act as a zeitgeber to the hippocampus, where local timekeeping mechanisms may be entrained (Gritton et al. 2013). Moreover, we previously found that Cry1/Cry2 double knockout mice were unable to master TPL, while Per1/Per2 double mutant mice showed cTPL similar as wild-type mice (Mulder et al., 2013a; Van der Zee et al., 2008). Whether Cry, but not Per genes are essential for temporal coding in the hippocampus remains to be further investigated, for example by using hippocampus specific Cry and Per knockout mice.
Reference List


