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Research article

How demanding is the brain on a reversal task under day and night conditions?

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HIGHLIGHTS

- We studied day and night effects on spatial reversal learning.
- The metabolic brain consume by cytochrome *c*-oxidase (CO) was shown.
- No behavioral differences in the time of the day between groups were found.
- CO differences were shown in cortical regions, hippocampus, striatum and thalamus.
- Orbitofrontal cortex was revealed as a key structure in the process.

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ABSTRACT

Reversal learning has been studied as the process of learning to inhibit previously rewarded actions. These behavioral studies are usually performed during the day, when animals are in their daily period rest. However, how day or night affects spatial reversal learning and the brain regions involved in the learning process are still unknown. We conducted two experiments using the Morris Water Maze under different light-conditions: naïve group (CN, $n = 8$), day group (DY, $n = 8$), control DY group (CDY, $n = 8$) night group (NG, $n = 8$), and control NG group (CNG, $n = 7$). Distance covered, velocity and latencies to reach the platform were examined. After completing these tasks, cytochrome *c*-oxidase activity (CO) in several brain limbic system structures was compared between groups. There were no behavioral differences in the time of day when the animals were trained. However, the metabolic brain consumption was higher in rats trained in the day condition. This CO increase was supported by the prefrontal cortex, thalamus, dorsal and ventral striatum, hippocampus and entorhinal cortex, revealing their role in the performance of the spatial reversal learning task. Finally, the orbitofrontal cortex has been revealed as a key structure in reversal learning execution.

1. Introduction

Circadian rhythms have been broadly studied on learning and memory processes in animal models that include both vertebrates and invertebrates [1]. However, the identified patterns of diurnal variation of memory performance differ among previous studies. One study in mice showed that memory performance was better during the light period than the dark in fear conditioning, whereas no effect was observed on place memory [2] or novel object recognition [3]. Contrarily, performance of the novel location recognition task was better in the dark period than the light period in rats [3]. One possible explanation for this discrepancy is the different brain regions involved in each task, and how they are affected by the time of day when the training takes place. For example, a diurnal variation in hippocampal long-term potentiation has been observed (LTP) [4] pointing out these circadian effects.

Other explanation could be the presence of diurnal/nocturnal activity rhythms which affect to several neurotransmitters release, especially the dopaminergic pathway [5] with consequences on behavioral performance.

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Moreover, it has been shown that melatonin also affects the firing rate of hippocampal CA1 neurons [6] and the appearance of circadian hormonal modulation of neuronal firing as a general mechanism throughout the brain has been revealed [7].

In line with this, the purpose of this work was to explore the way day or night affects spatial reversal learning and the brain regions involved in this learning process. For this purpose, the Morris Water Maze (MWM), a task commonly used to study spatial learning in rodents [8], was used in our experiments. To study metabolic activity, w used CO histochemistry. CO is a mitochondrial enzyme that catalyzes the transfer of electrons to oxygen generating ATP via the coupled process of oxidative phosphorylation [9]. CO activity reflects changes in the brain metabolic capacity induced by energy requirements, and CO activity is regulated by and closely correlated with brain functional activity [10,11].

Several authors demonstrated CO changes in memory circuits associated with spatial memory after several experimental manipulations. Hence, it was applied to discern how different structures modify their metabolic demands in subjects solving working memory tasks [12] or under other experimental manipulations [13,14].

2. Methods

2.1. Subjects

A total of 39 adult male Wistar rats from the animalarium of Oviedo University were used (250–300 g, three months age) at the start of the experiment. Rats were housed in groups of three to five, three weeks prior to the beginning of the experiments and maintained under standard laboratory conditions: *ad libitum* food and tap water, 22 ±2 °C, 65–70% relative humidity and an artificial light–dark cycle of 12 h (08:00–20:00/20:00–08:00). The procedures and manipulation of the animals used in this study were carried out according to the Directive (2010/63/EU), Royal Decree (1201/2005) of the Ministry of the Presidency and the local committee for animal studies (Oviedo University). The animals were randomly distributed into five groups: the naïve group (CN, $n = 8$); the night group (NG, $n = 8$), trained from 00.00 to 4.00 am; and the day group (DY, $n = 8$), trained from 8.00 to 12.00 pm. All the experiments were performed in the same frame of time for each experimental group. In addition, two swim control groups (one for each experimental group) were composed of rats that were placed in the maze the same number of times and days and at the same hour as their respective experimental groups, but without an available escape platform (CNG $n = 7$ and CDY, $n = 8$).

2.2. Reference and reversal task in the Morris water maze

The animals were trained in the MWM. The maze was made of fiberglass and measured 150 cm in diameter with a 40 cm high wall. The water level was 30 cm, and its temperature was 22 ±2 °C. The MWM was in the center of a 16 m^2 lit room (two halogen lamps of 4000 lx) and was surrounded by panels on which the spatial cues were placed. The behavior of the animal in the MWM was recorded by a video camera (Sony V88E) connected to a computer equipped with an EthoVision Pro program.

The learning protocol consisted of 6 days. The first day being destined for habituation, in which the animals carried out four trials with a visible platform in the center of the pool. During the following 4 days, the animals were subjected to four acquisition trials in which the platform was hidden in the center of quadrant D. After this training, the platform was moved to the opposite quadrant, C.

In each trial, the rats were allowed to swim to locate the platform, or were placed on it after 60 s, where they remained for 15 s before they were placed in the cage for 30 s. Latency to find the platform, defined as escape latency, were recorded. Daily, at the end of each learning session, rats were given a 25-s probe test without platform to measure the percentage of time spent in each quadrant of the pool.

On the sixth day, the escape platform was moved to the center of the opposite quadrant (quadrant C). In this case, cognitive flexibility was trained in one single session of 8 trials with the same experimental conditions as the other days. Latencies and time of permanence in each quadrant during the probe test were recorded.

2.3. Cytochrome oxidase histochemistry

Ninety minutes after the last training day, the animals were decapitated. Brains were removed, frozen rapidly in *N*-methylbutane (Sigma–Aldrich, Madrid, Spain) and stored at −40 °C [13]. To quantify enzymatic activity and control staining variability across different baths, sets of tissue homogenate standards from the Wistar rats' brains were cut at different thicknesses (10, 30, 40 and 60 μm) and included with each bath of slides. Quantification of CO histochemical staining intensity was done by densitometric analysis, using a computer-assisted image analysis workstation (MCID, Interfocus Imaging Ltd., Linton, England. The regions of interest were anatomically defined according to Paxinos and Watson's atlas [15] from bregma: +3.20 mm for the prefrontal cortex (infralimbic, prelimbic and cingulate cortex) and the orbitofrontal cortex (lateral and ventrolateral); −1.32 mm for the thalamus (anterodorsal, anteroventral and mediodorsal); +0.24 mm for the striatum (anterodorsal, anteromedial and anterolateral), the accumbens core and shell and the parietal cortex; −1.20 mm for the dorsal hippocampus (dentate gyrus, CA1 and CA3 areas); and −5.04 mm for the entorrhinal cortex.

2.4. Statistical analysis

Data were analyzed by SigmaStat 3.2 software (Systat Software, Chicago, USA). The distance covered and the velocity of the trained groups were compared using a two-way repeated-measures ANOVA. A one-way ANOVA was carried out to analyze the time spent in the target quadrant for each group. Tukey's tests were applied as *post hoc* tests when significant differences were observed. Differences in mean CO activity among the different groups for each brain region were analyzed with a two-way ANOVA (factors: group (CDY, CNG, CN, DY, NG) and time of the day (day or night) and expressed as mean ± SEM. Finally, Pearson product-moment correlations between CO activity and behavioral data were calculated using as the performance index the percentage of permanence in the reinforced quadrant during the last probe test for the two experimental groups (DY and NG groups). The results are considered statistically significant at $p < 0.05$.

3. Results

3.1. Behavioral learning

The two-way repeated-measures ANOVA revealed significant differences in distance covered ($F_{(1,79)} = 9.337$, $p = 0.009$) between the DY (571.743 ± 48.698) and NG (399.194 ± 30.519) groups in day 1 ($p = 0.021$) and day 2 ($p = 0.008$). No differences between DY (18.350 ± 0.589) and NG (18.199 ± 0.486) groups were found in velocity ($F_{(1,79)} = 0.0337$, $p = 0.857$). Moreover, significant differences between CDY (332.596 ± 45.012) and CNG (289.813 ± 26.510) group were found in distance covered ($F_{(1,79)} = 1.071$, $p = 0.318$) in day 1 ($p = 0.002$) and day 5 ($p = 0.038$). Velocity showed differences between CDY (10.725 ± 0.620) and CNG (14.525 ± 0.682) groups ($F_{(1,79)} = 45.150$, $p < 0.001$) in day 1 ($p = 0.005$), day 2 ($p < 0.001$), day 3 ($p = 0.008$) and day 5 ($p < 0.001$).