Light at night: Effect on the daily clock, learning, memory, cognition, and expression of transcripts in different brain regions of rat

Methods

Y-Maze: The standard protocol was followed for the Y-maze test (Havekes et al. 2007). Briefly, animals were first habituated to the testing room, examiner, and empty maze with doors open for three days. After three days of habituation, food was placed in both arms, with all the doors remaining open. Rat placed at the bottom of the Y chooses an arm to explore and gets the food reward. Once the food is consumed, it is placed again at the bottom of the Y for the second time to choose to go down of either arm. If it chooses the same arm, then it does not get the food reward and chooses incorrectly. If it chooses the other arm, then it gets the food reward. This test was continued for ten consecutive days. The apparatus was cleaned thoroughly between each animal trial using 70% ethanol. The number of times the rat chooses the correct arm to explore and get the food reward was recorded.

Novel Object Recognition (NOR) Test: Protocol proposed by Lueptow (Lueptow 2017) was followed. Here learning and memory are assessed with object recognition and measured by the time spent exploring the novel object compared with the familiar object. The objects were selected to be different enough to be easily discriminated by rats but have a similar degree of complexity. It minimizes any potential object preference that may bias the results. A square box (of about 40 cm x 40 cm x 40 cm) made from smooth white plywood was used as an arena for training. The experimental procedure involved habituation, training (T1), and testing (T2). In habituation, the rat was removed from its home cage, placed in the middle of the open empty arena, and allowed to explore the arena for 5 minutes freely. During training (T1), two identical

objects were placed in two corners of the arena. Twenty-four hours after habituation, the rat was removed from its home cage and placed in the arena facing the wall, equidistant from the two identical objects. Free exploration was allowed for 10 min, and video recording was done. After 10 minutes the rat was placed back in its home cage. The apparatus was cleaned after each animal trial with 70% ethanol. Twenty-four hours after training (T1), Testing (T2) was conducted. Here, one familiar object from Training (T1) and one novel object was placed in the exact locations used during training (T1) for each rat. The same steps were followed as in training (T1) for 10 minutes with video recording. The apparatus was cleaned thoroughly between each animal trial using 70% ethanol. At the end of the test, the rat was removed and placed in its home cage. Time spent exploring Nobel object was recorded.

Hole board (HB) test: This test allows the evaluation of a variety of behavioral dimensions (Boissier and Simon, 1962). The hole-board apparatus consisted of a square open field made up of four smooth plywood walls and a floor with 9 holes, each 4 cm in diameter, equidistant from each other. To record the animals, a video camera was mounted on a tripod, placed at a short distance from the hole-board. The rats were previously exposed to the hole-board before experimentation. The experiment was conducted after one hour of lights off during the dark phase to maximize the animal activity, under low red filtered light. The food rewards were placed in each hole to motivate the animal's exploration. The animals were placed in the corner of the hole-board facing the wall and allowed to freely explore for 10 minutes, and recorded by a video camera. After each animal, the hole-board was cleaned with ethanol (70%) to remove all scent traces and feces. The video recordings were analyzed, and the number of Head dip and unexplored holes was noted.

Morris water maze: The protocol for the Morris water maze test was adopted from Vorhees et al (Vorhees et al. 2006) with the modifications mentioned below. A circular steel tank of 6 feet in diameter (filled with water having a temperature of $26 \pm 2°C$) and a height of 3 feet was used for this experiment. A circular rescue platform of 10.5 cm diameter was placed inside the tank a little away from the center in the northeast direction. The camera was mounted on a tripod to monitor animals during trials. During the training, the rescue platform was exposed one inch above the water surface to be visible to the animals. Each animal had undergone four consecutive trials for ten days with different starting positions such as north, south, east, and west. The animal was placed by supporting it with hands and bringing it down gently facing the pool's wall into the water with its tail-end first. The animal was left to swim freely and search for the platform for a maximum of 60 seconds. Once the rat reached the platform, the time taken was recorded. If it does not find the platform in 60 seconds, the animal was gently guided with a hand and let the animal sit on the platform for 15 seconds. If it falls or jumps off, it was gently guided back to the platform. This would train the animal that to stay on the platform and be rescued from the pool. The same procedure was repeated for three more trials, starting in different directions for each trial. Once the animal had completed all four trials, it was dried off with a towel and put back in its cage. Water maze testing started ten days after the training phase. The tank was filled up with water, so the platform was one inch below the surface of the water. Milk powder was used to make the water opaque, and the rescue platform became invisible to the rat. To locate the rescue platform, the rat had to navigate through the pool using extra-maze cues. The rescue platform remained in the same position as it was during training. With different starting locations, each animal had undergone four trials. Each trial lasted for 60 seconds. The animal was monitored until it reached the platform, and the time taken was recorded.

Elevated plus maze test: The elevated plus maze consisted of four elevated arms of 50 cm long, 10 cm wide, which radiate from a central platform, forming a plus shape. Two of the opposite arms were walled by 20 cm in height and the remaining two opposite arms were opened apart from the platform itself. The experiment was conducted after one hour of lights off during the dark phase to maximize the animal activity, under low red filtered light. To record the animals a video camera was mounted on a tripod placed at a short distance from the maze apparatus. During the experiment, a rat was placed in the central area, and then it was left to explore the maze for 10 Minutes. After each animal trial, the apparatus was cleaned with ethanol (70%). The time spent in the walled arms was compared to the amount of time spent in open arms as a measure of anxiety or fear.

Passive avoidance test: Passive avoidance tests are behavioral tasks that are commonly used to determine fear-based conditioned avoidance learning in rats. The protocols rely on a common experience and behavioral adaptations, such as inhibition of innate activities or learned habits which have led to aversive consequences. It is based on the idea that a biologically probable response that was previously ended by an unpleasant event will be inhibited in the future (Herrnstein, 1969). In passive avoidance tests, an animal learns to avoid an aversive event by suppressing a particular behavior. It was carried out in a box with a light chamber and a dark chamber separated by a doorway. The illuminated environments are unpleasant to rodents, by their innate photophobia when placed in the light chamber, they are led into the preferred dark compartment. During the habituation phase, they are allowed to move freely into a dark chamber, but in the conditioning phase of the passive avoidance test, the animal receives a foot shock (0.1mA-1mA) when crossing into the dark chamber. Their innate behavior of seeking a darker environment will thereby become associated with an aversive stimulus. After the

conditioning phase when placed in the light chamber, the animal will avoid entering the dark, shock-associated chamber (Bures et al., 1976). The time from releasing the animal into the light chamber to its stepping into the dark compartment (step-through latency) is measured.

Radial arm maze test: A standard 8-arm radial arm maze made of smooth white plywood was used. It consisted of an octagonal central chamber with eight equal arms 50 cm in length, 10 cm in width, and 20 cm in height. The entrance of each arm consists of a door that allows the rats to move freely but prevents the visibility of food. After each animal trial, the apparatus was cleaned thoroughly with 70% ethanol. Rats were maintained on a restricted food diet (12 hours without food) to motivate them to search for the food placed in the maze. The experiment began with the four days habituation period where each rat was placed for 10 minutes in the maze with scattered food, which motivated the rat to explore the maze. After the habituation period, testing was conducted for ten consecutive days. Each rat was placed in the central chamber allowing free access to all arms. At the end of each arm, a food reward was placed. Each rat was allowed to remain in the maze until it entered all eight arms or for 10 min, whichever occurred first. An entry was considered if an animal had placed all of its four paws inside an arm of the maze. During data analysis, the first entry into an arm during a daily test session was scored as a correct choice, whereas a repeated entry into the same arm was scored as a working memory error. The time required to visit all eight arms, the number of correct arm choices and the number of unvisited arms were also noted for analysis.