

2022

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Key words

Morris water maze, amnesia, spatial working memory, procedural memory, palliative, treatment

received 21.06.2022

accepted 29.08.2022

published online 27.10.2022

Bibliography

Drug Res 2023; 73: 40–45

DOI 10.1055/a-1937-9064

ISSN 2194-9379

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ABSTRACT

Studies have shown the ability of benzodiazepine drugs to cause memory loss in animals and humans. Midazolam is a benzodiazepine commonly administered intravenously during surgical procedures because it reacts rapidly, causes anterograde amnesia, and has few side effects. It has also been used in palliative medicine where, among others, an oral route has been employed for chronic administration of the drug. The current study evaluated the effects of chronic orally administered midazolam on spatial working memory and procedural memory in control and experimental female rats over a three-week experimental period utilizing the Morris water maze. Sample and test run times to a submerged platform in the maze were recorded daily. In addition, activity wheels attached to each cage were employed to monitor daily circadian activity of the animals. Spatial working memory was not impaired in either group. However, procedural memory amnesia occurred in animals receiving the drug indicative of a consolidation or retrieval problem. Concerning circadian rhythms, a phase-shift was noted in experimental animals possibly indicating that time of day of drug administration is important. The findings of the present study could shed insight into altered reactions observed in humans who have received midazolam as a component of treatment in palliative medicine.

Introduction

Drugs in the benzodiazepine class are known for their depressant effects on the central nervous system and are used for procedural sedation, anxiety, insomnia, muscle spasms, seizures and treatment of withdrawal symptoms [1, 2]. Benzodiazepines cause numerous side effects, one of which is anterograde amnesia – a loss of memory for events occurring forward in time [3].

Midazolam is the generic name for a benzodiazepine which is widely used in medicine because of its ability to enter the brain ex-

tremely fast [4]. Once midazolam crosses the blood-brain barrier, it binds to specific gamma-aminobutyric acid-A receptors (GABA-A) causing an inhibition of excitable neurons and accumulation of GABA, which has calming effects on the brain and body [1]. This inhibition of cell firing may also affect the transfer of information from short-term memory to long-term memory [5], but information that is stored before the ingestion of the drug is not affected [3].

A type of memory upon which midazolam may have an effect is spatial working memory [6]. All rodents use navigation as a means

for survival and one type of navigation is using distal cues also known as allocentric wayfinding which involves the creation of spatial working memory. Spatial navigation is regulated in the hippocampus and the entorhinal cortex which is where a neural map of the environment is formed. Altogether, the place, grid, head direction and border cells of the entorhinal cortex and place cells of the hippocampus form a network that creates an external map. This network has been shown to increase firing in rodents in the Morris water maze (MWM) [7]. Another type of memory that may be affected by midazolam is procedural memory [6]. This memory involves the retrieval of previously learned procedural information.

Midazolam has become very popular in procedural use because of its properties. Most studies have been conducted on the amnesic effects of sedation via intravenous injection since midazolam is very compatible with intravenous solutions [4]. The drug, however, in the oral form, is also one of the most frequently administered drugs in palliative medicine [2, 8]. According to Capezuti et al. [9], two unmanaged problems encountered in palliative care are disruption of sleep and circadian rhythms. A closer correlation between time of drug administration and the circadian rhythm of the sleep/wake cycle of patients would help to alleviate these problems. The amnesic effects of orally administered midazolam have not been studied in depth, possibly due to low bioavailability of the drug when given by this route [2, 10].

The current study focused on the effects of chronic oral administration of midazolam on spatial working memory and procedural memory utilizing the MWM in female control and experimental rats. In addition, the effect of midazolam on circadian rhythms was measured in all animals. It was hypothesized that the midazolam-treated group would have impaired spatial working memory and procedural memory, as well as a shift in circadian rhythm patterns during drug action compared to the control group.

Method

All techniques were approved by the local Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines.

Subjects and Diet

Twelve Long-Evans female rats (Envigo, Indianapolis, IN), weighing between 80–100 grams, were placed in individual cages equipped with a running wheel connected to a computer that recorded daily activity every five minutes using VitalView software (Starr Life Sciences Corp., Oakmont, PA). Food (Lab Diet, Richmond, IN) and water were provided ad-libitum to the animals throughout the experiment. Upon arrival at the laboratory, rats were weighed and separated into control and experimental groups based on body weight, so that the mean weight of the six rats in each group was similar. A 12 hr/12 hr light/dark cycle was maintained with room temperature held at 21–23 °C.

Apparatus

Spatial working memory and procedural memory of the animals were tested using the MWM. The maze was a circular pool measuring 1.5 meters in diameter and 60 centimeters in depth. The water

in the maze was kept at room temperature and monitored with a thermometer. The maze was divided into eight segments with three towels placed over the side in a permanent location throughout the experiment. These towels acted as a cue for the rats to help them find the platform which was submerged 1 cm below the surface of the water and moved randomly every day forcing the rats to use their spatial working memory instead of memorizing a permanent location of the platform [11].

Methodology

The rats were initially habituated to their environment for one week. At the beginning of the dark cycle each day, all rats received a 250 µL “treat” of condensed milk in a glass dish which they consumed completely within five minutes. The condensed milk served as a carrier for the drug or placebo during the experimental period.

During a 3-week experimental period at the beginning of the dark cycle of each day, the experimental group received midazolam (Sigma Aldrich, St. Louis, MO) diluted in distilled water at a dose of 9 mg/kg, which was based upon a study done by Silveira et al. [12]. In addition, a study conducted by Pieri [13] showed that the response to different oral midazolam dosages on rats had no adverse side effects for the 9 mg/kg dose. The drug was placed in the condensed milk. The control group was given a placebo of distilled water in the condensed milk. All animals completely consumed the drug or placebo solution within five minutes.

With oral administration, midazolam is rapidly absorbed from the digestive system and has a T_{max} of one hour and an elimination half-life of 23–50 minutes [14]. Thus, each rat was given twenty minutes to digest and absorb the drug or placebo before it was assessed by the MWM. The MWM protocol was as follows: Each rat was placed in the water facing the rim of the tub and allowed to discover the location of the platform by trial and error while being timed. If the rat took more than 90 seconds to find the platform, it was guided to it. The rat was then allowed to rest on the platform for 15 seconds. This first trial was called the sample trial. The second trial, called the test trial, occurred after a 15-second break. The rat was placed at the same starting location as the first trial. If the rat recalled the location of the platform, it swam faster to it, thus, exemplifying enhanced spatial working memory. After the test trial was completed, the rat was gently patted with a paper towel to remove any water and returned to its cage. Platform location and starting position of the rat were changed every day of the experiment, as proposed by Vorhees & Williams [11]. The maze protocol was repeated each day during the experimental period. Procedural memory was exhibited when the animal recalled the underlying mechanism utilized in locating the platform in the sample trial and, thus, reached it in a shorter period of time.

Statistical Analysis

Data were statistically analyzed with SPSS (Chicago, IL) and R programming language. Repeated measures Analysis of Variance (ANOVA) test plus Tukey’s HSD post hoc test were used for mean MWM sample and test run times for both treatment groups. Permutation ANOVA was performed on MWM control and experimental group mean sample run times and mean test run times separately with bootstrap follow-up analysis. Wilks’ Lambda statistic

was used to report significance of the multivariate tests. A p value of <0.05 was considered significant for all statistical analyses.

Results

A 2×3 repeated measures ANOVA with 2,000 permutations test for MWM mean sample run time between the control and experimental groups during the 3 week experimental period revealed a significant effect of the experimental weeks on mean sample run time [$F(2, 20) = 19.088$, $p < 0.001$] and no effect of treatment groups on mean sample run time. A significant interaction between the two groups and the 3 experimental weeks was also discovered [$F(2, 20) = 3.896$, $p < 0.05$]. To investigate the suggested significant interaction, data was transformed into a multivariate structure and fitted with a repeated measures ANOVA model. The fitted model 7 and pairwise test function were used to get estimates of the pairwise differences between the groups for each experimental week, were wrapped in a bootstrap function that generated 500 repetitions and outputted 97.5% bootstrap confidence intervals (CI) with Bonferroni corrected p -values. The bootstrapped results showed a significant difference of mean sample run time between control and experimental groups within experimental week 3 ((97.5% CI -20.834 , -2.325), $p < 0.05$). No significant differences were shown in experimental weeks 1 and 2 between the groups. ► **Fig. 1** presents the control and experimental groups for 3 weeks, with week 3 showing the experimental group significantly deviating from the control group. A significant effect of the different weeks is observed in both groups as their mean sample times decrease from one week to the next but plateau for the experimental group during weeks 2 and 3. The same analysis methodology was performed on mean test time runs for both groups and the experimental weeks. A significant effect of the experimental weeks on mean test runs was found [$F(2, 20) = 4.7384$, $p < 0.05$]. Pairwise comparisons and bootstrapping did not find any significant inter-

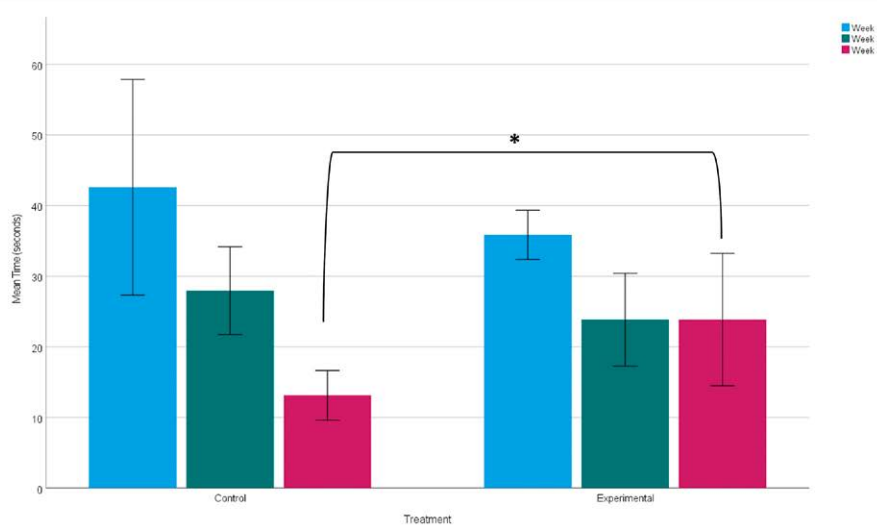
actions. ► **Fig. 2** shows changes of mean test run times over 3 weeks for both groups.

The 2×3 ANOVA for the MWM revealed an interaction between runs (sample and test) and experimental weeks for the control group. There was a significant difference between sample and test runs during experimental week 1 [$F(2,4) = 48.062$, $p < 0.05$] and week 2 [$F(2,4) = 48.062$, $p < 0.01$] with sample runs taking a longer time than test runs. There was no significant difference in week 3 between sample and test runs. It was also shown that there was a significant difference between sample runs of week 2 and week 3 [$F(2,4) = 34.514$, $p < 0.01$] with the sample run of week 2 taking a longer time.

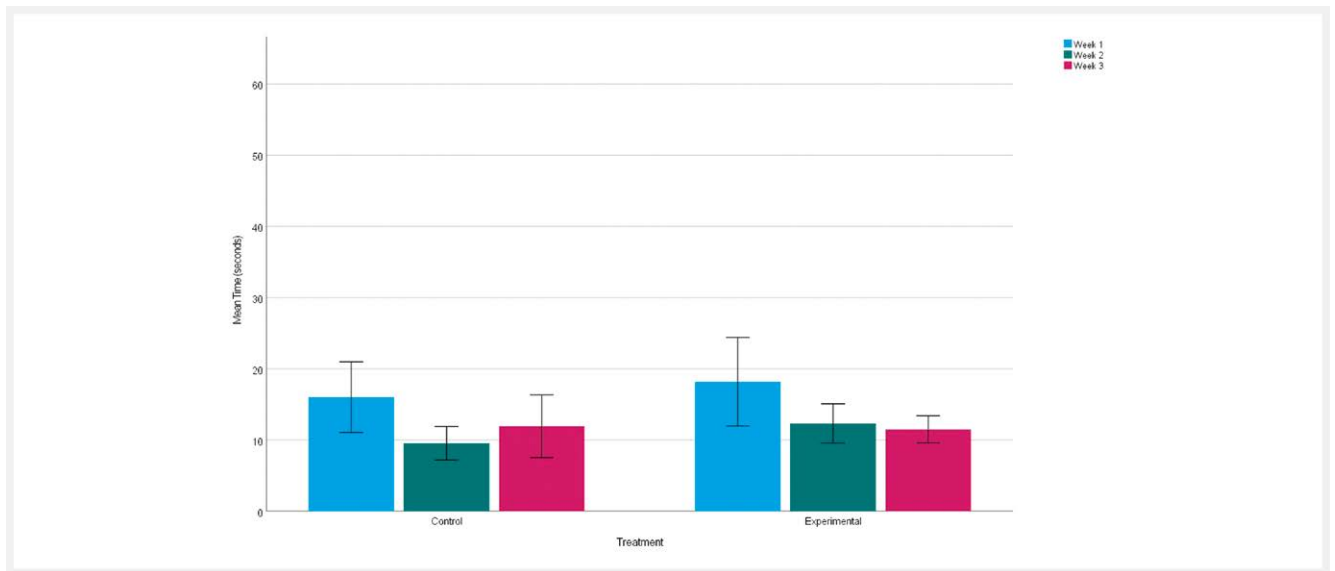
The 2×3 ANOVA revealed no interactions between runs (sample and test) and experimental weeks for the experimental group; therefore, the pattern of sample run and test run was similar over the three weeks. There was, however, a significant difference between week 1 sample and week 2 sample runs [$F(2,4) = 166.111$, $p < 0.001$] with the sample run in week 1 taking a longer time than the sample run of week 2. There was no significant difference between the sample run or the test run of week 2 and week 3.

Concerning the pattern of circadian activity, ► **Fig. 3** displays the mean activity pattern for each group during experimental week 3. This figure shows the delayed nocturnal response of the experimental animals compared to the control animals.

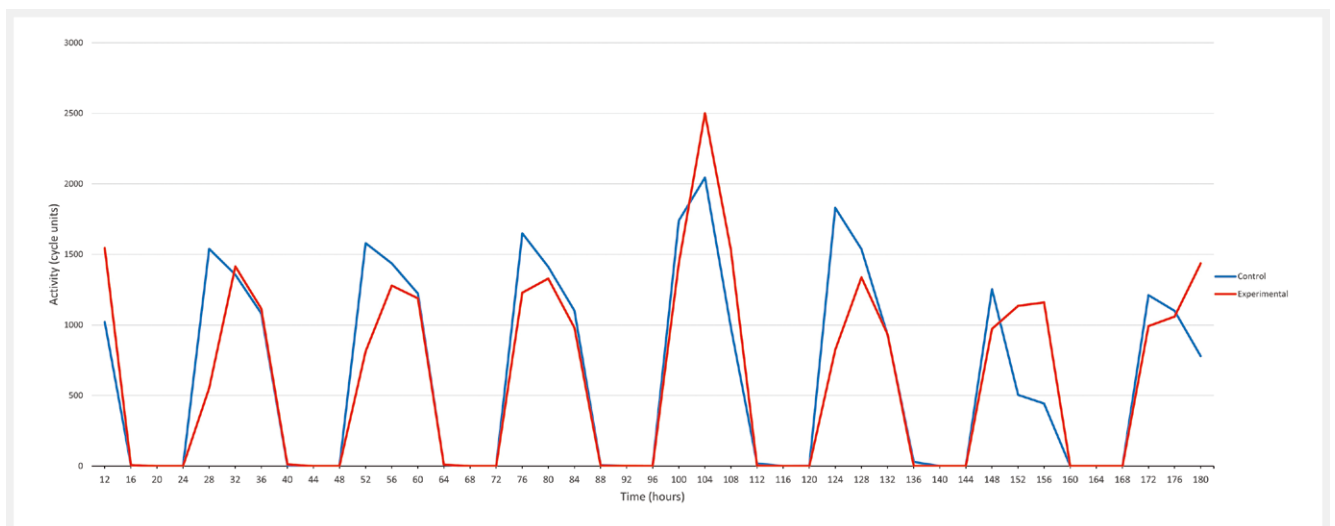
To examine this difference more closely, a 36-hour portion of the week was expanded to reveal details of the modified circadian rhythm observed (► **Fig. 4**). In the control rat the first peak of activity was higher than the second peak and both peaks occurred during the dark cycle. In the experimental rat, however, the first peak of activity, following drug administration, was lower than the second peak which developed later in the dark cycle and extended into the light cycle.



► **Fig. 1** Mean sample times in the MWM of control and experimental groups during the three experimental weeks (7 days/week) * $p < 0.05$



► **Fig. 2** Mean test times in the MWM of control and experimental groups during the three experimental weeks (7 days/week)

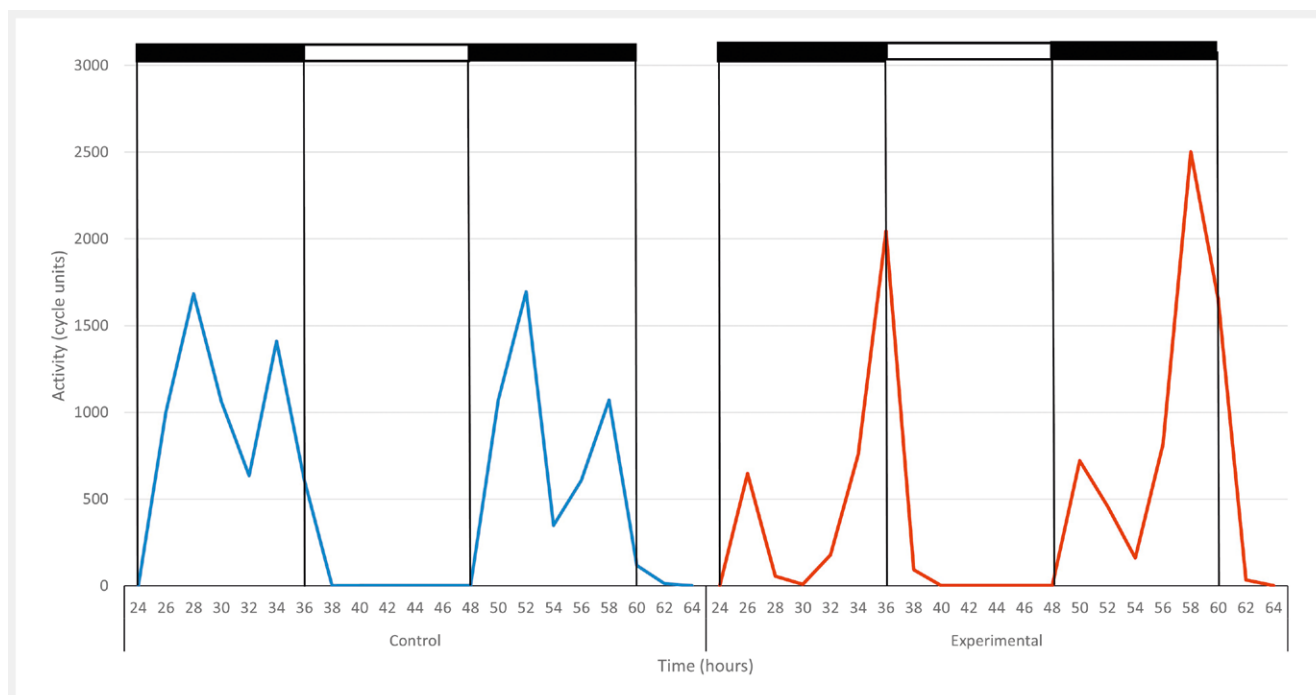


► **Fig. 3** Pattern of mean activity of both control and experimental groups during experimental week 3 (12–180 hours)

Discussion

The purpose of this study was to determine the effects of chronically administered oral midazolam on spatial working memory and procedural memory as well as circadian activity patterns in female rats. Utilizing the MWM, both the control and the experimental groups displayed similar test trial times indicative of functional spatial working memory (► **Fig. 2**), a finding contrary to the hypothesis of the current study and to the findings of Timic et al. [6]. It should be noted that the MWM technique of Timic et al. was conducted in the light (resting) phase of the circadian cycle; whereas, the current study took place in the dark (active) phase of the circadian cycle. The sample trials, however, were different in the two groups, especially during the third experimental week where the

control rats immediately found the platform and the experimental rats did not (► **Fig. 1**). This phenomenon exhibited by the control rats may have involved a memory system in charge of encoding and/or storing of the procedure involved in the MWM. On the other hand, the procedural memory system in the experimental rats may have been blocked by midazolam producing amnesia in these animals. Another explanation may be that the control rats learned after three weeks that the MWM test did not present a threat to their wellbeing and, thus, the initial stress of exposure to the water maze was minimized [15]. The experimental rats, however, receiving midazolam, may have exhibited procedural memory amnesia that was brought about by the impairment of the consolidation processes (inability to store new events over time) and could not



► **Fig. 4** Pattern of activity of a representative control rat and experimental rat during experimental week 3 (24–64 hours). (Black horizontal bar at the top of the graph indicates the dark component of the light/dark cycle.)

retrieve the former memories of the maze, thus, took more time for the sample test. Beracochea [16] has raised the question as to whether impairments induced by midazolam are not solely due to a weakness of the consolidation phase but could also reflect a pure deficit of retrieval processes. It would appear that the neuronal pathway involved in midazolam activity in sample to test trials (a rapid occurrence) and repetitive daily testing (a retrieval occurrence of trial conditions) may be different. Midazolam causes an increase of GABA inhibition in the basolateral amygdala neurons involved in storage memory [17]. The hippocampus and the amygdala work together to form spatial memory [18]. The hippocampus is known for creating spatial maps and the amygdala is known for its involvement in the development of emotion [18]. The amygdala has also been shown to play a role in emotional memory which is triggered by external stimuli like fear or anxiety [19]. This emotional memory is then sent to the hippocampus where it can be used to enhance spatial memory [19]. Studies have shown that learning and memory can be affected by the side effects of midazolam such as inducing an anxiolytic-like state [16]. Therefore, the experimental rats may not have been as stressed as the control rats when trying to find the platform. If the control rats experienced brief periods of stress while trying to find the platform, the hippocampus may have been stimulated and formed memories of the surroundings to try and navigate faster [18]. This conclusion would fit the natural survival instinct of animals because rodents need navigation to survive and they will try to remember surroundings especially in situations that are stressful or life-threatening [17].

In addition, the pattern of circadian activity of control and experimental rats during week three showed that the control rats had

a high peak followed by a lower peak during the dark period of the circadian cycle (► **Fig. 4**). This pattern is consistent with the findings of Bishop et al. [20]. The experimental rats, however, showed a low peak during the drug absorption time followed by a high peak after the drug had worn off (► **Fig. 4**). One explanation for this effect is that midazolam is a very fast-acting drug that produces a calm state but has a short half-life [12]; therefore, rats are only calm for about an hour before the drug effects begin to wear off. Wee and Turek [21] found that the drug produces alterations in the circadian cycle of free-running hamsters when exposed to a single dose of midazolam under constant light. Specifically, they reported that the phase-shifting actions of midazolam were mediated by benzodiazepine receptors.

The finding of a disrupted circadian rhythm produced by midazolam resulting in sleep problems for rats has a meaningful aspect when applied to palliative care. In a study conducted in New York State [22] utilizing information from facilities that provide palliative care services to a total of 5000 residents, respondents specifically recommended individualization of care practices in various areas with the first area being adjusting patient medication. Thus, knowing, that midazolam affects circadian rhythms, it can be given to patients at times that will not interfere with normal sleep patterns.

Conclusion

The results of this experiment do not support the hypothesis that orally administered midazolam impairs spatial working memory in female rats. However, even though spatial working memory was

not impaired, procedural memory was adversely affected in animals receiving the drug. In addition, the drug altered circadian rhythms in experimental animals. It should be noted that midazolam given intravenously is a popular drug administered only once before medical treatments mainly because of its ability to calm and produce amnesia and, thus, prevent a patient from remembering the procedure and any negative emotions felt during the procedure. On the other hand, the present study is related to the chronic daily oral use of midazolam over an extended time period as in palliative medicine and how the drug may affect spatial working memory, procedural memory, and amnesia. Finally, it has been demonstrated that midazolam alters circadian rhythms which is related to the time of day when the drug should be administered.

Conflict of Interest

The authors declare that they have no conflict of interest.

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