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(RESEARCH ARTICLE)

Acquisition of a spatial navigation task in the padding pool induces an increase of GABA level in the hippocampus of Swiss mice

Isabela Cristina Sena Romano [1] and Angela Maria Ribeiro [2,*]

[1] *Programa de Pós-graduação em Neurociências, Laboratório de Neurociências Comportamental e Molecular, LaNeC, Universidade Federal de Minas Gerais, Belo Horizonte 31270-010, Brasil.*

[2] *Departamento de Bioquímica e Imunologia – Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte 31270-010, Brasil.*

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Abstract

The balance between excitatory and inhibitory glutamatergic and GABAergic systems, respectively, is crucial for the maintenance of complex cognitive functions such as learning. Using Swiss mice as experimental model, the aims of the present study were to evaluate cognitive performance in a shallow water maze (SWM) and the effects of training in this spatial navigation task on hippocampal GABA and glutamate levels. In addition, correlations between neurochemical and behavioural data, and between glutamate and GABA levels were assessed. Forty-six three-month-old mice were divided into three groups: Learning, n=18: animals submitted to the SWM task; Active, n=14: animals exposed to the SWM, without the demand of performing a cognitive task and Control, n=14: the animals were kept in the vivarium without contact with the SWM. There was significant training effect indicating that the Learning group animals have learned the task. Regarding neurochemical data, the findings of the present work show for the first time that the task learning process in SWM has a significant effect on GABA levels in the hippocampus. The relationship between the two neurotransmitters, observed in the control animals, was adjusted by a significant increase in hippocampal GABA levels caused by the spatial training performed by the animals from the Learning group. However, the relationship observed in control condition is disrupted by a subsequent exposure to the maze in the absence of a spatial cognitive demand, as was the case of the Active group. These data open new perspectives to explore the involvement of the inhibitory and excitatory systems in the molecular mechanisms associated with different types and steps of learning processes.

Keywords: Spatial Learning; SWM; Hippocampus; GABA; Glutamate; Swiss mice

1. Introduction

Spatial memory, conceptualized initially by Olton et al. in 1979 [1] consists of a system involved in obtaining spatial information through repetition of a task and involves the ability to encode, store for long periods and retrieve information about spatial locations, settings or routes [2]. One reason that further reinforces the importance of studying the neurobiological substrates of learning and spatial memory is that these cognitive processes are one of the first to be impaired in physiological aging and neurodegenerative diseases. The advances in methods and techniques over the past decades have resulted in many important discoveries in the field of the neurobiology of cognitive processes [3–5]. However, many questions remain unclear, including the role of excitatory and inhibitory neurotransmitter systems, glutamatergic and GABAergic, respectively, in the functioning of the spatial learning process.

* Corresponding author: Angela Maria Ribeiro

Departamento de Bioquímica e Imunologia – Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte 31270-010, Brasil.

Several authors [6–10], including our group [11–13], have shown evidence of the involvement of different neurotransmitter systems in cognitive aspects, including that the balance between excitatory and inhibitory stimuli of glutamatergic and GABAergic systems, respectively, is crucial for both development and maintenance of complex cognitive functions such as learning and memory. However, the role of these neurotransmitters in the spatial learning process remains unclear.

Functional balance of these two systems is involved in various physiological processes as neurogenesis, cell migration and neuronal plasticity [6,7,9]. Using proteomic analysis and experimental models of neurodegeneration, our group showed [14] that the treated animals displayed a spatial cognitive deficit compared to control and, among the various molecular alterations, we point a significant change in the expression of the enzyme responsible to produce GABA from glutamate.

Most of the neurobiological data obtained in the studies mentioned above, are obtained using samples from the hippocampus and/or neocortex. The hippocampus is an elongated structure on the dorsoventral axis in rodents and on the anteroposterior axis in primates, and since the discovery of place cells in this region [15], the hippocampus has been a focus in studies of how spatial information is represented in the nervous system and how spatial knowledge is used for navigation in the environment. Lesions in the hippocampus have been found to impair animal performance on tasks that require solving spatial learning cues [16-19].

The central hypothesis of the present study is that a cognitive spatial experience changes the levels of GABA and/or glutamate in the hippocampus of the subjects, and that this alteration is related to the subjects' performance on the cognitive task.

2. Material and methods

2.1. Subjects

Forty-six three-month-old male Swiss mice were used in the present study. All experimental procedures were approved by the ethical committee on animal research at the Universidade Federal de Minas Gerais (protocol 161/2014).

2.2. Experimental procedure

The study consisted of one experiment and one replica, performed independently, counting a total of 46 mice divided into three groups: Control (n = 14): animals were kept in the vivarium without any contact with the Shallow Water Maze (SWM), Active (n = 14): animals were exposed to the SWM, over seven daily sessions/four trial per session, with no demand to perform the cognitive task, and Learning (n = 18): animals were submitted to the cognitive task in the SWM, with four trials/per session on seven consecutive days. After the fifth session, the animals were submitted to the Probe Trial, in which the reinforcement was removed, and the animals could stay in the maze for 2 min. After the probe trial (day 5), two other training sessions were performed in the days 6 and 7 to ensure that the mice that had eventually extinguished the behaviour during the probe test (fifth session), reacquire the task before being euthanized. The hippocampal samples were collected to determine neurochemical parameters two hours after the 7th session, after the mice reached maximum performance on the task. The hippocampal samples were weighed and kept at -80 °C for subsequent biochemical assay, which were performed within a maximum of sixty days. Details are described below.

2.3. Behavioural Assessment

2.3.1. Apparatus

The behavioural assessments were performed using the SWM, a mice maze built according to Deacon et al. [16], with modifications as detailed below. The maze consisted of a circular wooden platform, measuring 70 cm in diameter, covered with black rubber material and covered with a 2.5 cm high layer of water. In this way, when walking on the platform the animal left no trail. On the circular edge of the platform, a transparent acrylic wall composed of 12 plates 17 cm high and 30 cm wide, comprising a dodecagon. At each junction between two consecutive acrylic plate, there is an exit through black plastic tubes with 40mm diameter. However, only one of the 12 exits is open through which the animal can escape the maze [real exit], the other 11 exits are false. That is, they are sealed with black plastic to not allow the mouse to exit through the tube. From the inside of the maze all false exits and the single true exit looked the same. The SWM is in a 3.7 m2 room with brightness around 560 lux at a height of 30 cm above the floor. The room has a variety of extra-maze distal cues, used by the mouse to locate the real exit. The maze did not have any internal clues, marks or signals, which could serve as an intra-maze clue. In other words, the animal could only find its way out through the spatial cues in the environment.

2.3.2. Spatial Memory training and probe trial

Training: during the training, the true exit was located in a constant position relative to the room, in the North quadrant [the target quadrant]. Each trial consisted of placing the animal in the center of the maze with its head facing one of the quadrants, varying the quadrant throughout the four trials. The mouse was left in the maze for a maximum of 60 seconds. In the case of the Learning Group mice, the trial was completed when the mouse found the true output or if the 60-second time limit was reached. In the latter case, the mouse was gently led to the true exit by the experimenter. Once found or guided to the true exit, the mouse was removed from the tube and led back to the cage. Latency, the time in seconds taken by the mouse to find the true exit, was used as a quantitative unit of the mouse's performance during training in the SWM. Another way to express the animal's performance is the number of errors made. It was considered an "error" every time the animal introduced its head into a false exit. The latency in seconds and the errors committed were obtained by calculating the median values of the four trials of each session for each mouse, and the group average was obtained from the medians.

Probe Trial: two hours after the fifth session, the probe trial was performed to evaluate spatial reference memory. In this test, the real exit was blocked and the SWM apparatus was rotated 180° relative to room, each mouse was placed once in the maze and remained there for a total time of two minutes. The time the animal remained in the target quadrant was recorded and expressed as a percentage of preference for the target quadrant compared to the time they remained in the other quadrants. The time spent in the target quadrant, recorded during the first minute, was used as a measure of the mice's performance during the spatial memory test.

2.4. GABA and glutamate assay

The derivatisation procedure was performed as previously described by Mengerink et al. [20]. Briefly, the derivatisation was made by mixing 100 µL brain homogenate, 20 µL of daily prepared methanolic o-phthalaldehyde, 75 µL borate buffer (pH 9.9) and 5 µL 3-mercaptopropionic acid. The resulting solution was vortexed and analysed by HPLC after one minute at room temperature.

The HPLC system consisted of a Shimadzu chromatograph (LC-10AD, Tokyo, Japan) with a 200 L loop (Rheodyne 7725-I, California, USA) and a fluorescence detector (FLD- Shimadzu spectrofluorometric detector RF-551, Tokyo, Japan), coupled to an LC-10 AD PUMP. The system was equipped with a 3 µm particle size (150mm×4.6mm, ID) C18 analytical column (Hibar-Futigsanle RT) and a pre-packed column (RT 250-4 E. Merck, Darmstadt E.R., Germany). An integrator (Shimadzu C-R7Ae plus) was used to analyse the chromatographic data. The chromatography conditions were performed as previously described [13]. Briefly, the isocratic mobile phase consisted of a 0.05 M solution of sodium acetate, tetrahydrofuran and methanol (50:1:49, v/v), pH 4.0. The flow rate was 1mL/min. The wavelengths of excitation and emission used were 337 and 454 nm, respectively. The GABA and glutamate concentrations in ng/g and µg/g of hippocampus, respectively, were calculated according to the peak areas and their curve standard.

2.5. Data analysis

Data analysis was performed using GraphPad Prism 5.0 / 2007. Data normality was verified through the Kolmogorovi-Smirnov and Shapiro-Wilk tests. The behavioural data from the learning trials were analysed by ANOVA with repeated measures. For non-parametric data (Friedman test) and the data obtained during the probe trial, time spent in the target quadrant, were analysed using Kruskal-Wallis followed by Dunn's post hoc multiple comparisons test. Biochemical measurements were performed in triplicate and the mean was analysed by one-way ANOVA followed by post-hoc Tukey's multiple comparison test. These two last tests were also used to evaluate the ratio between glutamate/GABA concentrations in comparing the groups. All values are expressed as mean ± standard deviation (S.D) Differences were considered significant at 5% level ($p < 0.05$]) for all tests. Pearson Correlation Coefficient for parametric data and Spearman Correlation Test for nonparametric data were used to assess the correlation between the different parameters, such as GABA vs. glutamate, as shown in Figure 3, and between each and performance on the cognitive task (data not shown).

3. Results

Training: Figure 1 (panels A and B) shows the performance of the mice during the acquisition of the spatial task in the SWM. Friedman test showed significant effect of training ($F(6,21)= 34.54$ and $p= 0.0001$). Dunn's post-hoc test showed a significant difference between days five ($p= 0.001$), six ($p= 0.01$) and seven ($p= 0.0001$) when compared to the first day of training. The effect of training was also significant ($F(6,21)= 40.23$, $p= 0.0001$) when performance is expressed as number of errors made by the mice across sessions. Dunn's post-hoc test showed a statistical difference between days three ($p= 0.01$), four ($p= 0.01$), five ($p= 0.0001$), six ($p= 0.0001$) and seven ($p= 0.0001$) when compared to the first

day of training. Probe Trial: Figure 1 shows in panels C and D the percentages of time that the animals remained in the quadrants during the first and second minutes of the probe trial, respectively. The Kruskal-Wallis test indicated an effect (H = 16.93; p=0.0007) of training. Dunn's post-hoc test showed that there is a significant difference between the times in the quadrants, confirming that the mice spent significantly more time in the target quadrant when compared to the percentages staying in the other quadrants. The Kruskal-Wallis test showed that there was no effect (H= 6.973; p=0.0728) of training on the time spent in the quadrants during the second minute of the probe trial.

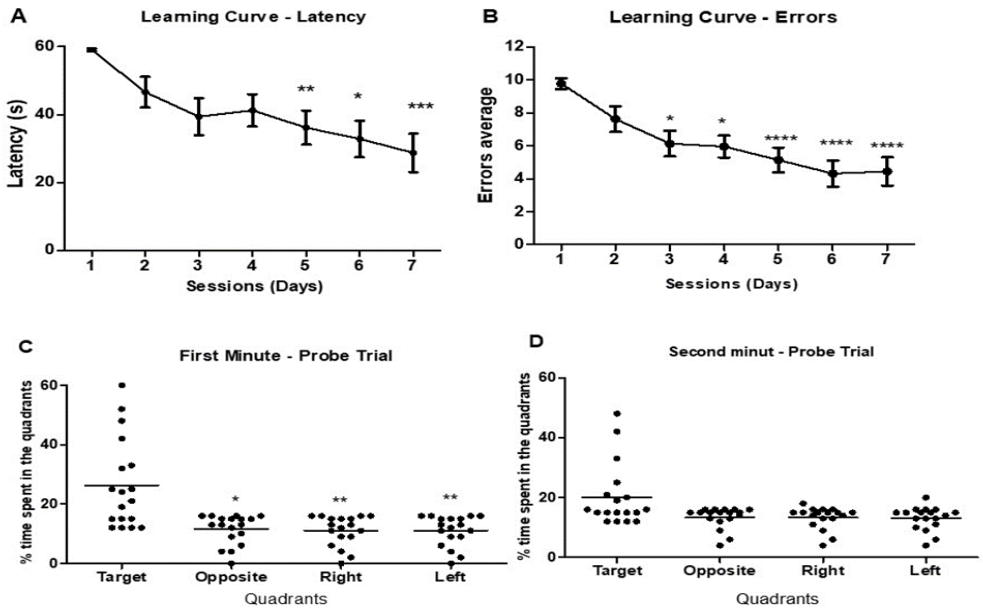

Figure 1 Performances of animals from the Learning group (n=18) on the spatial navigation task during acquisition (panels A and B) and Probe Trial (panels C and D), expressed as: latency (s) to find the escape (panel A); number of errors made (panel B); percentage of time in each quadrant in the first minute of the probe trial (panel C) and percentage of time in each quadrant in the second minute of the probe trial (panel D). (*) p<0.05; (**) p<001; (***); (p<0.001)

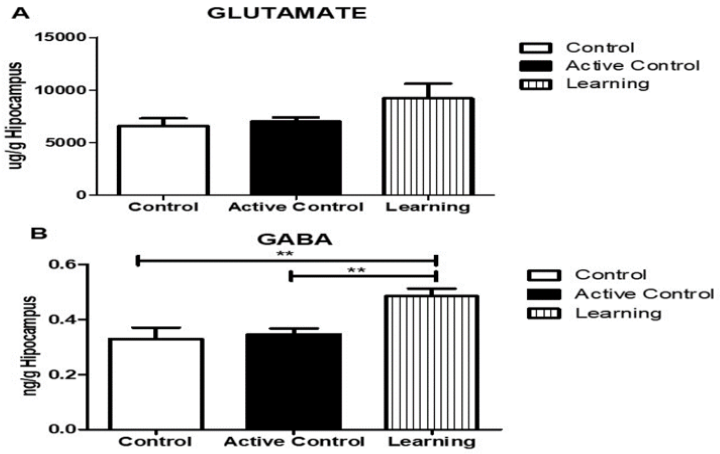

Figure 2 shows the levels of glutamate, µg/g, (panel A) and GABA, ng/g, (panel B) in the hippocampus of the Swiss mice for the three groups. The concentrations of both glutamate and GABA were analysed by one-way ANOVA with Tukey post-hoc, which showed no effect (F(2,43)=1.82; p=0.17) of training on Glutamate concentration (Figure 2A) in the hippocampus. On the other hand, training significantly affected the concentration of GABA (Figure 2B) in the hippocampus (F(2,43)=8.37; p=0.008) . The post-hoc test showed significant difference between the learning group when compared to the Control (p=0.01) and Active (p=0.01) group. The Kruskal-Wallis test found no significant effect (H= 3,206; p= 0.20) of training on [glutamate]/[GABA] ratios (data not shown).

Figure 3 shows the Scatter plots for the glutamate versus GABA data. Pearson's correlation test showed a positive linear correlation between glutamate and GABA concentrations in the hippocampus of the control (Figure 3A) (r=0.35; p=0.012) and learning (Figure 3C) (r=0.33; p=0.009) mice, but for the Active group a significant correlation was not verified (r=0.002; p=0.85) (Figure 3B). If the data from the Control and Learning groups (Figure 4D) are put together there is also a significant correlation (r=0.35; p=0.012).

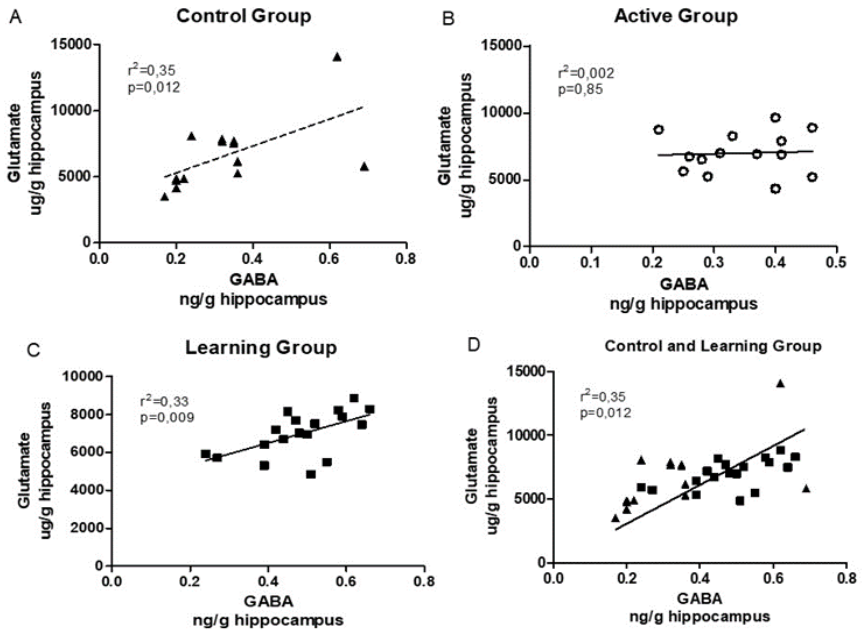

Figure 3 Scatter plot between hippocampal concentrations of glutamate and GABA. Panel A: Control Group (Black triangle, n=14). Panel B: Active Group (empty circle, n=14), Panel C: Learning Group (black square, n=18). Panel D: Data from the Control and Learning groups plotted together.

4. Discussion

The results of the present study show that the mice were able to learn the spatial navigation task and confirm with the probe test data that they used extra-labyrinth cues to solve the maze. The data also corroborate what was shown by Deacon et al. [16] about the task performed on the SWM equipment as a suitable method to assess the spatial cognitive performance of mice. A large-scale validation of the SWM to study hippocampus-based spatial cognition in mice was performed by Sankowski et al. [21], confirming that this test represents an important tool not only to study behavioural aspects, but also to assess the function of specific brain regions, such as the hippocampus.

During the SWM training we observed a significant effect of time, showing, as mentioned above, that the mice could learn the spatial task, indicating that they were able to elaborate a spatial map of the environment to locate the true exit. Despite the similarities between the learning curves observed here and the one obtained by Deacon et al. [16], it is important to note that the two curves show some differences in relation to the first sessions that can be explained by the protocols used. Deacon et al. used a protocol in which the animals, before having contact with the SWM, were submitted to a task to learn the escape principle. Thus, the curve presented by Deacon et al. shows on its first day, latency and number of errors committed much lower than those verified in the present study. However, as mentioned, even without the pre-training step the mice were equally able to learn the task.

To verify that the mice had indeed learned the task using extra-maze spatial strategies, a probe-trial was performed. Analysis of the animals' behaviour during the first minute of the test showed that the animals remembered the location of the reinforcement (exit), staying significantly longer in the target quadrant, where the exit used to be. The animals' staying in the correct quadrant in the first minute of the probe-trial indicates that they were using extra-maze spatial cues and, therefore, they used a spatial cognitive map of the environment. However, along the test, since they were no longer being reinforced, it would be expected that extinction of this behaviour would occur. When we analyse the behaviour of the animals during the second minute of the test, we observe that there is no significant difference among the time spent in the quadrants, indicating that the mice have extinguished the behaviour of looking for the exit in the

target quadrant. The choice of a two-minute duration time to perform the probe-trial was based on previous data obtained by our group [11,12], which showed that rats trained in the Morris Water Maze in five sessions (four trials per session) and tested 24 hours after the last session, could remember the task in the 1st minute of the test and extinguish the behaviour in the 2nd minute of the probe-trial. It is known that the extinction of a behaviour depends on several variables [16] such as the learning scheme, type of reinforcement, temporal scheme of reinforcement presentation, and it is also plausible to hypothesize that there are differences between species regarding the extinction of the same behaviour learned in a similar context. Evidence that extinction is a complex behaviour that depends on many variables, including individual variability. Figure 1, panels C and D shows the existence of variability among mice regarding extinction. It is interesting to note that the animals that remained longer in the target quadrant during the first minute of the test were those that persevered more in the search for the exit in the second minute of the test. This variability seems to be unrelated to hippocampal GABA levels, since we found no significant relationship between these two parameters, time spent in the quadrants and GABA levels (data not shown). In a previous work, we found significant correlations between both hippocampal AChE activity and extinction index [11-12]. Therefore, an indirect effect of GABA, such as via cholinergic components, on the extinction of this behaviour cannot be ruled out.

Regarding neurochemical data, the findings of the present work show for the first time that the task learning process in SWM has a significant effect on GABA levels in the hippocampus. Although a significant effect on glutamate levels was not observed, considering the significant correlation between GABA and glutamate concentrations, one can infer the occurrence of an adjustment of neurochemical circuit activities in the hippocampal region, induced by the cognitive activity generated during the spatial learning process. One should also consider the possibility that this adjustment is secondary to an alteration in other circuits, such as cholinergic, which is known to play an important role in learning and memory processes [11] Using rats as experimental models, Chiang and Liang [22] [xx]showed evidence of the role of cholinergic and GABAergic systems in contextual memory consolidation. In the same direction, there is evidence on the establishment of synapses between cholinergic terminals in the hippocampus and GABAergic neurons [23]. Our group showed that a decrease in hippocampal cholinergic parameters, induced by insults such as thiamine deficiency and/or ethanol consumption, interferes with aspects of spatial reference memory [11]. There are several articles in the literature showing evidence of GABA involvement in spatial cognitive functions [4, 10, 13, 23, 24]. However, to our knowledge, the present study is the first to show evidence of a significant effect of training on a spatial navigation task on GABA levels in the hippocampus. A better understanding of the role of GABAergic modulation in hippocampal function related to spatial learning represents an important step in understanding the neurobiological substrates associated with this process. It is known that in the hippocampus, a large body of work has identified an unprecedented diversity of GABAergic interneurons with pronounced anatomical, molecular, and physiological differences [25].

The chromatographic technique for the determination of glutamate and GABA concentrations used in the present study is well established in the literature and has been used by many authors [12,13,17-19]. The mean values of GABA (6000 ng/g) and glutamate (0.3 μg/g) concentrations obtained in the hippocampus samples are in accordance with those observed by other authors [10]. Training in SWM did not significantly affect glutamate levels in the hippocampus, but significantly increased GABA levels in animals from the "Learning" Group when compared to the other two groups. The lack of detection of a significant effect on glutamate concentration could be explained by the greater variability of the data obtained in these measurements. However, despite the lack of effect of training on glutamate concentrations, it seems that some adjustment in hippocampal glutamate levels occurred following the changes in GABA levels, since there was a significant and positive correlation between glutamate and GABA concentrations in the hippocampus of mice from the Control and "Learning" groups. The dispersion line between hippocampal glutamate and GABA concentrations for the "Learning" Group has a similar slope to that obtained for the Control Group. The difference is that for the animals of the "Learning" Group there was a shift toward higher levels of the two neurotransmitters, indicating a modulatory adjustment of the two systems induced by the execution of the cognitive task. The roles of these two systems in synaptic plasticity, learning and memory have already been demonstrated [10, 26- 27].

The only difference between the animals in the Active and Learning groups is the spatial cognitive demand. We could also consider that subsequent exposure to the maze would result in non-associative learning, such as habituation for animals in both the Active and Learning groups. An interesting observation is that the correlation between GABA and glutamate in the hippocampus is not detected for the animals of the Active group, suggesting that possibly the habituation phenomenon could had occurred and disrupted the association between the two neurotransmitters in the hippocampus. The mechanisms responsible for this disruption could be the subject of further studies in the future. This hypothesis needs to be tested, but the different results between the Learning group and the Active group suggest a dissociation between the two types of learning, associative and non-associative, respectively, at least with respect to the role of GABA in the hippocampus. Sanderson et al. [28] showed a dissociation between spatial reference memory and habituation, with distinct involving of the glutamatergic component, Glu1 AMPA receptor. Some authors suggest that more important than the change in the concentrations of inhibitory and/or excitatory neurotransmitters, would be the

balance between them, whose dysfunction could lead to neuronal apoptosis related to worse cognitive performance. Gao et al. [10] showed that stressed rats present impaired performance in the LAM and an increased GABA/glutamate ratio in the hippocampus when compared to the control group. Considering these data, we could also hypothesize that the increase in GABA observed here was a consequence of a stress induced by SWM exposure. However, this hypothesis can be discarded, because this effect was not observed for the animals in the Active group, that like the animals from the Learning group were also exposed to SWM and showed values of GABA concentration and GABA/glutamate ratios different compared to the animals that performed the spatial navigation activity associated with the maze solution task. Considering the above, the data obtained here, raise interesting questions to be tested in future studies.

5. Conclusion

The data indicate for the first time that the cognitive activity required to perform a spatial task increase significantly the GABA levels in the hippocampus. Although there was no significant change in hippocampal glutamate levels, the absence of effect on the ratio between glutamate and GABA concentrations and the significant correlation between these two neurochemical parameters suggest the occurrence of a possible regulatory adjustment in neurochemical circuits in the hippocampus induced by the acquisition process. The fact that the change in GABA level in the hippocampus was detected after the end of training suggests that they may be involved with the encoding and/or maintenance of learned information.

Furthermore, the results obtained here open new perspectives for further studies on the molecular mechanisms focusing on the involvement of both GABAergic and glutamatergic systems in different types and stages of the learning processes. While our understanding of hippocampal function, from the molecular to the system levels, has increased over the last years, this effort has not yet clarified many questions about the physiological and pathological mechanisms associated with the spatial cognitive process.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

Authors hereby state that there is no conflict of interest.

Statement of ethical approval

All experiments were performed according to the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA/UFMG), approved on 12/08/2014, Protocol no. 161 / 2014.

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