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Ethanol- and Diazepam-Induced Cytochrome Oxidase Activity in Mammillary Bodies

S. RUBIO, A. BEGEGA, L. J. SANTÍN AND J. L. ARIAS[1]

Laboratory of Psychobiology, Faculty of Psychology, Oviedo University, Aniceto Sela s/n, 33005 Oviedo, Spain

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RUBIO, S., A. BEGEGA, L. J. SANTÍN AND J. L. ARIAS. *Ethanol- and diazepam-induced cytochrome oxidase activity in mammillary bodies.* PHARMACOL BIOCHEM BEHAV **55**(2) 309–314, 1996.—This study aims to analyze the effect of the administration of diazepam and alcohol on cytochrome *c* oxidase activity (COX) in the mammillary bodies (MB) with a quantitative densitometry method. The histochemical reaction of the COX is used as a reflection of energy consumption. Our results show an increase in the COX activity after treatment with diazepam in the different nuclei of MB: medial medial nucleus (MMNm), lateral medial nucleus (MMN1), and lateral nucleus (LMN) of the MB, the MMNm and LMN being significantly more active compared to the MMN1. Furthermore, the consequences of administering these drugs become manifest in spatial learning (water T maze). The performance in a spatial discrimination task did not prove to be impaired. **Copyright © 1996 Elsevier Science Inc.**

Diazepam Ethanol Mammillary bodies Cytochrome *c* oxidase

MAMMILLARY bodies (MB) are found in the basal portion of the diencephalon and occupy the caudal part of the hypothalamus (20). This structure has been involved in many different functions, among which the following are included: memory and spatial learning (5,35,40), spontaneous alternation (2), sexual behavior (23), defensive behavior (29), and emotional processes (6). The MBs are components of the Papez circuit, a neuroanatomic substrate of emotion (18,31). They receive afferents from the ventromedial hypothalamus and emit two large projections to the anterior thalamus and dorsal and ventral tegmentum, regions that are related to behavioral suppression.

The MB are considered as an antianxiety center (44,45), a potential site of action of the benzodiazepines (18,33). This involvement in the regulation of anxiogenic behavior is related to the existence of GABA receptors in this nucleus. Moreover, the existence of GABAergic terminals in the mammillary bodies arising from subicular and septal connections has been demonstrated. The GABAergic system could inhibit a posterior hypothalamus neural population. Activation of the posterior hypothalamus is responsible for physiological and behavioral responses associated to states of emotional activation, experimental anxiety, and stress (21). Thus, posterior hypothalamus $GABA_a$ receptors seem to regulate the level of experimental anxiety (37). The GABA mediated inhibitions are amplified with the intervention of the benzodiazepines that increase the affinity of the receptor to the GABAa agonists; thus, smaller concentrations of these are necessary. This is due to the fact that the benzodiazepinic receptors are located in the neural membrane and form a part of the macromolecular complex of the $GABA_a$ receptor (22,27,41).

The MBs are also affected by chronic consumption of alcohol; thus, patients with Korsakoff's syndrome present structural alterations in the mammillary region (24). Alcohol acts selectively on certain proteins of the membrane. The $GABA_a$ and $GABA_b$ receptors are found among the molecular complexes. Like the benzodiazepines, alcohol strengthens the action of GABA (17,30,38). In this work, we have aimed to quantify neural energy consumption by the cytochrome *c* oxidase activity (COX) after different treatments with diazepam and alcohol. COX is measured in the following nuclei of the MB according to the classification of Allen and Hopkins (3): medial medial nucleus (MMNm), lateral medial nucleus (MMN1), and lateral nucleus (LMN). Cytochrome *c* oxidase is an enzyme involved in oxidative phosphorylation, a mechanism by which the necessary energy is extracted for the neural metabolism (43). Hence, the amount of COX will be an index of neural functional activity (16). Furthermore, we have studied the existing relation between the administration of two anxiolytics, diazepam and alcohol, in spatial learning per-

[1]To whom requests for reprints should be addressed.

formed in a water T maze. Most of the studies regarding the effects of anxiolytic drugs in animal learning and memory have used aversive paradigms, mainly two-session passive avoidance task. Recent studies (25,26,28,46) use the Morris Water Maze (MWM). In the MWM several rates of learning and performance can be measured independently. Following this idea, in the present work we propose water T maze as a useful model to detect the effects of the drugs that were administered during the process. It is an aversive paradigm where cold water (18–22°C) is used to motivate the animal. We will use 20°C, as in former research we have confirmed that modification of the water temperature is directy linked with the degree of anxiety and with the task performance. The temperature of 20°C seems appropiate, as it motivates the animal without preventing the animal from learning the task. With inferior temperatures (14°C) the escape latencies decrease but they hinder laterality behavior in the T maze (33). This spatial discrimination task allows the animal to use a response pattern from its behavior repertory that does not require long learning sessions. Furthermore, it avoids the manipulation of appetitive behaviors such as deprivation of food and water, which could have interfering effects on the nuclei studied. By using this type of task, the effects of the contaminating variables are eliminated.

METHOD

Subjects

Forty-two male Wistar rats of the *Rattus norvegicus* species from the vivarium of the University of Oviedo were used. The animals were kept on a 12 L:12 D schedule (0830–2030 h) at a constant temperature (21 ± 1°C) and with free access to food and water. All of the animals used were adults (age: 60 days old), with an approximate weight of 250 g. For the behavioral test, the animals were randomly distributed into the following groups: 1) control group 1, which received no treatment; 2) group treated with diazepam (Valium 10 mg/2 ml injectable ampuls, Roche, Basel, Switzerland). This was administered intraperitoneally (IP) (2 mg/kg) 30 min before each learning session; 3) control group 2: saline solution (2 mg/kg) was injected IP 30 min before beginning the learning. Other authors (36) have already demonstrated that the cerebral metabolic effects of the vehicle of diazepam (propylene glycol 40%, benzyl alcohol 1.5%, sodium benzoate 5%) in the MBs do not differ from those of the saline solution; 4) alcoholized group: the alcoholization progress was progressive, beginning with a 2% proportion of ethyl alcohol in the drink when the rats were 21 days old. This quantity was doubled weekly until 20% alcohol was reached during the second month. In this way, alcohol levels in blood of 25.7 ± 2.79 mg/dl were obtained (10).

Apparatus and Testing Procedures

The water T maze was used for the spatial learning task. This was constructed in metacrylate, was 40 cm high × 10 cm wide and the length of each one of its arms was 50 cm. The walls and the bottom of the maze were black so as to prevent external influences from interfering with the performance of the task. For this same reason, the escape platform was also hidden. It was constructed in transparent metacrylate, located on one of the arms, and was maintained 1 cm under the water surface. The water height was 12.5 cm. The water T maze was kept in an isolated room, with constant temperature and light during the process.

The learning acquisition process took place at the same time of the day (0900 h) for 3 consecutive days. Prior to this, there was an habituation phase in which the same environmental conditions as those that would form a part of the learning were maintained. The purpose of this habituation was to prevent the animal from adapting to the task. Thus, both platforms were placed on each of the arms submerged under the water and the water was maintained at a constant temperature of 20 ± 1°C. To prevent the animal from developing a preference for one arm, the arm was blocked if the same platform was chosen two consecutive times and the animal was forced to choose the other arm. During this period of habituation, the animal received the same number of trials and with the same interval between them as in the case of the later learning. During the learning process, the daily trials were distributed in forced and free trials. By forced trial, we understand that in which, by blocking the right arm, the animal is forced to choose the correct arm, that is, the left one, in which the escape platform is found. By free trial, we understand that in which the animal can choose either of the two arms. Therefore, the animal may make the wrong choice and commit an error. The variables recorded were escape latency and number of errors (in the case of free trials). The trials were distributed according to the following: habituation—with a platform on both arms; first day: four forced and two free trials; second day: two forced and four free trials; third day: six free trials. There was an interval of 2 min between trials.

The criterion established for the acquisition of learning, that of 80% correct responses, was reached by all of the animals on the last day. After the last learning session, the animals were anesthetized with ethyl ether and vascularly perfused by 0.1 M phosphate buffer (pH 7.6). Their encephalons were immediately extracted and cryoprotected for storage at −70°C.

Histology

Sections of the mammillary region were obtained with a freezing microtome (Model 2800 Frigocut of Reichert-Jung) at a temperature of −19°C. The cuts were coronal, done according to the Atlas of Paxinos and Watson (1982) and were 20 micrometers thick. The histochemical technique used was the enzymatic detection of the cytochrome *c* oxidase described by Carrol and Wong-Riley (8) and modified by Sukekawa (39). Prior to the staining, the sections were fixed in glutaraldehyde (1% Merck) and washed in phosphate buffer. After the washing, the sections were submerged in cytochrome at a temperature of 37°C for 2 h. The method followed for the preparation of the cytochrome solution is briefly described in the following: preparation of the solution for 100 c.c.: 50 mg of diaminobenzidine, 15 mg of cytochrome *c*, 4 g of sacarose, phosphate buffer 0.1 M (pH 7.4).

Quantification

To quantify CO activity, the method previously described by Gonzalez-Lima and Cada (11,12) was used with several modifications introduced by us (13). These are briefly described in the following: rats were perfused with 50 mM phosphate buffer (0.1 M) and their brains removed and homogenized in ice. Half of the brain paste obtained was heated in a water bath at 60°C for 5 h to inactivate all enzymes present, while the other half were refrigerated in ice for the same period of time. Different mixtures were made that contained increasing percent weight proportions of active brain homogenate (25, 50, 75, and 100%). An aliquot was extracted from

each one of the standards to quantify CO activity using the spectrophotometric method of Wharton and Tzagoloff (42), which briefly described consists in measuring the decrease of absorbance at 550 nm produced when the reduced cytochrome *c* is oxidized by the CO for 1 min at 23°C. In our case, the reaction mixture was 10 ml of brain paste in phosphate buffer of a previously known concentration with 990 ml of cytochrome *c* (Sigma, St. Louis, MO) that was previously reduced with sodium hydrosulfite. The final concentrations of the tissue and the cytochrome *c* in the reaction mixture were, respectively, 4.3 mg/ml and 59 mM. As the relation between the extinction coefficients for reduced and oxidized cytochrome *c* at 550 and 565 nm (19.6 $mM^{-1}cm^{-1}$ at 23°C) is known, the CO activity was calculated in specific units (mM of cyt. *c* oxidized per minute and gram of tissue in humid weight at 23°C). The measurement was repeated at least three times for each standard and then sections of 20 mM of the already determined standards of CO activity were done. These sections were stained together with the rest of the sections obtained from the regions being studied for CO. Once the staining was done, their optical density was measured by a computer image analysis system (IMCO-2, Microm Spain) coupled to the microscope to obtain an equivalence curve between relative optical density (O.D.) and CO activity. Finally, the O.D. of the brain regions of interest selected on the computer screen was measured; this O.D. was automatically converted to real CO activity by applying the calibration curve previously obtained with the brain paste. A total of nine measures per structure and animal as well as sections of different thickness of the paste (20, 25, 40, 60, and 80 mm) were done to verify the linearity of the relation between the histochemical reaction and O.D.

Statistical Treatment of the Data

Two variables were recorded in the spatial task: errors made and escape latencies. In both cases, a two-way ANOVA (treatment groups and days of learning) with repeated measures was applied. Then a post hoc test, the honestly significant difference of Tukey, was applied. Regarding the enzymatic activity, the ANOVA model applied is made up of one independent variable (the different groups) and one dependent variable (the mammillary nuclei). The post hoc test used is the honestly significant difference of Tukey.

RESULTS

The results of the analysis of the error show (see Fig. 1) significant differences between the days of learning, $F(2, 72) = 8.5961$, $p \leq 0.001$. This difference specifically occurs between the first and third day of the task, thus reflecting learning between these days. No differences in acquisition, $F(3, 36) = 1.7866$, $p = 0.1671$, were found between the different groups. We also have not observed any interaction between both factors (treatment groups and days), $F(6, 72) = 0.7565$, $p = 0.6063$).

The fourth group differs in its escape latencies (see Fig. 2), $F(3, 36) = 9.7848$, $p \leq 0.0001$. The groups treated (with diazepam and alcohol) present significantly lower times ($p \leq 0.05$; $p \leq 0.01$) than the control with injection of saline solution; and the ethanol group differs significantly from the control group without injection of saline solution ($p \leq 0.001$).

Significant differences were seen during the days of learning, $F(2, 72) = 3.9442$, $p \leq 0.05$. There is a significant decrease in latencies ($p \leq 0.05$) between the first and second day, a reflection of the previously mentioned learning. Interaction should be rejected again, $F(6, 72) = 0.3501$, $p = 0.9076$.

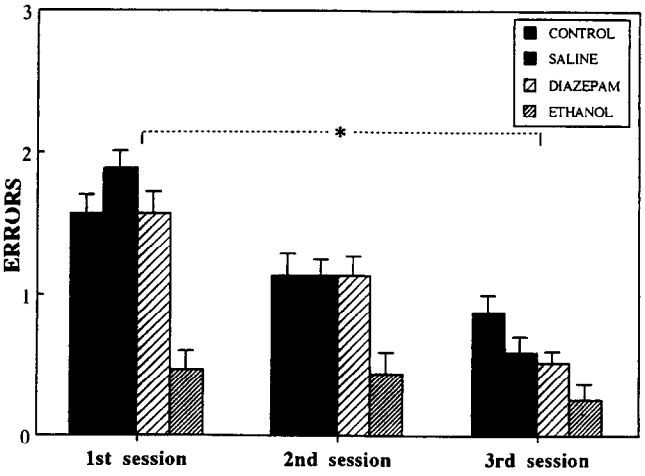

FIG. 1. Transformed percentage of errors during the learning process. There are significant differences between the first and third session (*$p \leq 0.001$). The standard errors are shown as vertical bars.

Regarding the analysis of the enzymatic activity (see Fig. 3), the different groups of treatment have an activity gradient in the following order: diazepam > alcohol > control. These differences, $F(2, 18) = 4.8211$, $p \leq 0.05$, are only significant between the group treated with diazepam and the control group ($p \leq 0.05$).

We have found statistically significant differences between the nuclei, $F(3, 54) = 102.4358$; $p \leq 0.0001$. The LMN and MMNm nuclei present a great activity in contrast to the MMN1, that has less activity ($p \leq 0.001$).

DISCUSSION

Our results show an increase in COX in the different treatments with diazepam and alcohol. This increase is significant

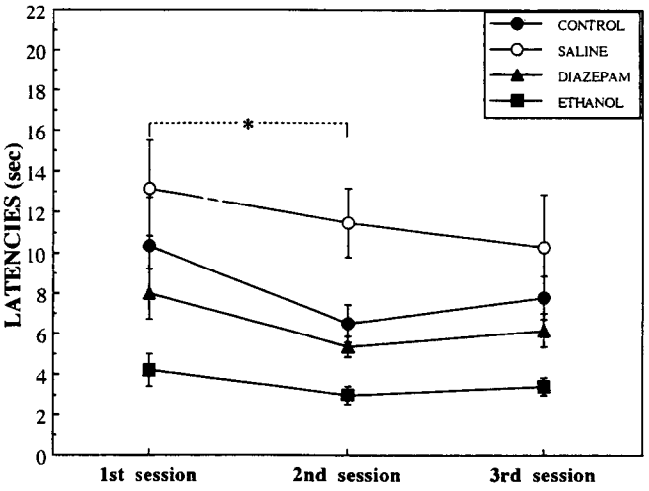

FIG. 2. Escape latencies in spatial learning. There are significant differences between the first and second sessions ($p \leq 0.05$). The saline group presents higher times than the diazepam and ethanol groups ($p \leq 0.05$), ($p \leq 0.01$); the latter group also differs significantly from the control group ($p \leq 0.001$). The standard errors are shown as vertical bars.

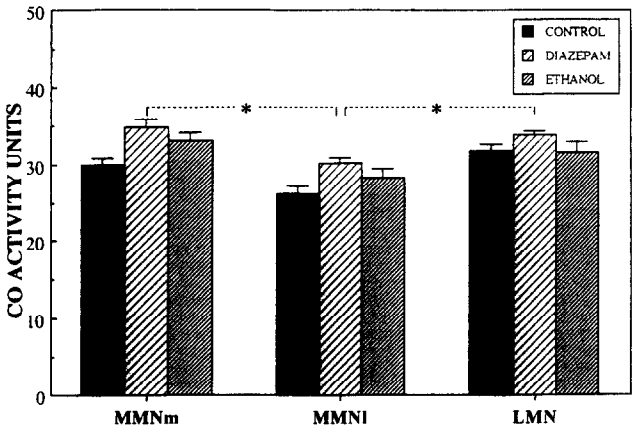

FIG. 3. Quantified cytochrome *c* oxidase activity in mM/min/g tissue w/w, in the different mammillary nuclei by treatment group. There are significant differences between the LN and MMN compared to the MLN ($p \leq 0.001$). There are also significant differences ($p \leq 0.05$) between the control group and that treated with diazepam. The standard errors are shown as vertical bars.

in the case of the group treated with diazepam compared to the control one. Furthermore, we found differences in the activity of the different nuclei, the MMNm and LMN being the most active and the MMNl the least active. This pattern of COX activity remains constant in the different treatments used. The fact that we found a COX increase after a period of four days of treatment is important and seems to indicate that this technique is valid to reflect changes in intervals of less than 15 days.

The variation found in the COX activity between the mammillary nuclei can be related to the distribution of the connections in the MB because most of the energy required, reflected in the COX activity, is related to the synaptic activity. We remind you that the MB mainly receives excitatory afferents from the subicular area of the hippocampus and inhibitory afferents from the tegmental area (4). The greater COX activity in the MMNm can be interpreted by the large number of fibers from the fornix and main mammillary tract, while the low COX activity of the MMNl observed is probably due to a greater dispersion of fibers in this nucleus (14,15).

It has already been demonstrated in different encephalic structures that the COX activity is a reflection of the synaptic activity associated to the maximum concentration of mitochondria that is found mainly in dendrites and axonic terminals (11,13). Notwithstanding, it is still unknown to us if the COX activity is associated to the type of neurotransmitter participating in the synapses, although it is supposed that the excitatory synapses produce a high COX activity (43). However, the GABAergic neurons of the cerebral cortex have been described as highly reactive while these same neurons are not so in the lateral geniculate nucleus (11).

Therefore, the increase in COX activity could be related to the connections present in the MB. This complex is considered to be a component of the Papez circuit, a neural substrate of emotion (31). Benzodiazepines supposedly produce their anxiolytic effect by acting on the MB-anterior thalamus-frontal cortex route (18). Their action would inhibit the MB, which through the anterior thalamus, would act on the cortex. The result of this inhibition would be the facilitation of performance on a behavioral level. Thus, we should remember that MBs have been related to the mediation in the behavioral suppression as other authors have already mentioned (44,45).

Another study (9) in which the uptake of 2-deoxy-D-glucose (2-DG) was used as an index of activity shows the same variation in metabolic activity of the MBs after chronic exposure to alcohol. This metabolic measurement agrees with the results in COX activity that we obtained. On the other hand, other authors (1,36) have found that a decrease in cerebral glucose utilization in the mammillary bodies is produced after exposure to diazepam. In both cases, the decrease in glucose consumption is accompanied by a decrease in protein synthesis, as we have verified (10).

These results suggest that both techniques, 2-DG and COX, reveal the same process in a different way. Thus, the COX reflects the levels of metabolic activity that remain in the brain structures over time and confirms that this technique can detect the effects produced by chronic treatments of days or months and relates them to the metabolic demands that such treatments produce, while the 2-DG technique makes it possible to visual the glucose utilization in the short run and, thus, the levels of activity of these same structures. On the one hand, the 2-DG detects the uptake of glucose for the production of ATP, and on the other hand, the COX catalyzes the transport of electrons from the substrate to oxygen as the final step in the respiratory chain in the formation of H_2O and ATP. It is clear that the 2-DG and COX are different methods.

Regarding the behavioral results, we must consider the anxiolytic properties of the drugs, the possible memory impairment and the impairments that could have been caused. In other authors, we find data that show that diazepam, in similar doses (26), and ethanol when administered postnatally (19) impair spatial learning in MWM. On the contrary, in a different work, using the MWM, the behavioral results show that diazepam does not impair the reversal or the learning after familiarization (46). Nor does one research see any difficulty in the treated groups (as shown by the number of errors in the performance) to acquire the task. A possible reason for this discrepancy is that training procedures are different in every investigation. The water T maze might require the use other types of strategies to locate the platform and not only the spatial strategies required in MWM (7).

The effects of drugs on the task seem to be mediated more by their anticonflict/anxiolytic activity than by a direct action on learning and memory processes. Thus, benzodiazepines lead to an atenuation of the fear and anxiety elicited by the experimental procedure. Such atenuation has been suggested as a participant of the aparent deficit caused by these drugs in the MWM spatial learning (26). These anxiolytic effects of ethanol and diazepam have been actually reflected in the discriminatory task performed in the water T maze. Their action seems to adequately decrease the arousal state, facilitating the correct performance. Thus, a 2 mg/kg dose of diazepam seems to improve performance in comparison to the control animals while the chronic consumption of ethanol also seems to favor the correct performance of the test, as is shown by the lower number of errors committed in these groups compared to the control group (see Fig. 1).

We should also consider that the drugs used produce hypothermia, which would interact with scape from cold water. Former studies have shown that the reduction of body temperature caused by diazepam and cold water was so low that it provoked amnesia simply by itself (32). Our data discarded this possibility, as the animals treated do not make more mistakes than the control ones. Therefore, is not possible to believe that the drugs had brought the amnesia about. Therefore,

the improvement in performance should be attributed to the tranquilizing effect of the treatments, no deterioration being produced in the locomotive capacity because the escape latencies decrease in regard to the controls (see Fig. 2). Thus, the performance should not be impaired because, if we consider our data, animals seem to use mainly a motor strategy. This strategy is effective when the exit site and goal are maintained in the same relative position as it happens in the water T maze. This motor strategy would be benefitted as the doses used decrease anxiety but do not interfere with the motor capacities or the swimming speed as far as we have observed. Other studies in which specific locomotive tasks have been used have verified that the animals have the right locomotive activity after these treatments (45). Thus, the animals could learn to perform a specific sequence of movements. This strategy could be simultaneously combined with other behavioral strategies.

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