EFFETS OF ETHANOL AND DIAZEPAN ON Ag-NOR NEURONAL ACTIVITY IN THE MEDIAL MAMMILLARY NUCLEUS

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It is intended here to study the disorders produced on mammillary bodies at both behavioural and morphological levels after diazepam and alcohol administration. For this purpose, 3 groups of rats were used, testing them in the water T-maze trial. One of the groups was treated with diazepam, another group alcoholized and the third was control group. The reaction time was recorded and also the errors commited in the last day. Histological analysis was made by evaluation of the number of Ag-NORs and the surface of these in each neuronal nucleus. The results show a statistically significant decrease in reaction time throughout the learning period, with almost no errors. There are not any differences in the estimated NOR surface of the experimental groups. However, differences were reported concerning the number of NOR quantified among the different treated groups in relation to control group.

Key words: Alcohol; Diazepam; Ag-NOR; Mammillary Body; Rat.

Efectos del etanol y el diazepan sobre la actividad Ag-NOR neuronal en el núcleo mamilar medial. Se pretende estudiar las alteraciones producidas en los mamilares tanto a nivel conductual como a nivel morfológico tras la administración de diazepam y alcohol. Para ello se emplearon 3 grupos de ratas testadas en la prueba del laberinto en T de agua. Uno de los grupos tratado con diazepam, otro grupo alcoholizado y un tercer grupo control. Se registraron los tiempos de reacción y los errores cometidos el ultimo día. El estudio histológico consistió en la determinacion del número de Ag-NORs y el área de los mismos en cada núcleo neuronal. Los resultados muestran una disminución en el tiempo de reaccion estadísticamente significativa a lo largo del aprendizaje, sin existir apenas errores. No aparecen diferencias en el área NORs estimada en los grupos experimentales. Sin embargo, se muestran diferencias en el número de NORs cuantificados entre los distintos grupos tratados en relación al grupo control.

Palabras clave: Alcohol; Diazepam; Ag-NOR; Cuerpo Mamilar; Rata.

Since the description by Cajal (1911) of the Mammillary Body (MB) (Hypothalamus) detailed morphological descriptions have been carried out accepting nowadays the division by Allen and Hopkins (1989) that establish the following nuclei: Medial Mammillary(MM), Lateral Mammillary, Premammillary, Supramammillary and Tuberomammillary.

Their connections with the frontal cortex have been described (Wouterlood, Steinbusch, Luiten and Bol, 1987), but also they show even more with the anterior tha-

lamus (Seki and Zyo, 1984), mesencephalon (Shibata, 1987) and hippocampal formation (Guillery, 1956; Swanson and Cowan, 1977). These connections allow us to know about their important contribution in memory processes (Weiskrantz, 1978; O'Keefe and Nadel, 1978), the acquisition of spatial alternation (Irle and Markowitsh, 1982), reversal of the latter (Aggleton and Mishkin, 1985), defensive behaviour (Shibata and Furukawa, 1988), fluid and endocrine regulation (Morales and Puerto, 1988).

Moreover, classical neuroanatomical studies (Kriekhaus, 1966) have shown the MB implication in pathologies such as Wernicke-Korsakoff syndrome, common in patients with a case history of chronical alcoholism (Aggleton and Mishkin, 1985; Lindboe, Erichsen and Strom, 1989) and Korsakoff's psychosis, the chronic phase of the Wernicke-Korsakoff syndrome, distinguished by several degrees of both retrograde and anterograde anmesia; confabulation frequently disappears in chronic stages of this disease.

Ethanol is one of the most used neurotoxic drugs and it is also most commonly found in Korsakoff's patients. The working model of ethanol in the MB takes place specifically on the neuronal membrane function and consequently on the whole neurotransmission due to the specific effects of acetaldehyde on the metabolism of biogenic amines (Ollat, Parvez and Parvez, 1988).

On the other hand, the MB show the presence of GABA-ergic receptor densities, being involved, therefore, in the chemical action of certain drugs like benzodiazepines, anxiolytic drugs. The MB is a potential site of the antianxiety action of benzodiazepines (Kataoka, Shibata, Gomita and Ueki, 1982). They seem to disinhibit the behavioural suppression by reducing the activity of the periventricular system through gamma aminobutyric acid (GABA)-ergic and serotonergic neurons (Stein, Wise and Belluzzi, 1975).

Taking into account these contributions, showed by neuroanatomical and experimental studies in humans, one might raise the question of possible structural and functional changes in the nuclei under the chemical effect of different drugs and the consequent behavioural repercussions after those changes.

We have chosen to study the fibrillar centers (FCs) in the nucleolus because of their importance on the neural ribosomal RNA synthesis (Puvion-Dutilleul, Mazan, Nicoloso, Pichard, Bachellerie and Puvion, 1992). —The nucleolar structure is made up of five components: nucleolar chromatin, FCs, dense fibrillar component, granular component and nucleolar interstices (Goessens, 1984)—

In the interphasic cells, the FCs represent the morphological correlation of the nucleolar organizer regions (NORs) in the mitotic chromosomes, (Hernandez-Verdum, 1983) which are made up of ribosomal desoxyribonucleic acid (rDNA) and proteins, some of which are argyrophile (Troster, Spring, Meissner, Schultz, Onder and Trendelenburg, 1985). Thus, this silver stained structure is called Ag-NOR.

The present survey was undertaken to examine the potential effects of performance in a water "T" maze on the MM, measured in terms of Ag-NOR activity, since this is a neuronal activity index under the effect of several drugs: ethanol (ETH) and diazepam (DZP).

MATERIALS AND METHODS

Animals

An amount of 30 wistar strain *Rattus norvergicus* adult males have been used, weighing 240+30 g. They were maintained during the experiment on a light-dark cycle

of 12 hours (8:00-20.00) at a constant temperature of 21±1 °C., with food and water available ad libitum.

They were divided into three groups in this way:

—Group 1, with administration of benzodiazepine (diazepam) (n=10)

—Group 2, consuming alcohol as the only available drink (n=10)

—Group 3, control (n=10)

—Group 1 was injected i.p. with 2mg/kg diazepam (Widgiz and Beck, 1990). After a period of 30 minutes began their learning session.

—Group 2 had drunk in a progressive way from the first weaning day during a 2 month period. The alcohol amount was increased beginning with a 2% solution and doubled weekly during the first month up to a 16% concentration. They had a 20% solution during the second month.

—Group 3 was also injected i.p. with 2 mg/kg distillated water to determine the possible effects of injection on the performance of the spatial task. After a period of 30 minutes began their learning session.

Apparatus

The learning process took place in a water "T" shaped maze made of plexiglas 50 cm wide x 40 cm high. The maze was painted black, enabling this the isolation from possible external stimuli.

Water temperature was mantained at 20±1 °C, filling the maze of tap water up to 1 cm above the two platforms, each one at the end of the arms.

Procedure

The learning process was developed for 5 consecutive days, receiving a daily amount of 12 trials. They were distributed between "forced" and "free" trials, meaning by "forced" the blockage of the incorrect arm, that bore no platform; whereas "free trial" is defined as that in which the animal can swim both arms, since no blockage occurs and it is then possible to have fails when the rat choose one of the arms (Castro, C. A, R. Paylor and J. W. Rudy; 1987).

The recorded variable, in this case, was the reaction time spent until it reaches the escape platform.

The procedure was as follows:

1.-Habituation, a period in which the animals are submitted to the same number of trials as during the subsequent period. In this case, the animal was allowed a free choice, with the two platforms placed. However, to prevent a bias towards one of the arms, it was blocked the one that had been chosen in two consecutive trials.

2.- 1st learning day, they had 12 trials, distributed as follows: 9 forced trials and 3 free trials.

3.- 2nd learning day: in this case, the distribution was the same as above.

4.- 3rd learning day: it was reversed the number of forced and free trials. So the animals had 3 forced trials and 9 free trials.

5.- 4th learning day: All were free trials.

Perfusion

Once finished learning period, the animals were anaesthetized by ethilic ether inhalation and then intracardially perfused, using 10% formaldehyde in phosphate buffer 0.1 M, pH 7.4.

It was also extracted 1 ml blood to determine the alcohol level in the animals corresponding to Group 2 by means of an enzymatic-polarized fluorescency technique. The brain was quickly removed and the mammillary bodies dissected, remaining during postfixation in the same fixer for several days. After this, the mammillary body was dehydrated by using a raising concentration chain of alcohols and then embedded into paraffin.

Sectioning and staining: 4 μm thickness sections were obtained using a microtome (MICROM, Heidelberg). These sections were later stained.

Staining was made using silver nitrate (Merck) with an amount of 0.5 g disolved in 1 ml of distilled water plus 0.5 ml of 2% gelatine. Once finished the hydration of the sections, they were maintained in a silver solution for 20 minutes, dehydrating and processing them for later examination.

Histological evaluation:

Quantifying was made using an Image Analysis system (IMCO lO,MICROM). Enabling this to determine the neuronal Ag-NOR surfaces sampled, in a parallel way for quantitative evaluation of the number of Ag-NORs contained in each chosen neuronal nucleus. The quantified sample was of 100 cells for each animal of the different groups.

RESULTS

Learning

All alcoholic animals reached an average alcohol in blood index of 25.7±2.79 mg/dl by means of the above described procedure.

As for the *reaction time*, it is observed in all groups a slightly decrease of this, statistically significant, all through the learning days for each treatment group (Student's t for paired data; $p<0.05$ between days 2 and 3/DIAZ-t_9=3.22, ETH-t_9=2.85, CTR-t_9=2.52).

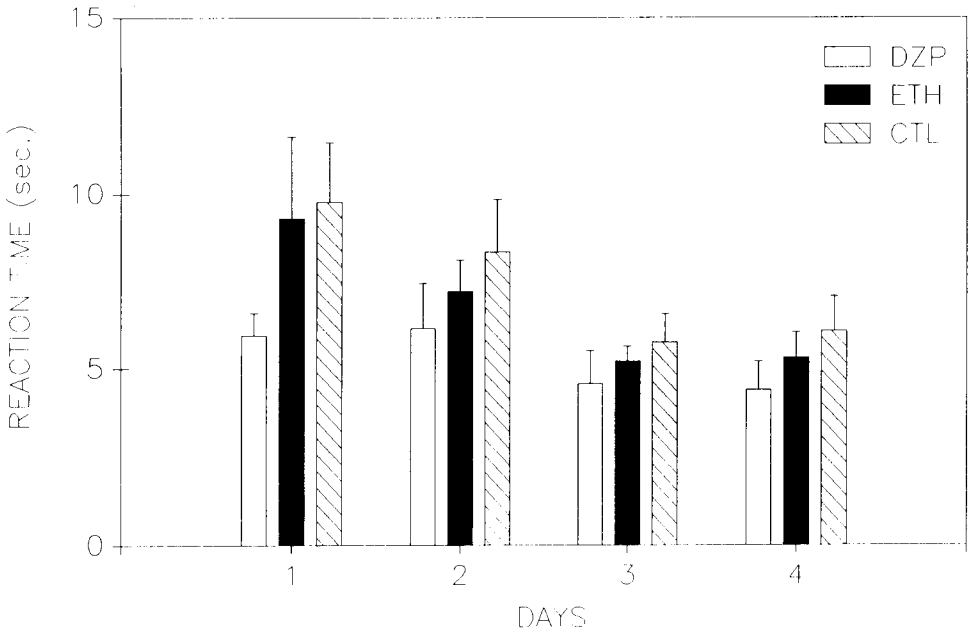

Figura 1. *Mean reaction time* for each one of the 4 learning days in the treatment gropups (n=10). It was found a significant decrease in this throughout the learning days in each group ($p< 0.05$, see text). Vertical bar indicates S.E.M. —Key; DZP: Diazepam group, ETH: Ethanol, CTR: Control—

However, between the penultimate and the last day appears a slightly increase of this variable, although it is non significant.

On the other hand, analyzing the effect of *treatment*, it is found in each day that the shortest time belongs to Group 1 *(see above)*, followed by Group 2 and finally Group 3. Nevertheless, in *no day* these differences were significant, (one-way ANOVA; $F_{2,27}$=1.42, 0.77, 0.53, 0.87 for each one of the days, respectively; p>0.05).

As for the number of errors commited, they were detected in practically none of the experimental groups.

Histology

Quantifying of several morphometric parameters stated the existence of statistically significant differences. Also, concerning the number of AgNORs quantified, come into sight statistically significant differences between Group 3, with the maximum number, and the rest of treatment groups. The latter showed a decrease in the NOR number per sectioned cell.(Student's t test for unpaired data; t_{14}=-5.96 -DZP/CTR-, t_{14}=2.031 -ETH/DZP-, t_{14}=-3.91 -ETH/CTR-: p<0.05).

There are no significant differences in the area occupied by NORs (Student's t test for unpaired data; t_{14}=-0.071 DZP/CTR-, t_{14}=-0.025 -ETH/DZP-, t_{14}=-0.108 -ETH/CTR-: p>0.05), that is to say that the surface of the NOR regions do not show any changes under different experimental situations.

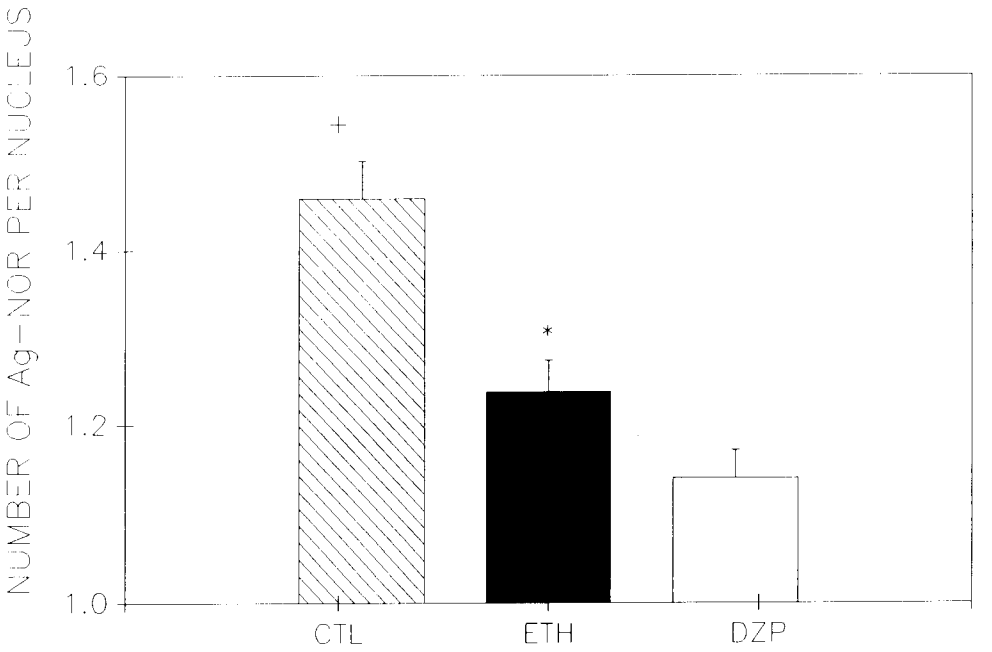

Figura 2. *Mean number of Ag-NOR per nucleus* found in each treatment gropup (n=8). +Indicates significancy of CTR group (p< 0.05 vs. ETH and p< 0.01 vs. DZP). *Indicates significancy (p= 0.062 vs. DZP).

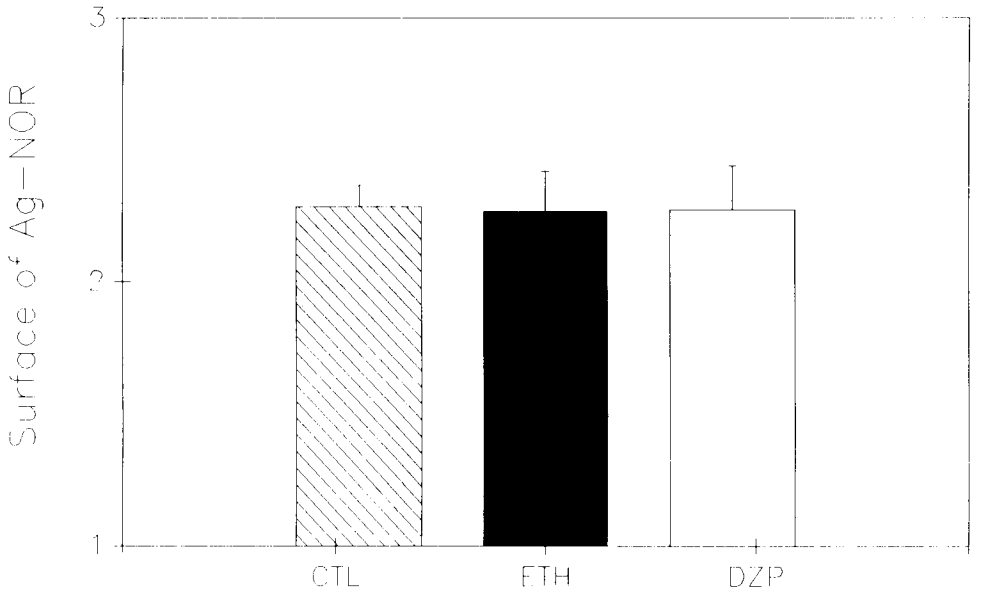

Figura 3. *Mean surfgace of Ag-NOR* (μm^2) for each treatment group (n=8).

DISCUSSION

The present survey demostrated that the region of the MB reflects the action of ETH and DZP on Ag-NOR behaviour. That's why it is possible to relate the NORs activity with behavioural aspects in memory tasks.

Anatomically, many studies concerning fibers of the MM in the rat have been performed (Seki and Zyo, 1984; Shibata, 1987; Allen and Hopkins, 1989) proving its close relation with the limbic system, receiving descending afferent fibers of subicular complex, infralimbic cortex, retrosplenial cortex, nucleus of diagonal band of Broca, lateral septal nucleus and anterior hypothalamic nucleus. The MM receives ascending afferents from ventral tegmental nucleus of Gudden (TV) and the pars compacta of the superior central nucleus(CC). The MM sends efferent fibers to the anteromedial and anteroventral thalamic nuclei, TV,CC medial pontine nucleus and nucleus reticularis tegmenti pontis. But few histochemical studies have shown which is the content of these fibers (Kiyama, Shiosaka, Sakamoto, Michel, Pearson and Tohyama, 1986) enabling us this in the future a suitable interpretation of the participation level that the MM has in these behaviours.

The number of nucleoli is a feature of species, whereas the Ag-NOR number and surface can also change as a result of fluctuations in transcriptional activity (Lopez-Iglesias and Arias, 1987). With increased transcriptional activity, there is increased synthesis of argyrophile NORAPs (NOR Associated Proteins) (Underwood, 1991). The diameter of Ag-NOR dots is almost certainly increased by the level of transcriptional activity causing the accumulation of larger pools of

argyrophilic NORAPs, and it is reduced by the dispersion of individual Ag-NOrs within large confluent argyrophile structures.

Our results suggest that DZP lowers more the number of Ag NORs than ETH (see fig. 2). The surface of the Ag-NORs in the three experimental groups, does not present any differences. Being the Ag-NORs per neuron in the MM cells treated with DZP bigger than the Ag-NORs of the ETH neuronal cells. Therefore, control neurons (CTL) present bigger transcriptional activity, average activity the ETH MM neurons and the lowest activity the DZP MM neurons.

The technique performed to measure the area occupied by the Ag-NOR proteins by means of an image processing system, was chosen among others, according to Derenzini and Trere (1991). Even though, with this we do not measure the number of Ag-NOR but the dispersion because some Ag-NOR are sumed up since they are close together (Underwood, 1991).

These results agree with the hypothesis suggesting that the MB would act as an "amplifier" of anterior thalamic neuronal responses to the ascendent cholinergic afferencies that, in turn, would be specifically involved in the discrimination aspects of avoidance learning performance (Schemker, Kubota, Mignard, Cappernel, Swanson and Gabriel, 1987). Other authors have found that the MB participate in memory disorders in male mice of the Balb/c strain of chronic alcoholics (Beracochea, Micheau and Jaffard, 1992). Our preliminary results would go this way, but a greater number of animals must be studied for its confirmation. We do not rule out that this behaviour could be produced by its anxiolytic action (Koob, Wall and Schafer, 1987).

We must remember that the cellular action of alcohol would be ubiquitously generated, disturbing the neuronal membrane function on a phospholipid level and consequently on the whole neurotransmission due to the specific effects (see Introduction). It is seen in this animals a GABAergic hypofunction in which may be involved GABA-B receptors, with recent studies demonstrating an implication of these in the neurobiochemical mechanisms of depression (Ollat, Parvez, 1988). This mechanism would affect by causing an increase in GABA release and an inhibition of GABA reuptake.

Whereas the action of DZP would cause behaviourally the initial increase and subsequent decrease in exploration, answering this way to a biphasic model of DZP action (Widgiz and Beck, 1990). Although the amnesic effects of benzodiazepines have been reported in a wide variety of clinical and experimental tests, its effect and potency on spatial tasks has not been widely studied. Evidence of the effect of benzodiazepines on spatial learning is equivocal. Acquisition impairment was observed under clordiazepone (McNaughton and Morris, 1987), but no drug effect was found in another study using a radial-arm maze (Higara and Iwasaki, 1984).

The evaluation of the diazepam effects seems to modify itself according to the used dose; thus, changes in the dosage have modified the achieved results in spatial memory tasks(Widgiz and Beck, 1990; Kalynchuk and Beck, 1992).

Its action is performing specifically on GABA-A receptors, which are found at high densities in the MB (Squieres, 1984; Sanchez, Dietl, De Blas and Palacios, 1991), causing a decrease in the percentage of correct responses in the delayed nonmatching-to-sample task (Kalynchuk and Beck, 1992). It would produce this way a fear-reduction in the animal that would enable it to have a greater familiarization with the water T maze, helping this to understand our behavioural results, that show no increase of hipoactivity all through the testing days.

To summarize, the action of DZP is more specific than that of the ETH, which

might be reflected on the neuronal activation, decreasing its Ag-NOR number, as a direct consequence of a decrease in ribosomic proteins and protein synthesis.

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