

## BRAIN OXIDATIVE METABOLISM AND LEARNING IN THE MORRIS WATER MAZE

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By using a recently developed quantitative histochemical technique for measuring cytochrome oxidase activity (CO) in the CNS, a mitochondrial enzyme whose levels are closely related to 2-deoxyglucose incorporation and with brain functional activity, the levels of oxidative metabolism were obtained in the mammillary bodies from the hypothalamus of the rat. The study included a spatial learning procedure with the Morris circular pool, in which the animals must find a hidden platform below cloudy water, with the starting point being changed in each trial in order to avoid intra-maze cues. The results show a progressive statistically significant decrease ( $p < 0.0000001$ ,  $N=10$ ) of the escape latencies across the 4 learning days, reaching a steady state from the third day on; the transfer test in the 5th day, where the platform is removed, show significant ( $p \leq 0.05$ ) longer time spent in the quadrant that had the platform during learning. By comparing the CO levels 28 days after learning in the chosen nuclei from the mammillary bodies, with a control group ( $N=8$ ) that was untrained, there were statistically significant differences across nuclei from this region in both groups ( $p < 0.00001$ ); however, there were no differences between the trained and control groups in the CO activity from each one of the nuclei studied.

*Metabolismo oxidativo cerebral y aprendizaje en el laberinto de agua de Morris.* Mediante el uso de una reciente técnica histoquímica cuantitativa para la medida de la actividad citocromo oxidasa (CO) en el SNC, un enzima mitocondrial cuyos niveles están relacionados estrechamente con la incorporación basal de 2-desoxiglucosa y con la actividad funcional cerebral, se obtuvieron los niveles de metabolismo oxidativo en los cuerpos mamilares del hipotálamo de la rata. El estudio incluyó un procedimiento de aprendizaje espacial con la piscina circular de Morris, en la que los animales deben encontrar una plataforma oculta bajo la superficie de agua teñida, cambiándose el punto de salida en cada ensayo para evitar pistas intralaberínticas. Los resultados muestran una disminución progresiva estadísticamente significativa ( $p < 0.0000001$ ,  $N=10$ ) de las latencias de escape durante los 4 días de aprendizaje, alcanzando un valor constante a partir del tercer día; la prueba de transfer en el 5º día, en el que se quita la plataforma, muestra un mayor tiempo de permanencia en el cuadrante que tenía la plataforma ( $p \leq 0.05$ ). Comparando los niveles de CO pasados 28 días desde el aprendizaje en los núcleos seleccionados de los cuerpos mamilares, con un grupo control ( $N=8$ ) en el que no se realizó ningún aprendizaje, se hallaron diferencias significativas entre los núcleos de esta región en ambos grupos ( $p < 0.00001$ ); sin embargo, no hubo diferencias entre los grupos control y experimental en la actividad CO en ninguno de los núcleos estudiados.

However, it is possible actually to use histochemical methods that do not require from radioactive materials, thereby avoiding the problem of handling and storing those radioisotopes. Wong-Riley (1984) introduced an histochemical technique that made it possible to stain cytochrome c oxidase (CO; ferrocytochrome c oxygen oxidoreductase; EC 1.9.3.1) in nervous tissue; this substance is a mitochondrial enzyme present in the neurons from CNS; it is responsible for oxidation of food materials (mainly glucose) by oxygen, in a process known as cellular respiration.

The relative activity of this enzyme detected in histological sections from encephalon has been related to functional activity of their different regions, which show very different levels in basal situation; furthermore, a metabolic relationship does exist with basal utilization of glucose; in this way, CO represents sustained variations over longer period of time (weeks) in energy metabolism activity (Di Rocco, Kageyama and Wong-Riley, 1989). This activity would depend on both the levels of CO protein synthesized and the activation of each individual CO molecule, which could change its metabolic activity (turnover rate) thanks to alterations in its structure; given that the half-life of the DNA that encodes this enzyme in the rat is 30 days, those changes in their levels would be long-termed. Moreover, the recent development of quantitative methods for cytochrome oxidase (Gonzalez-Lima and Garrosa, 1991), it is possible to make precise measures of real CO activity from brain regions with a great anatomical resolution.

On the other hand, it has been possible to find changes in CO activity in animals under brain electrical stimulation (Nobrega et al., 1993), with ageing (Martínez, Ferrándiz, De Juan and

Miquel, 1994) and even though after sensorial stimulation (Gonzalez-Lima and Jones, 1994).

However, the studies of learning performed after stimulation of brain regions include several types of learning like conditioning or habituation; there are still no data about the use of this technique to evaluate the involvement of brain structures in other learning processes. In this work, we chose the Morris water maze (1981), in order to perform spatial learning in rats. This method is based on the location of an submerged escape platform by the animals; the water is clouded with powdered milk, so this platform cannot be seen by the rats and they must use "distal" cues to find it, that is, those that are far from the goal, and can not directly guide the animal to find the goal object.

This maze is today widely used to study spatial orientation processes, due to three main advantages of this procedure against other methods: the speed of spatial learning in several tasks (8 trials), because it is obtained a more complex kind of learning than with others (T- and radial-mazes, etc.), and being this similar to the natural condition, because the task cannot be solved by using nonspatial strategies, such as the "fixed-response" ones; it requires also the involvement of several brain regions. Finally, the water avoids the problem of using olfactory cues, that are very difficult to manage by the observer (Rudy, Stadler-Morris and Albert, 1987).

The structures involved in this learning are the frontal cortex and hippocampus, whose roles in memory and learning are well known (for revision see: Brandeis, Brandys and Yehuda, 1989). However, it is still not well known the role of non cortical structures in learning; to make this point clear, we chose the mammillary

bodies (MB), an hypothalamic structure included in the Limbic System (Papez, 1937). They are located in the caudal surface of hypothalamus and are divided by the fornix fibers into a medial mammillary nucleus (Mm: pars medialis, Ml: pars lateralis) and two lateral mammillary nuclei (L), according to Gonzalo-Ruiz et al. (1992).

Their implication in memory and learning seems to be clear, due to the severe amnesia caused in chronic alcoholics who suffer from the Korsakoff syndrome, revealing necrosis in the mammillary region (Brierley, 1977). By using other learning procedures, like the T- or radial mazes, it has been possible to involve these MB in those functions, although the results are still contradictory (Aggleton, Hunt and Shaw, 1990).

In the present work, it is used for the first time the quantitative histochemistry for CO in animals after spatial learning with the Morris water maze, in order to know the feasible implication of the MB in such kind of tasks.

## Materials and method

### *Animals*

A total sample of 18 Wistar rats was used. They were all adult males weighing 250-320 g at the beginning of the experiment. 10 of them were used for spatial learning, experimental group (E) and the 8 remaining ones were considered as control group (C). 28 days after finishing the spatial test, 8 animals from group E and 8 from group C were anesthetized by ether inhalation and transcardially perfused with 0.1 M phosphate buffer (pH 7.6). Their brains were quickly removed and thick (4 mm) coronal sections were made including the caudal part of hypothalamus. These sections

were protected with O.C.T. compound (Miles, USA) and frozen into Freon-22 during 30 seconds. They were stored later in a -70°C refrigerator.

### *Learning*

For spatial orientation test it was used, as already mentioned, the experimental protocol developed by Morris in 1981. In our case, we have used a circular pool made of fibreglass, painted black and 1.5 m diameter, 40 cm high. The water level was set at 30 cm and its temperature at  $23 \pm 1$  °C. The water was made opaque with a nontoxic product (Lytron 641) to avoid that the transparency of the water would let the animal to see the escape platform; this was located 2 cm below the water level and 15 cm from the pool walls. Besides this, the equipment included a video camera (Sony V88E) attached to the ceiling, just over the center of the pool, and a video recorder (Panasonic NV-F70) with a TV monitor (Sony KV-M14E).

With this equipment, the 10 animals from group E were trained in an easy spatial learning task (the subjects must find an invisible platform located in a certain area from the pool), during 4 days, between 10,00 and 15,00 hours. Each day, two sessions of 4 trials were done, with an inter-session interval of 2 hours. In each trial, the animal was placed in a different start point (position A, B, C, D), which corresponded with the conceptual division of the pool into 4 quadrants. The intertrial interval was 30 s, and the time spent since the animal was placed until it reached the escape platform was recorded. If the elapsed time was 90 s, the animal was placed onto the platform, remaining there for 15 s (time spent always on the platform). At day 5, it was performed a "transfer" or recall test (Morris, 1981), by removing the

escape platform; the subject was placed in the pool for 1 min starting in the opposite side to the quadrant that had the platform. The transfer test was video recorded and later the time spent in each quadrant was calculated.

### Histochemistry

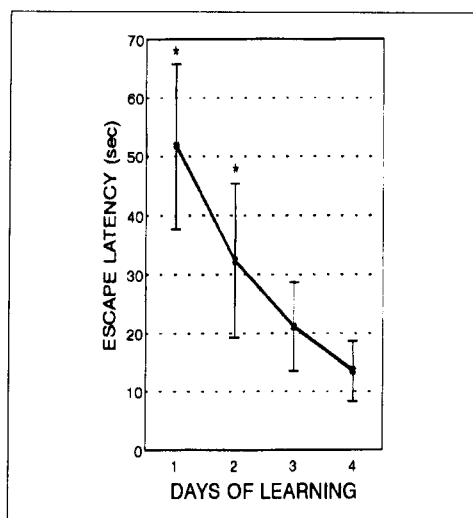
The frozen tissue was cut in series of 20  $\mu\text{m}$  sections at  $-20^{\circ}\text{C}$  in a cryostatic microtome (Frigocut-2800, Reichert-Jung) and the sections were fixed in a solution of 0.1M phosphate buffer (pH 7.3) and 1.5% glutaraldehyde (Merck) during 5 min. After rinsing them three times with the same buffer solution, they were stained for CO. For this purpose, the sections were incubated in darkness and with continuous shaking during 2 hours at  $37^{\circ}\text{C}$  in a solution that contained: 100cc 0.1M phosphate buffer (pH 7.3), 50 mg diaminobenzidine (Sigma),

15 mg cytochrome c (Merck) and 4 g sucrose. Together with the sections it was added a control of homogeneized brain paste of a previously known CO activity. After incubation period, they were rinsed again with three 5 min baths of phosphate buffer, dehydrated and coverslipped.

Quantification of the CO was done by using computerized optical densitometry (IMCO-2 system, Microm Spain). This allow us to determine real CO activity if we obtained previously a calibration curve between the optical density of sections of brain paste stained for CO and real CO activity determined spectrophotometrically. Thus, the quantification of real CO activity (expressed as specific units:  $\mu\text{M}$  cytochrome c oxidized/minute/g of tissue (w/w) at  $23^{\circ}\text{C}$ ), was done by using the method of Gonzalez-Lima and Garrosa (1994) with some modifications introduced by our team (González-Pardo et al., 1995); so, with the help of an electronic cursor (mouse) and a digitizer table it was possible to select the brain nuclei in order to quantify their CO activity, obtaining a mean grey tone (or optical density), that afterwards is translated to CO activity units.

Figure 1

Escape latencies across the four learning days (mean  $\pm$  S.D.). \*statistically significant differences vs. the remaining days.

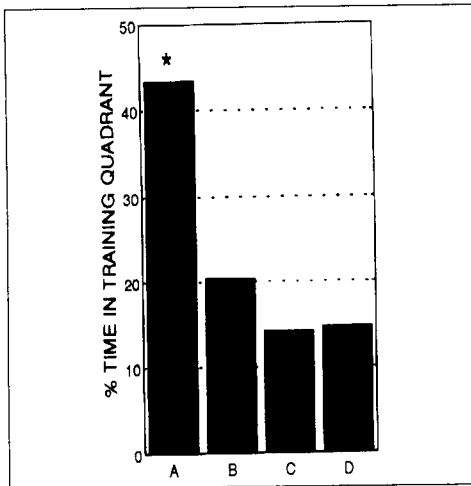


### Results

The data of the spatial learning test for group E were collected in two variables. On the one hand, the escape latencies during the 4 days of the test, and on the other, the transfer test. Taken into consideration the analyzed escape latencies, we found significant differences between the days (Fig. 1) after performing an ANOVA ( $F_{3,27} = 36.6969$ ;  $p < 0.0000001$ , with the time spent during the first two days being significantly longer than the rest of the days (Tukey test,  $p < 0.05$ ).

Figure 2

Percentage of the time spent in each quadrant (total time: 60 s). A: quadrant that contained the platform during learning; B: opposite quadrant. \*significant differences vs. the remaining quadrants.



In the transfer test, the obtained results show differences in the time spent in each quadrant ( $F_{3,27} = 12.33$ ;  $p < 0.00003$ ). After doing a post-hoc test (Tukey), the time spent in quadrant A, from which the platform was previously removed, was significantly longer when comparing with the rest ( $p \leq 0.05$ ) (Fig. 2).

The CO activity measured do show differences between the mammillary nuclei in both groups by using a two-way mixed ANOVA ( $F_{2,28} = 120.312$ ;  $p < 0.00001$ ), although there are no statistically significant differences between the C and E groups. Thus, the Mm and L nuclei show a similar CO activity, being this lower in the MI (Figs. 3 and 4).

### Discussion

The MB are involved in several neurovegetative responses, such as the control of water intake or thermic regulation.

But they are also involved with learning and spatial memory as already stated in studies with both animal models (Saravis et al., 1990; Sziklas and Petrides, 1990) and human beings (Linboe, 1989; Squire and Zola-Morgan, 1991).

The patients who suffer from Korsakoff psychosis show deficiencies in the retention of recent events and display an anterograde amnesia together with spatial and time disorientation. These patients show some alterations in the MB with an important loss of grey matter and also a decrease in glucose utilization (Squire et al., 1990).

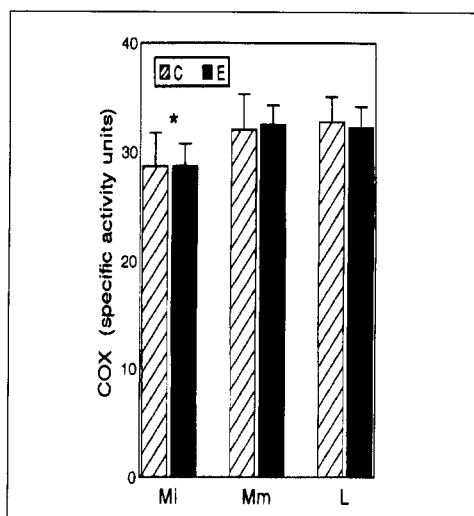
This clear involvement in spatial memory processes has allowed us to use the Morris water maze in our study. This procedure is one of the most widely used to determine cognitive deficiencies in animals under different experimental conditions; the animals use during their performance sensory-motor variables too, like those present in other learning models (Gallagher and Pelleumonte, 1988; Brandeis et al., 1989). As seen in our work, the animals gradually improve their performance, with the escape latencies decreasing and reaching a steady level from the third learning day on. Although, the transfer test allows us to determine consolidation and retention of the location of the escape platform used in the acquisition phase; in fact, the rat swims freely during longer time in the quadrant where the escape platform was hidden. In this model, the animal uses place strategies and not motor ones, based on remembering a motor pattern to reach the platform, but uses distal cues because the platform is not visible (Goodlett et al., 1988).

But also the MB are considered together with the amygdala as an antianxiety center. The presence of GABA<sub>A</sub> receptors and the effects that the administration of diazepam has on protein synthesis of the neurons from MB (García-Moreno

Figure 3

Cytochrome oxidase activity (see text for units) in the three nuclei from the mammillary bodies.

**C:** control group, **E:** trained group, **Ml:** medial mamillary n., pars medialis, **Mm:** medial mamillary n., pars lateralis and **L:** lateral mammillary n. \* significant differences in both groups of Ml vs. Mm and Ml.



et al., 1991) state their implication in such kind of responses.

The CO activity quantified in the two groups and in the different nuclei from the MB does not show the effect of this kind of spatial learning, and besides this, the functional differences between the nuclei are maintained in both groups, as regards to the energy metabolism. These differences in CO activity in the Mm, Ml and L mammillary nuclei are based on the neural circuits between these nuclei and other brain areas such as tegmental and tuberal regions, etc. Thus, taking into account the tegmental projections, the L has connections of asymmetric type (based on the classification from Gray, 1959), with round vesicles and having excitatory effects. Histochemical studies

show a great number of tegmental neurons, that are reactive to acetylcholinesterase (an enzyme that inactivates acetylcholine); this fact supports the existence of acetylcholine in their terminals, a possible excitatory neurotransmitter in the above-mentioned projections.

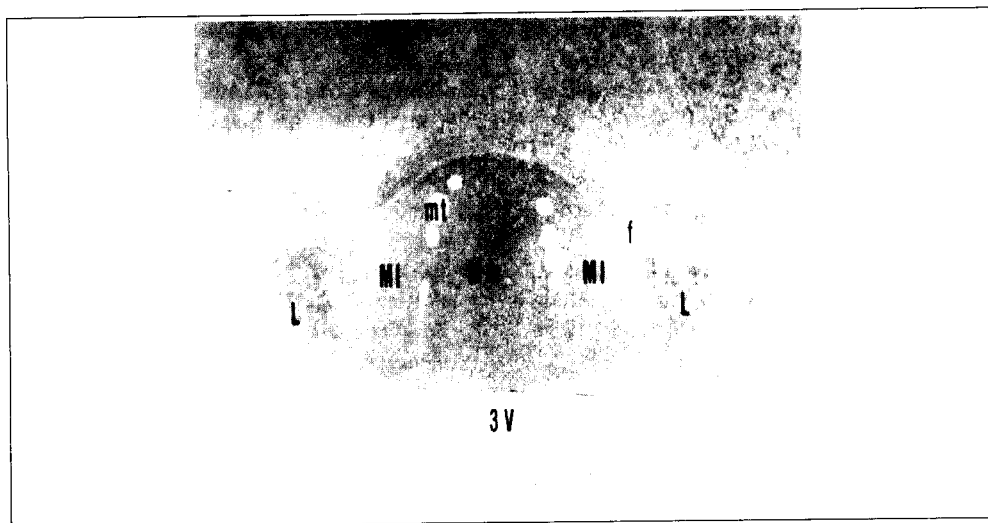
There are also dopaminergic terminals (with excitatory effects) in this MB which arise from the supramammillary nucleus towards the L, with minor projections to the medial mammillary nucleus (both Mm and Ml) (Gonzalo Ruiz et al., 1992).

However, in the case of the medial nucleus, the tegmental projections are mainly of symmetric type (inhibitory effect) with pleomorphic vesicles and containing GABA. The presence of GABA receptors, over all GABA<sub>A</sub>, are seen very often in the medial regions. This is why the energy metabolism would be different between medial and lateral regions. Nevertheless, inside this medial region we have detected differences between Mm and Ml, with a greater activity in the former. This difference could be explained on the one hand, to a lower neuronal density of the Ml than in the Mm, and on the other, the fact that the tuberomammillary nucleus projects histaminergic fibers widely to the medial regions and to a lesser extent to the lateral regions of the MB, with a greater excitatory effect over the Mm.

To summarize, our results do not show any effect of learning in the CO activity from the mammillary nuclei. This could be explained with three possibilities: first, the involvement of the MB in the Papez circuit together with hippocampal formation and thalamic regions, being the circuit itself responsible for the acquisition of learning, with no specific contribution of a single

Figure 4

Coronal section of the mammillary body stained for cytochrome oxidase. **f**: fornix. **mt**: mammillothalamic tract. **3V**: third ventricle. (see Fig. 3 for other abbreviations)



nucleus (Irle and Markowitsch, 1982); the hippocampal formation is essential for spatial learning tasks like those used here by us, because it is required from the animals to build a series of associations between visual stimuli and also spatial configurations in order to perform correctly this task. Second, this task does not by itself involve additional anxiolytic responses from the MB, because the stress level is not high. In this sense, there are reports where the relation between swimming activity and water temperature of the pool supporting the existence of an U-type of relation for both variables; so, extreme temperatures would cause a greater swimming response in the animals as a result of an increase in stress level. Third, one may consider the suitability of the chosen period of time after learning to quantify CO activity; even though several authors have found differences with certain treatments after 28 days (Nobrega et al., 1993; Hevner and

Wong-Riley, 1990), those are based on the mean half-life of the DNA that encodes for CO. However, we do not discard that this change could arise in shorter periods of time (less than 3 days), because there are other metabolic mechanisms which could be faster, regulating the CO activity by covalent modifications of the individual molecules of CO, changing its turnover rate (Hevner and Wong-Riley, 1993).

Future papers should still unravel the functional meaning of the changes in energy metabolism of CNS structures related with complex behaviors, such as those involved in learning and memory.

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