c-Fos expression in supramammillary and medial mammillary nuclei following spatial reference and working memory tasks

L.J. Santín, J.A. Aguirre, S. Rubio, A. Begega, R. Miranda, J.L. Arias

Abstract

To investigate brain substrates of spatial memory, neuronal expression of c-Fos protein was studied. Two groups of rats were trained in two spatial memory tasks in the Morris water maze, where the rats have to apply a reference memory rule or a working memory rule. In addition to the experimental groups, two control groups were used to study c-fos activation not specific to the memory processes studied. After immunohistochemical procedures, the number of c-Fos positive neuronal nuclei was quantified in the mammillary body (MB) region (medial mammillary nucleus [MMn] and supramammillary nucleus [SuM]). The results have shown that some MMn neurons expressed c-Fos nuclear immunoreactivity related to spatial working memory but not to spatial reference memory. The increased number of c-Fos immunoreactive neuronal nuclei in the SuM was related to spatial training but not to either working or reference memory demands of the tasks.

Keywords: c-Fos immunoreactivity; Spatial memory; Mammillary bodies; Supramammillary nucleus

1. Introduction

A great deal of research has focused on studying the neurobiologic substrate of spatial memory [1–8]. Following Olton’s group research [5,6,9] about reference memory and working memory in the radial arm maze, several authors have focused their studies in the brain substrate underlying these two kinds of memory. Reference memory (RM) is trial independent and is used to learn the general rules required for the performance of a task. The information available for solving reference memory tasks is constant throughout the trials [10] and is reinforced by repeated training [11]. Working memory (WM) is a temporary memory that is trial dependent (it is only relevant for one trial) [10]. These two kinds of memory can be assessed in the rodents using spatial tasks [7,12]. Moreover, the specific role of the different brain regions in spatial RM and WM is still unclear.

Several studies have shown the involvement of the mammillary bodies (MBs) in memory processes, both in humans [13] and in rodents [14]. In this way, memory disorders have been observed following MB lesions [3,7,14–20]. The effect of MB lesions on spatial memory could, at least in part, depend on the location of the lesion. Lesions restricted to medial zones of the MB might not affect spatial memory processes while more extensive lesions would produce spatial memory impairments. Many studies have reported spatial memory impairments after lesions of the MB and neighboring fibres and zones (mammillary region) [14,18–20]. In these works, one of the nuclei damaged was the supramammillary nucleus (SuM). This hypothalamic nucleus receives projections from the medial mammillary nucleus (MMn) and the lateral mammillary nucleus (LMn) [21]. The connection between these nuclei suggests the combined involvement of both the SuM and the MB in spatial memory. However, in previous studies memory impairments were observed with lesions restricted to medial portions of the MB [7].

The aim of this study was to assess the involvement of the MMn and the SuM in both spatial reference memory and
spatial working memory. Specifically, we studied the effect of spatial training, with reference and working memory demands to solve the tasks, on c-fos expression by immunohistochemical detection of the c-Fos protein.

The c-fos gene activation is one of the earliest transcriptional events to follow neuronal activation. In the last few years, several studies have suggested that c-Fos immunoreactivity (c-fos IR) can be used as a marker of neuronal activity that provides information on brain regions underlying learning and memory, possibly associated with neuronal plasticity required for memory processes [22,23]. However, some events frequently associated with training, such as stress, motor activity and novelty can also induce changes in c-fos gene expression [24–27]. For this reason, two control groups were included in the study (spatial reference memory control and spatial working memory control) that permitted c-fos activation not specific to the spatial working memory tasks associated with the training process, to be ruled out.

2. Method

2.1. Animals

Twenty-eight male Wistar rats, weighing 302 ± 33 g, from the central vivarium of the University of Oviedo were used. All rats were given free access to food and water. Rats were housed individually in a temperature-controlled colony (20 ± 2 °C) on a constant light–dark cycle (lights on 0800–2000). Animals were divided into four groups: RM (n = 7), RM control (n = 7), WM (n = 7) and WM control (n = 7). The care and use of animals were in accordance with the Spanish regulation for the use of animals in research.

2.2. Apparatus

The apparatus consisted of a circular pool with the following dimensions: diameter: 150 cm, walls: 43 cm high. The pool was filled with water (21 ± 2 °C) that was made opaque with nontoxic white paint. The goal platform (11 cm diameter) could be placed anywhere in the pool at a distance of 30 cm from the pool edge. The platform was submerged to a depth of 2 cm beneath the surface of the water. The pool was placed in an experimental room furnished with several extramaze cues. The pool remained immobile in the room throughout the experimental period. An automatic video system (Ethovision, Noldus) was used to record the animals’ movements in the pool.

2.3. Behavioral tasks

The day before starting the behavioral experiments all the animals were submitted to two 60-s sessions of free exploration.

2.3.1. Spatial reference memory task

The pool was divided into four quadrants (A, B, C and D). The rats were able to escape from the water using the submerged platform. The platform was placed in the center of quadrant B where it remained throughout the experiment. The rats were introduced into the pool from one of the four release positions (quadrant A, B, C or D). Each animal was submitted to six trials. The trial finished when the animal found the platform. When a rat did not find the platform within 60 s, the experimenter placed the animal on the platform where it remained for 15 s. After this period the rat was returned to its cage for 30 s after which it was introduced into the pool again. The control group was submitted to a period of free exploration, during the first day, in the circular pool without the escape platform.

2.3.2. Spatial working memory task

The animals were submitted to two trials per day, one acquisition and one retention trial. In the acquisition trial the animal had to find a hidden platform to escape from the water. If the animal did not find the platform in 60 s the experimenter placed the animal on the platform where it remained for 15 s before being placed in its cage for 30 s. After this interval the animal was again introduced into the circular pool for the retention trial. The same exit and escape quadrants were used for the acquisition and the retention trial on the same day but this varied pseudorandomly over 8 days. In this task, the control group was submitted to a daily 30-s trial in the circular pool in the absence of the escape platform.

2.4. Immunohistochemical analysis

Ninety minutes after the end of the behavioral task, the animals were deeply anaesthetized with equithesin (3 ml/kg) and perfused via the ascending aorta with cold physiological saline solution followed by a cold formalin buffer (4% paraformaldehyde in 0.16 M phosphate buffer, pH 6.9). The perfusion was continued for 5 min and the brains were postfixed in the same fixative for 2 h. They were then transferred successively into phosphate-buffered saline (PBS, pH 7.2) containing 10%, 20% and 30% sucrose until they sank for cryoprotection. Coronal sections (16-μm) of the brain were cut at –20 °C in a cryostat. The slices were mounted on gelatinized slides. c-Fos antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect c-Fos protein. The avidin–biotin complex (ABC, Vector Laboratories) immunoperoxidase method was used to visualize c-Fos IR. Briefly, the slides containing the sections were washed in PBS followed by a wash in a solution of 0.1 M PBS containing 0.3% Triton X-100 and 1% normal goat serum. The sections were then incubated at 4 °C in c-Fos primary antiserum (diluted 1:10,000 in the same solution) overnight. The antiserum was a rabbit polyclonal antibody directed against the amino acids 3–16 of the N-terminal region of the human c-Fos p62. It is not cross-reactive with...
c-Fos, Fos B, Fra-1 or Fra-2. Sections were washed in PBS and then incubated in biotinylated donkey anti-rabbit secondary antibody (Pierce, Illinois; diluted 1:200 in incubating solution) for 2 h. They were further washed in PBS and incubated in an avidin-biotinylated horseradish peroxidase complex (Vector Laboratories standard kit: 1:100 in incubating solution). After two washes in PBS, the reaction was visualized treating the sections for about 5 min in an immunopure metal-enhanced diaminobenzidine tetrahydrochloride (0.025%) substrate solution (Pierce). The reaction was terminated by washing the sections in cold PBS. Finally, the slides were dehydrated through a graded series of alcohols and coverslipped for microscopic observation.

2.5. Quantification of c-Fos IR

The number of c-Fos IR neuronal nuclei was quantified in the MMn and SuM (Fig. 1). Brain regions were located using the stereotaxic atlas of Paxinos and Watson [28]. Two sections of the MMn and SuM were sampled. c-Fos IR nuclei were counted with a computerized system (Leica QWIN) and the results were expressed as number per cubic micrometer (Nv). The quantification was done by systematically sampling each of the regions selected in each section using frames superimposed over the preparations. To obtain a comparable metric unit the following formula was used: \( N_v = N/V_{ref} \) or \( N_v = \frac{Q}{\sum a_{fra}} h \), where \( Q \) is the total number of c-Fos IR nuclei counted in all the frames used; \( a_{fra} \) is the area of the frames used and \( h \) is the thickness of the section [29]. The thickness of the sections was determined using a microcator (Heidenhain, Germany).

2.6. Statistics

Trend analysis was performed to analyze the reference memory task. Student t test for paired samples was used to compare the acquisition trial with the retention trial in the working memory task. The results obtained by c-Fos IR sections.
quantification were analyzed with the Student $t$ test for independent samples (comparisons between trained and control groups) and paired samples (comparisons between reference and working memory tasks in each of the groups).

3. Results

3.1. Behavioral results

3.1.1. Spatial reference memory task

A reduction in the escape latencies $[F(1,6) = 7.2, P \leq .05]$ and distances swam by the animals in the Morris water maze $[F(1,6) = 6.8, P \leq .05]$ was observed along the training (Fig. 2). These results show that the rats have learned to locate the hidden platform and were able to learn the reference memory rule.

3.1.2. Spatial working memory task

The results show that the animals can successfully learn to locate a place (hidden platform) in a task with a daily acquisition and retention trial in the pool. The rats reduced their escape latencies ($t = 3.039, P \leq .05$) and distances swam ($t = 3.25; P \leq .05$) in the retention trial compared to the acquisition trial (Fig. 3).

3.2. Quantification results

The statistical results show that the trained animals in the working memory task had an increase in the number of c-Fos IR neuronal nuclei compared with the control group ($t = 3.623; P \leq .05$) (Fig. 4). Moreover, a c-Fos IR increase in the SuM in these same rats was not observed ($t = 1.087; P \geq .05$) (Fig. 4). The rats trained in the reference memory task did not show any changes, neither in the MMn ($t = 1.568; P \geq .05$) nor in the SuM ($t = 0.509; P \geq .05$) (Fig. 5).

The supramammillary c-Fos IR, was greater in the rats trained in the working memory task ($t = 2.5; P \leq .05$) and their control animals ($t = 3.58; P \leq .05$) than in the rats trained in the reference memory task and their control rats (Figs. 4 and 5). In contrast, no difference was observed...
between the rats included in neither training groups \( t = 0.29; P \geq 0.05 \) nor control groups \( t = 0.48; P \geq 0.05 \) in the amount of the c-Fos IR neuronal nuclei, in the MMn (Figs. 4 and 5).

4. Discussion

Some studies have demonstrated an increase in \( c\text{-}fos \) expression in several brain regions after behavioral tasks. For example, a rise in \( c\text{-}fos \) mRNA was found in an aversive conditioning task in rodents [30,31] and during the first training session in the learning of an active avoidance task [31]. Zhu et al. [32] determined \( c\text{-}fos \) expression in different brain regions associated with recognition memory. They observed a rise in the amount of c-Fos IR in the anterior cingulated cortex associated with the recognition of new objects.

Nevertheless, taken together these data do not demonstrate that the changes observed in \( c\text{-}fos \) gene expression are directly related to learning and memory. Although immunohistochemical techniques for determination of the c-Fos protein cannot explain the role played by the \( c\text{-}fos \) gene in learning, they could reveal the relationship between some neurons and certain learning and memory tasks [22,23]. In this way, the results obtained in our study provide information about neuronal activity in two hypothalamic regions associated with spatial working and reference memory. To date, the relation between the mamillary region and the spatial memory has been studied using other experimental approaches. In this sense, lesion studies in animals indicate a clear participation of the MB in spatial memory tasks [3,7,14–20], and electrophysiological studies have shown that both the MMn and SuM are involved in theta-frequency activity around the limbic circuitry [33]. These works strongly suggest that these nuclei would play an important role in some spatial memory processes.

Our results also suggest the involvement of both regions in spatial memory but in different ways. The increase in the number of c-Fos positive neuronal nuclei in the MMn become evident in the animals trained in the spatial WM task but not in the rats trained in the spatial RM task (Figs. 4 and 5). In spite of a rise in the number of c-Fos IR cells in the spatial RM group compared with its control, these differences are not statistically significant. The variability between subjects in the spatial RM group could possibly mask the existence of clear differences between the two groups. Nevertheless, on the basis of the statistically differences found, our results support those of previous experiments, which suggests a greater participation of the MB in spatial WM compared to spatial RM [7,14].

In contrast, the rats trained in the WM task (and their control animals) showed a greater number of c-Fos IR in the supramammillary neurons than the rats submitted to the RM task (Figs. 4 and 5). Possibly, the differences recorded in \( c\text{-}fos \) activation between the tasks reflect the relationship between the SuM and spatial working memory processes or perhaps only the relationship between this nucleus and the processing of spatial information. This later suggestion is supported by the increase of c-Fos IR in those animals that explored the environment for several days. This c-Fos IR increase was observed in both control and experimental groups (Figs. 4 and 5). The obtained results seem to support those obtained by electrophysiological records, which indicate that the SuM primarily determines the frequency of hippocampal theta rhythm [33], suggesting that it plays a role in processing spatial information.

The results described in this work reflect the participation of both the MMn and SuM in spatial memory in different ways. These results are generated by comparing the numerical densities of c-Fos IR neurons in the experimental and control groups. Inclusion of the control groups in this study reveals that some of the c-Fos IR neurons are possibly associated with stimuli and responses present
during training (motor activity, sensorial stimulation, etc.) not directly related to memory demands. Nevertheless, although these control groups were used, possibly the total increase in c-Fos IR neurons in the experimental groups is not only due to memory demands or spatial processing during training. Therefore, in the reference memory task the experimental animals are trained in six consecutive trials and remain for several seconds on the platform and in the homebox. During the working memory task, the experimental group is trained in two daily trials and also remain for several seconds on the platform and in the homebox. However, the two control groups are not trained in the circular pool with these periods of intertrial intervals and reinforcement. These differences in the training of the groups in the two behavioral tasks could possibly be reflected in some c-Fos IR neurons not being involved in the memory processes during the tasks.

In conclusion, our results show that the regions studied by immunohistochemical analysis of the c-Fos protein play a different role in spatial RM and WM processes in the MWM. Therefore, MMn neuron activation suggests a relationship with spatial WM but not with spatial RM. Expression of c-Fos protein in the SuM was greater in spatial WM groups than in the spatial RM groups. This result suggests the participation of SuM neurons in general spatial processing. In addition, the role of the MMn in the spatial working memory could require the spatial information processed by the SuM, to be able to correctly develop its mnesic function in spatial tasks.

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References

