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Brain c-Fos immunocytochemistry and cytochrome oxidase histochemistry after a fear conditioning task

Nélida M. Conejo, Héctor González Pardo, Matías López, Raúl Cantora and Jorge L. Arias
Universidad de Oviedo

The involvement of the basolateral and the medial amygdala in fear conditioning was evaluated using different markers of neuronal activation. The method described here is a combination of cytochrome oxidase (CO) histochemistry and c-Fos immunocytochemistry on fresh frozen brain sections. Freezing behavior was used as an index of auditory and contextual fear conditioning. As expected, freezing scores were significantly higher in rats exposed to tone-shock pairings in a distinctive environment (conditioned; COND), as compared to rats that did not receive any shocks (UNCD). CO labeling was increased in the basolateral and medial amygdala of the COND group. Conversely, c-Fos expression in the basolateral and medial amygdala was lower in the COND group as compared to the UNCD group. Furthermore, c-Fos expression was particularly high in the medial amygdala of the UNCD group. The data provided by both techniques indicate that these amygdalar nuclei could play different roles on auditory and contextual fear conditioning.

Inmunocitoquímica c-Fos e histoquímica citocromo oxidasa cerebral tras una tarea de condicionamiento al miedo. Se analiza la implicación de la amígdala basolateral y medial en el condicionamiento al miedo empleando diferentes marcadores de activación neuronal. El método que describimos es una combinación de la histoquímica citocromo oxidasa (CO) y la inmunocitoquímica c-Fos en secciones de tejido cerebral. La conducta de inmovilización se usó como índice del condicionamiento auditivo y contextual. Las puntuaciones obtenidas fueron significativamente mayores en ratas expuestas a emparejamientos tono-descarga en un ambiente distintivo (grupo COND), comparadas a las obtenidas en ratas que nunca recibieron descargas eléctricas (UNCD). La actividad CO se incrementó en la amígdala medial y basolateral del grupo COND. Sin embargo, la expresión c-Fos en estos núcleos fue inferior en el grupo COND comparado al grupo UNCD. Además, los niveles de la proteína c-Fos fueron más altos en la amígdala medial del grupo UNCD. Nuestros resultados muestran que los núcleos amigdalinos podrían jugar diferentes papeles en el condicionamiento al miedo auditivo y contextual.

Fear conditioning is a form of learning that has been extensively studied, because it is acquired quickly and its neural substrate is well characterized. The brain region most consistently associated with fear conditioning in mammals is the amygdala (Maren & Quirk, 2004; Fanselow & LeDoux, 1999). The basolateral nucleus of the amygdala (BL) has been the focus of many studies, because it is considered as the site where the association between an aversive unconditional stimulus (US) and a conditioned stimulus (CS) occurs during fear conditioning (Maren, 2001; Fanselow & LeDoux, 1999). In particular, the BL amygdala is involved in auditory and contextual fear conditioning (Scicli, Petrovich, Swanson, & Thompson, 2004; Goosens & Maren, 2001). On the other hand, the medial nucleus of the amygdala (Me) has been related with unconditioned fear, but it may also participate in contextual fear conditioning (Li, Maglinao, & Takahashi, 2004). However, some authors argue that the amygdala does not play a significant role in the acquisition and storing of fear memories (Cahill, 1999; Cahill & McGaugh, 1998). This discrepancy may be related not only with the different behavioral paradigms used to induce fear conditioning, but also with the diversity of techniques applied to study brain function.

In order to clarify the contribution of these particular amygdalar nuclei to contextual and auditory fear conditioning, we have developed a functional brain mapping method to simultaneously evaluate stimulus-evoked neuronal activation using c-Fos immunocytochemistry, and changes in brain metabolic capacity using cytochrome oxidase histochemistry. Cytochrome oxidase (CO; ferrocytochrome c oxygen oxidoreductase; EC 1.9.3.1) is a mitochondrial enzyme that catalyzes the transfer of electrons to oxygen generating ATP via the coupled process of oxidative phosphorylation (González-Lima 1992). CO activity is regulated by and closely correlated with neuronal functional activity (Wong-Riley, 1989). Sustained changes in synaptic activity are associated with altered ion pump activity, energy demand, and, ultimately, CO activity. Therefore, CO activity is an index of the oxidative metabolic capacity of the

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Correspondencia: Nélida M. Conejo
Facultad de Psicología
Universidad de Oviedo
33003 Oviedo (Spain)
E-mail: conejonelida@uniovi.es

nervous tissue, which can change over a period of hours to days (González-Lima, 1992). CO histochemistry has been previously used to map funcional changes in brain metabolism associated to learning and memory processes in different animal species (Conejo, González-Pardo, Vallejo, & Arias, 2004; Deglise, Dacher, Dion, Gauthier, & Armengaud, 2003; Agin, Chicher, & Chichery, 2001; López et al., 1995; Poremba, Jones, & González-Lima, 1998).

On the other hand, *c-fos* is an immediate-early gene that be induced rapidly and transiently in neurons after applying a variety of stimuli (Herrera & Robertson, 1996). Expression of the immediate early gene *c-fos* and its protein product c-Fos has been extensively used to map stimulus-evoked functional activity in the brain (Nikolaev, Kaczmarek, Zhu, Winblad, & Mohammed, 2002). Thus, the immunocytochemical detection of the c-Fos protein in cell nuclei has been used as an indicator of neuronal activation. In this regard, c-Fos immunocytochemistry has been previously applied to study the effects of fear conditioning on neuronal activation of amygdalar nuclei (Holahan & White, 2004; Scicli et al., 2004; Conejo et al., 2004; Milanovic et al., 1998). CO histochemistry was also used to evaluate the metabolic capacity of several brain regions after fear conditioning (Conejo, López, Cantora, González-Pardo, López, Begega, Vallejo, & Arias, 2005a, 2005b; Poremba et al., 1998). However, both techniques provide different information on the brain function associated with learning and memory processes. Changes in neuronal metabolic capacity are associated with the entire training period, whereas c-Fos protein expression reflects only short-term neuronal activation lasting a few hours. Therefore, the application of different markers of neuronal function represents an interesting approach to understand the neuronal processes underlying behavior. In the present study, we used a new method to simultaneously evaluate CO activity and c-Fos expression in the BL and Me amygdalar nuclei following fear conditioning.

Materials and methods

Animals

Twelve male Wistar rats 453-538 g were used in this experiment. Each group had six rats. All animals were housed in pairs in clear plastic cages, and kept in a temperature-controlled room (21 ºC) with a 12-h light/dark cycle (lights on between 08:00 and 20:00 h). After being housed, the rats were handled daily for 7 days to habituate them the researchers. The experiment was conducted during the light phase of the cycle. Food and water were available *ad libitum* in the home cage. All experimental procedures were in accordance with guidelines for the care and use of laboratory animals of the European Council Directive (86/609/EEC) and Spanish regulation RD-1201/2005 regarding the care and use of animals.

Fear conditioning

This study relied on previously reported methods of Pavlovian fear conditioning using freezing behaviour as the measure of fear (Célérier, Ognard, Decorte, & Beracochea, 2000; Lee, Dickinson, & Everitt, 2004). In a conditioning chamber, presentation of a tone (CS) that predicts the occurrence of an aversive footshock (US) causes conditioned freezing during later retention test in a novel context. Freezing was defined strictly as the absence of all movement of the animal, except that related to respiration, while animal was in a stereotyped crouching posture.

Apparatus

Fear conditioning and behavioral observation occurred in one experimental chamber (28 cm width × 24 cm length × 33 cm height) housed in a controlled acoustic box. A loudspeaker connected to a tone generator was inserted in the front wall of the chamber for the delivery of the auditory cue (CS, 1-kHz tone, 70 dB). A background noise (60 dB) made by the ventilation of the sound-attenuating box and the illumination provided by a soft light (approximately, 45 lux) centered in the ceiling of the attenuating cubicle were continuously supplied in the conditioning chamber. Two distinct contexts were prepared in this apparatus -designated «COND» and «NOV». The COND context, for fear conditioning, was characterized by the presence of a metal grid-floor, and connected to an electric shock generator for the delivery of footshock (US, 0.5 mA, 0.5-s duration). The NOV context, used for novel context and auditory tests, had a wooden floor with holes instead of a metal grid-floor. In addition, the chamber was surrounded by white-black striped plates to emphasize the visual differences between conditioning and novel contexts. A previous pilot study showed a lack of preference for one floor over the other.

The behavior of the rats was recorded by videotape recorder with an infrared camera. Two observers who were blind to groups assignment measured the freezing behavior in the test session for auditory fear conditioning or contextual fear conditioning (Pearson's correlation coefficient, r= 0.996; p= 0.01).

Procedure

The experiment was performed on two consecutive days. During the fear conditioning session (Day 1), the rats assigned to the conditioned group were placed into the conditioning apparatus (COND context) for 14 min. After a 4-min period of habituation to the chamber, the animals were given four trials of tone-footshock pairings with an inter-trial interval of 90 s. In each trial, a 20-s tone CS was paired with a 0.5-s 0.5 mA footshock. The shock was presented during the last 0.5 s of the tone CS. Animals remained 4 min after the termination of the last conditioning trial in the conditioning chamber, and they were then returned to their home cage. The procedure was the same for rats in the unconditioned group except that they did not receive any footshock.

Twenty-four hours later (Day 2), contextual fear conditioning was assessed by replacing rats in both groups in the COND context, the initial conditioning chamber, for 4 min. Each rat was scored to be freezing or not once every 10 s. There were a total of 24 observations during the 4-min period. The percentage of observations spent freezing was calculated for each rat (no. of observations freezing/total observations × 100). Conditioned fear to the tone CS was assessed the same day, 1.5 h later, by placing subjects in the NOV context, the neutral chamber, for 10 min and giving the auditory CS during the last 6 min. Freezing was rated again at 10-s intervals during the 4 min preceding the occurrence of the CS and during the first 4 min of the CS. Percentage of the observations with freezing was used as index of the fear conditioning.

Tissue preparation

Each animal was decapitated exactly 90 min after completion of the behavioral tests. Its brain was removed and immediately frozen by slowly immersion in isopentane (2-methylbutane, Sigma, Spain) at -40 ºC, and stored at the same temperature until sectioned. Frozen brains were introduced in a cryostat microtome (*Microm* HM-505E, Heidelberg, Germany) and coronal sections through the right and left amygdala regions were cut at 30 µm at -20 ºC. A series of sections was picked up with chrome-alum gelatinized slides and allowed to air-dry at room temperature on the outer surface of the cryostat. These sections were used for c-Fos immunohistochemistry. An adjacent set of sections for C.O. histochemistry was thaw-mounted on clean slides and maintained at -20 ºC in the cryostat chamber during sectioning. To obtain the two series of adjacent sections, when a section was mounted on a gelatinized slide, the following section was mounted on a clean slide. On average, three sections were collected on each slide. All sections were stored at -40 ºC until processed.

c-Fos immunohistochemistry

Slides for immunohistochemistry were brought to room temperature and fixed for 30 min in 4% paraformaldehyde dissolved in a phosphate buffer solution (0.1M, pH 7.4). The slides were rinsed twice in phosphate-buffered saline (PBS; 0.01M, pH 7.4) for 30 min. Endogenous peroxidase activity was blocked by placing the slides in a solution containing 3% hydrogen peroxide in PBS, followed by several washes in PBS. Unspecific immunoreactivity was prevented by incubation for 30 min in a solution of 3% bovine serum albumin (Sigma, Spain) dissolved in PBS. Slides were then incubated in a rabbit anti-c-Fos antibody (1:10000; Santa Cruz, USA) diluted in PBS-T for 24 h at 4 ºC in a humid chamber. The next day, slides were washed several times with PBS-T, and incubated in a goat anti-rabbit biotinylated IgG (1:200; Pierce, USA) for 2 h at room temperature. Thereafter, slides were washed several times with PBS and then reacted with avidin-biotin peroxidase complex (Vectastain ABC Ultrasensitive Elite Kit, Pierce) for 1 h, and revealed with a commercial nickel-cobalt-intensified diaminobenzidine kit (Pierce). The slides were dehydrated through a series of graded alcohol baths, cleared with xylene and coverslipped with Entellan (Merck, USA).

The total number of c-Fos positive nuclei was quantified in at least three alternate sections containing the BL and Me nuclei of the amygdala according to the Paxinos and Watson atlas (1986). The profiles of the amygdala nuclei were first outlined in the slides using a permanent marker and then copied on a paper using a microscope (Nikon SE, Japan) equipped with a drawing tube. Counting of c-Fos positive nuclei was visually performed by an investigator who was unaware of the experimental groups, using a microscope (Olympus BH-2, Japan). Finally, the mean density of c-Fos positive nuclei was calculated dividing the total number of c-Fos positive nuclei by the estimated area quantified. Significant differences in the density of c-Fos positive nuclei between the experimental groups for each brain region, was calculated using Student's t tests.

Cytochrome oxidase histochemistry

The quantitative histochemical method described by Gonzalez-Lima and Cada (1994) was essentially followed with minor modifications. Alternate sections were preincubated for 10 min in a solution containing 0.1 M Tris buffer (pH 7.4) containing 0.0275% cobalt chloride, 0.005% DMSO and 10% sucrose. After rinsing the sections, they were incubated during one hour at 37 ºC in a staining solution containing: 25 g sucrose, 10 mg catalase (Sigma, USA), 37.5 mg cytochrome c (Sigma,USA), 250 mg DAB, 1.25 ml DMSO in 500 ml of 0.1 M phosphate buffer (pH 7.6). The tissue was then fixed in 10% buffered formalin with 10% sucrose for 30 min. The slides were then dehydrated with alcohols and coverslipped. A set of sections of rat brain homogenates cut at different thicknesses (15, 30, 60 and 80 µm) were included in each staining bath as staining standards. They were used to control for variability among different staining baths. Relative optical density (ROD) readings were performed using an image analysis system (Leica Q-550, Wetzlar, Germany). The mean ROD of the BL and Me nuclei of the amygdala was measured. ROD readings were also taken from each brain homogenate standard. A correlation curve was calculated between section thickness and the mean optical density measured in each standard. This curve was used to standarize the ROD readings of the brain regions from different staining baths. Optical density measurements were performed by investigators blind to the treatment assignment.

Statistical analysis

The percentage of freezing behavior recorded in the conditioning context was analyzed using two-way repeated measures ANOVA with group (COND versus UNCD) as independent variable and Day (Day 1 versus Day 2) as the repeated measure. For comparisons of conditioned freezing to the tone in the novel context, a two-way repeated measure ANOVA with factor group (COND versus UNCD) and tone (tone off versus tone on) was performed.

Each nucleus of the amygdala was considered separately for the statistical analysis of immunocytochemical and histochemical measures. Student's *t*-tests were used to evaluate significant group differences in c-Fos positive nuclei density and the ROD. A $p<0.05$ was considered statistically significant.

Results

Freezing behavior

On the session of aversive conditioning with the tone (Day 1), there were no significant differences between the groups in exploratory behavior before the CS-US pairings. However, exploratory activity was replaced by conditioned freezing after fear conditioning. Figure 1 displays the data (expressed as freezing percentage) during testing in both the original conditioning context and the novel context (with the tone on or off), respectively. As shown in Fig. 1A, the subjects in Group COND exhibited significantly more conditioned freezing after aversive conditioning in the COND context than did animals in Group UNCD. The repeated measures ANOVA revealed significant effects of Group (F (1,10)= 34.950; $p<0.001$) and Day factors [F (1,10)= 21.556; p= 0.001], together with a Group × Day interaction [F (1,10)= 21.556; p= 0.001]. It can be concluded from this test that context in which the tone-shock pairings occurred acquired aversive properties and elicit conditioned freezing.

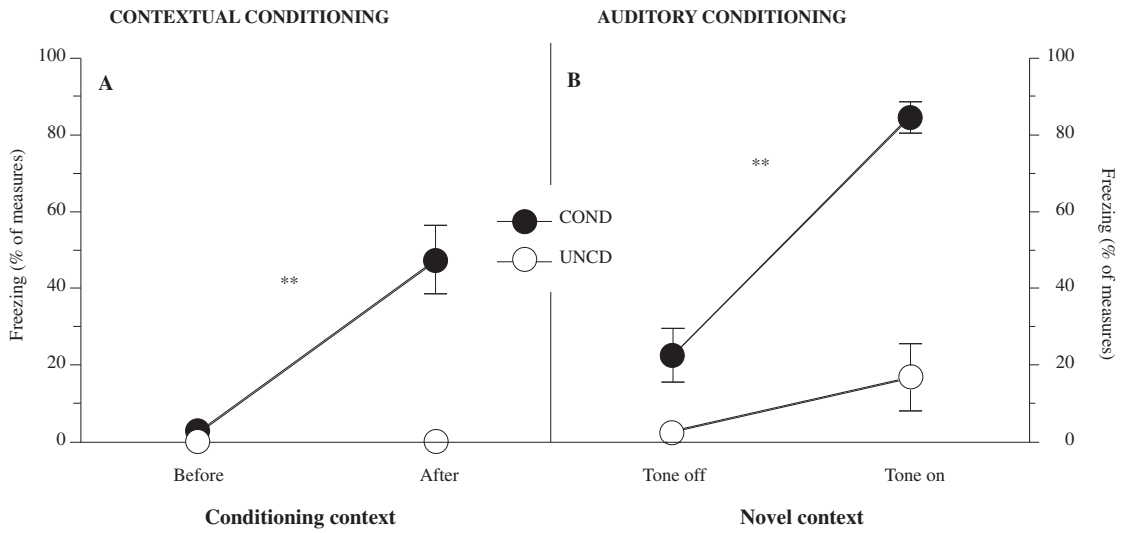

Figure 1. *Conditioned freezing (values expressed as a percentage of observations at 10s intervals) to the COND context before and after tone-shock pairings (panel A), and to the NOV context with the tone off and the tone on (panel B). **Significant differences (p<0.01) between experimental groups*

Fig. 1B displays the freezing data for novel context and tone tests. As expected, the rats in the Group COND showed increased conditioned freezing to the tone CS as compared to animals in Group UNCD. The ANOVA using repeated measures (Tone On and Tone Off) showed a significant main effect of Group [F (1,10)= 75.182; p<0.001], and Tone [F (1,10)= 36.132; p<0.001], as well as a significant interaction between these two factors, [F (1,10)= 14.556; p= 0.003]. Further comparisons (t-test) indicated that there was a difference between groups for freezing to both the novel context with the tone absent [t (10)= 2.690; p= 0.023], and to the novel context with the tone present [t (10)= 7.043; p<0.001].

CO histochemistry and c-Fos immunocytochemistry

Statistically significant differences were observed between the experimental groups in both the density of c-Fos positive cells and the levels of CO staining (see Figure 2). Specifically, a higher density of c-Fos positive cells was quantified in the BL (t_8= 2.4; p<0.05) and the Me (t_{10}= 4.1; p<0.01) amygdala in the UNCD group. Conversely, the analysis of the CO data showed significantly higher CO staining intensity in the BL (t_{10}= 2.3; p<0.05) and the Me (t_8= 2.4; p<0.05) amygdala of the COND group as compared to the UNCD group.

Discussion

The behavioral results of this study indicate that rats in the conditioned group exhibited freezing responses to both the auditory stimulus and the conditioning context, and to a lesser extent fear responses to the novel context. In addition, the neuronal activity data suggest that the BL and Me nuclei of the amygdala are involved in contextual and auditory fear conditioning in different ways. First, CO activity was higher in the BL and Me of animals previously exposed to tone-shock pairings as compared to the animals exposed only to the tone. Due to the cumulative nature of CO histochemistry, it is difficult to attribute the changes in CO labeling of the COND group to contextual fear conditioning, auditory fear conditioning, or both. It has previously reported that auditory fear conditioning increases CO activity in brain regions associated with the CS processing (Poremba et al., 1998). Moreover, we have also reported the involvement of particular limbic regions in fear conditioning using CO histochemistry (Conejo et al., 2005b). Conversely, a study using a related metabolic mapping technique, 2-deoxyglucose (2-DG) utilization, reported global increases in brain 2-DG uptake after contextual fear conditioning in rats (Bryan & Lehman, 1988). Consequently, the higher CO activity of the BL and Me in the COND group as compared with the UNCD group would support a general involvement of these amygdalar nuclei in fear conditioning.

Second, c-Fos protein expression was detected in the BL and the Me of both groups. Furthermore, the UNCD group showed a higher density of c-Fos positive nuclei than the COND group in the BL and Me. The BL has been consistently involved in auditory fear conditioning (Maren, 2001; Vazdarjanova & McGaugh, 1999) and it has also been related with contextual fear conditioning (Huff, Frank, Wright-Hardesty, Sprunger, atus-Amat, Higgins, & Rudy, 2006; Malin & McGaugh, 2006; Huff & Rudy, 2004). In particular, other authors have reported an increased c-Fos expression in the BL associated with contextual conditioning (Baker & Kim, 2004; Scicli et al., 2004; Holahan & Whit, 2004) and auditory fear conditioning (Hall, Thomas, & Everitt, 2001; Sotty, Sandner, & Gosselin, 1996).

On the other hand, the UNCD group showed c-Fos activation in the BL amygdala. Exposure to a no conditioned tone in the absence of any learning or to a novel context also elicits c-Fos expression in the BL (Holahan & White, 2004; Stork, Stork, Pape, & Obata, 2001; Beckett, Duxon, Aspley, & Marsden, 1995). Therefore, our results would support a general role of the BL in the representation of unconditioned aversive events, rather than the CS-US association. It should be considered that pattern of c-Fos expression found is a result of the neuronal activation taking place during the second day. Therefore, the lower c-Fos expression detected in the amygdala nuclei of the COND group as compared with the UNCD group, may be related with to a partial extinction

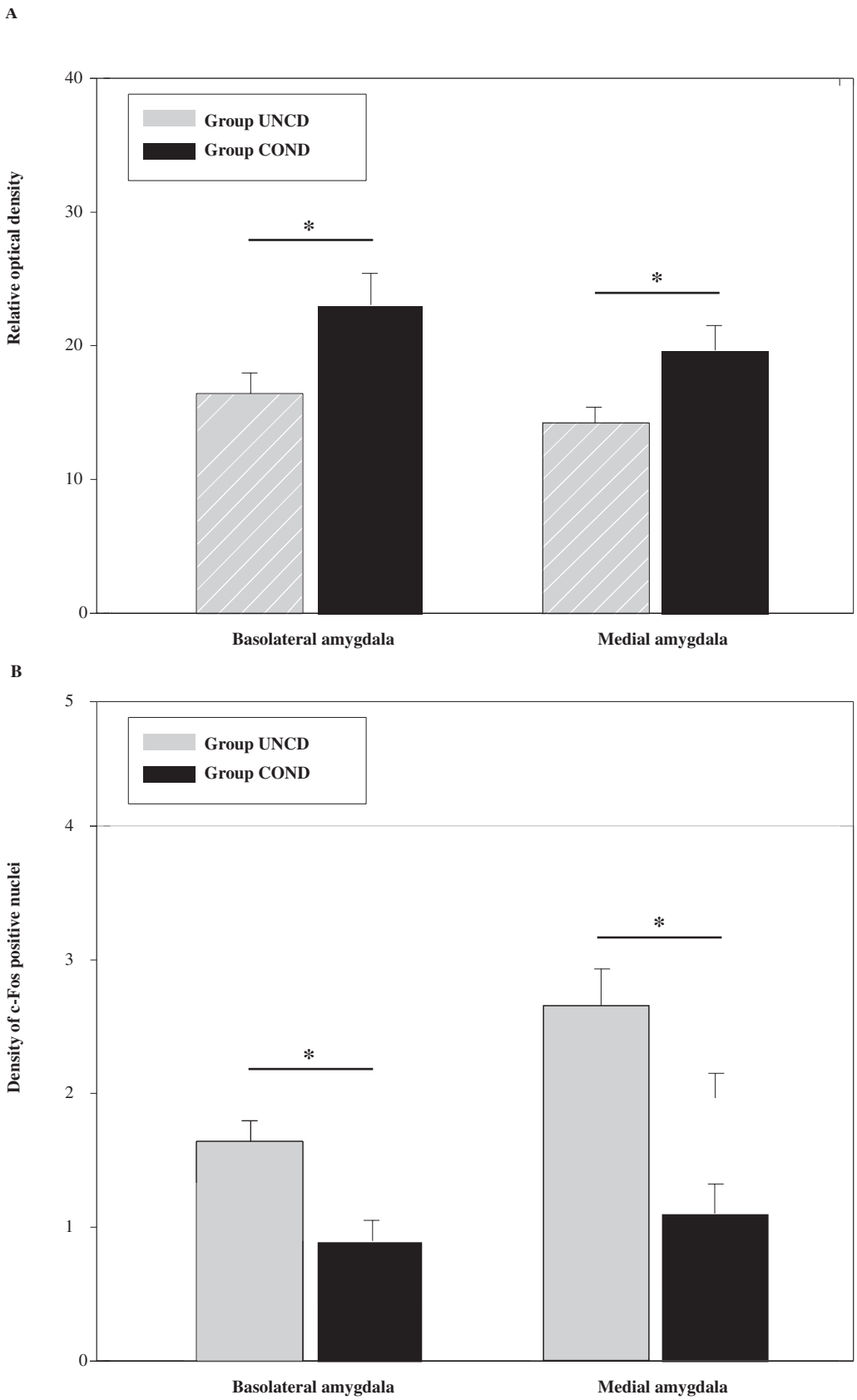

Figure 2. *Mean CO staining intensity (A) and mean density of c-Fos positive nuclei (B) measured in the basolateral and medial amygdala in the COND and UNCD groups. Data represent mean ± s.e.m. * Significant differences ($p<0.05$) between experimental groups*

of the CS properties during the first exposure. Despite the high freezing scores of the COND group after the tone, c-Fos expression in the amygdala has been shown to be dissociated from freezing behavior (Holahan & White, 2004; Milanovic, Radulovic, Laban, Stiedl, Henn, & Spiess, 1998).

However, the role of the Me on fear conditioning is less clear. Some authors reported c-Fos induction in the Me after fear conditioning (Campeau, Falls, Cullinan, Helmreich, Davis, & Watson, 1997; Rosen, Fanselow, Young, Sitcoske, & Maren, 1998), but other studies indicate that this nucleus is more related with unconditioned odor-induced fear (Müller & Fendt, 2006; Takahashi, Nakashima, Hong, & Watanabe, 2005; Li et al., 2004). Moreover, the expression of c-Fos protein in the Me has been reported to remain unaltered after exposure to a contextual CS (Holahan & White, 2004; Rosen et al., 1998). Our results are consistent with the hypothesis suggesting an involvement of the Me not only in conditioned fear, but also in unconditioned fear, due to the increases in c-Fos expression in the Me of the COND and UNCD groups. In particular, the UNCD group showed a larger increase in c-Fos expression in the Me as compared to the BL. This result would suggest a greater involvement of the Me in unconditioned fear than the BL.

CO activity and c-Fos expression seem to be unrelated in the brain under normal circumstances, although their relationship is inverse after hypoxia, since a decline in oxidative phosphorylation is paired with an increase of c-Fos expression (Nomura, Ueta, Serino, Yamamoto, Shibuya, & Yamashita, 1999). CO is cytoplasmatic enzyme used as marker of brain oxidative metabolism whereas c-Fos is a nuclear protein expressed only under particular circumstances involving neuronal activation. In this regard, changes in CO activity do not necessarily parallel those found using c-Fos expression, although both techniques reflect polysinaptic activation in response to particular stimuli (Wong-Riley, 1989). An absence of CO changes in a particular brain region after behavioral manipulation indicates that this is not critically involved in such behavior, but this may be not be the case for c-Fos expression (Konkle and Bielajew, 2004). Thus, c-Fos is sometimes expressed in a particular brain region, but it may be not detected in other behaviorally related regions, as opposed to other metabolic markers like CO or 2-deoxyglucose uptake (Sharp, Sagar, & Swanson, 1993). Further studies are required to evaluate the function of amygdalar nuclei during particular time points of fear conditioning. This task could be certainly facilitated using different functional approaches in the same experimental subject, as reported here.

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