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Intracranial self-stimulation induces expression of learning and memory-related genes in rat amygdala

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Intracranial self-stimulation (ICSS) in the lateral hypothalamus improves memory when administered immediately after a training session. In our laboratory, ICSS has been shown as a very reliable way to increase two-way active avoidance (TWAA) conditioning, an amygdala-dependent task. The aim of this work was to study, in the rat amygdala, anatomical and molecular aspects of ICSS, using the same parameters facilitating TWAA. First, we examined the activation of ipsilateral and contralateral lateral (LA) and basolateral (BLA) amygdala, the main amygdalar regions involved in the TWAA, by the immunohistochemical determination of c-Fos protein expression. Second, we tested the effects of the ICSS treatment on the expression of 14 genes related to learning and memory processes using real-time polymerase chain reaction. Results showed a bilateral increase in c-Fos protein expression in LA and BLA nuclei after ICSS treatment. We also found that *Fos*, brain-derived nerve growth factor (*BDNF*), *Arc*, inducible cAMP early repressor (*ICER*), *COX-2*, *Dnajb1*, *FKpb5* and *Ret* genes were upregulated in the amygdala 90 min and 4.5 h post ICSS. From this set of genes, *BDNF*, *Arc* and *ICER* are functionally associated with the cAMP-responsive element-mediated gene transcription molecular pathway that plays a pivotal role in memory, whereas *Dnajb1* and *Ret* are associated with protein folding required for plasticity or neuroprotection. Our results suggest that ICSS induces expression of genes related with synaptic plasticity and protein folding functions in the rat amygdaloid area, which may be involved in the molecular mechanisms by which ICSS may improve or restore memory functions related to this brain structure.

Keywords: Amygdala, ICSS, immunohistochemistry, protein folding, real-time PCR, synaptic plasticity

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Intracranial self-stimulation (ICSS) is a form of deep brain stimulation with electrodes implanted in specific areas belonging to the brain reward system, such as the medial forebrain bundle (MFB) at the lateral hypothalamus (LH). In agreement with the original idea proposed by Huston *et al*. (1977), many works have shown that ICSS is a reliable way to induce facilitation of learning and memory processes in rats. In particular, post-training ICSS at the LH can improve learning and memory in a wide variety of tasks, related to both explicit (Ruiz-Medina *et al*. 2008a; Soriano-Mas *et al*. 2005) and implicit memories (Coulombe & White 1982; Huston & Mueller 1978; Ruiz-Medina *et al*. 2008b), suggesting that post-training ICSS facilitates memory consolidation in different memory systems. However, knowledge of underlying cellular and molecular mechanisms on how electrical stimulation can improve learning and memory is limited and studies are required to clarify it. In the hippocampus, ICSS can induce long-lasting structural changes in dendrites and synapses (Rao *et al*. 1999). Moreover, we have shown that LH ICSS upregulates the expression of genes in hippocampus related to learning and memory, neurogenesis and neuroprotection (Huguet *et al*. 2009).

In our laboratory, ICSS has been shown to be a very reliable way to increase two-way active avoidance (TWAA) conditioning, an amygdala-dependent task. Using this task, we have shown that ICSS, besides being especially effective in subjects with a naturally low learning capacity (Aldavert-Vera *et al*. 1996) or a cognitive deficit caused by ageing (Aldavert-Vera *et al*. 1997), ICSS is also able to improve TWAA deficits caused by extensive bilateral lesions of the parafascicular nucleus of the thalamus (Redolar-Ripoll *et al*. 2003) or the basolateral (BLA) amygdala (Segura-Torres *et al*. 2010). In this context, we were interested in studying the activation of specific amygdala nuclei, and possible molecular basis, in this brain area, which could be implicated in the improved ICSS effects on amygdala-dependent tasks.

Among the molecular mechanisms of learning and memory processes in amygdala, cAMP-dependent signal transduction machinery, which included activation of cAMP-responsive element (CRE)-dependent gene expression, has been widely implicated in neuronal plasticity underlying learning and memory across species (Wang *et al*. 2006). As modulators of this pathway, CRE modulator (CREM) and inducible cAMP early repressor (ICER) may have a significant role in this process (Borlikova & Endo 2009). The neural plasticity-related brain-derived nerve growth factor (*BDNF*) and *Arc* genes (activity-regulated cytoskeletal-associated protein) have also been involved in memory processing in amygdala (Datta *et al*. 2008; Rattiner *et al*. 2005).

The aim of this work was to study anatomical and molecular aspects of the ICSS in the rat amygdala, using the same parameters facilitating TWAA. First, we examined the activation of ipsilateral and contralateral lateral (LA) and BLA amygdala, the main amygdalar regions implicated in active avoidance conditioning (Amorapanth *et al*. 2000; Savonenko *et al*. 2003), by the immunohistochemical determination of c-Fos protein expression. Second, we tested gene expression regulation 90 min and 4.5 h post ICSS by real-time polymerase chain reaction (PCR). We analysed *BDNF*, *Arc*, *CREM* and *ICER* genes as well as 10 other genes related to synaptic plasticity and/or protein folding, which we previously found significantly regulated by ICSS in the hippocampus (Huguet *et al*. 2009).

Material and methods

Subjects

A total of 45 male Wistar rats, obtained from our laboratory breeding stock, with a mean age of 93.6 days (SD = 4.1) at the beginning of the experiments, and a mean weight of 403.12 g (SD = 37.9) at the time of surgery, were used. All rats were housed singly, always kept under conditions of controlled temperature (21–23°C) and humidity (40–70%), and subjected to an artificial light/dark cycle of 12/12 h (lights on at 0800 h). All experiments were carried out in compliance with the European Community Council Directive for care and use of laboratory animals (CEE 86/609) and the Generalitat de Catalunya decree (Departament de Medi Ambient; Generalitat de Catalunya, 1995; protocol number 2381).

Intracranial self-stimulation

Stereotaxic surgery

Under general anesthesia induced by 110 mg/kg Ketolar® (ketamine hydrochloride; Parke-Davis S.L. Pfizer, Madrid) and 0.08 ml/100 g Rompun® (xylazine 23 mg/ml, intraperitoneal; Bayer, Barcelona), rats in the ICSS and control-sham groups were implanted with a monopolar stainless steel electrode (150 μm in diameter) aimed at the LH, into the fibres of the MFB, with the incisor bar set at −2.7 mm below the interaural line and according to coordinates from the stereotaxic atlas of Paxinos and Watson (1998): anteroposterior (AP) = −2.56 mm; $L = 1.8$ mm (right hemisphere) and $P = -8.5$ mm, with the cranium surface as dorsal reference. ICSS electrodes were anchored to the skull with jeweller's screws and dental cement. In the post-surgery recovery period (7 days), the animals were weighed and handled daily. Rats in the naive group were not submitted to the surgery procedure.

ICSS behaviour establishment

Rats in the ICSS group were taught to self-stimulate by pressing a lever in a conventional Skinner box (25 × 20 × 20 cm) constructed of Plexiglas. Electrical brain stimulation consisted of 0.3-s trains of 50 Hz sinusoidal waves at intensities ranging from 10 and 400 μA. The ICSS behaviour was shaped for each subject to establish the range of current intensities that would support responding on a continuous reinforcement schedule. On two consecutive days, the animals were trained in ICSS to establish the individual optimum current intensity of ICSS (as described in Segura-Torres *et al*. 1991). The mean of the two current intensities that gave rise to the highest response rate (responses/min) was considered as the optimum intensity (OI) of ICSS for each rat. Rats in the control-sham group were handled and allowed to explore the ICSS box for 20 min on two consecutive days, but without ICSS. Rats in the naive group were not manipulated.

ICSS treatment

Twenty-four hours after the last ICSS establishment session, animals in the ICSS group were allowed to self-administer 2500 trains of electrical stimulation at the 100% of their OI, parameters that have shown a facilitative effect on TWAA in our previous works (Aldavert-Vera *et al*. 1996; Ruiz-Medina *et al*. 2008b). Animals in the control-sham group were equally placed in the ICSS box for 40 min but did not receive stimulation (sham session). Immediately after the ICSS treatment session or the sham session, rats were returned to their home cages. These procedures were conducted during the first half of the light cycle. Treatment duration (min) and total number of lever pressings in the treatment session (total responses) were also recorded.

c-Fos immunolocalization

Immunohistochemistry

For c-Fos immunolocalization, 70 min after the end of the ICSS treatment or the sham session, rats in the ICSS ($n = 5$) and control-sham ($n = 5$) groups were sacrificed by decapitation. Naive rats ($n = 4$) remained in their home cages until they were sacrificed. Brains were dissected and stored in at −80°C until used for cryosectioning. Coronal sections (20 μm) were obtained in a cryostat at −20°C, mounted onto SuperFrost/Plus slides (Menzek-Gläser, Braunschweig, Germany) and dried at room temperature (rt). The sections were fixed for 20 min in freshly prepared 2% formaldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.4, permeabilized with 0.1% Triton X-100 plus 0.1% sodium citrate in PBS for 5 min, incubated in 0.3% H_2O_2 in PBS for 20 min to block endogenous peroxidase activity and then in 5% goat serum in PBS for 30 min. To determine the immunohistochemical localization of c-Fos in the rat brain, we used a specific rabbit anti-c-Fos sc-52 polyclonal antibody (Santa Cruz Biotech, Santa Cruz, CA). Incubation with 1:900 diluted rabbit anti-c-Fos antibody plus 1:900 goat serum (Sigma) in PBS was performed for 4 h at rt and overnight at 4°C. Next, the sections were incubated with goat anti-rabbit IgG 1:200 plus 1:200 horse serum in PBS for 3 h and 30 min at rt and then incubated for 50 min with avidin–biotin–peroxidase complex, prepared according to the manufacturer's instruction and diluted 1:3 in PBS just before application (Immunopure ultra-sensitive ABC Rabbit IgG Staining Kit, Pierce, Rockford, IL). Sections were incubated for 3 min with Immunopure metal-enhanced diaminobenzidine tetrahydrochloride substrate kit (Pierce, Rockford IL) prepared according to manufacturer's instruction and then diluted 1:2 with PBS. Sections were washed with 0.01 M phosphate buffer, pH 7.4, and air dried before mounting with Vectamount (Vector Laboratories Inc., Burlingame, CA). No staining was detected when the primary antibody was omitted.

Image acquisition and analysis

Images were obtained with a digital camera (Olympus DP-70) coupled to a microscope (Olympus BX-41) from LA and BLA nuclei and piriform (Pir) cortex. A ×10 magnification was used and special care was taken to ensure that all images were taken under the same lighting conditions.

Quantification of c-Fos immunopositive nuclei was performed using the freeware ImageJ 1.38 software (http://rsb.info.nih.gov/ij/), using an automated algorithm installed on a macro described elsewhere (Boix-Trelis *et al*. 2006; Huguet *et al*. 2009). Briefly, for each brain area, a region of interest (ROI) was drawn and stored to be used in all the animals. This ROI was specific for each AP coordinate between bregma −2.3 and −3.6 in the case of LA and BLA nuclei. For the Pir cortex, the ROI was composed of four circular areas that were individually situated in order to have the complete set of equidistant circular areas adjusted to the standard shown in Fig. 1. To remove noise and to avoid differences between immunostaining batches, each image was digitally smoothed and subtracted from the original one. Appropriate grey threshold and particle size were set for each area and maintained for all subjects. To control the efficacy of the automatic quantification, a final step was included in the macro, which consisted of merging, in different colours, the original image and the image resulting from the particle detection. All regions were

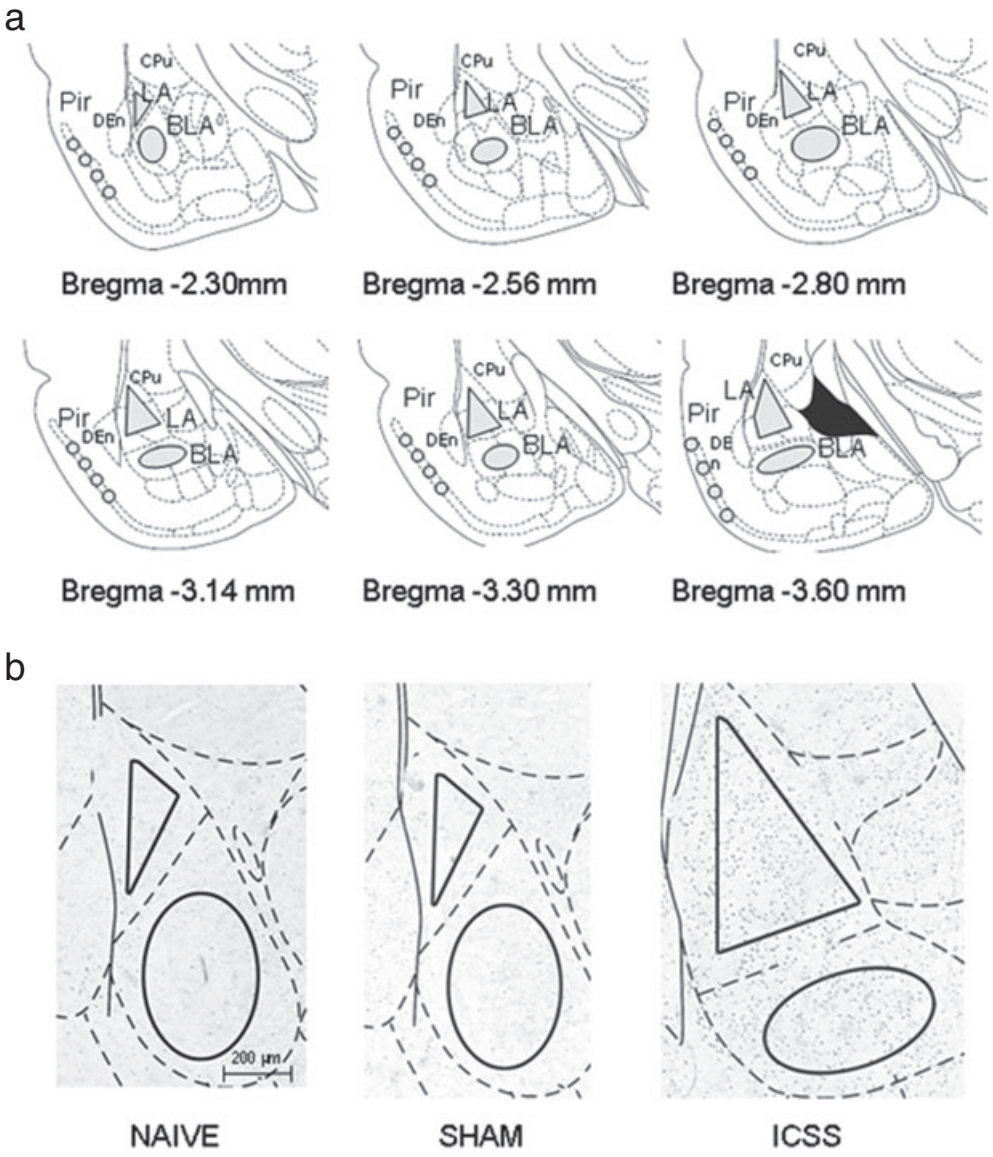

Figure 1: c-Fos immunolocalization. (a) Defined ROIs for BLA and LA and for the Pir cortex. The standard placement is superimposed on coronal sections adapted from Paxinos and Watson's atlas (2007), corresponding to coordinate AP between −2.30 and −3.60 mm to bregma. (b) Representative photomicrographs (with the corresponding adapted ROIs) of c-Fos immunohistochemistry in the ipsilateral side to the electrode placement (scale bar represents 200 μm). CPu, caudate-putamen; DEn, dorsal endopiriform nucleus.

bilaterally counted in two sections for each rat. The mean number of immunostained nuclei per mm^2 for these two sections of each hemisphere was used for the statistical analyses. Data were analysed using a repeated-measures ANOVA with one between-group factor, the *treatment condition* (ICSS, control-sham or naive) and one within-group factor, the *hemisphere* (ipsilateral or contralateral to electrode placement).

Gene expression study

Amygdala dissection and tissue collection

The animals were killed by decapitation 90 min or 4.5 h after the end of the ICSS treatment or the sham session. Brains were rapidly removed from the skull and sliced with a brain matrix (Stoelting, Wood Dale, IL). Slices between −2.8 and −3.8 anteroposterior to bregma were used to dissect the ipsilateral amygdaloid area with respect to the electrode. Anatomical regions were identified by comparison with Paxinos and Watson (1998) brain atlas plates, and dissection of the amygdaloid area was performed according to the scheme adapted by Koks (Koks *et al*. 2004; Lindblom *et al*. 2006) (Fig. 2). Dissected tissue were subsequently stored in RNA later (Ambion, Austin, TX) for 48 h at 4°C and then stored until RNA isolation at −20°C.

RNA isolation and cDNA synthesis

Total RNA from each sample was prepared with an RNeasy Lipid Tissue Mini kit according to the manufacturer's instructions (Quiagen, Valencia, CA) and quantified using the NanoDrop ND-1000

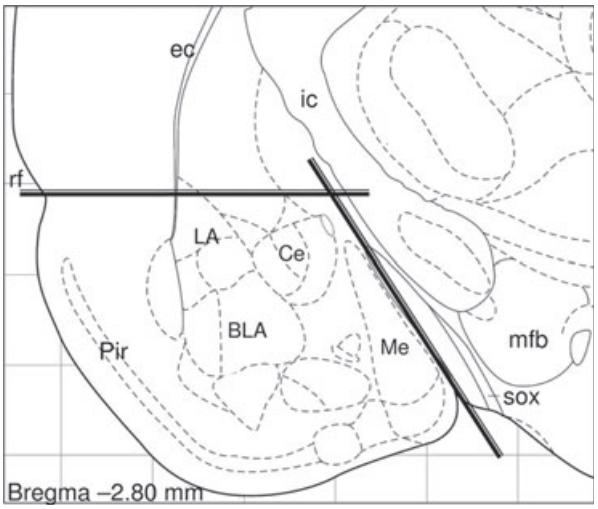

Figure 2: Amygdaloid area used for real-time PCR analysis delimited in the figure by double-solid lines. Ce, central amygdala nucleus; ec, external capsule; ic, internal capsule; Me, medial amygdala region; mfb, medial forebrain bundle; opt, optic tract; rf, rhinal fissure; sox, supraoptic decussation.

spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was assessed by running an agarose gel of each sample. Total RNA samples were then treated with the DNA free kit (Ambion, Austin, TX) to remove any contaminating genomic DNA and cDNA was synthesized by reverse transcription (RT) from 100 ng of total RNA in a two-step protocol using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), dNTP Mix (Applied Biosystems, Foster City, CA), random 15mers primers (Invitrogen, Carlsbad, CA) and RNase out inhibitor (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The samples were incubated at 65°C for 10 min, 4°C for 1 min, 20°C for 10 min, 50°C for 1 h, 85°C for 5 min and cooled at 4°C in a Biometra Thermocycler. 'No RT' controls were carried out using the same RT reaction mix but substituting H_2O-diethyl pyrocarbonate for RT enzyme.

Real-time PCR analysis

Fourteen candidate genes were analysed by real-time PCR comparing the ICSS vs. control-sham conditions for 90 min and 4.5 h after the treatment. The number of samples used for these experiments varied from $n = 6$ to $n = 8$ rats, depending on the variability of the expression for each specific gene. For *Cart* gene, the primers previously reported by Marie-Claire *et al*. (2003) were used. For the other genes, specific primers were designed with Primer Express 2.0 (Applied Biosystems, Foster City, CA) (Table 1). Hypoxanthine guanine phosphoribosyl transferase (*Hprt*) gene was used as a reference gene.

PCR cocktails (20 μl) contained cDNA, Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA), water and custom primers (Invitrogen, Carlsbad, CA). The PCRs were performed in an ABI PRISM 7300 Sequence Detector System (Applied Biosystems, Foster City, CA). Each reaction was performed in triplicate and 'negative' controls (without sample) were included in each plate to exclude potential contamination. 'No RT' controls were also analysed to discard genomic DNA contamination. The mRNA abundances for each candidate gene were calculated as: relative transcript abundance $= (E_{\text{target}})^{\Delta \text{Ct target(control - sample)}} / (E_{\text{reference}})^{\Delta \text{Ct reference(control - sample)}}$ (Pfaffl 2001), where E is real-time PCR efficiency calculated as efficiency $= 10^{(-1/\text{slope})}$, and control is the mean of Ct of control-sham samples. Data were analysed using one-side Mann–Whitney U test of ICSS vs. control-sham fold changes ($P < 0.05$).

Table 1: Primer sequences used for quantitative real-time PCR assays

Gene name	5′–3′ primer sequence	GenBank ID*
Fos	f: CCAAGCGGAGACAGATCAACTT	NM_022197
	r: TCTTTCAGTAGATTGGCAATCTCG	
BDNF	f: TAAAAGGAGCCCCATCACAATC	NM_012513
	r: TGCGGAGGGTCTCCTATGAA	
Arc	f: GGCATCTGTTGACCGAAGTGT	NM_019361
	r: CACATAGCCGTCCAAGTTGTTCT	
CREM	f: TTCCTCTGATGTGCCTGGTATTC	NM_01110860
	r: TGCCCCGTGCTAGTCTGATAT	
ICER	f: CTCTGTATGCAAAAGCCCAACA	NM_017334
	r: TCTGGTAAGTTGGCATGTCACC	
COX-2	f: ATCAAATTACCGCTGAAGCCC	NM_017232
	r: ATGTTCCAGACTCCCTTGAAGTG	
Cart	f: GCCAAGTCCCCATGTGTGAC	NM_017110
	r: CACCCCTTCACAAGCACTTCA	
Sgk	f: TCGGGGCTGTCTTGTATGAG	NM_019232
	r: GTGCCTTGCTGAGTTGGTGA	
Adcyap1	f: CCTACGCCTTTACTACCCAGC	NM_016989
	r: TTTGCGTAGGCTTCGTTAAG	
Pde1a	f: CATGGTTGGTTTGACATATCCTG	NM_030871
	r: TATGCTCCCCGCTTGCTT	
Hspa1a	f: GAGGAGGTGGATTAGAGGCTTTTC	NM_031971
	r: TTGATAAGAATCGTGCACCAGC	
Dnabj1	f: GCCGAATGTTTTCCACAGAAT	XM_341663
	r: TTCCCACCGCCTGCCTAT	
Ret	f: ATTGGGTGTTGCCCTTGCT	NM_012643
	r: GTTCTGGTCTGCGGGTAGGT	
Fkbp5	f: GAGCCGTTTGTCTTTAGCCTTG	NM_001012174
	r: GCCAGCAGAGCCGTAAGC	
Hprt	f: AAAGGACCTCTCGAAGTGTTGG	NM_012583
	r: AAGTGCTCATTATAGTCAAGGGCA	

f, forward primer; r, reverse primer.
*GenBank identification available at http://www.ncbi.nlm.nih.gov/.

Results

ICSS behaviour

Some of the rats used in these studies underwent small seizures (one in the immunohistochemical study and one in the PCR-90 min) and were thus not included in the overall statistical analysis described next and are not part of the specified number of animals used in these experiments. The mean values (±SD) of ICSS variables for the rats used in the immunohistochemistry experiment ($n = 5$) were OI (227 ± 112.06 μA), highest response rate (79 ± 17.12 responses/min), treatment duration (72.15 ± 34.37 min) and total responses (3079.1 ± 302.96) lever pressings). The mean values (±SD) of the same ICSS variables for the rats used in the real-time PCR analysis were *PCR-90 min* ($n = 8$), OI (81.11 ± 26.19 μA), highest response rate (83.00 ± 20.89 responses/min), treatment duration (52.63 ± 12.47 min), and total responses (3346.25 ± 530.21 lever pressings); *PCR-4.5 hours* ($n = 8$), OI (96.25 ± 30.67 μA), highest response rate (69.37 ± 17.20 responses/min), treatment duration

(57.35 ± 10.16 min), and total responses (3114.87 ± 315.33 lever pressings). Correlation analyses showed no relationship between the ICSS variables and number of positive c-Fos cells in LA and BLA.

c-Fos immunohistochemistry

ANOVA showed a statistically significant higher number of c-Fos immunopositive cells in ICSS rats compared with the control-sham and naive rats in BLA ($F_{1,6} = 12.1$; $P = 0.013$; $F_{1,6} = 10.33$; $P = 0.018$, respectively) and LA ($F_{1,6} = 8.07$; $P = 0.030$; $F_{1,6} = 9.77$; $P = 0.020$, respectively). The differences between groups were observed independently of the hemisphere. Comparisons between hemispheres only showed a higher expression of the ipsilateral hemisphere in the ICSS group at the BLA ($F_{1,6} = 7.98$; $P = 0.030$) (Figs. 1b and 3). No differences were observed between the non-stimulated groups (control-sham and naive). In the Pir cortex, no statistically significant differences were observed between groups or hemispheres (Fig. 4).

Real-time PCR analysis

Statistical analysis showed significant changes in the expression of the *Fos*, *BDNF*, *Arc*, *ICER*, *COX-2*, *Dnajb1*, *Fkbp5* and *Ret* genes 90 min and 4.5 h following ICSS (Fig. 5). The expression of these genes was 1.4-fold to 4.3-fold higher in ICSS-stimulated rats compared with control-sham animals after 90 min and 1.3-fold to 2.9-fold at 4.5 h following the session. In addition, we showed that the mRNA-encoding Hspa1a is only significantly upregulated at 90 min and mRNA CREM only at 4.5 h following ICSS. In contrast, *Sgk*, *Cart*, *Adcyap1* and *Pde1a* genes showed mRNA levels similar in ICSS-stimulated and control-sham animals both at 90 min and 4.5 h after ICSS.

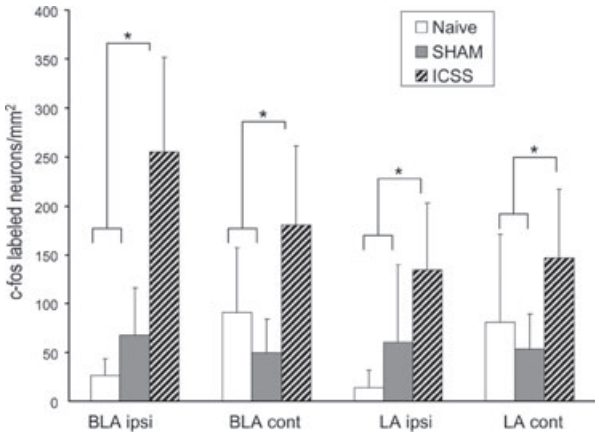

Figure 3: Effects of ICSS on the c-Fos expression in amygdala. Bar graph depicting the mean number of c-Fos immunopositive cells (neurons/mm^2 ± SD) in BLA and LA nuclei and in the ipsilateral and the contralateral hemispheres with respect to the electrode placement.

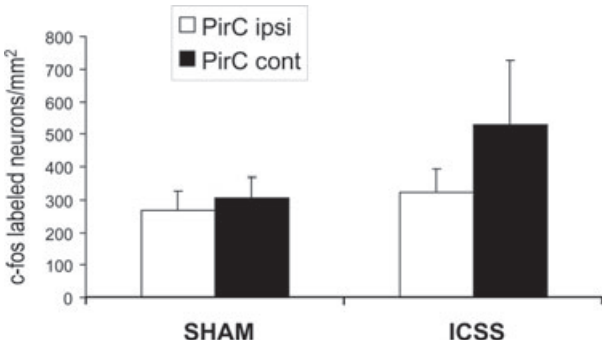

Figure 4: Effects of ICSS on the c-Fos expression in Pir cortex. Bar graph depicting the mean number of c-Fos immunopositive cells (neurons/mm^2 ± SD) in the Pir cortex in the ipsilateral and the contralateral hemispheres with respect to the electrode placement.

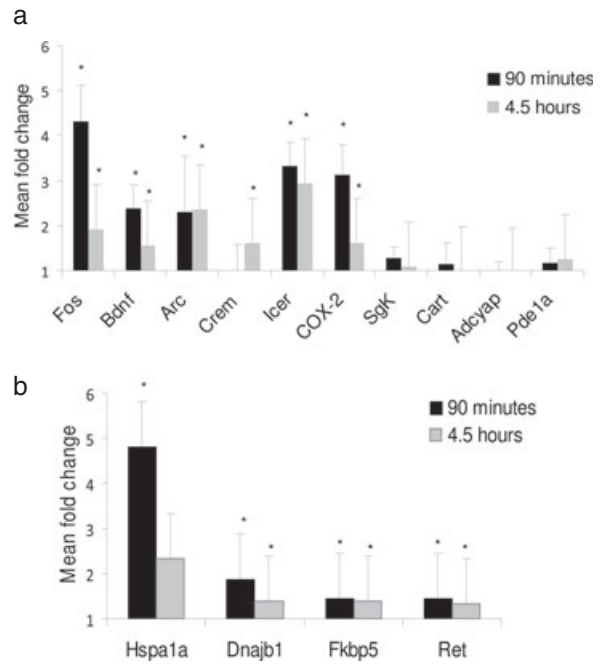

Figure 5: Effects of ICSS on gene expression in amygdaloid area. Relative amygdalar mRNA levels for (a) synaptic plasticity and (b) protein folding-related genes in ICSS vs. control-sham rats, as determined by RT-PCR assay. Data are presented as mean fold change (±SD). Number of samples used were $n = 8$ (*Adcyap1*, *Cart*, *Sgk* and *Pde1a*) or $n = 6$ (for the remaining tested genes) ($^*P < 0.05$).

Discussion

This is the first study showing that LH ICSS treatment induces a bilateral increase in c-Fos protein expression in LA and BLA nuclei and expression changes in synaptic plasticity and protein folding-related genes in the amygdaloid area. It

is important to point out that the ICSS parameters used are the same that we have previously shown to improve the acquisition and short (24 h) and long-term memory (10 and 30 days) of a TWAA conditioning (Aldavert-Vera *et al*. 1996; Segura-Torres *et al*. 1991).

Our immunohistochemical results agree with previous studies showing that one similar ICSS session increases the number of c-Fos-like immunoreactive neurons in the rat amygdaloid complex 15 and 120 min later (Flores *et al*. 1997; Nakahara *et al*. 1999). Although there are no previous data reporting c-Fos immunoreactivity in specific amygdalar nuclei, autoradiography studies showed that ICSS was able to increase the activation of BLA and central amygdala (Esposito *et al*. 1984; Porrino *et al*. 1990). Interestingly, intense labelling was observed on the stimulated and unstimulated sides of the brain, even though an ipsilateral predominance was shown in the BLA nucleus. Starting from the hypothesis that the brain regions with exclusively ipsilaterally increased c-Fos staining are closely related to neural substrates mediating the rewarding effect of the MFB stimulation (Waraczynski 2006), the pattern of activation observed in the amygdala suggests a non-critical role for the ICSS behaviour. In this sense, it has been shown that electrolytic lesions of the amygdala complex in general (Waraczynski *et al*. 1990), or BLA nucleus, in particular (Segura-Torres *et al*. 2010), did not affect ICSS reward effectiveness. Similar patterns of c-Fos expression have been observed after ICSS in other brain regions not directly involved in the reward system, such as the hippocampus (Huguet *et al*. 2009). However, a c-Fos immunoreactivity increase was observed in the amygdala, but not in the Pir cortex, suggesting an anatomical-specific ICSS response.

Because no differences were observed between naive and control-sham groups in BLA and LA, we suggest that variables, such as the amount of handling, the stereotaxic intervention or the ICSC box exposure did not significantly affect amygdalar activation at the time it was evaluated. Moreover, the observed increase in c-Fos expression in BLA and LA does not seem to be attributable to motor activity inherent to the ICSS treatment, because no correlation between c-Fos expression and any motor measure of the rats' ICSS behaviour (as response rates or total number of responses performed in the treatment session) were observed.

How could LH ICSS have activated amygdala? On one hand, it has been shown that basal forebrain neurons, including the ones in the amygdala, are antidromically driven by LH stimulation (Murray & Shizgal 1996), suggesting that many of the labelled cells could be directly activated by the electrical stimulation delivered at the tip of the electrode. On the other hand, LH ICSS induces a significant increase in the number of Fos-immunopositive cells in several brainstem monoaminergic nuclei, such as the locus coeruleus (Arvanitogiannis *et al*. 1997; Ishida *et al*. 2001), that in turn activate the amygdala (Samuels & Szabadi 2008). Thus, these data indicate that some amygdalar neurons also can be trans-synaptically activated by ICSS.

Taking into account that the present ICSS treatment activates BLA and LA nuclei, which are important for active avoidance conditioning (Amorapanth *et al*. 2000; Savonenko *et al*. 2003), and that TWAA conditioning also results in a marked increase in c-Fos mRNA and protein in the amygdala (Savonenko *et al*. 1999), we suggest that ICSS treatment would facilitate TWAA, activating the same memory system implicated in this conditioning. This idea agrees with previous behavioural results indicating that ICSS effects could accelerate memory consolidation, in a similar way to extended training (Major & White 1978; Redolar-Ripoll *et al*. 2002). Several data support that other memory systems, in special the hippocampal one, could also mediate in a synergistic or cooperative way the facilitative effects of ICSS on TWAA. In this way, ICSS induces a widespread increase in the expression of c-Fos in hippocampus (Huguet *et al*. 2009) and other memory-related brain areas such as prefrontal cortex and dorsal striatum (Arvanitogiannis *et al*. 1997, Arvanitogiannis *et al*. 2000).

Moreover, increases in c-Fos expression and CRE binding (CREB) phosphorylation have been observed in the rat hippocampus after TWAA training trials (Datta *et al*. 2009, Nikolaev *et al*. 1992, Saha & Datta 2005), suggesting an hippocampal contribution to TWAA. In support of such idea, Datta *et al*. (2009) recently showed that the increase in BDNF expression in the dorsal hippocampus, also increased in the amygdala in this work, is essential for the improvement of TWAA memory. In addition, the activation of the hippocampus by the ICSS treatment results in the expression of some genes, such as *Adcyap1*, *COX-2* or *Sgk* (Huguet *et al*. 2009), that have been shown to be functionally related to TWAA (Adamik & Telegdy 2005) or other tasks that are dependent on the hippocampal system (Rall *et al*. 2003, von Hertzen & Giese 2005). In our opinion, the hypothesis of a synergic or cooperative activation of different memory systems by ICSS could also explain the memory restorative or compensatory capacity of the treatment in amnesic subjects bearing damage in some memory-related brain region, as the BLA amygdala (Segura-Torres *et al*. 2010) or the parafascicular thalamic nucleus (Redolar-Ripoll *et al*. 2003).

As regards the changes in gene expression after ICSS, we chose to test 14 genes, 10 directly related to synaptic plasticity (*Fos*, *BDNF*, *Arc*, *ICER*, *CREM*, *COX-2*, *Sgk*, *Adcyap1*, *Cart* and *Pde1a*) and 4 related to protein folding required for plasticity or neuroprotection (*Hspa1a*, *Dnajb1*, *Fkbp5* and *Ret*). All of them, except *BDNF*, *Arc*, *ICER* and *CREM*, were tested in the hippocampus in our previous work showing regulation at 90 min following ICSS (Huguet *et al*. 2009). In this study, the gene expression was analysed at two time points after ICSS: 90 min and 4.5 h, in order to obtain information on the immediate-early and also on the slightly delayed early genes that could be involved in different periods of the memory consolidation process that may be modulated by the ICSS treatment.

In accordance with the early increased c-Fos protein expression detected in the immunolabelling study, Fos showed an early mRNA increase at 90 min post ICSS that was also detected at 4.5 h. These results agree with the idea that this gene may control long-term changes in the cellular functioning (Kaczmarek 2002) and may suggest that it could be one of the genes involved in the long-term memory facilitation of TWAA task induced by ICCS.

The present results also showed a significant increase in the expression of the synaptic plasticity-related genes, *CREM*, *ICER*, *BDNF* and *Arc* by ICSS. All these genes are functionally associated with the CRE-mediated gene transcription molecular pathway, which play a pivotal role in memory (Wang *et al*. 2006; Won & Silva 2008). ICER is an endogenous antagonist of CREB, inhibiting the CRE-mediated transcription, whereas CREM acts as cognates of CREB. However, BDNF transcription is regulated by the CREB family (Josselyn & Nguyen 2005) and *Arc* is another downstream cAMP-inducible gene (Datta *et al*. 2008). Increased mRNA levels of these genes have been detected in the amygdala after emotional learning (Borlikova & Endo 2009; Monfils *et al*. 2007; Rattiner *et al*. 2005) and, in particular, *BDNF* and *Arc* after TWAA (Datta *et al*. 2008). Moreover, a very recent study shows induction of neuronal activity-dependent genes following both long-term potentiation (LTP) induction and fear conditioning in the LA amygdala, which are likely calcium, extracellular-regulated kinase/mitogen-activated protein kinase and CREB dependent (Ploski *et al*. 2010). Taken together, these findings suggest CREB signalling cascade in the amygdala as a target for the ICSS memory facilitative effects.

In contrast to BDNF, Arc and ICER, the expression of CREM was only increased at 4.5 h following ICSS. Similarly, a delayed CREM expression in comparison with c-Fos has been observed after a visual conditioning (Konopka *et al*. 1998). Thus, the present results indicate that these four genes are heterogeneously activated after ICSS treatment during periods compatible with memory stabilization processes in the amygdala (Ressler *et al*. 2002; Rodrigues *et al*. 2004). As with our previous work, these four genes were not detected at 90 min in hippocampus after the same ICSS treatment. However, the *COX-2* gene is induced in both brain areas, the amygdala (present results) and the hippocampus (Huguet *et al*. 2009). Evidences showing COX-2 participation in synaptic plasticity and memory acquisition (Shaw *et al*. 2003; Sharifzadeh *et al*. 2006; Yang & Chen 2008) include LTP induction in the LA nucleus of the amygdala (Albrecht 2007). Thus, there could be an important role of BDNF, Arc, CREM, ICER and COX-2 in mediating ICSS effects in the amygdala with specific temporal activation.

Another set of genes, *Sgk*, *Adcyap1*, *Cart* and *Pde1a*, which also play important roles in learning tasks and plasticity (Adamik & Telegdy 2005; Blokland *et al*. 2006; von Hertzen & Giese 2005), did not show changes in their expression in the amygdaloid area. However, we had previously observed hippocampal expression changes in these genes (Huguet *et al*. 2009). One possible explanation could be that ICSS induces a different regulation for these genes in the hippocampus and amygdala. Nevertheless, we cannot rule out that the differences were not able to be detected on analysing the entire amygdaloid area. In this sense, Zirlinger *et al*. (2001) reported that the majority of amygdala-enriched genes exhibited boundaries of expression corresponding to cytoarchitectonically defined amygdala subnuclei. Thus, further detailed studies would be necessary to verify if these ICSS-regulated genes may also present nuclei-specific regulation.

Interestingly, ICSS treatment also increased the mRNA abundance of the four tested genes associated with protein folding required for plasticity or neuroprotection (Bingol & Schuman 2005; Chen *et al*. 2005; Powers & Workman 2007). They included the chaperones, Hspa1a and Dnajb1, both members of heat shock proteins (Hsps) family, the co-chaperone Fkbp5, and Ret, a receptor belonging to the receptor-like tyrosine kinase superfamily. Different works provide evidence that Hsps are related to learning and memory processes (Ammon-Treiber *et al*. 2008; Lin *et al*. 2004; Pizarro *et al*. 2003; Su *et al*. 2009) and the two tested chaperones are jointly involved in synaptic protection (Brown 2007; Chen *et al*. 2005). Fkbp5 may interact with Hsps and has antiapoptotic properties (Giraudier *et al*. 2002), and Ret promotes protein folding in response to cellular stress through the activation of the heat shock element (HSE) gene promoter region. Interestingly, *Hspa1a* and *Dnajb1* genes present this promoter region (Myers & Mulligan 2004). Glial-derived nerve growth factor-Ret signalling has been correlated with cognitive enhancement in rats following traumatic brain injury (Bakshi *et al*. 2006).

In conclusion, the present results suggest that ICSS could activate synaptic plasticity and protein folding-related molecular mechanisms in the amygdalar region, which may contribute to improving and/or restoring learning and memory functions related to this brain structure.

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