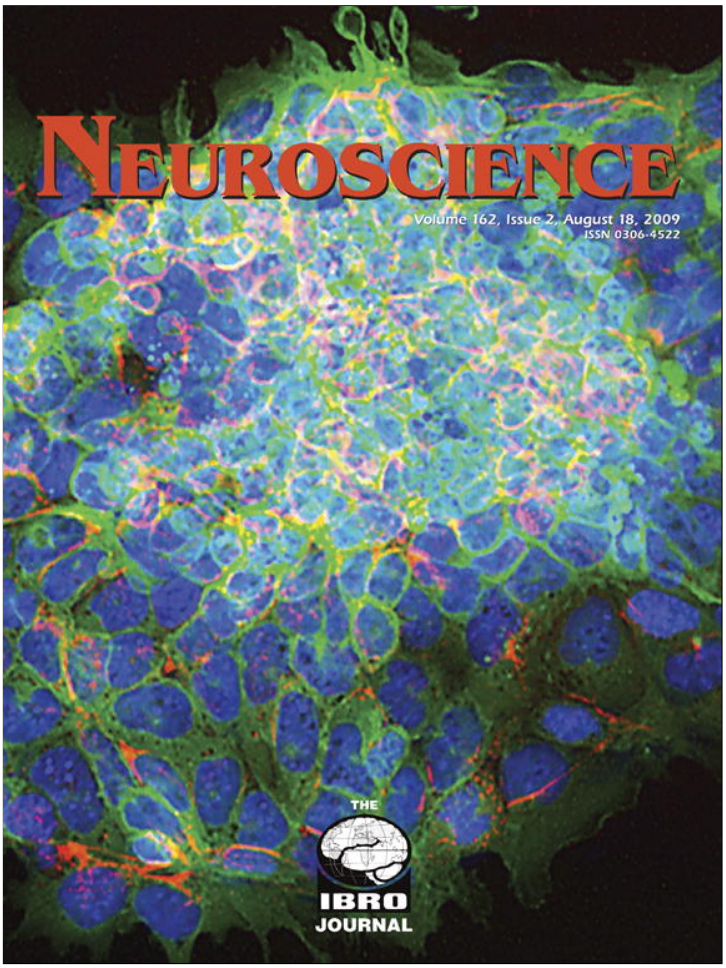

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INTRACRANIAL SELF-STIMULATION TO THE LATERAL HYPOTHALAMUS, A MEMORY IMPROVING TREATMENT, RESULTS IN HIPPOCAMPAL CHANGES IN GENE EXPRESSION

G. HUGUET,[a]* L. ALDAVERT-VERA,[b] E. KÁDÁR,[a] S. PEÑA DE ORTIZ,[c] I. MORGADO-BERNAL[b] AND P. SEGURA-TORRES[b]

[a]*Universitat de Girona, Departament de Biologia, Facultat de Ciències, Campus de Montilivi, 17071 Girona, Spain*

[b]*Universitat Autónoma de Barcelona, Departament de Psicobiologia i de Metodologia de les Ciències de la Salut, Institut de Neurociències, 08193 Bellaterra, Barcelona, Spain*

[c]*University of Puerto Rico, Department of Biology and Functional Genomics Research Center, P. O. Box 233360, San Juan, Puerto Rico 00931-3360*

Abstract—Intracranial self-stimulation (ICSS) within the medial forebrain bundle of the lateral hypothalamus (LH) facilitates consolidation of implicit and explicit memories for a variety of learning paradigms in rats. However, the neural and molecular mechanisms involved in memory facilitation by ICSS are not known. Here, we investigated the influence of ICSS treatment on hippocampal gene expression in order to identify potential signaling pathways and cellular processes involved in ICSS-mediated cognitive improvements. Immunohistochemistry studies demonstrated that ICSS caused a rapid induction of c-Fos expression in hippocampal cornu ammonis (CA) 3 and dentatus gyrus areas. Moreover, using microarray or quantitative real-time polymerase chain reaction (PCR) analysis, we showed that ICSS modulates the expression of 62 hippocampal genes shortly after training. Most of the proteins encoded by these genes, such as calmodulin-dependent-phosphodiesterase 1 A (Pde1a), are part of signal transduction machineries or are related to anti-apoptosis, as heat shock 70 kDa protein 1A (Hspa1a). Importantly, 10 of the regulated genes have been previously related with learning and memory or neural plasticity, including the cocaine and amphetamine-regulated transcript (Cart), adenylate cyclase activating polypeptide 1 (Adcyap1), serum/glucocorticoid regulated kinase (Sgk), Ret proto-oncogene (Ret), and Fos. The fact that the Fos gene was differentially expressed in our microarray experiments validated our findings from our immunohistochemical studies mentioned above. In addition, using quantitative real-time PCR, we confirmed the observed expression changes for several of the genes identified by our microarray analyses. Our results suggest that ICSS may facilitate learning and memory by regulation of multiple signaling pathways in the hippocampus that may promote neuroplasticity.

Key words: c-Fos immunohistochemistry, deep brain stimulation, microarray, neural plasticity, neuroprotection, intracranial self-stimulation.

Intracranial self-stimulation (ICSS) is a form of deep brain stimulation (DBS) in which experimental animals press continuously a lever to receive electrical stimulation through electrodes implanted in specific areas belonging to the reward brain system, such as the lateral hypothalamus (LH). Different experiments have been done to explore the nature of such a phenomenon and its functional implications. Many of them investigated whether ICSS could serve to improve cognitive processes. Particularly, ICCS of the medial forebrain bundle (MFB) of the LH can consistently facilitate learning and memory in a variety of paradigms of both implicit (Aldavert-Vera et al., 1996; Huston and Mueller, 1978; Redolar-Ripoll et al., 2002; Ruiz-Medina et al., 2008b) and explicit hippocampus-dependent tasks in rats (Ruiz-Medina et al., 2008a; Soriano-Mas et al., 2005; Yoganarasimha et al., 1998). ICSS at the LH has been also able to reverse memory deficits caused by aging (Aldavert-Vera et al., 1997), brain damage (Redolar-Ripoll et al., 2003; Yoganarasimha and Meti, 1999) or stress-induced CA3 dendritic atrophy in rats (Ramkumar et al., 2008).

It has been found that ICSS to the LH-MFB increased Fos-like immunoreactivity in some areas associated with memory processes, such as the amygdala (central and basolateral nuclei) or the medial prefrontal cortex (see Waraczynski, 2006). However, previous studies have not reported data showing c-Fos induction by ICSS in the hippocampal memory system. Interestingly, it has been shown that ICSS induces long-lasting structural changes in dendrites and synapses in the hippocampus (Rao et al., 1993; Shankaranarayana Rao et al., 1999), and that such structural changes could be associated to the mechanisms of ICSS memory facilitation (Yoganarasimha et al., 1998). Thus, it can be expected that ICSS induces changes in

*Corresponding author. Tel: +34-972-418171; fax: +34-972-418150.
E-mail address: gemma.huguet@udg.edu (G. Huguet).

Abbreviations: Bag3, Bcl2-associated athanogene 3; CA, cornu ammonis; Cart, cocaine- and amphetamine-regulated transcript; Cryab, Crystallin, alpha B; DBS, deep brain stimulation; DG, dentate gyrus; DGlb, lateral blade of the dentate gyrus; DGmb, medial blade of the dentate gyrus; Dnajb1, DnaJ-Hsp40-homolog subfamily B member 1; EST, expressed sequence tag; FDR, false discovery rate; FKbp5, FK506 binding protein 5; GC, glucocorticoid; Gda, guanine deaminase; GDNF, glial-derived neurotrophic factor; Grem1, Gremlin 1 homolog, cysteine knot superfamily; Hprt, hypoxanthine guanine phosphoribosyl transferase; HSE, heat shock element; Hspa1a, heat shock 70kDa protein 1 A; Hspe1, heat shock 10 kDa protein 1; ICSS, intracranial self-stimulation; LH, lateral hypothalamus; LTP, long-term potentiation; MFB, medial forebrain bundle; OI, optimum intensity; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Plcl1, phospholipase C-like 1; Plekhf1, pleckstrin homology domain containing family F- with FYVE domain-member 1; Ret, Ret proto-oncogene; ROI, region of interest; rt, room temperature; SD, standard deviation; Sgk, serum/glucocorticoid regulated kinase; SSPE, saline sodium phosphate–EDTA; Tsc22d3, TSC22 domain family 3; Ubqln1, Ubiquilin 1.

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gene expression in the hippocampus, as well as in the other brain structures mentioned above.

In the present study we asked the following specific question: does ICSS regulate hippocampal genes that have been previously associated to learning and memory processes? To answer this question we used one session of ICSS treatment, using parameters that are within the range of values obtained in our previous studies showing enhancement of hippocampus-dependent or independent learning and memory (Aldavert-Vera et al., 1996; Ruiz-Medina et al., 2008a,b; Segura-Torres et al., 1991; Soriano-Mas et al., 2005). Here, we initially focused on the induction of c-Fos within different hippocampal regions following ICSS of the LH. Next, in order to begin to understand what molecular signaling pathways affected by ICSS could be involved in learning and memory facilitation, we analyzed hippocampal gene expression by oligonucleotide microarrays shortly after ICSS of the LH. The present study constitutes a first approximation to the knowledge of ICSS effects on genomic mechanisms in the hippocampus.

EXPERIMENTAL PROCEDURES

Subjects

A total of 59 male Wistar rats, obtained from our laboratory breeding stock, with a mean age of 93.12 days (SD=3.4) at the beginning of the experiments and a mean weight of 410.21 g (SD=29.3) at the time of surgery, were used. All rats were singly housed, always kept under conditions of controlled temperature (21–23 °C) and humidity (40%–70%), and subjected to an artificial 12-h light/dark cycle of (lights on at 08:00 h). Gene expression changes involving c-Fos and other genes have been observed in the hippocampus due to factors such as stress, exercise or novelty (Chen et al., 2006; Cotman and Berchtold, 2002; Lee et al., 2003; Romanelli et al., 2007; Wirtshafter, 2005). Thus, in order to evaluate more specifically the effects of ICSS treatment we included a Control-sham group identical to the ICSS treated group but without ICSS stimulation, and also a Naive group. The sample size was determined taking into account statistical and ethic criteria. A supervision protocol, approved by the Ethical Committee of the Autonomous University of Barcelona, was used to control the animals' welfare. 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).

ICSS

Stereotaxic surgery. Under general anesthesia induced by 110 mg/kg Ketolar® ketamine chlorhydrate (Parke-Davis S.L. Pfizer, Madrid, Spain) and 0.08 ml/100 g Rompun® xylazine 23 mg/ml; i.p. (Bayer, Barcelona, Spain), 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 fibers 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): 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 jeweler'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 behavior 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 behavior 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 (ICSS treatment). 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=4) groups were sacrificed with a guillotine. Naive rats (n=4) remained in their home cages until they were sacrificed. Brains were hand dissected and stored in at −80 °C until used for cryosectioning. Fresh frozen 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 Biotechnology, Santa Cruz, CA, USA). 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 manufacture and diluted 1:3 in PBS just before application (Immunopure Ultra-sensitive ABC Rabbit IgG Staining Kit, Pierce, Rockford, IL, USA), Sections were incubated for 3 min with ImmunoPure metal enhanced DAB substrate kit (Pierce) prepared according to manufacturer 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, USA). No staining was detected when the primary antibody was omitted.

Image acquisition and analysis. Images were obtained with a BX-41 Olympus microscope coupled to a DP-70 Olympus digital camera with 10× magnifications and numerical aperture 0.25 from different hippocampal subfields such as cornu ammonis (CA) 1, CA3 and the medial and lateral blade of the dentate gyrus (DGmb and DGlb, respectively). Quantification of c-Fos immunopositive

nuclei was performed using the freeware ImageJ software (http://rsb.info.nih.gov/ij/). Briefly, for each brain area, a region of interest (ROI) was drawn and stored. Each ROI was composed by some circular areas (three or five), depending on the hippocampal field to analyze. For every section, each component of the ROI was individually situated in order to have the complete set of equidistant circular areas adjusted to the standard showed in Fig. 1A for each hippocampal field. The mean of the quantification in two sections of each hemisphere into each hippocampal subfield, for each rat was used for the statistical analyses. To remove noise and to avoid differences between immunostaining batches, each image was digitally smoothed and subtracted from the original one. Appropriate gray threshold and particle size were set for each area and maintained for all subjects. All brain regions were bilaterally counted in two sections for each rat between bregma −2.80 and −3.80 (Paxinos and Watson, 2007). Cell counts per mm^2 were analyzed for each HPC subfield using a MANOVA analysis with one between-group factor, the *treatment condition* (ICSS, Control-sham or Naive) and one within-group factor, the *hemisphere* (ipsi- or contralateral to electrode placement). The estimation of the effect size of the ICSS treatment was determined on the basis of the Glass Δ statistic [$\Delta=(\text{Mean}_{\text{reference group}}-\text{Mean}_{\text{comparison group}})/\text{SD}_{\text{reference group}}$] (Glass, 1976), taking the standard deviation of the ICSS group to be the standardizer. The interpretation of this measurement (Δ) was made in accordance with the criteria defined by Cohen (1988); which is that a value of 0.2 indicates a small effect, 0.5 a medium effect and 0.8 a large effect.

Brain dissection and RNA extraction for gene expression studies

For gene expression studies, 90 min after the end of the ICSS-treatment or the sham session, ICSS ($n=20$) and Control-sham ($n=20$) rats were sacrificed by decapitation as above. Brains were hand dissected and sliced with a brain matrix (Stoelting, Wood Dale, IL, USA). Slices between bregma −2.30 and −3.56 (anteroposterior) were used to dissect the ipsilateral hippocampi respect to the electrode. The tissue used as a reference in the first microarray experiment consisted of pooled hippocampal, amygdalar and cortical brain tissue of Naive ($n=1$), Control-sham ($n=2$) and ICSS ($n=1$) rats. This tissue combination was chosen as reference to ensure that genes expressed in Control-sham or ICSS samples were also expressed in some degree in the reference tissue, allowing us to better identify fold changes in expression. All tissues were conserved in RNA later (Ambion, Austin, TX, USA) for 48 h at 4 °C. Total RNAs were prepared with an RNeasy Lipid Tissue Mini kit according to manufacturer's protocol (Qiagen, Valencia, CA, USA). RNA was quantified by using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and quality was assessed with a 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

Microarray procedures

Three samples of ICSS hippocampi and three samples of Control-sham hippocampi were used for gene expression comparisons using oligonucleotide microarray analysis. In order to obtain enough mRNA for these studies, every sample consisted of four pooled ipsilateral hippocampi. Pooling has the additional advantage of improving accuracy and reducing biological variability allowing a reduction in the number of arrays required, even when fewer than three samples are used, as demonstrated by Kendziorski et al. (2005). Two microarray experiments were performed with the same samples, one with a common reference design, and the other with a direct comparison design. A diagram of the comparisons performed in the two microarrays experiments is depicted in Fig. S1 of the supplementary material. In the first microarray experiment, each cRNA sample (three from ICSS and three from Control-sham), was labeled with Cy5 and hybridized against the reference cRNA labeled with Cy3. In the second microarray analysis, three direct comparisons (biological replicates), each of an ICSS sample against a Control-sham sample in two-color hybridizations were performed and two additional technical replicates were also carried out using dye reversal. Thus, a total of 11 rat oligonucleotide microarrays from Agilent (G4130B), containing 22,000 probes, were hybridized: six in the first design and five in the second design. Briefly, 500 ng of total RNA from each sample were amplified by oligo-dT-T7 reverse transcription and labeled by *in vitro* transcription with T7 RNA polymerase in the presence of Cy5-CTP or Cy3-CTP using the Low Input RNA labeling kit (Agilent) and purified using RNAeasy columns (Qiagen). After fragmentation, 750 ng of labeled cRNA from each of the two samples were co-hybridized in *in situ* hybridization buffer (Agilent) for 17 h at 65 °C and washed at rt 1 min in 6× SSPE (saline sodium phosphate–EDTA) pH 7.4+0.005% sarcosine, 1 min at rt in 0.06X SSPE+0.005% sarcosine, 1 min in acetonitrile and 30 s in Dye Stabilization and Drying solution (Agilent).

The images were generated on a confocal microarray scanner (G2565BA, Agilent) at 10 μm resolution and quantified using GenePix 6.0 (Molecular Dynamics). Spots with signal intensities twice above the local background, not saturated and not flagged by GenePix were considered reliable and with a weight of 1 for normalization purposes, whereas the rest were given weights of 0.01. Extracted intensities were subtracted from the local background and the $\log_2$ ratios were normalized in an intensity-dependent fashion by the global lowess method with a span parameter of 0.3. Normalized $\log_2$ ratios were scaled between arrays to make all data comparable. Raw data were processed using MMARGE, a web implementation of limma (Smyth, 2005), a microarray analysis library developed within the Bioconductor project in the R statistical environment (Gentleman et al., 2004).

From the first experiment, where each sample was hybridized against a common reference, direct comparisons between ICSS hippocampi and control hippocampi were retrieved by subtracting the corresponding $\log_2$ ratio values. Such ICSS versus control $\log_2$ ratios were calculated for the same pairs of samples as were hybridized together in the second experiment. Hence, the combined data set used for statistical analyses consisted of three ICSS versus control $\log_2$ ratio samples from the first experiment and the same three comparisons plus two additional technical replicates from the second experiment. These data are given in the supplementary Table S2.

A linear mixed model was applied to analyze differential expression in the combined data set using the limma package (Smyth, 2005). Differences in expression between ICSS hippocampi and control hippocampi were assessed by testing the intercept of the linear model for a deviation from zero. An effect-coded covariate indicating in which experiment each sample was processed was included in the model in order to adjust for a possible batch effect of the two different experiments. Furthermore, the mixed model approach allows (using the limma function duplicateCorrelation) accounting for the fact that technical replicates are supposed to be more similar than biological replicates. The repeated use of the same biological samples in the second experiment as well as the dye-swap hybridizations were considered as technical replication. *P*-values were adjusted for multiple testing using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995). A fold change cutoff of 1.2 and a q-value of 0.05, setting an FDR of 5%, were used to choose relevant genes. The R code used for the differential expression analysis described above and $\log_2$ ratio data used in this analysis are given in the supplementary file S3 and S4 respectively.

Quantitative real time–polymerase chain reaction (PCR) analysis

Quantitative RT-PCRs were carried out comparing the ICSS versus Control-sham conditions using independent samples from the

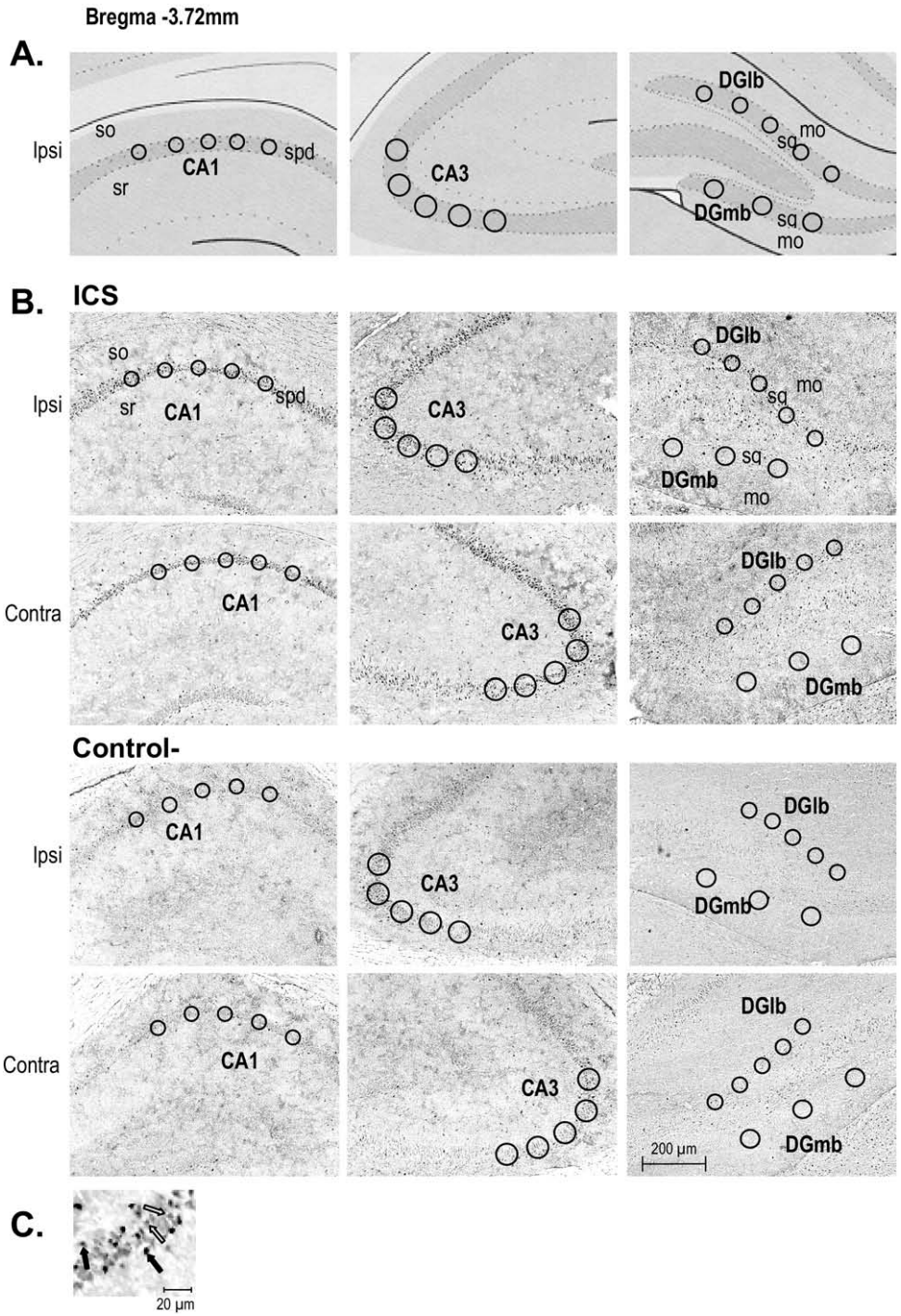


Fig. 1. c-Fos immunolocalization. (A) Defined ROIs, composed of several circular areas, for each hippocampal subfield studied, in the ipsilateral hemisphere to the ICSS electrode location. The standard placement is superposed to a coronal section adapted from Paxinos and Watson's atlas (2007), corresponding to coordinate AP −3.72 mm to bregma. (B) Representative photomicrographs (with the corresponding adapted ROIs) of c-Fos immunohistochemistry from one subject from the ICSS group and one from the Control-sham group in hippocampal subfields (scale bar=200 μm; stereotaxic coordinates AP between −2.80 and −3.80 to bregma). (C) High magnification of a CA1 region photomicrograph corresponding to an ICSS subject (scale bar=20 μm). Black arrows indicate some high c-Fos immunoreactive neurons, counted as positive cells. White arrows indicate some low c-Fos immunoreactive neurons, counted as non-positive cells. CA1: field CA1, Ammon's horn; CA1so: stratum oriens; CA1spd: pyramidal layer, deep; CA1-sr: stratum radiatum; CA3: field CA3, Ammon's horn; DGlb-mo: DGlb-molecular layer; DGlb-sg: DGlb-granule cell layer; DGmb-mo: DGmb-molecular layer; DGmb-sg: DGmb-granule cell layer.

microarray experiments. The number of samples used for these experiments varied from an $n=3$ to an $n=8$, depending on the variability of the expression of each specific gene. Every sample consisted of two to four pooled ipsilateral hippocampi. For cocaine

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

Gene name	5′–3′ Primer sequence	GenBank ID
Hspa1a	f: GAGGAGGTGGATTAGAGGCTTTTC	NM_031971
	r: TTGATAAGAATCGTGCACCAGC	
Fos	f: CCAAGCGGAGACAGATCAACTT	NM_022197
	r: TCTTTCAGTAGATTGGCAATCTCG	
Pgst2	f: ATCAAATTACCGCTGAAGCCC	NM_017232
	r: ATGTTCCAGACTCCCTTGAAGTG	
Ret	f: ATTGGGTGTTGCCCTTGCT	NM_012643
	r: GTTCTGGTCTGCGGGTAGGT	
Cart	f: GCCAAGTCCCCATGTGTGAC	NM_017110
	r: CACCCCTTCACAAGCACTTCA	
Dnajb1	f: GCCGAATGTTTTCCACAGAAT	NM_001108441
	r: TTCCCACCGCCTGCCTAT	
Sgk	f: TCGGGGCTGTCTTGTATGAG	NM_019232
	r: GTGCCTTGCTGAGTTGGTGA	
FKbp5	f: GAGCCGTTTGTCTTTAGCCTTG	NM_001012174
	r: GCCAGCAGAGCCGTAAGC	
Adcyap1	f: CCTACGCCTTTACTACCCAGC	NM_016989
	r: TTTGCGTAGGCTTCGTTAAG	
Pde1a	f: CATGGTTGGTTTGACATATCCTG	NM_030871
	r: TATGCTCCCCGCTTGCTT	
Hprt	f: AAAGGACCTCTCGAAGTGTTGG	NM_012583
	r: AAGTGCTCATTATAGTCAAGGGCA	

(f) Forward primer; (r) reverse primer. GenBank identification available at http://www.ncbi.nlm.nih.gov/.

and amphetamine-regulated transcript (Cart) gene the primers previously reported by Marie-Claire et al. (2003) were used and for the other genes, specific primers were designed with Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) (Table 1). We used hypoxanthine guanine phosphoribosyl transferase (Hprt) gene as our control housekeeping (reference) as this had not changed in expression level in the study. Total RNA was treated with the DNA free kit (Ambion) and cDNA was synthesized from 100 ng of total RNA using Superscript III (Invitrogen) reverse transcriptase, random 15mers primers and RNase out inhibitor (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR reaction cocktails (25 μl) contained cDNA, Power SYBR Green PCR master mix (Applied Biosystems) water and custom primers (Invitrogen). The PCR reactions were performed in an ABI PRISM 7300 Sequence Detector System (Applied Biosystems). Each reaction was performed in triplicate. The mRNA abundances for each candidate gene were calculated using the following formula: Relative Transcript Abundance$=$(E target)$^{\Delta Ct\ target(control-sample)}$/(E reference)$^{\Delta Ct\ reference(control-sample)}$ (Pfaffl, 2001), where E is real-time PCR efficiency calculated as Efficiency$=10^{(-1/slope)}$, and control is the mean of Ct of Control samples. Data were analyzed using one side *t*-test of $\log_{10}$ ratio, and differences were considered significant at $P<0.05$.

RESULTS

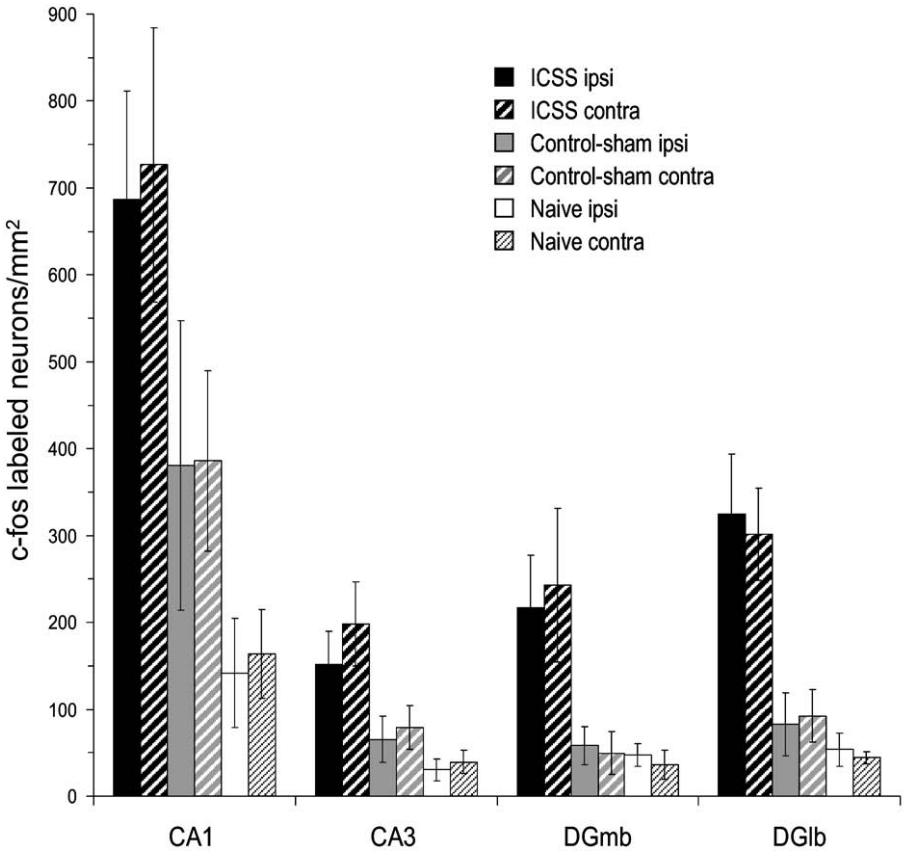
ICSS behavior

All rats in the ICSS groups rapidly learned to press the lever, indicating the rewarding effects of the brain stimulation. The mean values ($\pm$SD) of ICSS variables for the rats used in the immunohistochemistry experiment ($n=5$) were OI (343.33$\pm$72.34 μA), highest response rate (70.66$\pm$21.07 responses/min), treatment duration (66.00$\pm$36.29 min) and total responses (2917.42$\pm$116.44 lever pressings). The mean values ($\pm$SD) of the same ICSS variables for the rats used in the gene profiling studies ($n=21$) were OI (173.33$\pm$120.97 μA), highest response rate (76.43$\pm$18.36 responses/min), treatment duration (50.76$\pm$12.71 min), and total responses (3174.44$\pm$430.10 lever pressings). Some of the rats used in these studies underwent small seizures 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. Correlation analyses showed no relationship between the ICSS variables and number of positive c-Fos cells in any hippocampal subfield (see Table S5 in the Supplementary Material). These results imply that neither the motor activity during ICSS treatment (measured as the highest response rate and the total number of lever pressings in the treatment session) nor the intensity of stimulation (OI) seems to determine the level of c-Fos expression in the hippocampus. Importantly, the parameters of the ICSS treatment (100% OI, frequency and number of trains of the electrical stimulation, the animal's rate of response and mean time spent to self-administer the treatment) used here are within the range of values obtained in our previous studies showing enhancement of both hippocampus-dependent or independent learning and memory (Aldavert-Vera et al., 1996; Ruiz-Medina et al., 2008a,b; Segura-Torres et al., 1991; Soriano-Mas et al., 2005).

c-Fos immunohistochemistry

We analyzed c-Fos immunolabeling in the hippocampal subfields CA1 (pyramidal layer), CA3 (pyramidal layer), DGmb (granule cell layer), and DGlb (granule cell layer), in the ipsilateral and contralateral hemispheres to the electrode placement. Immunoreactive cells exhibited a dark brown nucleus clearly detectable from the surrounding background tissue. We compared the number of immunopositive nuclei among hippocampus of ICSS, Control-sham and Naive groups of rats by using the ImageJ processing program.

The quantitative results of c-Fos immunolabeling (neurons/mm^2) in the CA1, CA3, DGmb and DGlb subfields for ICSS, Control-sham and Naive groups are summarized in Fig. 2 (see also Table S6 in the Supplementary Material). In our analyses, we aimed to determine if there was a difference in the number of c-Fos immunopositive nuclei in the various hippocampal subfields among the three experimental groups, also considering the expression in ipsilateral versus contralateral areas. In the MANOVA analysis, 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), were used. First of all, the MANOVA analyses showed a statistically significant higher number of c-Fos immunopositive cells in ICSS rats compared with the Control-sham and Naive rats in CA3 [$F(1,10)=8.71$; $P=0.014$; $F(1,10)=16.16$; $P=0.002$ respectively], DGmb [$F(1,10)=6,37$; $P=0.03$; $F(1,10)=7.25$; $P=0.023$ respectively] and DGlb [$F(1,10)=14,37$; $P=0.004$; $F(1,10)=19.69$; $P=0.001$ respectively]. Although, the plotted data suggested similar tendencies for c-Fos induction within the CA1 hippocampal



		CA1		CA3		DGmb		DGlb	
		ipsi	contr	ipsi	contra	ipsi	contr	ipsi	contr
Δ de GLASS Index	ICSS *vs.* Control-sham	0,92	1,65	1,62	2,39	3,6	3,91	3,33	3,45
	ICSS *vs.* Naive	4,34	5,49	4,83	5,75	6,33	6,20	7,08	19,58

Fig. 2. Effects of ICSS on the c-Fos expression in hippocampus. Bar graph depicting the mean number of c-Fos immunopositive cells (neurons/mm^2±SD) in CA1, CA3, DGmb and DGlb hippocampal subfields, in the ipsilateral and the contralateral hemispheres respect to the electrode placement (numerical values are expressed in Table S6 of Supplementary Material). MANOVA analyses showed that ICSS treatment increased the number of c-Fos positive cells in all the hippocampal subfields with respect to the Naive subjects, and in CA3 and DG (medial and lateral blades) with respect to the Control-sham subjects. Table shows the Glass Δ statistic of standardized differences between ICSS and both non-stimulated groups (Control-sham and Naive).

subfield, this effect was only significant between ICSS and Naive rats [$F(1,10)=10.76$; $P=0.008$], but did not reach statistical significance between ICSS and Control-sham groups [$F(1,10)=3.67$; $P=0.085$]. No differences were observed between the nonstimulated groups (Control-sham and Naive). Fig. 2 also shows the values of the Glass Δ statistic of standardized differences between ICSS and Control-sham and Naive groups. In general, Glass Δ values were very high (between 0.93 and 2.17) suggesting that, based on the criteria defined by Cohen (Cohen, 1988), the effect of ICSS treatment on c-Fos expression in the hippocampus was of a large magnitude.

Second, our quantitative analyses confirmed our qualitative assessments that ICSS caused similar levels of c-Fos induction ipsilaterally and contralaterally in all three hippocampal subfields. No statistically significant differences were observed between the hemispheres ipsilateral and contralateral to the electrode location in any hippocampal region for any group. Moreover, differences between groups were observed independently of the hemisphere (interaction group by hemisphere factor was not significant) thus, it can be concluded that the activating effect of ICSS treatment on c-Fos induction was bilateral.

Fig. 1B shows differences of c-Fos hippocampal expression between ICCS rats and Control-sham animals. Interestingly, not all cells in every one of the analyzed hippocampal regions had the same intensity of c-Fos labeling and only a proportion of them showed detectable ICSS-induced increases of c-Fos immunoreactivity (see Fig. 1C), suggesting that not all cells contribute in the same level to the hippocampal ICSS gene regulation response. In contrast, for the group of rats that experienced seizure

activity (see above) during ICSS treatment we found that most of CA1, CA3, and dentate gyrus (DG) hippocampal neurons displayed similar c-Fos immunoreactivity (data not shown). Overall, these findings suggest that ICSS leads to the activation of gene transcription in discrete cells of the hippocampal formation.

Gene profiling in the hippocampus after the ICSS treatment

To understand what molecular signaling pathways affected by ICSS could be involved in learning and memory facilitation, we analyzed hippocampal gene expression. In these studies we used a more delayed time point than in the c-Fos immunohistochemistry analyses in order to identify not only immediate-early genes, but also slightly delayed early genes.

We performed an ICSS regulation gene profiling study using oligonucleotide microarrays. Three samples of Control-sham and three of ICSS hippocampal mRNA were compared by dual color-hybridization using a total of 11 rat oligonucleotide (22,000 probes) microarrays as detailed in the Experimental Procedures. Rats were sacrificed 90 min after ICSS or sham treatments. Data of relative expression ratios between ICSS and Control-sham samples of all the 11 hybridizations were analyzed as described above and a maximum stringency of a *P*-value of 0.05, was used to choose relevant genes. As suggested by our c-Fos immunohistochemistry labeling results, not all cells are stimulated in the same way by ICSS and do not contribute in the same dosage to the total changes in hippocampal gene expression. Moreover, very low increments of signaling proteins may exert significant effects (Roberto et al., 2001). For these reasons, we decided to set a criterion that would select as genes of interest those that showed a fold change starting from a 1.2 threshold-intensity ratio, which represents an increment of 20% labeling intensity in the total hippocampal cell population. Data of the microarray analysis is provided in the Supplementary Material (Table S2). With this criterion, a total of 60 expressed sequence tags (ESTs) from the microarrays were found to be differentially expressed, representing 59 different genes, as some genes are spotted in a duplicate fashion within the array. Thus, 0.27% of the 22,000 genes examined were determined to show differential hippocampal expression associated to ICSS. Forty-five genes were upregulated in the hippocampus of ICSS-treated rats, compared to controls, and 14 were downregulated. For our subsequent analyses, we focused exclusively on the 35 ESTs representing defined or predicted genes that encoded proteins for which a function is known or inferred (see Table 2A). The complete list of differentially expressed genes identified in our studies, including those of unknown function, is shown in Table S2 of the Supplementary Material.

Interestingly, we found numerous genes encoding proteins related to signal transduction machinery, some of which have been associated with learning and memory or neuroplasticity. Among such genes we report those encoding different neuropeptides (Cart) or intercellular signaling molecules (Gremlin 1 homolog, cysteine knot superfamily, *Grem1*), receptors (Ret proto-oncogene, Ret), transcription factors (c-Fos), molecules of the signal transduction machinery (serum/glucocorticoid regulated kinase, *Sgk*; phospholipase C-like 1, *Plcl1*; and pleckstrin homology domain containing family F with FYVE domain-member 1, *Plekhf1*), and other enzymes that may strongly contribute to signal transduction (guanine deaminase, *Gda*). The ICSS-induced Fos mRNA overexpression is in accordance with the c-Fos protein overexpression observed in several hippocampal areas in our immunohistochemistry experiments. In addition, other genes identified as modulated by ICSS in the hippocampus may be associated to cell stress responses that might be associated to neuroprotective mechanisms. These genes encode protein chaperones and co-chaperones (heat shock 70 kDa protein 1 A, *Hspa1a*; DnaJ-Hsp40-homolog subfamily B member 1, *Dnajb1*; crystallin, alpha B, *Cryab*; heat shock 10 kDa protein 1, *Hspe1*; Bcl2-associated athanogene 3, *Bag3*; and FK506 binding protein 5, *Fkbp5*), antiapoptotic proteins (the transcription factor TSC22 domain family 3, *Tsc22d3*), and a regulatory protein of proteasomal degradation (Ubiquilin 1, *Ubqln1*).

For further validation of the gene expression changes caused by ICSS with quantitative real time-PCR we only focused our attention in those genes that could influence learning and memory, lead to the neural plasticity changes required for long-term memory, or collaborate in the memory restoring capacities of ICSS. We tested seven representative genes that showed significant differential expression in our arrays, Hspa1a, Fos, Ret, Cart, Dnajb1, Sgk, FKbp5. We also tested three genes, encoding signaling proteins relevant in learning and memory, that appeared among the differentially expressed genes only in the second microarray experiment, but not in the combined analysis: prostaglandin-endoperoxide synthase 2, *Ptgs2*, which has a significant role in hippocampal-dependent tasks (Rall et al., 2003; Teather et al., 2002; Sharifzadeh et al., 2006), adenylate cyclase activating polypeptide 1, *Adcyap1*, which facilitates the extinction of active avoidance response (Adamik and Telegdy, 2005), and calmodulin-dependent-phosphodiesterase 1 A, *Pde1a*, which belongs to the family of phosphodiesterases, reported to regulate memory tasks (Blokland et al., 2006).

To confirm the microarray results, we performed quantitative real time-PCRs with new hippocampal samples from the same brain area as in the microarrays experiments (biological replicates). The results of this quantitative real time-PCR study corroborated the observed differential expression for the seven genes arising from our microarray analysis, validating the results obtained from our microarray experiments and data analyses. Ptgs2, Adcyap1 and Pde1a, when analyzed with a higher number of samples with quantitative real-time PCR (see Fig. 3), where validated as differentially regulated, and thus we do consider these three candidate genes to be among those that are influenced by ICSS to the LH in the hippocampus (Table 2B). Fig. 3 illustrates the relative hippocampal mRNA levels for these genes between the ICSS versus Control-sham conditions as determined by the quantitative

Table 2A. Genes differentially expressed in the rat hippocampus 90 min after ICSS demonstrated by microarray analysis, and for which a protein of known or predicted function has been defined

Gene symbol	Gene name (*) genes confirmed by real time-PCR	Aliases	GenBank ID	FC		Learning and memory	LTP	Other forms of synaptic plasticity	Neurite outgrowth	Neurogenesis	Neuroprotection	Apoptosis	HSE	GC
Hspa1a	*Heat shock 70 kD protein 1 A	HSP70-1A	NM_031971	5.02	UP	ind (1)					+(2)	anti (3)	+(4)	
Bag3	Bcl2-associated athanogene 3	CAIR-1	NM_001011936	1.91	UP						+(5)	anti (6)		
Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	GPDH	NM_022215	1.76	UP									
Cryab	Crystallin, alpha B	HSPB5	NM_012935	1.65	UP						+(7)	anti (7)	+(8)	ind (9)
Fos	*FBJ murine osteosarcoma viral oncogene homolog		NM_022197	1.64	UP	+(10,11)	+(11)		+(12)					
Tsc22d3	TSC22 domain family 3	GILZ, Dsipi	NM_031345	1.57	UP							anti (13)		ind (13)
Cart	*Cocaine- and amphetamine-regulated transcript		NM_017110	1.55	UP				+(14)		+(15)	anti (15)		ind (16)
Sgk	*SERUM/glucocorticoid regulated kinase	Sgk1	NM_019232	1.50	UP	+(17)	+(18)	+(18)			+(19)	anti (19)		ind (19)
Ret	*Ret proto-oncogene		NM_012643	1.48	UP				+(20)	+(21)	+(4,22)	anti (4)		
Upp1	Uridine phosphorylase 1		NM_001030025	1.45	UP									
Fkbp5	*FK506 binding protein 5	FKBP51	NM_001012174	1.45	UP							anti (23)		ind (24)
Per2	Period homolog 2 (*Drosophila*)		NM_031678	1.42	UP									
Errfi1	ERBB receptor feedback inhibitor 1	MIG6, GENE 33	NM_001014071	1.39	UP									ind (25)
Cnksr3	Cnksr family member 3	MAGI1	NM_001012061	1.37	UP									
S100a9	S100 calcium binding protein A9 (calgranulin B)	CAGB	NM_053587	1.34	UP									ind (26)
Cacybp	Calcyclin binding protein	SIP	NM_001004208	1.32	UP									
Klf15	Kruppel-like factor 15		NM_053536	1.30	UP									ind (27)
Hspe1	Heat shock 10 kDa protein 1 (chaperonin 10)	HSP10	NM_012966	1.30	UP							anti (28)	+(4)	
Grem1	Gremlin 1 homolog, cysteine knot superfamily (*Xenopus laevis*)		NM_019282	1.30	UP					+(29)				
Dnajb1	*DnaJ (Hsp40) homolog, subfamily B, member 1	HSP40	XM_341663	1.29	UP						+(2)	anti (2)	+(4)	

Table 2A. continued

Gene symbol	Gene name (*) genes confirmed by real time-PCR	Aliases	GenBank ID	FC		Learning and memory	LTP	Other forms of synaptic plasticity	Neurite outgrowth	Neurogenesis	Neuroprotection	Apoptosis	HSE	GC
Usp54	Ubiquitin-specific protease 54		BC097982	1.28	UP									
Plcl1	Phospholipase C-like 1	PRIP-1	NM_053456	1.26	UP			+(30)						
Nid67	Putative small membrane protein NID67		AF313411	1.26	UP									
Plekhf1	Pleckstrin homology domain containing, family F member 1	LAPF	NM_001013148	1.26	UP	ind (31)						pro (32)		
Jmjd1a	Jumonji domain containing 1 A		NM_175764	1.24	UP									
Ubqln1	Ubiquilin 1		NM_053747	1.21	UP						+(33)	anti (33)		
Bnip1	BCL2/adenovirus E1B 19 kDa-interacting protein 1		NM_080897	−1.22	DW							pro (34)		
Slc22a8	Solute carrier family 22 (organic anion transporter), member 8		NM_031332	−1.22	DW									
Ca4	Carbonic anhydrase 4		NM_019174	−1.24	DW									
Gda	Guanine deaminase	Cypin, cytoplasmic PSD95 interactor	NM_031776	−1.28	DW				−(35)					
Slco1a4	Solute carrier organic anion transporter family, memb 1a4		NM_131906	−1.35	DW									
Ly6h-predicted	Lymphocyte antigen 6 complex, locus H (predicted)		XM_235426	−1.36	DW									
Ddc	Dopa decarboxylase		NM_012545	−1.44	DW									
Trt	Transthyretin		NM_012681	−3.91	DW									

(A) Genes differentially expressed demonstrated by microarray analysis. Genes with differential gene expression that had been verified with real-time PCR are indicated with (*) in the Gene Name column. In (A) FC represents the fold change between gene expressions of ICSS and Control groups from microarray analysis and in we also include information indicating if particular genes had been previously reported to promote or inhibit memory and learning, different forms of neuroplasticity and neuroprotective functions. Identification of the HSE within the gene promoter is indicated (+) and reported GC gene induction or inhibition are also indicated (ind). (+) gene induction promotes this function or gene downregulation inhibits it; (−) gene induction inhibits this function or gene downregulation promotes it; (ind) induces the gene expression; (inh) inhibits the gene expression; (anti) antiapoptotic; (pro) proapoptotic. References: (1) Pizarro et al. (2003); (2) Takeuchi et al. (2002); (3) Giffard et al. (2004); (4) Myers and Mulligan (2004); (5) Carra et al. (2008); (6) Doong et al. (2002); (7) Ousman et al. (2007); (8) Somasundaram and Bhat (2004); (9) Scheier et al. (1996); (10) (Countryman et al., 2005b); (11) Fleischmann et al. (2003); (12) Gil et al. (2004); (13) Yachi et al. (2007); (14) Wu et al. (2006); (15) Mao et al. (2007); (16) Koylu et al. (2006); (17) von Hertzen and Giese (2005); (18) Ma et al. (2006); (19) Humbert and Saudou (2005); (20) Zhang et al. (2006); (21) Barlow et al. (2003); (22) Bakshi et al. (2006); (23) Giraudier et al. (2002); (24) Park et al. (2007); (25) Xu et al. (2006); (26) Hsu et al. (2005); (27) Teshigawara et al. (2005); (28) Lin et al. (2004); (29) Katoh and Katoh (2005); (30) Mizokami et al. (2007); (31) Park et al. (2006); (32) Chen et al. (2005); (33) Wang and Monteiro (2007); (34) Zhang et al. (2003); (35) Charych et al. (2006).

Table 2B. Genes differentially expressed in the rat hippocampus 90 min after ICSS demonstrated by quantitative analysis

Gene symbol	Gene name	Aliases	GenBank ID	FC		Learning and memory	LTP	Other forms of synaptic plasticity	Neurite outgrowth	Neurogenesis	Neuroprotection	Apoptosis	HSE	GC
Ptgs2	Prostaglandin-endoperoxide synthase 2	COX-2	NM_017232	2.63	UP	+(36,37)	+(7)		+(8)	+(9)				
Adcyap1	Adenylate cyclase activating polypeptide 1	PACAP	NM_016989	1.55	UP	+(39)	+(40)	+(41)	+(42)	+(42)	+(43)	anti (43)		ind (44)
Pde1a	Phosphodiesterase 1 A, calmodulin-dependent	HCAM1	NM_030871	−1.23	DW	−(47)								

(B) Genes differentially expressed in the rat hippocampus 90 min after ICSS demonstrated by quantitative real-time PCR analysis. In (B) FC corresponds to fold change gene expression from real-time PCR analysis. We also include information indicating if particular genes had been previously reported to promote or inhibit memory and learning, different forms of neuroplasticity and neuroprotective functions. Identification of the HSE within the gene promoter is indicated (+) and reported GC gene induction or inhibition are also indicated (ind). (+) gene induction promotes this function or gene downregulation inhibits it; (−) gene induction inhibits this function or gene downregulation promotes it; (ind) induces the gene expression; (inh) inhibits the gene expression; (anti) antiapoptotic; (pro); proapoptotic. References: (7) Ousman et al. (2007); (8) Somasundaram and Bhat (2004); (9) Scheier et al. (1996); (36) Teather et al. (2002); (37) Sharifzadeh et al. (2006); (38) Albrecht (2007); (39) Kadoyama et al. (2001); (40) Sasaki et al. (2003); (41) Adamik and Telegdy (2005); (42) Matsuyama et al. (2003); (43) Roberto et al. (2001); (44) Falluel-Morel et al. (2007); (45) Brenneman (2007); (46) Yang et al. (2007); (47) Blokland et al. (2006).

Fig. 3. Validation of microarray data by quantitative reverse transcriptase-PCR (real time-PCR). For several genes that had been identified as being differentially expressed in the hippocampus after ICSS by using the microarray analysis, the changes in transcript levels were verified by quantitative PCR. Tree genes arising from a less stringent microarray analysis were also validated: Ptgs2, Adcyap1 and Pde1a. Shown is the bar graph depicting the relative hippocampal mRNA expression levels between the ICSS versus Control conditions for the genes in the microarray study (spotted areas) and the same genes determined by quantitative PCRs (black areas). Data are presented as log 10 of mean fold change and standard deviations are indicated with the error bars. The number of samples used for each gene in real-time PCR assay was $n=3$ (Hspa1a), $n=5$ (Ptgs2, Ret, Dnajb1), $n=6$ (Fos, Cart, Adcyap1), $n=7$ (Sgk, FKbp5) and $n=8$ (Pde1a). * $P \leq 0.05$ versus control group.

PCRs and the microarrays studies (see also Table S7 in Supplementary Material). The results demonstrated the significant upregulation of Hspa1a, Fos, Ptgs2, Ret, Cart, Dnajb1, Sgk, FKbp5 and Adcyap1 (Student's *t*-test, $P<0.05$ each). In addition, we showed that the mRNA encoding Pde1a is downregulated in the hippocampus following ICSS (Student's *t*-test, $P<0.05$). Thus, all the genes tested were confirmed including genes with low a fold difference threshold in the microarray, such as Dnajb1 (X1.28). Overall, these results demonstrate that we were able to confirm the changes in expression seen in our microarray studies with the levels of stringency and threshold chosen, since all the genes tested were validated using a technique other than microarray analyses to assess changes in gene expression at the level of mRNA (real-time PCR).

DISCUSSION

Our studies presented here are the first to demonstrate that ICSS to the LH can induce a plurality of changes in hippocampal gene expression. Specifically, here we report that one ICSS session (2500 trains) (i) induces an early increase in c-Fos expression in several areas of the hippocampus (CA3, DGmb and DGlb) and (ii) modulates the expression of a set of 62 early genes in the hippocampus.

The nature of ICSS behavior, in which animals have to perform an operant response to obtain electrical stimulation in rewarding brain areas, involves several behavioral components and brain systems. This complexity makes it difficult to dissociate which component of ICSS is the main factor responsible for the neuronal activation in the hippocampus. In any case, our aim was to determine which changes in gene expression occur in hippocampus due to

the ICSS treatment as a whole. The parameters and conditions used for the ICSS treatment (electrode location, electrical current parameters, number of trains self-administered and number of sessions) are the same that we have previously demonstrated that enhance active avoidance memory, which takes place immediately after the training session (Aldavert-Vera et al., 1996; Redolar-Ripoll et al., 2002). Similar ICSS parameters also enhance hippocampus-dependent learning and memory (Ruiz-Medina et al., 2008a).

ICSS to the LH induces hippocampal increases in c-Fos expression

The ICSS treatment caused an increment of immunopositive nuclei for c-Fos immunochemistry in CA3 and DG (medial and lateral blades) compared with the two non-stimulated control groups (Control-sham and Naive). The changes in c-Fos expression in the CA1 subfield were also higher in the ICSS compared with the Naive condition, but only a tendency was observed compared with the Control-sham group.

Since no differences were observed between Naive and Control-sham groups in any hippocampal subfield, we can suggest that the amount of handling administered, the stereotaxic intervention or the ICSS-box exposure did not significantly affect hippocampal activation at the time it was evaluated. Moreover, because the Control-sham rats in the present study have been implanted, handled and allowed to explore the ICSS-box in a way similar to that of the ICSS rats, we can rule out factors, as exploratory behavior, exposure to novel context or contextual learning, as the main causes of the observed effects. Likewise, we also can rule out the possibility that increases in c-Fos expression were caused by the operant response because task-dependent increases in c-Fos-labeled nuclei only have been observed after initial ICSS training and not following complete acquisition (Bertaina-Anglade et al., 2000). Since in the present study the ICSS-related operant response is acquired very fast (only about 25 trains of stimulation are enough for the establishment of a stable operant response), and since rats had learned the ICSS behavior two days before the ICSS treatment, it can be assumed that at the time of sacrifice ICSS rats have a complete acquisition of the operant response and no hippocampal c-Fos expression would be expected due to this variable. The phase for gene analyses in the hippocampus was that of expression of the acquired operant response. On the other hand, the observed increment in c-Fos expression in hippocampal subfields does not seem attributable to motor activity inherent to the ICSS treatment, since no correlation between c-Fos expression and any motor measure of the rats' ICSS behavior (as response-rates or total number of responses performed in the treatment session) was observed. It is important to mention that motor activity related to bar pressing is probably not involved in the observed hippocampal changes in gene expression. Previous studies involving electrical stimulation of other brain regions, such as the central thalamus, that does not imply motor activity (since stimulation is administered by the experimenter), also enhances cognitive performance and activates specific regulation of gene expression in the hippocampus (Shirvalkar et al., 2006). Thus, motor activity does not seem to be associated with the changes in hippocampal gene expression of our present studies. In any case, since ICSS implies both, reward and motor activity, we cannot rule out that hippocampus modulation could be due to possible additive effects of both.

The present findings suggest that different hippocampal areas seem to respond with differential sensibility to our ICSS-LH paradigm (no differences were found between ICSS and Control-sham group in CA1). We should note that no differential connections between LH and the any of the hippocampal subfields have been shown. However, LH lesions produced extensive cellular loss specifically in CA3 (Leach et al., 1980), and ICSS-LH induces neuronal plasticity (such as promoting increase in dendritic length and in the numerical density of synapses) also in CA3 field (Rao et al., 1994, 1999).

Moreover, the pattern of ICSS-induced c-Fos expression, with discrete cells responding to ICSS stimulation in every one of the analyzed hippocampal subfields, may indicate a cellular specific ICSS response. This is in contrast to what occurred in the rats that experienced seizures, which displayed a massive unspecific response, in terms of c-Fos induction. Thus, specific networks associated to learning and memory may be activated by ICSS in the absence of seizure activity. There are several ways by which ICSS-LH could modulate hippocampal activity. First, the hippocampus receives inputs from the dopaminergic mesolimbic pathway, originated into the ventral tegmental area and activated by ICSS-LH (Schultz, 2000). Moreover, the hippocampus could be activated indirectly by projections from other arousal-related systems, also activated by LH rewarding stimulation (Arvanitogiannis et al., 1997; Sarter and Bruno, 2000). Finally, recent data suggest that the HPC could be also directly activated by the LH stimulation through the fornix (Saunders and Aggleton, 2007).

Although we do not know of previous studies about the same kind of induction in the hippocampus, c-Fos has been induced by rewarding brain stimulation in other brain areas, such as the amygdala and the medial prefrontal cortex (see Waraczynski, 2006). Increases in c-Fos expression in the DG subfield have been also observed after thalamic brain stimulation capable of remediating cognitive disability (Shirvalkar et al., 2006).

ICSS affects early expression of genes related to learning and memory, neural plasticity, and neuroprotection

In the reported gene expression studies we identified a total of 62 ICSS-regulated genes in the hippocampus, 59 of them arising from the microarray analysis and three from independent quantitative real-time analysis. More specifically, results from our gene expression studies showed that 51% of the 37 genes that encode proteins of known or predicted function expressed by the ICSS memory-facilitative treatment may promote directly or indirectly learning and memory or neuroprotection (see Table 2, Fig. 4). As

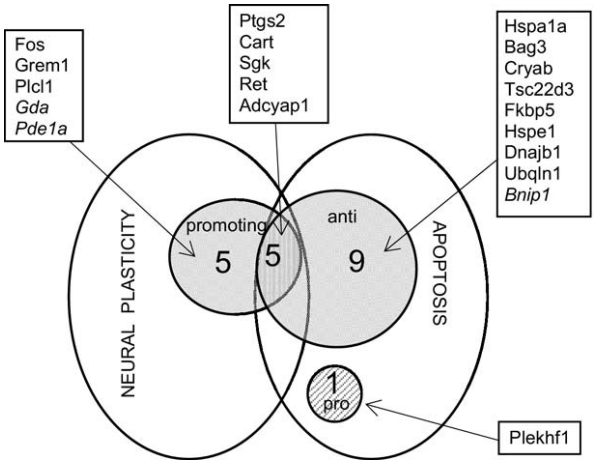


Fig. 4. Diagram showing a functional distribution of the ICSS-regulated genes related to neural plasticity and cell survival functions (apoptosis), and their interactions. The regulated set promoting neural plasticity includes upregulated genes reported to enhance neural plasticity and downregulated genes described as inhibitors. The regulated set promoting cell survival includes upregulated antiapoptotic genes and a downregulated apoptotic genes. Downregulated genes are represented in cursive. Numbers represent the number of genes included in each condition. (pro) proapoptotic; (anti) antiapoptotic.

expected, since we examined gene expression 90 min after the ICSS treatment, we found numerous genes encoding proteins of the signal transduction machinery and, more surprisingly, another set of early expressed genes related to neuroprotection. We discuss the identified genes according to their known or possible relation with such processes, focusing on behavioral and neural plasticity, including possible roles on neurogenesis and on neuroprotection. Both neurogenesis and neuroprotection are mechanisms that could be related to the memory facilitation and restorative effects of the ICSS.

Genes related to learning and memory. Some of the ICSS-upregulated genes observed in the present study (see Table 2) have been directly related to explicit or relational learning and memory in rats. In general, induction of these genes is observed in the hippocampus after training in hippocampal-dependent tasks, and impairment of these tasks is observed after specific gene-inhibition. For example, Fos, expressed in both the present microarray and immunolabeling studies, is required in hippocampus-dependent spatial and associative learning tasks (Countryman et al., 2005a; Fleischmann et al., 2003). Ptgs2, also known as Cox 2, has a significant role in acquisition (Rall et al., 2003), consolidation (Teather et al., 2002) and retrieval of spatial memory (Sharifzadeh et al., 2006). Similarly, the Sgk protein kinase was shown to play an important role in both consolidation and reconsolidation of contextual fear conditioning (von Hertzen and Giese, 2005). Interestingly, Hspa1a, the gene encoding heat shock protein 70, was identified as an ICSS-induced gene in our studies and has been shown to be upregulated in the hippocampus after spatial learning in the Morris water maze (Pizarro et al., 2003).

Other upregulated genes in our work have been observed in other brain regions related to implicit learning and memory. For example, the neuropeptide Adcyap1 (also known as PACAP) facilitated the extinction of an active avoidance response (Adamik and Telegdy, 2005), whereas the signal transduction adaptor protein, Plekhf1, was induced in the cerebellum after training in classic eye-blink conditioning (Park et al., 2006). Finally, from the observed downregulated genes, the implication of the Pde1a in learning and memory has not been studied but the inhibition of other phosphodiesterases has been reported to improve memory as well (Blokland et al., 2006).

As we discuss below, these and other genes expressed in our gene expression studies promote molecular and/or neural changes, such as synaptic potentiation or depression, dendritic branching, neurogenesis or neuroprotection, that could be directly or indirectly related to learning and memory improvement by ICSS.

Genes related to neural plasticity. Some upregulated genes of the present work could participate in several forms of neural plasticity required for learning and/or short- and long-term memory consolidation and extinction (Wang et al., 2006). Genes such as Adcyap1, Fos, Ptgs2 and Sgk (see Table 2) have been implicated in long-term potentiation (LTP) in the hippocampus and amygdala. Plcl1, which expresses a phospholipase homologous to PLC-delta, but with no catalytic activity, Adcyap1 and Sgk have been related to other synaptic changes in the hippocampus (see Table 2).

Of relevance to structural neural plasticity is the fact that several of the overexpressed genes in the hippocampus after ICSS have been shown to promote neurite outgrowth (Adcyap1, Cart, Ret, Ptgs2 and Fos), see Table 2. The downregulated Gda, a post-synaptic density 95 interactor, can also be involved in regulation of dendritic branching (Charych et al., 2006). Then, it is possible to suggest that these and other genes could mediate the observed dendritic changes induced by ICSS reported by Shankaranarayana Rao et al. (1999).

Genes related to neurogenesis. Adult neurogenesis in the hippocampus may also play an important role in hippocampus-dependent learning and memory (Aimone et al., 2006; Leuner et al., 2006). Curiously, some recent data showed increased hippocampal neurogenesis with place learning in poor, but not good, learner rats. It is suggested that increased hippocampal neurogenesis in poor place learners may act to improve the efficiency of a poorly functioning hippocampus (Epp et al., 2007). Interestingly, our previous work has also showed a higher capacity of post-training ICSS to facilitate learning and memory in poor versus good learner rats (Aldavert-Vera et al., 1996). In agreement with these experimental observations, the present microarray study has also found ICSS upregulated genes that may be involved in neurogenesis and then, indirectly, could be related to the learning and memory improvements seen with ICSS. Thus, Adcyap1 (Falluel-Morel et al., 2007), Grem1 (Katoh and Katoh, 2005), Ret (Barlow et al., 2003), and Ptgs2 (Sasaki et al., 2003) have

been shown, using gene targeting or over-expression approaches, to promote neurogenesis and play important roles in neural cell fate determination.

Genes related to neuroprotection. Neuroprotective-related genes configure another important group among the ICSS-upregulated genes of our microarray work (see Table 2). These genes could contribute to the survival of newly generated neurons after neurogenesis that seems to be directly related to the level of successful hippocampal-dependent learning (Dalla et al., 2007). Another possible significance is that such genes might be important for neuroprotection of preexistent neurons. Both forms of neuroprotection could serve to maintain neuronal integrity in order to prevent or ameliorate some forms of cognitive impairment. Among the genes in this group, are those related to protein folding function, that is the chaperones Hspa1a, Dnajb1, Cryab, Hspe1, and the co-chaperones, Bag3, Fkbp5, all of which have been demonstrated to have antiapoptotic properties, and some of them (Hspa1a, Dnajb1, Cryab, and Bag 3) have also been proven to exert neuroprotective functions (see Table 2). Signaling through the upregulated gene Ret, the glial-derived neurotrophic factor (GDNF) receptor, may favor protein folding by activating the gene promoter region HSE (heat shock element) (Myers and Mulligan, 2004), present in the five chaperones upregulated in our array study (Table 2). Ret has been related to antiapoptotic and neuroprotective responses (Myers and Mulligan, 2004; Bakshi et al., 2006) and GDNF-Ret signaling has been correlated with cognitive enhancement in rats following traumatic brain injury (Bakshi et al., 2006). We also report upregulation of a gene related to regulation of protein degradation that may be neuroprotective, Ubqln1, that may reduce protein aggregates and toxicity of expanded polyglutamine proteins (Wang and Monteiro, 2007). As protein aggregation is considered to be part of the etiology of chronic neurodegenerative diseases, such as Alzheimer's, or stroke (Giffard et al., 2004), proteins promoting protein folding or preventing aggregation appear to be important for conferring neuroprotection, being proposed as possible approaches to prevent or treat neurodegenerative diseases (Fujikake et al., 2008) and could be implicated in the therapeutic benefits reported for DBS (Hamani et al., 2007). Concerning ICSS's learning and memory enhancing properties, protein folding related mechanisms may be a relevant since protein synthesis is a pivotal aspect allowing the consolidation of long-term memories. Thus, we cannot rule out that some of the mentioned chaperones could collaborate in this function, as was suggested for Hspa1a in spatial learning consolidation (Pizarro et al., 2003). Overall, the numerous set of genes encoding proteins that may be neuroprotective could be involved in the mechanisms underlying the potential of ICSS for restoring learning and memory capacities observed in aging and brain-damaged rats (Aldavert-Vera et al., 1997; Redolar-Ripoll et al., 2003).

Future studies may determine the mechanisms by which ICSS to the LH induces hippocampal changes in gene expression. The c-Fos immunolabeling study showing discrete cells responding to ICSS stimulation suggests that specific networks are activated by ICSS. Other candidates to participate in the facilitating effect of ICSS on learning and memory could be the glucocorticoids (GCs), since many of the present regulated genes by ICSS that may promote either neural plasticity or neuroprotection have been previously shown to be regulated by GCs (see Table 2). In fact, it has been reported that ICSS activates the hypothalamus–pituitary–adrenal axis leading to elevated levels of circulating GCs (Nakahara et al., 2000) and moderate increases in GCs facilitate performance on hippocampal dependent memory tasks (Roozendaal et al., 2006).

The present work provides results that contribute to studies examining gene expression changes induced by DBS techniques. There is little knowledge about the molecular mechanisms of DBS techniques currently used for treatment of Parkinson's disease, chronic pain and various affective disorders (Shirvalkar et al., 2006; Henning et al., 2007; Salin et al., 2002). Only one previous study using gene expression profiling in response to intracranial stimulation has been reported, but the electrical stimulation was given to the subthalamic nucleus and was not a self-stimulation paradigm (Henning et al., 2007). Moreover, this previous study limited the gene expression analyses to the stimulation area, contrasting with our study where we were interested in determining the effects of LH ICSS in a remote brain area involved in cognitive processes, the hippocampus. The ICSS-induced gene expression changes observed by us, involving specific signaling pathways related with neuroplasticity and neuroprotection, points to the hippocampus as being an interesting area of study for establishing neural and molecular mechanisms activated by DBS techniques applied to neurodegenerative or cognitive diseases.

CONCLUSION

The present work showed that ICSS in the LH modulates the hippocampus activity, a well-known component of the brain memory systems. ICSS regulated genes in the hippocampus encoding components of the signal transduction machinery involved in learning and memory, neural plasticity, neurogenesis and neuroprotection. Moreover, among the learning- and memory-related genes, we showed that c-Fos, an immediate-early transcription factor, is upregulated in specific hippocampal regions as a result of ICSS in the LH. These findings led us to suggest that ICSS may promote cognitive improvement by enhancing cellular and molecular mechanisms related to neural plasticity, and neurogenesis and neuroprotection appear as other possible candidate mechanisms in this facilitation. The present study represents, therefore, a new approach to investigate the neural and cognitive effects of rewarding intracranial stimulation, and other DBS therapies. New experiments are now necessary to test the hypotheses discussed here and clarify the mechanisms by

which electrical stimulation can improve learning and memory.

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REFERENCES

Adamik A, Telegdy G (2005) Effects of pituitary adenylate cyclase polypeptide (PACAP) on extinction of active avoidance learning in rats: involvement of neurotransmitters. Regul Pept 127:55–62.

Aimone JB, Wiles J, Gage FH (2006) Potential role for adult neurogenesis in the encoding of time in new memories. Nat Neurosci 9:723–727.

Albrecht D (2007) Angiotensin-(1-7)-induced plasticity changes in the lateral amygdala are mediated by COX-2 and NO. Learn Mem 14:177–184.

Aldavert-Vera L, Costa-Miserachs D, Massanes-Rotger E, Soriano-Mas C, Segura-Torres P, Morgado-Bernal I (1997) Facilitation of a distributed shuttle-box conditioning with posttraining intracranial self-stimulation in old rats. Neurobiol Learn Mem 67:254–258.

Aldavert-Vera L, Segura-Torres P, Costa-Miserachs D, Morgado-Bernal I (1996) Shuttle-box memory facilitation by posttraining intracranial self-stimulation: differential effects in rats with high and low basic conditioning levels. Behav Neurosci 110:346–352.

Arvanitogiannis A, Flores C, Shizgal P (1997) Fos-like immunoreactivity in the caudal diencephalon and brainstem following lateral hypothalamic self-stimulation. Behav Brain Res 88:275–279.

Bakshi A, Shimizu S, Keck CA, Cho S, LeBold DG, Morales D, Arenas E, Snyder EY, Watson DJ, McIntosh TK (2006) Neural progenitor cells engineered to secrete GDNF show enhanced survival, neuronal differentiation and improve cognitive function following traumatic brain injury. Eur J Neurosci 23:2119–2134.

Barlow A, de Graaff E, Pachnis V (2003) Enteric nervous system progenitors are coordinately controlled by the G protein-coupled receptor EDNRB and the receptor tyrosine kinase RET. Neuron 40:905–916.

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B 57:289–300.

Bertaina-Anglade V, Tramu G, Destrade C (2000) Differential learning-stage dependent patterns of c-Fos protein expression in brain regions during the acquisition and memory consolidation of an operant task in mice. Eur J Neurosci 12:3803–3812.

Blokland A, Schreiber R, Prickaerts J (2006) Improving memory: a role for phosphodiesterases. Curr Pharm Des 12:2511–2523.

Brenneman DE (2007) Neuroprotection: a comparative view of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Peptides 28:1720–1726.

Carra S, Seguin SJ, Lambert H, Landry J (2008) HspB8 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulator of macroautophagy. J Biol Chem 283:1437–1444.

Charych EI, Akum BF, Goldberg JS, Jornsten RJ, Rongo C, Zheng JQ, Firestein BL (2006) Activity-independent regulation of dendrite patterning by postsynaptic density protein PSD-95. J Neurosci 26: 10164–10176.

Chen Y, Fenoglio KA, Dube CM, Grigoriadis DE, Baram TZ (2006) Cellular and molecular mechanisms of hippocampal activation by acute stress are age-dependent. Mol Psychiatry 11:992–1002.

Chen W, Li N, Chen T, Han Y, Li C, Wang Y, He W, Zhang L, Wan T, Cao X (2005) The lysosome-associated apoptosis-inducing protein containing the pleckstrin homology (PH) and FYVE domains (LAPF), representative of a novel family of PH and FYVE domain-containing proteins, induces caspase-independent apoptosis via the lysosomal-mitochondrial pathway. J Biol Chem 280:40985–40995.

Cohen J (1988) Statistical power analysis for the behavioural sciences. New York: Academic Press.

Cotman CW, Berchtold NC (2002) Exercise: a behavioral intervention to enhance brain health and plasticity. Trends Neurosci 25:295–301.

Countryman RA, Kaban NL, Colombo PJ (2005a) Hippocampal c-fos is necessary for long-term memory of a socially transmitted food preference. Neurobiol Learn Mem 84:175–183.

Countryman RA, Orlowski JD, Brightwell JJ, Oskowitz AZ, Colombo PJ (2005b) CREB phosphorylation and c-Fos expression in the hippocampus of rats during acquisition and recall of a socially transmitted food preference. Hippocampus 15:56–67.

Dalla C, Bangasser DA, Edgecomb C, Shors TJ (2007) Neurogenesis and learning: acquisition and asymptotic performance predict how many new cells survive in the hippocampus. Neurobiol Learn Mem 88:143–148.

Departament de Medi Ambient. Generalitat de Catalunya (1995) Generalitat de Catalunya. LAW 5/1995 on protection of animals used for experimentation and other scientific purposes. DOGC num. 2073.

Doong H, Vrailas A, Kohn EC (2002) What's in the "BAG"—a functional domain analysis of the BAG-family proteins. Cancer Lett 188:25–32.

Epp JR, Spritzer MD, Galea LA (2007) Hippocampus-dependent learning promotes survival of new neurons in the dentate gyrus at a specific time during cell maturation. Neuroscience 149:273–285.

Falluel-Morel A, Chafai M, Vaudry D, Basille M, Cazillis M, Aubert N, Louiset E, de Jouffrey S, Le Bigot JF, Fournier A, Gressens P, Rostene W, Vaudry H, Gonzalez BJ (2007) The neuropeptide pituitary adenylate cyclase-activating polypeptide exerts anti-apoptotic and differentiating effects during neurogenesis: focus on cerebellar granule neurones and embryonic stem cells. J Neuroendocrinol 19:321–327.

Fleischmann A, Hvalby O, Jensen V, Strekalova T, Zacher C, Layer LE, Kvello A, Reschke M, Spanagel R, Sprengel R, Wagner EF, Gass P (2003) Impaired long-term memory and NR2A-type NMDA receptor-dependent synaptic plasticity in mice lacking c-Fos in the CNS. J Neurosci 23:9116–9122.

Fujikake N, Nagai Y, Popiel HA, Okamoto Y, Yamaguchi M, Toda T (2008) Heat shock transcription factor 1-activating compounds suppress polyglutamine-induced neurodegeneration through induction of multiple molecular chaperones. J Biol Chem 283: 26188–26197.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5:R80.

Giffard RG, Xu L, Zhao H, Carrico W, Ouyang Y, Qiao Y, Sapolsky R, Steinberg G, Hu B, Yenari MA (2004) Chaperones, protein aggregation, and brain protection from hypoxic/ischemic injury. J Exp Biol 207:3213–3220.

Gil GA, Bussolino DF, Portal MM, Pecchio AA, Renner ML, Borioli GA, Guido ME, Caputto BL (2004) c-Fos activated phospholipid synthesis is required for neurite elongation in differentiating PC12 cells. Mol Biol Cell 15:1881–1894.

Giraudier S, Chagraoui H, Komura E, Barnache S, Blanchet B, Le-Couedic JP, Smith DF, Larbret F, Taksin AL, Moreau-Gachelin F, Casadevall N, Tulliez M, Hulin A, Debili N, Vainchenker W (2002) Overexpression of FKBP51 in idiopathic myelofibrosis regulates the growth factor independence of megakaryocyte progenitors. Blood 100:2932–2940.

Glass GV (1976) Primary, secondary, and meta-analysis of research. Ed Res 10:3–8.

Hamani C, Neimat JS, Lozano AM (2007) Deep brain stimulation and chemical neuromodulation: current use and perspectives for the future. Acta Neurochir Suppl 97:127–133.

Henning J, Koczan D, Glass A, Karopka T, Pahnke J, Rolfs A, Benecke R, Gimsa U (2007) Deep brain stimulation in a rat model modulates TH, CaMKIIa and Homer1 gene expression. Eur J Neurosci 25:239–250.

Hsu K, Passey RJ, Endoh Y, Rahimi F, Youssef P, Yen T, Geczy CL (2005) Regulation of S100A8 by glucocorticoids. J Immunol 174:2318–2326.

Humbert S, Saudou F (2005) Huntington's disease: intracellular signaling pathways and neuronal death. J Soc Biol 199:247–251.

Huston JP, Mueller CC (1978) Enhanced passive avoidance learning and appetitive T-maze learning with post-trial rewarding hypothalamic stimulation. Brain Res Bull 3:265–270.

Kadoyama K, Takahashi Y, Higashida H, Tanabe T, Yoshimoto T (2001) Cyclooxygenase-2 stimulates production of amyloid beta-peptide in neuroblastoma x glioma hybrid NG108-15 cells. Biochem Biophys Res Commun 281:483–490.

Katoh Y, Katoh M (2005) Comparative genomics on SLIT1, SLIT2, and SLIT3 orthologs. Oncol Rep 14:1351–1355.

Kendziorski C, Irizarry RA, Chen KS, Haag JD, Gould MN (2005) On the utility of pooling biological samples in microarray experiments. Proc Natl Acad Sci U S A 102:4252–4257.

Koylu EO, Balkan B, Kuhar MJ, Pogun S (2006) Cocaine and amphetamine regulated transcript (CART) and the stress response. Peptides 27:1956–1969.

Leach L, Whishaw IQ, Kolb B (1980) Effects of kainic acid lesions in the lateral hypothalamus on behavior and hippocampal and neocortical electroencephalographic (EEG) activity in the rat. Behav Brain Res 1:411–431.

Lee TH, Jang MH, Shin MC, Lim BV, Kim YP, Kim H, Choi HH, Lee KS, Kim EH, Kim CJ (2003) Dependence of rat hippocampal c-fos expression on intensity and duration of exercise. Life Sci 72:1421–1436.

Leuner B, Gould E, Shors TJ (2006) Is there a link between adult neurogenesis and learning? Hippocampus 16:216–224.

Lin KM, Hollander JM, Kao VY, Lin B, Macpherson L, Dillmann WH (2004) Myocyte protection by 10 kD heat shock protein (Hsp10) involves the mobile loop and attenuation of the Ras GTP-ase pathway. FASEB J 18:1004–1006.

Ma YL, Tsai MC, Hsu WL, Lee EH (2006) SGK protein kinase facilitates the expression of long-term potentiation in hippocampal neurons. Learn Mem 13:114–118.

Mao P, Ardeshiri A, Jacks R, Yang S, Hurn PD, Alkayed NJ (2007) Mitochondrial mechanism of neuroprotection by CART. Eur J Neurosci 26:624–632.

Marie-Claire C, Laurendeau I, Canestrelli C, Courtin C, Vidaud M, Roques B, Noble F (2003) Fos but not Cart (cocaine and amphetamine regulated transcript) is overexpressed by several drugs of abuse: a comparative study using real-time quantitative polymerase chain reaction in rat brain. Neurosci Lett 345:77–80.

Matsuyama S, Matsumoto A, Hashimoto H, Shintani N, Baba A (2003) Impaired long-term potentiation in vivo in the dentate gyrus of pituitary adenylate cyclase-activating polypeptide (PACAP) or PACAP type 1 receptor-mutant mice. Neuroreport 14:2095–2098.

Mizokami A, Kanematsu T, Ishibashi H, Yamaguchi T, Tanida I, Takenaka K, Nakayama KI, Fukami K, Takenawa T, Kominami E, Moss SJ, Yamamoto T, Nabekura J, Hirata M (2007) Phospholipase C-related inactive protein is involved in trafficking of gamma2 subunit-containing GABA(A) receptors to the cell surface. J Neurosci 27:1692–1701.

Myers SM, Mulligan LM (2004) The RET receptor is linked to stress response pathways. Cancer Res 64:4453–4463.

Nakahara D, Nakamura M, Oki Y, Ishida Y (2000) Lack of glucocorticoids attenuates the self-stimulation-induced increase in the in vivo synthesis rate of dopamine but not serotonin in the rat nucleus accumbens. Eur J Neurosci 12:1495–1500.

Ousman SS, Tomooka BH, van Noort JM, Wawrousek EF, O'Connor KC, Hafler DA, Sobel RA, Robinson WH, Steinman L (2007) Protective and therapeutic role for alphaB-crystallin in autoimmune demyelination. Nature 448:474–479.

Park J, Kim M, Na G, Jeon I, Kwon YK, Kim JH, Youn H, Koo Y (2007) Glucocorticoids modulate NF-kappaB-dependent gene expression by up-regulating FKBP51 expression in Newcastle disease virus-infected chickens. Mol Cell Endocrinol 278:7–17.

Park JS, Onodera T, Nishimura S, Thompson RF, Itohara S (2006) Molecular evidence for two-stage learning and partial laterality in eyeblink conditioning of mice. Proc Natl Acad Sci U S A 103: 5549–5554.

Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates. San Diego: Academic Press.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Pizarro JM, Haro LS, Barea-Rodriguez EJ (2003) Learning associated increase in heat shock cognate 70 mRNA and protein expression. Neurobiol Learn Mem 79:142–151.

Rall JM, Mach SA, Dash PK (2003) Intrahippocampal infusion of a cyclooxygenase-2 inhibitor attenuates memory acquisition in rats. Brain Res 968:273–276.

Ramkumar K, Srikumar BN, Shankaranarayana Rao BS, Raju TR (2008) Self-stimulation rewarding experience restores stress-induced CA3 dendritic atrophy, spatial memory deficits and alterations in the levels of neurotransmitters in the hippocampus. Neurochem Res 33:1651–1662.

Rao BS, Desiraju T, Meti BL, Raju TR (1994) Plasticity of hippocampal and motor cortical pyramidal neurons induced by self-stimulation experience. Indian J Physiol Pharmacol 38:23–28.

Rao BS, Desiraju T, Raju TR (1993) Neuronal plasticity induced by self-stimulation rewarding experience in rats—a study on alteration in dendritic branching in pyramidal neurons of hippocampus and motor cortex. Brain Res 627:216–224.

Rao BS, Raju TR, Meti BL (1999) Increased numerical density of synapses in CA3 region of hippocampus and molecular layer of motor cortex after self-stimulation rewarding experience. Neuroscience 91:799–803.

Redolar-Ripoll D, Aldavert-Vera L, Soriano-Mas C, Segura-Torres P, Morgado-Bernal I (2002) Intracranial self-stimulation facilitates memory consolidation, but not retrieval: its effects are more effective than increased training. Behav Brain Res 129:65–75.

Redolar-Ripoll D, Soriano-Mas C, Guillazo-Blanch G, Aldavert-Vera L, Segura-Torres P, Morgado-Bernal I (2003) Posttraining intracranial self-stimulation ameliorates the detrimental effects of parafascicular thalamic lesions on active avoidance in young and aged rats. Behav Neurosci 117:246–256.

Roberto M, Scuri R, Brunelli M (2001) Differential effects of PACAP-38 on synaptic responses in rat hippocampal CA1 region. Learn Mem 8:265–271.

Romanelli P, Di Matteo L, Cobellis G, Varriale B, Menegazzi M, Gironi Carnevale UA, Ruocco LA, Sadile AG (2007) Transcription factor expression, RNA synthesis and NADPH-diaphorase across the rat brain and exposure to spatial novelty. Behav Brain Res 184:91–100.

Roozendaal B, Okuda S, Van der Zee EA, McGaugh JL (2006) Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. Proc Natl Acad Sci U S A 103:6741–6746.

Ruiz-Medina J, Morgado-Bernal I, Redolar-Ripoll D, Aldavert-Vera L, Segura-Torres P (2008a) Intracranial self-stimulation facilitates a spatial learning and memory task in the Morris water maze. Neuroscience 154:424–430.

Ruiz-Medina J, Redolar-Ripoll D, Morgado-Bernal I, Aldavert-Vera L, Segura-Torres P (2008b) Intracranial self-stimulation improves memory consolidation in rats with little training. Neurobiol Learn Mem 89:574–581.

Salin P, Manrique C, Forni C, Kerkerian-Le Goff L (2002) High-frequency stimulation of the subthalamic nucleus selectively reverses dopamine denervation-induced cellular defects in the output structures of the basal ganglia in the rat. J Neurosci 22:5137–5148.

Sarter M, Bruno JP (2000) Cortical cholinergic inputs mediating arousal, attentional processing and dreaming: differential afferent regulation of the basal forebrain by telencephalic and brainstem afferents. Neuroscience 95:933–952.

Sasaki T, Kitagawa K, Sugiura S, Omura-Matsuoka E, Tanaka S, Yagita Y, Okano H, Matsumoto M, Hori M (2003) Implication of cyclooxygenase-2 on enhanced proliferation of neural progenitor cells in the adult mouse hippocampus after ischemia. J Neurosci Res 72:461–471.

Saunders RC, Aggleton JP (2007) Origin and topography of fibers contributing to the fornix in macaque monkeys. Hippocampus 17:396–411.

Scheier B, Foletti A, Stark G, Aoyama A, Dobbeling U, Rusconi S, Klemenz R (1996) Glucocorticoids regulate the expression of the stress protein alpha B-crystallin. Mol Cell Endocrinol 123:187–198.

Schultz W (2000) Multiple reward signals in the brain. Nat Rev Neurosci 1:199–207.

Segura-Torres P, Portell-Cortes I, Morgado-Bernal I (1991) Improvement of shuttle-box avoidance with post-training intracranial self-stimulation, in rats: a parametric study. Behav Brain Res 42: 161–167.

Shankaranarayana Rao BS, Raju TR, Meti BL (1999) Self-stimulation rewarding experience induced alterations in dendritic spine density in CA3 hippocampal and layer V motor cortical pyramidal neurons. Neuroscience 89:1067–1077.

Sharifzadeh M, Tavasoli M, Soodi M, Mohammadi-Eraghi S, Ghahremani MH, Roghani A (2006) A time course analysis of cyclooxygenase-2 suggests a role in spatial memory retrieval in rats. Neurosci Res 54:171–179.

Shirvalkar P, Seth M, Schiff ND, Herrera DG (2006) Cognitive enhancement with central thalamic electrical stimulation. Proc Natl Acad Sci U S A 103:17007–17012.

Smyth GK (2005) Limma: linear models for microarray data. In: Bioinformatics and computational biology solutions using R and Bioconductor (Gentleman R, Carey S, Dudoit S, Irizarry R, Huber W, eds), pp 397–420. New York: Springer.

Somasundaram T, Bhat SP (2004) Developmentally dictated expression of heat shock factors: exclusive expression of HSF4 in the postnatal lens and its specific interaction with alphaB-crystallin heat shock promoter. J Biol Chem 279:44497–44503.

Soriano-Mas C, Redolar-Ripoll D, Aldavert-Vera L, Morgado-Bernal I, Segura-Torres P (2005) Post-training intracranial self-stimulation facilitates a hippocampus-dependent task. Behav Brain Res 160:141–147.

Takeuchi H, Kobayashi Y, Yoshihara T, Niwa J, Doyu M, Ohtsuka K, Sobue G (2002) Hsp70 and Hsp40 improve neurite outgrowth and suppress intracytoplasmic aggregate formation in cultured neuronal cells expressing mutant SOD1. Brain Res 949:11–22.

Teather LA, Packard MG, Bazan NG (2002) Post-training cyclooxygenase-2 (COX-2) inhibition impairs memory consolidation. Learn Mem 9:41–47.

Teshigawara K, Ogawa W, Mori T, Matsuki Y, Watanabe E, Hiramatsu R, Inoue H, Miyake K, Sakaue H, Kasuga M (2005) Role of Kruppel-like factor 15 in PEPCK gene expression in the liver. Biochem Biophys Res Commun 327:920–926.

von Hertzen LS, Giese KP (2005) Memory reconsolidation engages only a subset of immediate-early genes induced during consolidation. J Neurosci 25:1935–1942.

Wang H, Kubica N, Ellisen LW, Jefferson LS, Kimball SR (2006) Dexamethasone represses signaling through the mammalian target of rapamycin in muscle cells by enhancing expression of REDD1. J Biol Chem 281:39128–39134.

Wang H, Monteiro MJ (2007) Ubiquilin overexpression reduces GFP-polyalanine-induced protein aggregates and toxicity. Exp Cell Res 313:2810–2820.

Waraczynski MA (2006) The central extended amygdala network as a proposed circuit underlying reward valuation. Neurosci Biobehav Rev 30:472–496.

Wirtshafter D (2005) Cholinergic involvement in the cortical and hippocampal Fos expression induced in the rat by placement in a novel environment. Brain Res 1051:57–65.

Wu B, Hu S, Yang M, Pan H, Zhu S (2006) CART peptide promotes the survival of hippocampal neurons by upregulating brain-derived neurotrophic factor. Biochem Biophys Res Commun 347:656–661.

Xu D, Patten RD, Force T, Kyriakis JM (2006). Gene 33/RALT is induced by hypoxia in cardiomyocytes, where it promotes cell death by suppressing phosphatidylinositol 3-kinase and extracellular signal-regulated kinase survival signaling. Mol Cell Biol 26:5043–5054.

Yachi K, Inoue K, Tanaka H, Yoshikawa H, Tohyama M (2007) Localization of glucocorticoid-induced leucine zipper (GILZ) expressing neurons in the central nervous system and its relationship to the stress response. Brain Res 1159:141–147.

Yang TT, Tsao CW, Li JS, Wu HT, Hsu CT, Cheng JT (2007) Changes of dopamine content and cell proliferation by dexamethsone via pituitary adenylate cyclase-activating polypeptide in PC12 cell. Neurosci Lett 426:45–48.

Yoganarasimha D, Meti BL (1999) Amelioration of fornix lesion induced learning deficits by self-stimulation rewarding experience. Brain Res 845:246–251.

Yoganarasimha D, Shankaranarayana Rao BS, Raju TR, Meti BL (1998) Facilitation of acquisition and performance of operant and spatial learning tasks in self-stimulation experienced rats. Behav Neurosci 112:725–729.

Zhang HM, Cheung P, Yanagawa B, McManus BM, Yang DC (2003) BNips: a group of pro-apoptotic proteins in the Bcl-2 family. Apoptosis 8:229–236.

Zhang Y, Zhu W, Wang YG, Liu XJ, Jiao L, Liu X, Zhang ZH, Lu CL, He C (2006) Interaction of SH2-Bbeta with RET is involved in signaling of GDNF-induced neurite outgrowth. J Cell Sci 119: 1666–1676.

APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2009.04.074.

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