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c-Fos expression induced by electroacupuncture at the Zusanli point in rats submitted to repeated immobilization

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Abstract

In laboratory animals, acupuncture needs to be performed on either anesthetized or, if unanesthetized, restrained subjects. Both procedures up-regulate c-Fos expression in several areas of the central nervous system, representing therefore a major pitfall for the assessment of c-Fos expression induced by electroacupuncture. Thus, in order to reduce the effect of acute restraint we used a protocol of repeated restraint for the assessment of the brain areas activated by electroacupuncture in adult male Wistar rats weighing 180-230 g. Repeated immobilization protocols (6 days, 1 h/day and 13 days, 2 h/day) were used to reduce the effect of acute immobilization stress on the c-Fos expression induced by electroacupuncture at the Zusanli point (EA36S). Animals submitted to immobilization alone or to electroacupuncture (100 Hz, 2-4 V, faradic wave) in a non-point region were compared to animals submitted to electroacupuncture at EA36S (4 animals/subgroup). c-Fos expression was measured in 41 brain areas by simple counting of cells and the results are reported as number of c-Fos-immunoreactive cells/10,000 μm^2. The protocols of repeated immobilization significantly reduced the immobilization-induced c-Fos expression in most of the brain areas analyzed ($P < 0.05$). Animals of the EA36S groups had significantly higher levels of c-Fos expression in the dorsal raphe nucleus, locus coeruleus, posterior hypothalamus and central medial nucleus of the thalamus. Furthermore, the repeated immobilization protocols intensified the differences between the effects of 36S and non-point stimulation in the dorsal raphe nucleus ($P < 0.05$). These data suggest that high levels of stress can interact with and mask the evaluation of specific effects of acupuncture in unanesthetized animals.

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Key words
- Acupuncture
- Zusanli point
- Immediate early genes
- c-Fos
- Restraint
- Habituation

Introduction

Studies of the mapping of neuronal activity using immunocytochemistry to detect c-Fos have shown that electroacupuncture (EA) at the Zusanli point (36S) up-regulates c-Fos expression in several central nervous system (CNS) areas as compared to EA at non-acupuncture points (1). In contrast, other studies using either non-acupuncture points or simply noxious stimulation whether or not applied to an accupoint have failed to dem-

onstrate that these responses are, in fact, specifically associated with stimulation of acupuncture points (2,3). In laboratory animals, acupuncture must be performed on either anesthetized or, if unanesthetized, restrained subjects. Both procedures are known to induce changes in several physiological parameters and can thus mask specific acupuncture responses. It has been well established that both anesthesia and immobilization up-regulate c-Fos expression in several areas of the CNS (4-6), thus representing a major limitation for the assessment of EA-induced c-Fos expression.

Repeated immobilization can markedly reduce restraint-induced c-Fos expression, causing habituation of the response. However, the patterns of neuronal activity induced by habituation to repeated stress are stressor-specific (7,8), allowing the effect of a new stimulus to be observed in animals previously submitted to repeated stress, i.e., habituation does not prevent the effect of a new stimulus (7). Therefore, to attenuate the effects of acute restraint on c-Fos expression, we used a protocol of repeated restraint which allowed us to differentiate the brain regions particularly responsive to EA.

We investigate here the c-Fos expression induced by EA at the Zusanli point (compared to EA at a non-point region and to immobilization alone) in animals previously submitted to two protocols of repeated immobilization. The results showed that EA at the Zusanli point induced higher levels of c-Fos expression in the dorsal raphe nucleus, locus coeruleus, posterior hypothalamus and central medial nucleus of the thalamus. Furthermore, repeated immobilization protocols reduced immobilization-induced c-Fos expression and intensified the differences between the effects of 36S and non-point stimulation in the dorsal raphe nucleus.

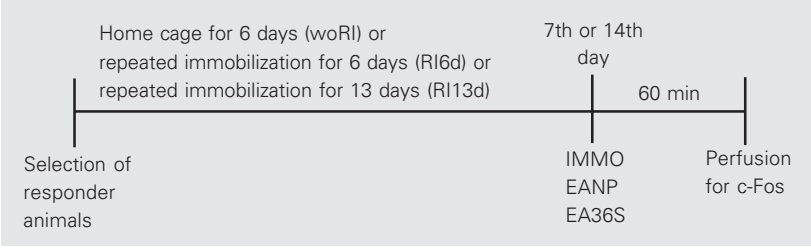

Figure 1. Experimental design. See legend to Table 1 for definitions of abbreviations.

Material and Methods

Subjects

Adult male Wistar albino rats, weighing 180-230 g, from the local breeding facilities (CEDEME-UNIFESP) were used in the present study. Animals were kept under conditions of controlled temperature (23 ± 2ºC) and illumination (12-h light-dark cycle, lights on at 7:00 am) and had free access to water and standard rat chow diet (Nuvilab®, Colombo, PR, Brazil). All experimental protocols were approved by the Animal Care and Use Committee of UNIFESP and complied with NIH guidelines on animal care (National Academy Press, Washington, DC, USA, 1996). Experiments were carried out between 9:00 am and 12:00 pm. The experimental design is shown in Figure 1.

Selection of responder animals

In order to reduce variations in the sensitivity to acupuncture, animals were initially classified as responders or non-responders to acupuncture. For this purpose, we performed a tail-flick (Ugo Basile®, Varese, Italy) test (9) after EA at the Zusanli point (36S) (described below). Rats exhibiting a significant increase (150%) in tail-flick latency were categorized as responders (1). Only responder animals were used in this study.

Experimental design

Responder animals were then assigned to one of three groups: i) without repeated immobilization (woRI): left undisturbed in their home cages for 6 days without any manipulation; ii) submitted to repeated immobiliza-

tion for 1 h/day for 6 days (RI6d), and iii) submitted to repeated immobilization for 2 h/day for 13 days (RI13d). On the 7th (woRI and RI6d groups) or 14th day (RI13d group), the animals were further divided into one of three subgroups: subjected to immobilization only (IMMO), immobilized and submitted to EA36S, and immobilized and submitted to EA at a non-acupoint site (EANP). Animals of all groups were then assessed for Fos-immunoreactivity in the CNS (4 animals/subgroup).

Immobilization and electroacupuncture stimulation

Animals were immobilized in a plastic cylinder that allowed performing the tail-flick test while maintaining access to all four limbs for the application of acupuncture needles. EA was bilaterally applied at point 36S or at a non-acupoint site. Stainless steel needles were inserted 5 mm deep into 36S, located between the tibia and the fibula, approximately 5 mm lateral to the anterior tubercle of the tibia (10). The non-acupoint site was located 5 mm lateral to the midline of the posterior surface of the hind limb. Each needle was independently stimulated for 60 min with an electrical current of faradic, bipolar and asymmetrical waves at 100 Hz using the asymmetric F1000 apparatus (Lautz, Rio Claro, SP, Brazil). Stimulus intensity was set at twice the threshold for a detectable muscle twitch (between 2 and 4 V) since this response is necessary for EA to be effective. This stimulus intensity is in the range of a previously published report (11). Animals of the IMMO groups were immobilized in the same apparatus as used for the EA groups and for the same period of time, but with no needle insertion or electrical stimulation.

Immunohistochemistry

One hour after the beginning of immobilization or EA stimulation, rats were deeply anesthetized (50 mg/kg thiopental, *ip*) and perfused transcardially with saline followed by 2.5% paraformaldehyde in 0.05 M potassium phosphate-buffered saline (KPBS), pH 7.4, at 4ºC. Brains were removed immediately after perfusion, stored at 4ºC in 30% sucrose and cut (32-µm coronal sections) with a freezing microtome. The sections were processed for the immunohistochemical detection of c-Fos protein using a conventional avidin-biotin-immunoperoxidase technique to localize antibodies raised against a synthetic N-terminal fragment of human Fos protein (Ab-5, Oncogene Sciences, San Diego, CA, USA). Briefly, free-floating sections were pretreated with hydrogen peroxidase, followed by sodium borohydride. Sections were treated with normal goat serum (1:100) and 0.3% Triton X-100 for 2 h and incubated with the primary antiserum at a dilution of 1:3000 in KPBS at room temperature for 24 h. Subsequently, the sections were incubated with a secondary antibody (goat anti-rabbit IgG 1:200; Vector Laboratories, Burlingame, CA, USA) for 90 min at room temperature, treated with 1:100 avidin-biotin complex (Vector) for 90 min, and submitted to a nickel-intensified diaminobenzidine reaction. Between steps, the sections were rinsed in 0.05 M KPBS, pH 7.4. The tissue was agitated in a rotary shaker between incubation and rinse steps. Sections were mounted on gelatin-coated slides, dried, dehydrated and coverslipped.

Counting c-Fos-positive nuclei and statistical analysis

The nomenclature and nuclear boundaries defined in Swanson's Stereotaxic Rat Brain Atlas were used in this study (12). Fos-immunoreactive nuclear profiles in different areas of the brain were counted using a BX50 Olympus microscope (Melville, NY, USA) coupled to a Macintosh-based image analysis system (Cupertino, CA, USA) and

Neurozoom software (La Jolla, CA, USA). The boundaries of the brain areas were identified using adjoining Nissl-stained sections. A template or outline was constructed for each brain nucleus or subnucleus based on the shape and size of the region (13). The location and relative size of each template are illustrated in Figure 2. The number of c-Fos-positive nuclei within each area was counted bilaterally (where possible) in two consecutive sections per animal and the mean value is reported as number of c-Fos- immunoreactive cells/10,000 μm^2. This counting procedure allowed a reliable time-effective analysis of c-Fos expression in 41 brain areas. Stereological methods were not employed in this study due to potential bias associated with counting nuclei in this manner, such as uncertainties as to the extent to which antiserum penetrates the thickness of the tissue sections and difficulties in defining the boundaries of the various cell groups of interest. Moreover, our interest was to make only relative comparisons of the strength of Fos induction as a function of treatment status (14).

Statistical analysis of c-Fos expression was performed by two-way ANOVA followed by Fisher's *post hoc* test, with main factors represented by repeated immobilization (woRI, RI6d, RI13d) versus acupuncture (IMMO, EANP, and EA36S). The level of significance was set at $P \leq 0.05$. All data are reported as mean ± SEM.

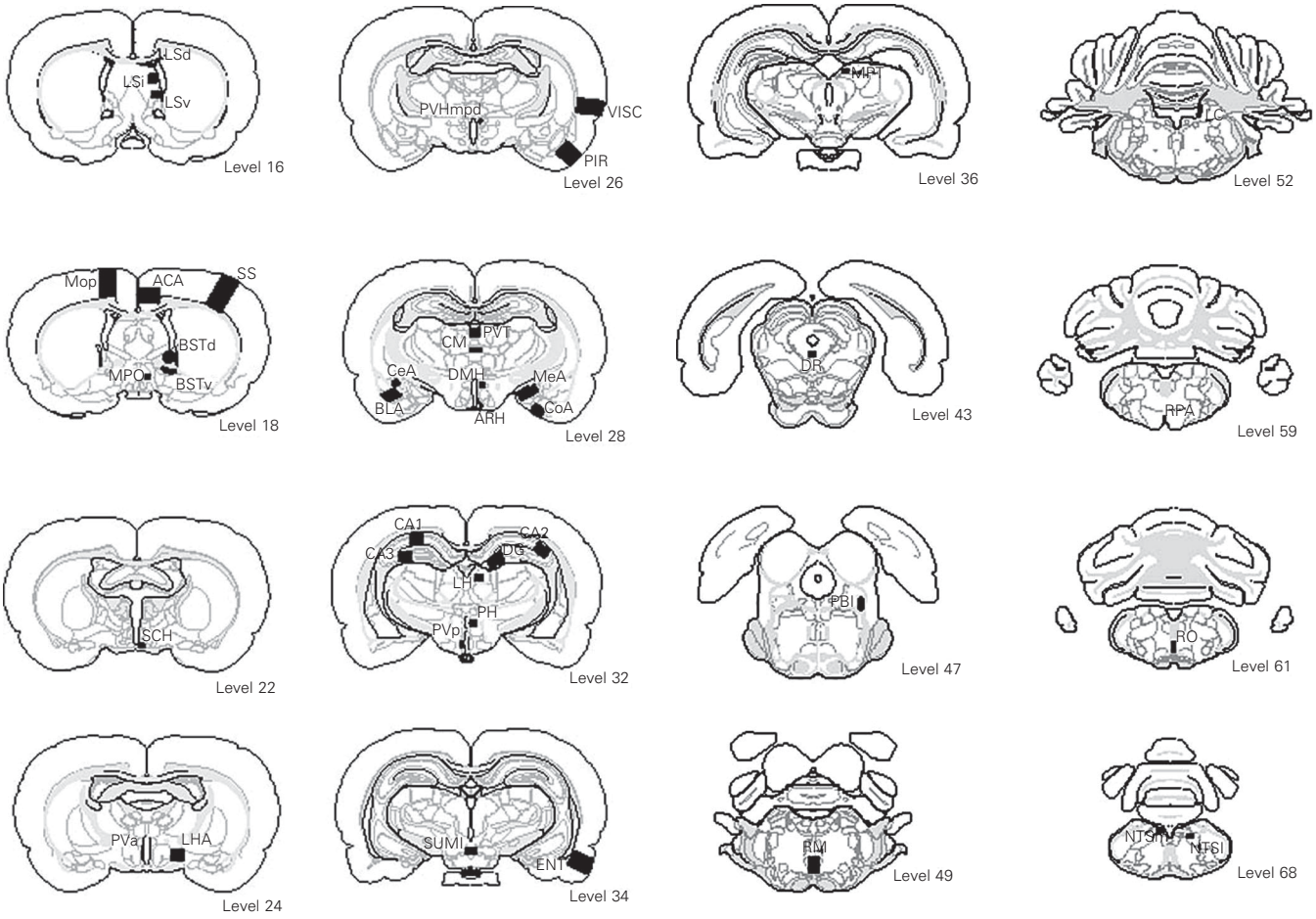

Figure 2. Diagrams illustrating the templates and relative sizes of the different brain areas subjected to counting of Fos-immunoreactive cells. The levels were based on Swanson's Stereotaxic Atlas of the Brain (12). Abbreviations are shown in the legend to Table 1.

Results

c-Fos-immunoreactive cells were detected in several brain areas of all animals, with a similar intensity in each cell, ranging from dark brown to black. Some Fos-immunoreactive cells stood out sharply from the background, whereas other stained neurons in the same nuclei showed less intense immunoreactivity. In animals submitted only to acute immobilization (IMMO woRI group), the pattern of c-Fos expression was similar to that previously described in the literature for stress-induced c-Fos expression (15,16), i.e., c-Fos expression was widely distributed in the brain, with moderate to intense staining in areas involved in the stress response (e.g., hypothalamus, locus coeruleus, amygdala, etc.). Under our laboratory conditions, intact naive animals (without any kind of manipulation) showed negligible c-Fos expression.

Effect of repeated immobilization

Quantitative analysis (two-way ANOVA) showed a clear and diffuse effect of repeated immobilization. In most of the studied areas, repeated immobilization protocols reduced immobilization-induced background c-Fos expression. In some regions, mainly those typically involved in the stress response (paraventricular nucleus of the hypothalamus (PVH), locus coeruleus, medial amygdala nucleus, cingulate cortex), the effect of repeated immobilization was highly significant ($P < 0.0001$) and *post hoc* analysis showed that both the RI6d and RI13d groups differed from the woRI group (Table 1 and Figure 3). In other brain areas such as hippocampal CA2 ($P = 0.03$), CA3 ($P = 0.01$) and dentate gyrus ($P = 0.03$), dorsolateral septum ($P = 0.003$), lateral habenula ($P = 0.04$), central medial nucleus of the thalamus ($P = 0.02$), and preoptic area ($P = 0.03$), differences were detected only between RI13d and woRI groups, while no statistical difference was detected between woRI and RI6d or between RI13d and RI6d. No detectable effects of immobilization were observed in the intermediate and ventral lateral septum, central nucleus of the amygdala, dorsomedial and supramammillary nuclei of the hy-

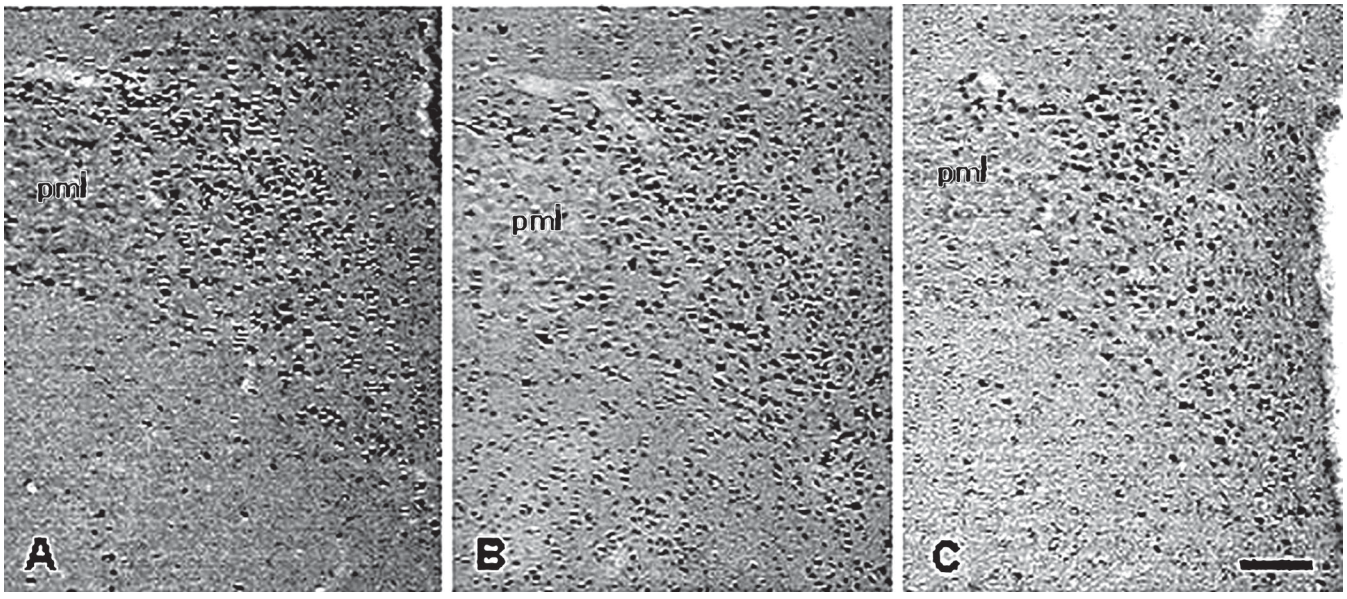

Figure 3. Photomicrograph illustrating the effect of repeated immobilization on c-Fos expression induced by immobilization in the paraventricular hypothalamic nucleus. *A*, Rats were left undisturbed in their home cages for 6 days; *B*, rats were submitted to 6 days of repeated immobilization (1 h/day); *C*, rats were submitted to 13 days of repeated immobilization (2 h/day). pml = posterior magnocellular part, lateral zone. Magnification bar: ~200 µm. Note a significant reduction in the number of c-Fos-immunoreactive cells in *B* and *C* (habituated groups) in relation to *A* (acute group).

Table 1. Eletroacupuncture-induced c-Fos expression in the brain of previously immobilized rats.

	woRI			RI6d			RI13d		
	IMMO	EANP	EA36S	IMMO	EANP	EA36S	IMMO	EANP	EA36S
Cortex									
ACA	0.50±0.08	0.38±0.09	0.443±0.08	0.194±0.03	0.192±0.05	0.203±0.04*	0.057±0.02	0.158±0.02	0.179±0.03*
Mop	0.48±0.04	0.393±0.09	0.394±0.07	0.153±0.02	0.217±0.03	0.194±0.03*	0.97±0.04	0.204±0.03	0.227±0.0*
SS	0.37±0.06	0.259±0.05	0.289±0.05	0.135±0.03	0.163±0.02	0.123±0.03*	0.098±0.05	0.145±0.03	0.199±0.03*
VISC	0.413±0.09	0.314±0.06	0.335±0.07	0.141±0.04	0.134±0.02	0.150±0.01*	0.098±0.05	0.128±0.02	0.179±0.05*
PIR	0.328±0.08	0.285±0.07	0.282±0.10	0.136±0.04	0.141±0.04	0.099±0.04*	0.082±0.03	0.170±0.2	0.256±0.80*
ENT	0.25±0.07	0.165±0.03	0.201±0.05	0.099±0.02	0.098±0.03	0.082±0.05*	0.045±0.01	0.146±0.03	0.164±0.05*
Hippocampus									
CA1	0.122±0.02	0.072±0.02	0.086±0.03	0.056±0.03	0.039±0.02	0.058±0.02*	0.044±0.02	0.041±0.01	0.05±0.01*
CA2	0.125±0.03	0.056±0.03	0.112±0.05	0.078±0.02	0.047±0.01	0.061±0.01	0.028±0.01	0.03±0.01	0.056±0.03*
CA3	0.117±0.02	0.086±0.02	0.113±0.03	0.091±0.01	0.081±0.02	0.083±0.01	0.061±0.02	0.042±0.01	0.055±0.03*
DG	0.091±0.02	0.076±0.02	0.08±0.02	0.067±0.02	0.052±0.02	0.058±0.01	0.036±0.01	0.031±0.02	0.053±0.02*
BST-amygdala									
BST (dorsal)	0.544±0.13	0.466±0.1	0.339±0.02	0.289±0.04	0.275±0.04	0.353±0.07*	0.158±0.04	0.2±0.04	0.239±0.06*
BST (ventral)	1.17±0.11	0.853±0.13	0.945±0.16	0.669±0.06	0.545±0.13	0.646±0.13*	0.333±0.06	0.427±0.06	0.519±0.06**
CeA	0.402±0.14	0.195±0.06	0.235±0.05	0.304±0.08	0.394±0.03	0.265±0.05	0.154±0.07	0.21±0.02	0.383±0.12
MeA	0.681±0.1	0.723±0.07	0.756±0.16	0.325±0.22	0.472±0.09	0.465±0.09*	0.109±0.04	0.320±0.05	0.455±0.12*
CoA	0.405±0.06	0.321±0.05	0.311±0.1	0.181±0.01	0.232±0.07	0.161±0.05*	0.076±0.03	0.230±0.05	0.343±0.11*
BLA#	0.525±0.07	0.338±0.03	0.364±0.06	0.20±0.03	0.294±0.07	0.250±0.05*	0.116±0.06	0.185±0.01	0.291±0.10*
Septal area									
LSd	1.219±0.406	1.070±0.235	1.312±0.398	1.055±0.358	0.609±0.143	0.992±0.424	0.445±0.027	0.492±0.023	0.771±0.209*
LSi	6.188±1.176	9.256±0.475	6.981±1.339	7.900±0.786	7.275±0.826	7.337±0.525	6.106±0.792	6.269±1.300	7.263±0.668
LSv	7.367±0.805	9.695±0.492	8.523±0.568	7.836±0.803	8.523±0.394	7.844±0.659	7.078±0.892	7.367±1.184	8.284±0.77
Thalamus-habenula									
LH	2.275±0.40	1.444±0.59	2.419±0.48	1.712±0.28	1.325±0.29	1.319±0.04	0.912±0.26	0.725±0.2	1.981±0.58*
CM+	1.480±0.23	1.062±0.23	1.551±0.27	0.789±0.18	0.832±0.25	1.297±0.32	0.426±0.05	0.602±0.09	1.219±0.38*
PVT	1.569±0.34	1.125±0.09	1.394±0.11	0.85±0.23	0.838±0.19	1.281±0.24	0.519±0.12	0.750±0.26	1.01±0.31*
Hypothalamus									
MPO	2.819±0.42	2.325±0.20	2.250±0.33	2.556±0.34	1.694±0.44	2.356±0.46	1.394±0.35	1.569±0.17	2.013±0.31*
SCH#	11.43±2.88	7.143±0.44	6.143±1.10	6.071±1.35	6.732±0.77	5.125±0.51*	2.804±0.25	6.714±1.05	5.196±1.13**
PVa#	10.04±0.57	6.104±1.24	7.208±0.47	5.438±0.64	3.771±0.90	6.250±1.2*	4.354±1.56	6.271±0.9	5.167±0.39*
LHA	2.097±0.21	1.350±0.23	1.538±0.25	1.562±0.89	0.997±0.34	1.253±0.34	0.953±0.25	1.112±0.16	1.481±0.19*
ARH	3.794±0.47	3.00±0.53	2.931±0.44	2.075±0.32	2.6±0.37	2.594±0.55*	0.831±0.26	1.838±0.36	2.044±0.38*
DMH	2.513±0.3	2.169±0.22	2.581±0.22	2.056±0.33	2.087±0.57	2.069±0.55	1.188±0.39	1.544±0.13	2.55±0.47*
PH+	1.756±0.36	0.881±0.12	1.456±0.16	1.04±0.11	0.762±0.23	0.869±0.13*	0.512±0.14	0.512±0.07	1.119±0.37*
PVp	3.150±0.28	2.625±0.32	3.487±0.99	1.913±0.38	2.00±0.36	2.213±0.39*	0.975±0.20	1.025±0.22	1.8±0.37**
SUMI	3.1±0.49	2.913±0.57	2.706±0.36	3.169±0.21	3.263±0.41	3.525±0.45	2.356±0.49	3.069±0.47	3.094±0.58
PVHmpd	31.04±2.08	29.35±1.9	32.92±3.1	20.75±3.78	18.06±4.42	21.12±1.47*	10.06±1.97	11.73±2.51	16.96±2.73**
Brain stem									
MPT	3.794±0.41	2.231±0.64	3.044±0.32	2.806±0.59	2.025±0.51	2.744±0.51	1.419±0.59	2.00±0.41	3.194±0.38
PBI	1.995±0.19	1.00±0.11	1.375±0.09	1.143±0.22	1.00±0.14	1.347±0.34	0.708±0.14	0.753±0.21	1.115±0.40*
LC+	3.812±1.02	3.516±0.25	4.117±0.23	1.898±0.38	1.922±0.36	3.148±0.43*	0.969±0.29	1.055±0.29	2.047±0.37**
NTS (lateral)	1.562±0.37	2.025±0.23	2.975±0.59	1.55±0.19	3.01±0.33	1.756±0.68	1.331±0.89	1.081±0.43	2.23±0.76
NTS (medial)	1.781±0.34	0.919±0.1	0.975±0.09	0.975±0.2	1.725±0.23	1.288±0.21	0.738±0.26	1.1±0.3	1.894±1.02

Continued on next page

Table 1 continued.

	woRI			RI6d			RI13d		
	IMMO	EANP	EA36S	IMMO	EANP	EA36S	IMMO	EANP	EA36S
Raphe									
DR#++	2.331±0.16	1.344±0.13	1.912±0.27	1.295±0.13	1.252±0.16	1.419±0.12*	0.526±0.17	0.657±0.21	1.27±0.24+**
RM	0.983±0.23	1.083±0.32	1.292±0.28	0.95±0.25	0.425±0.06	0.8±0.18*	0.125±0.06	0.792±0.26	0.925±0.2*
RO	1.146±0.34	0.875±0.38	1.167±0.27	0.854±0.12	1±0.09	0.875±0.18	0.521±0.19	0.708±0.07	0.812±0.22
RPA	13.67±3.57	10±2.62	15±4.94	11.5±1.91	15.83±3.5	14.5±1.89	10.08±2.48	10.17±1.25	14.67±2.45

woRI = animals without repeated immobilization.
RI6d = animals submitted to repeated immobilization for 6 days (1 h/day).
RI13d = animals submitted to repeated immobilization for 13 days (2 h/day).
IMMO = animals submitted to immobilization.
EANP = animals submitted to immobilization and electroacupuncture at a non-acupoint site.
EA36S = animals submitted to immobilization and electroacupuncture at the Zusanli point.

Data are reported as means ± SD for 4 animals in each subgroup. Values obtained for different brain areas have been grouped for each procedure (woRI, RI6d and RI13d) for statistical purposes.

*Indicates a significant difference (ANOVA, $P < 0.05$, Fisher PLSD *post hoc* test, $P < 0.05$) between the group and woRI.
**Indicates a significant group difference (ANOVA, $P < 0.05$, Fisher PLSD *post hoc* test, $P < 0.05$) between RI13d and RI6d.
#Indicates an interaction effect (ANOVA, $P < 0.05$, Fisher PLSD *post hoc* test, $P < 0.05$) between acupuncture (IMMO, EANP, EA36S) and immobilization procedure.
+Indicates a significant effect (ANOVA, $P < 0.05$) of the acupuncture treatment in the absence of an interaction effect.
++Indicates a significant effect (Fisher PLSD *post hoc* test, $P < 0.001$) of the acupuncture treatment (associated with interaction effect).

Cortex
ACA = anterior cingulate area; Mop = primary motor area; SS = somatosensory area; VISC = visceral area; PIR = piriform area; ENT = entorhinal area.

Hippocampus
CA1 = field CA1; CA2 = field CA2; CA3 = field CA3; DG = dentate gyrus.

BST-amygdala
BST = bed nucleus of the stria terminalis; CeA = central nucleus of the amygdala; MeA = medial nucleus of the amygdala; CoA = cortical nucleus of the amygdala; BLA = basolateral nucleus of the amygdala.

Septal area
LSd = lateral septal nucleus, dorsal part; LSi = lateral septal nucleus, intermediate part; LSv = lateral septal nucleus, ventral part.

Thalamus-habenula
LH = lateral habenula; CM = central medial nucleus of the thalamus; PVT = paraventricular nucleus of the thalamus.

Hypothalamus
MPO = medial preoptic area; SCH = suprachiasmatic nucleus; PVa = anterior periventricular nucleus of the hypothalamus; LHA = lateral hypothalamic area; ARH = arcuate nucleus of the hypothalamus; DMH = dorsomedial nucleus of the hypothalamus; PH = posterior hypothalamus; PVp = posterior periventricular nucleus of the hypothalamus; SUMl = supramammillary nucleus; PVHmpd = paraventricular nucleus of the hypothalamus, medial parvicellular part, dorsal zone.

Brain stem
MPT = medial pretectal nucleus; PBl = parabrachial nucleus, lateral part; LC = locus coeruleus; NTS = nucleus tractus solitarius.

Raphe
DR = dorsal raphe nucleus; RM = nucleus raphe magna; RO = nucleus raphe obscurus; RPA = nucleus raphe pallidus.

pothalamus, pretectal medial nucleus, nucleus of the solitary tract, or obscurus and pallidus nuclei of the raphe.

Effect of electroacupuncture

Analysis by two-way ANOVA indicated that the significant effect of acupuncture treatment was restricted to the central medial nucleus of the thalamus (P = 0.03), posterior hypothalamus (P = 0.04), locus coeruleus (P = 0.03), and dorsal raphe nucleus (P = 0.02). *Post hoc* analysis indicated that the nuclei of group EA36S differed significantly from those of EANP and IMMO and no difference was found between EANP and IMMO (Table 1 and Figures 4 and 5).

The interaction effect between repeated immobilization and acupuncture treatment was observed in the basolateral nucleus of the amygdala (P = 0.05), suprachiasmatic nucleus (P = 0.03), periventricular anterior area of the hypothalamus (P = 0.04), and dorsal raphe nucleus (P = 0.01) (Table 1 and Figure 5). However, only in the dorsal raphe nucleus was this effect observed together with the effects of acupuncture and repeated immobilization.

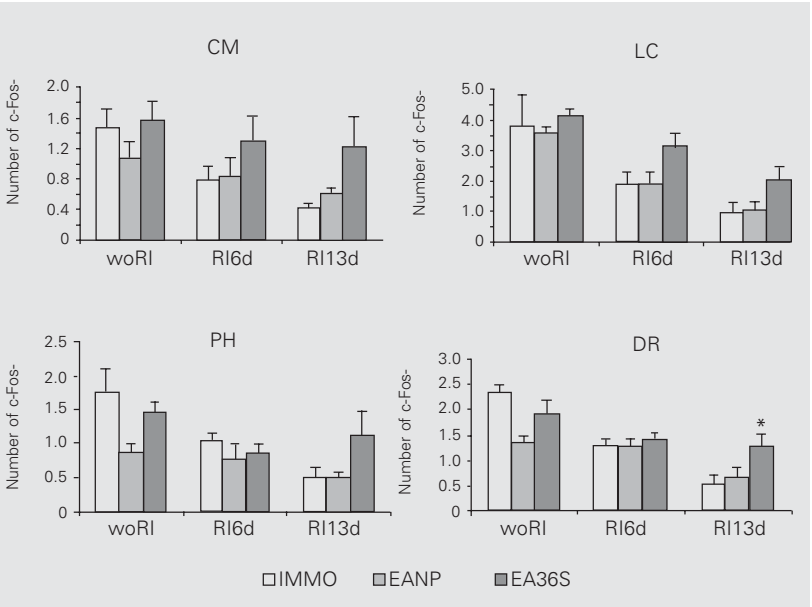

Figure 4. c-Fos expression induced by electroacupuncture in the central medial nucleus of the thalamus (CM), locus coeruleus (LC), posterior hypothalamus (PH) and dorsal raphe nucleus (DR) of animals previously submitted to repeated immobilization. Animals were previously subjected to 6 days of repeated immobilization (1 h/day), group RI6d, or to 13 days of repeated immobilization (2 h/day), group RI13d, or were left undisturbed in their home cages for 6 days, group woRI. On the 7th (woRI and RI6d groups) or 14th day (RI13d group), animals were further divided into 3 subgroups: only subjected to immobilization (IMMO), immobilized and submitted to EA36S, and immobilized and submitted to electroacupuncture at a non-acupoint site (EANP). *Different from RI13d-IMMO and RI13d-EANP (P < 0.05, ANOVA followed by Fisher's *post hoc* test).

Discussion

Habituation

Most of the brain areas analyzed showed a clear reduction of c-Fos expression after repeated immobilization, indicating that both the 6- and 13-day protocols of repeated immobilization are valuable tools in attenuating restraint-induced c-Fos expression in the rat brain (Figure 4). These results agree with previous work indicating a habituation to stress in specific brain areas after repeated restraint (7,17). However, there are some discrepancies over which areas are more sensitive to repeated stress. The PVH, locus coeruleus and dorsal raphe nucleus demonstrated the highest sensitivity to repeated immobilization by presenting a significant reduction of c-Fos expression after a 6-day protocol of repeated immobilization. Chen and Herbert (15) showed that the ventral lateral septum, lateral hypothalamus, locus coeruleus and lateral preoptic area exhibit c-Fos expression even after 10 sessions of restraint. In the present study, some structures such as the intermediate and ventral lateral septum and central nucleus of the amygdala still presented a sustained stress-induced c-Fos expression even after 13 days of repeated immobilization. In contrast, Melia et al. (7) showed habituation at the amygdala

and septum level after 9 sessions of restraint, although they did not specify the subareas. Moreover, Stamp and Herbert (18), using the same paradigm of repeated immobilization used by Chen and Herbert (15), described a clear habituation of the lateral ventral septum, central amygdala nucleus, PVH and nucleus of the solitary tract, and a sustained activation of the medial amygdala nucleus and locus coeruleus after 14 days of repeated stress. These discrepancies concerning the sensitivity of brain areas to repeated immobilization can be related to various factors, such as the immunocytochemistry assay (sensitivity of antibodies used), kind of stress (psychological versus physical stimulation/restraint or immobilization), previous life experience (group or individual housing, previous handling), duration and intensity of stress (18), and the rat strain used (Sprague-Dawley, Wistar or Lister Hooded rats were used in the cited studies; 7,15,17,18). Even for similar kinds of stressors, such as restraint and immobilization, the brain areas can show distinct sensitivity to habituation. Chowdhury et al. (19) have shown that induction and adaptation of brain Fos expression during stress depend on the intensity and duration of the stressors.

The reduced expression of c-Fos as a consequence of repeated stress is usually associated with the habituation of some behavioral and physiological responses. For some brain areas, however, increased c-Fos expression has been reported as a response to chronic stress (15). Induction of immediate early genes with repeated restraint or immobilization has been described in the PVH, as well as several extrahypothalamic cell groups, leading to the general conclusion that habituation is mediated by decreased neuronal activity in facilitatory afferents (7,8,17). In the present study, 31 of the 41 brain areas analyzed displayed decreased c-Fos expression after the repeated immobilization protocols. In general, the reduction of c-Fos expression was more evident in areas directly involved in neuroendocrine or autonomic responses to stress, such as the PVH and the locus coeruleus. However, some areas such as the ventral lateral septum, central nucleus of the amygdala and hypothalamic lateral area, which are also involved in stress responses, did not show any reduction of c-Fos expression after repeated stress. It has been suggested (7) that habituation may not be due to the stimulation of specific afferent channels since a novel psychological stressor using them might be effective in producing a response, but rather to a psychological factor ("habituation is a perceptual event"), the animal no longer feeling restraint as a stressor upon repetition. This issue is further discussed below.

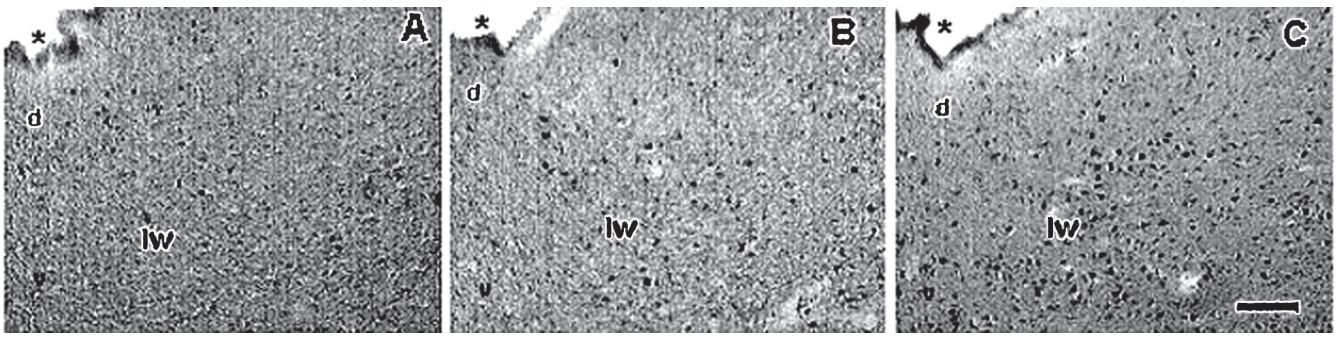

Figure 5. Photomicrograph illustrating the c-Fos expression induced by electroacupuncture (faradic wave, 2-4 V) in the dorsal raphe nucleus of animals previously submitted to 13 days of repeated immobilization. *A*, Rats only subjected to immobilization (IMMO); *B*, animals immobilized and submitted to electroacupuncture at a non-acupoint site (EANP); *C*, animals immobilized and submitted to EA36S (Zusanli point). * = brain aqueduct; d = dorsal part; lw = lateral wings of dorsal raphe nucleus; v = ventral part. Magnification bar: ~140 µm. Note that in *C* there is a higher number of c-Fos-immunoreactive cells in comparison with *A* and *B*.

Specific effect of stimulation at point 36S

In the present study, the effect of acupuncture was associated with specific c-Fos expression in the central medial nucleus of the thalamus, posterior hypothalamus, locus coeruleus and dorsal raphe nucleus. All of these areas have been involved in acupuncture analgesia. Using combined lesion techniques and evoked potentials, Takeshige et al. (20-24) reported that the dorsal periaqueductal gray (PAG) arcuate nucleus, lateral and posterior hypothalamus, lateral septum, dorsal hippocampus, habenula-interpeduncular tract, central medial nucleus of the thalamus and anterior hypothalamus are involved in acupuncture analgesia. Studies with mapping of neuronal activity using c-Fos immunohistochemistry in unanesthetized animals have shown that both 2 and 100 Hz EA can up-regulate c-Fos expression in several regions of the brain, including the lateral habenula, PVH, arcuate nucleus, PAG and gigantocellular nucleus (25,26). However, these studies did not evaluate c-Fos expression induced by non-point stimulation and showed a minimal or absent c-Fos expression in their naive and needle groups (the latter consisting of animals only subjected to needle insertion without electrical stimulation). These findings contradict most, if not all, of the published data because the manipulation and immobilization required for needle insertion *per se* should induce high levels of c-Fos expression, as those reported here. Here again it should be noted that the use of different techniques (electrophysiology versus c-Fos staining) might have greatly contributed to the reported differences. Lee and Beitz (1) used halothane-anesthetized animals and showed that EA at 36S induced higher c-Fos expression in the dorsal horn of the L2 spinal cord segment, lateral parabrachial nucleus, substantia nigra, nucleus raphe pallidus, dorsal raphe, locus coeruleus, posterior pretectal nucleus and lateroventral PAG than EA at a non-point. However, halothane, as well as acupuncture, have been shown to up-regulate c-Fos expression mainly in areas involved in analgesic responses, such as the PAG (4). Furthermore, in the cited study, the authors did not provide a more detailed description of their non-point group, and just mentioned that EA at non-point induced an inconsistent pattern of c-Fos expression (1). In contrast to those studies suggesting that EA can induce a specific pattern of neuronal activity, Pan et al. (27) showed that EA at 36S induced a pattern of c-Fos expression similar to that induced by a noxious stimulus, with staining found in pituitary gland, arcuate nucleus and other hypothalamic nuclei, such as the PVH, medial preoptic area, lateral hypothalamic area and ventromedial nucleus of the hypothalamus.

Our results also showed an interaction effect associated with repeated immobilization and acupuncture treatment on the dorsal raphe (Figure 5). In this structure, the differences between EA36S and EANP were more evident in the RI13d group. Although the interaction effect was only observed in the dorsal raphe, in most of the studied areas, c-Fos expression of animals not submitted to repeated immobilization (woRI group) was very similar among the IMMO, EANP and EA36S subgroups (Table 1 and Figure 3). Conversely, in animals submitted to repeated stress, there was a tendency to show clear differences between EA36S, EANP and IMMO (EA36S > [EANP = IMMO]). For instance, no difference between EA36S- and EANP-induced c-Fos expression in the central medial nucleus of the thalamus was observed in the woRI group, whereas this difference was 64% in the RI6d group and 186% in the RI13d group. The same pattern of differences between EA36S and EANP was also observed in the locus coeruleus: 8% in the woRI group, 61% in the RI6d group and 111% in the RI13d group. In fact, we speculate that the lack of significance for the interaction in the statistical analysis can be attributed to the small number of animals

in each group (N = 4) that might have precluded the observation of such difference.

The higher levels of c-Fos expression in the central medial nucleus of the thalamus, posterior hypothalamus, locus coeruleus and dorsal raphe nucleus induced by EA36S in animals previously submitted to repeated immobilization are consistent with the higher levels of analgesia induced by EA36S. Notably, all of these nuclei have been associated with analgesia. Thus, the central medial nucleus of the thalamus and posterior hypothalamus show high levels of c-Fos expression after acute injection of morphine (28) and are involved in the modulation of nociceptive information (29,30). Indeed, the intralaminar nuclei have been suggested to participate in the emotional aspects of pain perception (31,32). A number of studies have also reported that the locus coeruleus participates in pain modulation (33,34). Moreover, the dorsal raphe is also recognized as an important structure in pain modulation (35). Thus, stimulation of the dorsal raphe potentiates, whereas its lesion blocks, morphine-induced analgesia (35). In fact, similarly to the ventrolateral PAG, the dorsal raphe has also been regarded as a purely analgesic region, and stimulation of this area elicits potent analgesia without aversive behavioral side effects (36).

The fact that the differences between the woRI subgroups were more subtle than the differences between the RI13d subgroups suggests that in acute immobilization, EA36S, EANP and IMMO induced a closely similar pattern of neuronal activity. We speculate that the high levels of neuronal activity in the IMMO animals (control group) might mask some differences between EA36S and EANP. However, in "habituated" animals, there was a significant reduction of c-Fos expression in the controls, and we observed that EA36S induced higher levels of c-Fos than EANP in some brain areas previously cited as being involved in acupuncture analgesia. It might be speculated that even in acute experiments acupuncture might lead to significant responses. However, the concomitant existence of stress (restraint and needle puncture *per se*) activates pathways that overlap those activated by acupuncture, or that either afferent nervous pathway might be already occupied by the input generated by restraint. As a result, in experiments of neuronal activation, it may not be possible to distinguish the pathways activated by acupuncture from those activated by stress. After habituation, the pathways activated by stress might have been reduced in both intensity and extent and a different stimulus might then have afferent channels available and therefore activation induced by acupuncture might be observed. Moreover, in these "habituated" animals, EANP led to a pattern of c-Fos expression quite similar to IMMO, suggesting that analgesic and behavioral responses to EANP might share a common basis with that induced by IMMO alone (20). Therefore, EA36S can work as a novel stimulus (psychological) acting mainly on brain areas related to well-being, relaxation and pleasure, while EANP might work as a stress similar to immobilization.

Our results suggest that high levels of stress can interact with and mask the specific effects of acupuncture in unanesthetized animals. Despite its obvious limitations, repeated immobilization appears to be a good strategy for the assessment of specific effects triggered by acupuncture stimulation.

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