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ARTICLE

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Electroacupuncture reverses ethanol-induced locomotor sensitization and subsequent pERK expression in mice

Paula Fallopa[1]†, João Carlos Escosteguy-Neto[1]†, Patrícia Varela[1], Thiago Nogueira Carvalho[1], Angela M. F. Tabosa[2] and Jair Guilherme Santos-Junior[1,2,3]

[1] *Laboratory of Neurobiology, Group of Neuronal Plasticity and Psychiatric Disorders, Federal University of São Paulo, São Paulo, Brazil*

[2] *Division of Chinese Medicine – Acupuncture, Department of Orthopedics and Traumatology, Federal University of São Paulo, São Paulo, Brazil*

[3] *Department of Physiological Science, Faculty of Medical Science, Santa Casa of São Paulo, São Paulo, Brazil*

Abstract

Extracellular signal-regulated kinase (ERK) plays a role in neuronal changes induced by repeated drug exposure. Given that electroacupuncture reverses locomotor sensitization induced by ethanol, we investigated whether this effect is parallel to ERK signalling. Mice received daily ethanol (2 g/kg i.p), for 21 d. Electroacupuncture was performed daily, during four (subsequent) days of ethanol withdrawal. The stimulus of 2 Hz or 100 Hz was provided in combinations of two acupoints: Ea1 (ST-36/Zusanli and PC-6/Neiguan) or Ea2 (Du-14/Dazhui and Du-20/Baihui). The specificity of acupoint effects were assessed by the inclusion of additional groups: Ea3 (ST-25/Tianshu – acupoint used for other non-related disorders), Sham1 or Sham2 (transdermic stimulation near the respective acupoints). The control group was only handled during withdrawal and the saline group was chronically treated with saline and handled similarly to controls. At day 5 of withdrawal, each group was divided in two subgroups, according to the presence or absence of ethanol challenge. The animals were perfused and their brains processed for pERK immunohistochemistry. Only Ea1 at 100 Hz (Ea1_100) and Ea2 at 2 Hz (Ea2_2) reversed locomotor sensitization induced by ethanol. Ethanol withdrawal decreases pERK in the dorsomedial striatum. This decrease is not abolished by electroacupuncture. Conversely, ethanol challenge increases pERK in the dorsomedial striatum, infralimbic cortex and central nucleus of amygdala. The specificity of acupoint stimulation to reverse these increases was seen only for Ea2_2, in the infralimbic cortex and dorsomedial striatum. Therefore, behavioural effects of Ea2_2 (but not Ea1_100) depend, at least in part, on ERK signalling.

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Key words: Alcohol, electroacupuncture, locomotor sensitization, pERK.

Introduction

Repeated drug exposure promotes several neurochemical and structural changes in the brain motivational circuitry. This neuronal plasticity leads to sensitized responses to drugs and drug-associated stimuli (Kalivas & Stewart, 1991; Phillips & Shen, 1996; Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000). Given that the brain motivational circuitry plays a prominent role in determining the manner that stimuli is perceived as desirable (Berridge, 2007; Berridge & Robinson, 1998), repeated drug exposure markedly increases the salience of drugs and drug-associated stimuli. Moreover, according to the incentive-sensitization hypothesis, this exaggerated drug-seeking can lead to the compulsive pursuit and consumption of the drug without sensitization of the drug's subjective pleasurable effects (Robinson & Berridge, 1993, 2008).

In rodents, increase in psychomotor response to a drug challenge has long been considered a standard

Address for correspondence: Professor J. Santos-Junior, Rua Cesário Mota Jr, 61, 10 andar, 01221-020, São Paulo, Brazil.
Tel./Fax: 55 11 3331-2008
Email: guilherme.stos.jr@gmail.com
† These authors contributed equally to this work.

method to determine whether behavioural or incentive sensitization has occurred (Kalivas & Stewart, 1991; Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000). Moreover, some authors hypothesized that locomotor sensitization is a suitable model to study the neuronal plasticity that leads the transition between recreational use to drug addiction (De Vries *et al.* 1998; Deroche *et al.* 1999).

Extracellular signal-regulated kinase (ERK) plays a crucial role in the neuronal plasticity related to drug addiction (Girault *et al.* 2007; Zhai *et al.* 2008). It is involved in many aspects of neuronal function including control of cell growth, cell differentiation, neuronal survival, and synaptic plasticity (Grewal *et al.* 1999; Sweatt, 2004; Thomas & Huganir, 2004). ERK is widely expressed throughout the brain, including in the structures comprising the brain motivational circuitry, such as amygdala, prefrontal cortex, ventral tegmental area, striatum and hippocampus (Sweatt, 2004; Zhai *et al.* 2008). There is a positive correlation between cocaine-induced locomotor sensitization and increase in phospho-ERK (pERK) immunoreactivity in several encephalic nuclei, notably in the nucleus accumbens (NAc) (Corbillé *et al.* 2007; Janes *et al.* 2009; Marin *et al.* 2009; Pascoli *et al.* 2011; Valjent *et al.* 2010). However, the pattern of ERK expression in ethanol-induced locomotor sensitization remains to be elucidated.

There is a consensus that chronic ethanol exposure decreases pERK in the frontal cortex, dorsal and ventral striatum, hippocampus, amygdala and cerebellum. However, opposite effects have been described for acute withdrawal (first 24 h), with some regional variations in the levels and kinetics of induction (Sanna *et al.* 2002). Furthermore, in protracted abstinence, cue-induced reinstatement of ethanol self-administration was associated with an increase of pERK in the basolateral amygdala and nucleus accumbens shell (NAcS) (Schroeder *et al.* 2008). Therefore, despite the decrease in ERK expression induced by chronic ethanol exposure, there is a brief increase during acute withdrawal and after cue-induced reinstatement of ethanol self-administration.

A previous study from our laboratory showed that electroacupuncture inhibits the acquisition, expression and maintenance of locomotor sensitization induced by ethanol (Dos Santos *et al.* 2009). In particular, the effect of electroacupuncture on maintenance of locomotor sensitization has important clinical relevance. In this condition, the treatment could reverse the neuronal plasticity related to drug addiction, and as consequence, minimize the behavioural effects resulting from subsequent exposures to the drug. Furthermore, that study revealed that the behavioural effects were accompanied by decreases in mRNA *homer1a* changes induced by ethanol.

Homer1a is a scaffold protein that plays a crucial role in the plasticity of the glutamatergic system (Szumlinski *et al.* 2004, 2005). *Homer1a* is an immediate gene that inhibits the functional role of other constitutive isoforms of Homer proteins (Xiao *et al.* 1998). Given the positive relation between Homer expression and ERK activation (Kato *et al.* 2003; Mao *et al.* 2005; Sato *et al.* 2001), it is possible that electroacupuncture modulates ERK activation. In fact, in a recent study, Du *et al.* (2010) showed that electroacupuncture minimizes the neurological outcome and infarct volumes in an animal model of cerebral ischaemia. These effects were accompanied by increase in pERK expression. Interestingly, pretreatment with U0126 (an inhibitor of ERK1/2) disrupted the effects of electroacupuncture.

Another interesting characteristic of electroacupuncture is its differential effect when the stimuli are provided at a low (2 Hz) or high (100 Hz) frequency. At 2 Hz, electroacupuncture increases β-endorphin and enkephalin, while at 100 Hz it increases dynorphin. These peptides have distinct affinity to opioid receptors. β-endorphin and enkephalin interact more specifically with mu opioid receptors (MORs), while dynorphin has higher affinity to kappa receptors (KORs) (Di Chiara & Imperato, 1988; Spanagel, 1990*a*, *b*). Despite these differences, changes in opioid gene expression are thought to represent an initial step in the development of neuroplasticity underlying long-term profiles of drugs of abuse (Contet *et al.* 2003; Herz, 1997; Wee & Koob, 2010). Interestingly, both MORs and KORs activate ERK downstream, although by different molecular mechanisms (Belcheva *et al.* 2000, 2005; Schulz *et al.* 2004).

As stated above, a previous study from our laboratory showed that low-frequency electroacupucture disrupted the maintenance of locomotor sensitization induced by ethanol. Here, we investigated the role of the frequency stimulation (low *vs.* high) over the maintenance of the locomotor sensitization induced by ethanol. Furthermore, we addressed the importance of ERK signalling in these effects. Thus, we performed two experiments, according to the presence or absence (withdrawal) of ethanol challenge. The inclusion of these two experiments was due to the fact that the possible effects of electroacupuncture on pERK expression could be related to their actions on neuronal plasticity at the course of withdrawal, as well as, to their influences over the neurobiological mechanisms involved in the ethanol challenge.

Methods

Subjects

Swiss EPM-1 adult male mice (aged 12 wk, weighing 30–40 g) from the Center for the Development of Animal Models in Biology and Medicine, Federal University of São Paulo, were maintained in a number of standard home cages ($40 \times 34 \times 17$ cm) ($n = 10$ per cage) on a standard 12-h light/dark cycle (lights on at 07:00 hours) in a temperature- (20 ± 1 °C) and humidity- (50 ± 10%) controlled room, with free access to rodent chow pellets and tap water. The animal care and experimental procedures were conducted under protocols approved by the Animal Care and Use Ethics Committee of the University, according to the National Institute of Health's Guide for the Care and Use of Laboratory Animals, 1996.

Locomotor sensitization induced by ethanol

The protocol of locomotor sensitization was based on a previous study from our laboratory (Dos Santos *et al.* 2009). Animals were injected with saline and immediately placed in an automatic active cage (Insight, Brazil). The locomotor activity (baseline) was measured during 15 min. Two days later, mice were injected daily, for 21 d, with ethanol (15% v/v, at dose of 2 g/kg i.p.). After the 1st, 7th, 14th and 21st ethanol injections, the animals were placed in the active cage and the locomotor activity was measured again for 15 min. On the other days of ethanol treatment, the animals were returned to their home cages immediately after ethanol injections. The saline group was composed of mice similarly treated with saline. The animals which received ethanol were randomly allocated in 12 groups.

As stated, the possible effects of electroacupuncture over pERK expression could be related to its actions on neuronal plasticity at the course of withdrawal or to its effects on the neurobiological mechanisms involved in ethanol challenge. To address this issue, we performed two experiments of locomotor sensitization. In the first experiment, 5 d after withdrawal, all subjects (including those pretreated with saline) were injected with ethanol (1.4 g/kg i.p.) and then placed again in the active cage for 15 min (challenge locomotion). The second experiment was conducted similarly to the first, except there was no ethanol challenge. Furthermore, in the second experiment, we included only the electroacupuncture procedures (and their respective shams) that disrupted the maintenance of the locomotor sensitization in the first experiment.

Electroacupuncture procedures and the experimental groups

The electroacupuncture procedure consisted of 10-min sessions performed daily during the four days of ethanol withdrawal. The animals were immobilized and individual sterile needles (0.25×2 cm, Lautz, Brazil) were inserted at a depth of ~3 mm. Electroacupuncture stimulus was provided by an electrostimulator apparatus Plexus AP 585 (Accurate Pulse/Biotherapy, Lautz, Brazil), through an electrical current of faradic, bipolar and asymmetrical wave of 50 μA, at 2 or 100 Hz. At the end of the each session, the needles were removed and the animals returned to their respective home cages. We used the following experimental groups:

Ea1: To assess the effect of the combination of two acupoints and their 'energetic' synergism related to the central nervous system (O'Connor & Bensky, 1981): (i) ST-36 (Zusanli) in mice is located bilaterally ~1 mm lateral to the tibial tuberosity, according to anatomical references (Romita *et al.* 1997; Tabosa *et al.* 2002); (ii) PC-6 (Neiguan) in mice is located bilaterally ventral of the distal end of the forelimb, between the radius and ulna, ~1 mm from the wrist joint and just below the skin (as there is no great muscular mass under this area) (see Supplementary Material, Fig. S1, available online).

Ea2: To evaluate the specific action of the association between acupoints Du-14 (Dazhui) and Du-20 (Baihui). These acupoints were respectively located at the dorsal midline just below the spinous process of 7th cervical vertebra and at the dorsal midline in the skin of the skull, corresponding to bregma. They were selected considering their neurochemical effects over dopaminergic neurons (Liang *et al.* 2002), a main substrate of drug addiction (see Supplementary Material, Fig. S2).

Ea3: In order to assess the neurological specificity of the Ea1 and Ea2 procedures, we selected the ST-25 acupoint (Tianshu) given its significant peripheral (but not central) actions (O'Connor & Bensky, 1981). It is located on the abdomen, bilaterally, according to anatomical references described by Tabosa *et al.* (2004) (see Supplementary Material, Fig. S3).

For Ea1 and Ea2 groups, a corresponding Sham group was used, which received the same procedures performed in Ea1 and Ea2, except that the stimulation was done in non-acupoints described as follows.

Table 1. Experimental groups

Groups ($n=6$)	Moment of perfusion	Procedure
Saline	Challenge	Chronic saline injections and ethanol challenge
Control	Challenge	Chronic ethanol injections and ethanol challenge
Ea1_2	n.a.	Chronic ethanol injections, electroacupuncture at ST-36 and BP-6 at 2 Hz and ethanol challenge
Ea1_100	Challenge	Chronic ethanol injections, electroacupuncture at ST-36 and BP-6 at 100 Hz and ethanol challenge
Ea2_2	Challenge	Chronic ethanol injections, electroacupuncture at Du-14 and Du-20 at 2 Hz and ethanol challenge
Ea2_100	n.a.	Chronic ethanol injections, electroacupuncture at Du-14 and Du-20 at 100 Hz and ethanol challenge
Sh1_2	n.a.	Chronic ethanol injections, electroacupuncture at 2 Hz near ST-36 and BP-6 and ethanol challenge
Sh1_100	Challenge	Chronic ethanol injections, electroacupuncture at 100 Hz near ST-36 and BP-6 and ethanol challenge
Sh2_2	Challenge	Chronic ethanol injections, electroacupuncture at 2 Hz near Du-14 and Du-20 and ethanol challenge
Sh2_100	n.a.	Chronic ethanol injections, electroacupuncture at 100 Hz near Du-14 and Du-20 and ethanol challenge
Ea3_2	n.a.	Chronic ethanol injections, electroacupuncture at ST-25 at 2 Hz and ethanol challenge
Ea3_100	n.a.	Chronic ethanol injections, electroacupuncture at ST-25 and at 100 Hz and ethanol challenge
Saline_wd	Withdrawal	Chronic saline injections without ethanol challenge
Control_wd	Withdrawal	Chronic ethanol injections without ethanol challenge
Ea1_100_wd	Withdrawal	Chronic ethanol injections, electroacupuncture at ST-36 and BP-6 at 100 Hz without ethanol challenge
Ea2_2_wd	Withdrawal	Chronic ethanol injections, electroacupuncture at Du-14 and Du-20 at 2 Hz without ethanol challenge
Sh1_100_wd	Withdrawal	Chronic ethanol injections, electroacupuncture at 100 Hz near ST-36 and BP-6 without ethanol challenge
Sh2_2_wd	Withdrawal	Chronic ethanol injections, electroacupuncture at 2 Hz near Du-14 and Du-20 without ethanol challenge

n.a., Not available – perfusion was performed.

Sham1: Needles were inserted 5 mm distal, in a diagonal and lateral direction to ST-36 (Zusanli) and 3 mm proximal, in a diagonal and medial direction to PC-6 (Neiguan).

Sham2: Needles were inserted 5 mm distal, in a left diagonal direction to acupoint Du-14 (Dazhui), and 3 mm distal, in a right diagonal direction to acupoint Du-20 (Baihui).

Considering that the electric stimulus was performed at two different frequencies (2 Hz and 100 Hz), all experimental groups were divided in two subgroups ($n=6$ per subgroup). Moreover, because electroacupuncture required immobilization in order to be performed, it is possible that the stress resulting from immobilization could promote biases in the electroacupuncture effects. However, a previous study from our laboratory showed that a similar experimental schedule did not alter either the locomotor sensitization or mRNA *homer1a* expression resulting from chronic ethanol exposure (Dos Santos *et al*. 2009). Therefore, we did not include an additional control group submitted only to immobilization.

To summarize, the present study comprises the experimental groups described in Table 1.

Perfusion and immunohistochemistry

Perfusion was done 90 min after the ethanol challenge session (in the first experiment) or on day 5 of ethanol withdrawal (in the second experiment). The animals

were deeply anaesthetized with ketamine and xylazine, administered at doses of 75 mg/kg and 10 mg/kg i.p., respectively. After loss of corneal reflex, they were perfused via the intracardiac route, first with a PBS infusion and then with 4% paraformaldehyde (PFA). The brains were removed immediately after perfusion, stored in PFA for 24 h and then kept in a solution of 30% sucrose for 48 h. After this procedure, sections were cut (30-μm coronal sections) in a freezing microtome.

For immunohistochemistry, a conventional technique of avidin-biotin-immunoperoxidase was performed. Free-floating sections were pretreated with hydrogen peroxidase for 10 min followed by PBS for 30 min. Thereafter, sections were incubated overnight with a primary antibody (rabbit anti-pERK, 1:200, Cell Signaling, USA) in PBS-T solution (30 ml PBS, 30 μl Triton X-100). Subsequently, sections were incubated for 2 h with a secondary antibody (rabbit anti-goat IgG, 1:600, Vector, USA) at room temperature. The sections were then treated with avidin-biotin complex for 2 h and submitted to nickel-intensified DAB reaction. Between steps, the sections were rinsed in PBS (pH 7.4) and agitated on a rotator between each incubation and rinse step. Sections were mounted on gelatin-coated slides, dried, dehydrated and coverslipped.

The stereotaxic mouse brain atlas (Franklin & Paxinos, 1997) was used to define the nomenclature and nuclear boundaries. The encephalic areas analysed were: prefrontal cortex [anterior cingulate (Cg1), prelimbic cortex (PrL) and infralimbic cortex (IL)], dorsal striatum [dorsal medial striatum (DMS) and dorsal lateral striatum (DLS)], amygdala [basolateral nucleus (BLA) and central nucleus (CeA)], ventral striatum [nucleus accumbens core (NAcC) and NAcS], ventral tegmental area (VTA) and hippocampus [CA1, CA2, CA3 and dentate gyrus (DG)], as depicted in Fig. 1. A Nikon microscope connected to a computer was used to capture images from each section. After capture, the image was saved for posterior analysis of pERK immunoreactivity. The cells were counted visually with aid of software (ImageJ, NIH Image, USA). A template or outline was developed for each brain nucleus or subnucleus based on the shape and the size of the region. The number of pERK immunoreactive cells within each area was counted bilaterally in three consecutive sections per animal and their average was expressed as the number of pERK immunoreactive cells per 2.5×10^3 μm^2.

Statistical analysis

One-way repeated-measures ANOVA was used to evaluate the locomotor activity, considering four distinct periods: baseline, days 1 and 21 of ethanol treatment, and finally, ethanol challenge. A similar analysis was performed for locomotor activity in the second experiment. However, in this case, we considered only three periods: baseline, days 1 and 21 of ethanol treatment. To analyse pERK expression, we performed two-way ANOVA, considering treatment (saline, control, Ea1_100, Ea2_2, Sh1_100, Sh2_2) and moment of perfusion (withdrawal, challenge). Newman–Keuls *post-hoc* test was used when necessary. The level of significance was set at $p<0.05$. Finally, we analysed the correlation between locomotor activity after ethanol challenge and pERK expression using Pearson's test.

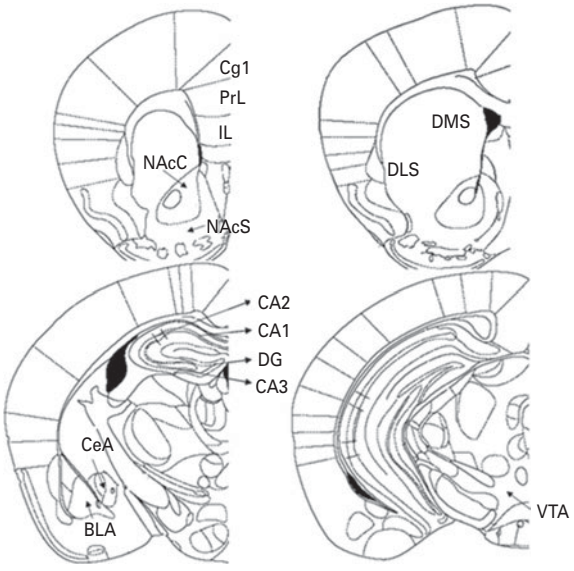

Fig. 1. Coronal sections of the mouse stereotaxic atlas. The arrows and acronyms indicate the regions analysed. Cg1, Cingulate anterior cortex; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; DMS, dorsomedial striatum; DLS, dorsolateral striatum; CeA, central nuclei of amygdala; BLA, basolateral nuclei of amygdala; CA1, 2, 3, cornu ammon 1, 2, 3; DG, dentate gyrus; VTA, ventral tegmental area. Coordinates in relation to Bregma (rostral-dorsal, respectively): +1.70, +1.10, −1.46 and −3.08 mm.

Results

The effects of electroacupuncture on locomotor sensitization induced by ethanol are depicted in Fig. 2. One-way repeated-measures ANOVA revealed significant difference in treatment ($F_{11,60}=4.67$, $p<0.01$) and period ($F_{3,180}=170.51$, $p<0.01$) factors. Moreover, there was an interaction of factors ($F_{33,180}=2.41$, $p<0.01$). As expected, no difference was seen between saline and all ethanol pretreated groups regarding baseline

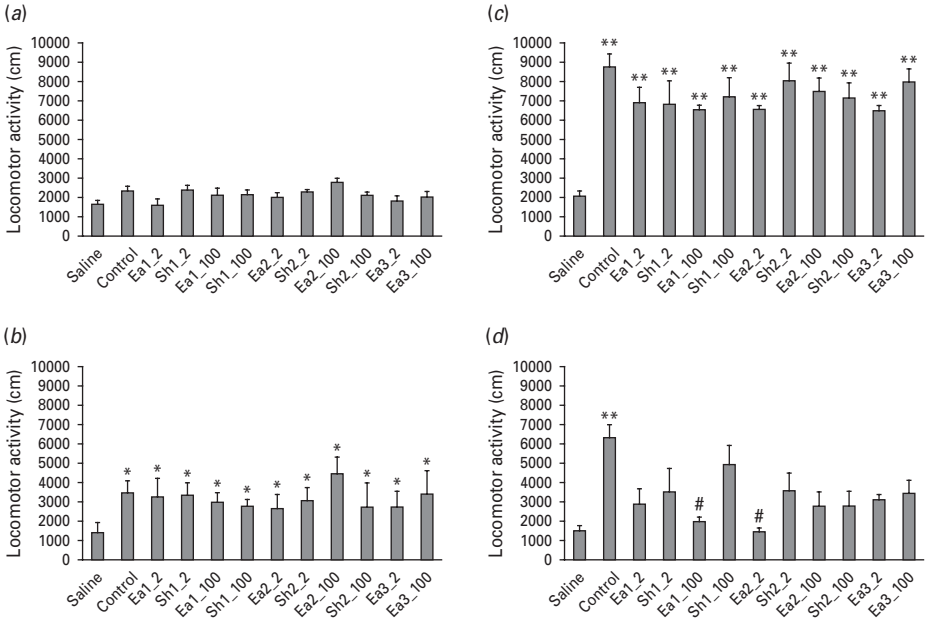

Fig. 2. Locomotor activity during a 15-min session in the activity cage, (*a*) before experiment (baseline), (*b*) after 1st ethanol injection, (*c*) after 21st ethanol injection, and (*d*) after ethanol challenge. The data are expressed as mean ± S.E.M. ($n = 6$ per group). * $p < 0.05$, ** $p < 0.01$ in relation to saline group. # $p < 0.05$ in relation to control group.

locomotion. Ethanol exposures induced a gradual but robust increase of locomotion similarly in all ethanol pretreated groups. These increases were maintained in the challenge session for the control group compared to the saline group ($p < 0.01$). In contrast, all experimental groups had similar locomotor activity after ethanol challenge compared to the saline pretreated group. However, only Ea1_100 ($p < 0.05$) and Ea2_2 ($p < 0.01$) had a decrease of locomotion compared to controls, despite the fact that these groups did not differ from their respective Sham and Ea3 groups. Therefore, electroacupuncture provided concomitantly at ST-36 (Zusanli) and PC-6 (Neiguan) (at high frequency) or at Du-14 (Dazhui) and Du-20 (Baihui) (at low frequency), reversed the locomotor sensitization induced by ethanol in mice.

Regarding the second behavioural experiment, one-way repeated-measures ANOVA revealed a significant difference in treatment ($F_{5,30} = 15.91$, $p < 0.01$) and period ($F_{2,60} = 201.21$, $p < 0.01$) factors. Furthermore, an interaction of the factors was seen ($F_{10,60} = 7.13$, $p < 0.01$) (data not showed). Similarly to the first experiment, all ethanol pretreated groups did not differ from saline (saline_wd group) regarding the baseline locomotion. Moreover, ethanol exposures induced a gradual and robust increase of locomotion in all groups. Therefore, in this second experiment, all groups developed locomotor sensitization, despite the different treatments which were performed during the four subsequent days of ethanol withdrawal.

Immunoreactivity of pERK on the different experimental groups is depicted in Table 2 and Figs 3 and 4. (For more details concerning the statistical parameters, see Table S1 in the Supplementary material.) Control group (animals chronically exposed to ethanol) differed from saline group, regardless of the moment of perfusion. During withdrawal, the control_wd group only had a decrease in pERK expression in the dorsomedial striatum ($p < 0.05$), compared to the saline_wd group. In contrast, after ethanol challenge, the control group had increases in pERK expression in the infralimbic cortex ($p < 0.05$), dorsomedial striatum ($p < 0.01$) and central nucleus of amygdala ($p < 0.01$), compared to the saline group. Paradoxically, there was a decrease in pERK in NAcC ($p < 0.01$). Therefore, locomotor sensitization induced by ethanol is accompanied by increases in pERK immunoreactivity in the infralimbic cortex, dorsomedial striatum and central nuclei of amygdala and a decrease in pERK immunoreactivity in the NAcC.

Electroacupuncture at specific acupoints did not alter the effects of ethanol withdrawal over pERK immunoreactivity. In contrast, it produced complex changes in pERK expression after ethanol challenge. The major finding is that only Ea2_2 minimized

Table 2. pERK expression in the experimental groups with or without (withdrawal) ethanol challenge

Nucleus		Saline	Control	Ea1_100	Sh1_100	Ea2_2	Sh2_2
mPFC							
PrL	Withdrawal	5.1±2.5	6.5±1.2	12.2±1.2**	14.1±1.4**	7.1±0.7‡‡	19.3±2.1*#‡‡
	Challenge	10.1±1.2	10.1±0.8	12.7±1.4##	14.3±1.4##	8.5±0.8‡‡	22.1±2.2*#‡‡
IL	Withdrawal	5.3±0.8	3.2±0.5	24.2±3.2*#‡‡	25.2±1.4*#‡‡	11.3±0.5##‡‡	21.3±4.2*#‡‡
	Challenge	5.6±0.8	13.1±1.2*§§	11.3±1.2§§	14.2±1.1*#‡‡§	3.1±0.5§##‡‡	12.2±1.1*#‡‡
Cg1	Withdrawal	1.2±0.3	1.1±0.3	12.4±1.2*#‡‡	11.2±1.1*#‡‡	5.2±0.5‡‡	18.3±2.5*#‡‡
	Challenge	6.1±0.3	6.1±0.3	8.1±1.3	9.3±1.2	3.1±0.5	2.5±1.4
Striatum							
NAcC	Withdrawal	5.5±2.1	5.2±1.1	25.4±3.2*#‡‡	12.5±0.3*#	7.5±1.8	6.1±2.5
	Challenge	16.3±1.8§§	4.1±1.1**	1.2±0.3*§§	0.5±0.3*§§	0.5±0.3*§	13.2±3.1#
NAcS	Withdrawal	1.5±0.2	1.1±0.2	5.2±1.1	4.2±0.2	2.2±0.3	4.2±1.2
	Challenge	1.2±0.3	3.2±0.3	0.5±0.3§	1.3±0.2	1.2±0.3	8.1±1.1
DMS	Withdrawal	15.2±2.1	2.2±1.2*	16.4±1.3#	18.2±1.5#	0.5±0.5‡‡	18.3±2.1#
	Challenge	16.3±2.1	49.3±9.8*§§	5.2±0.9##‡‡	18.1±1.8##	3.2±0.5##‡‡	46.5±9.9*§§
DLS	Withdrawal	2.1±1.5	1.1±0.5	14.3±5.1*#‡‡	1.1±1.5	1.1±0.5	4.2±1.1
	Challenge	1.5±1.5	2.3±0.5	1.3±1.5§§	1.2±0.5	1.1±1.5	1.2±0.5
Hippocampus							
CA1	Withdrawal	0.5±0.2	0.5±0.2	11.2±1.4*‡‡	0.5±0.2	6.2±2.1*‡‡	1.2±0.6
	Challenge	0.5±0.2	0.5±0.2	1.1±0.2§§	2.2±0.5	0.5±0.2§§	0.5±0.5
CA2	Withdrawal	0.2±0.2	0.2±0.2	5.2±1.1*‡‡	0.2±0.2	3.3±0.8*‡	0.2±0.2
	Challenge	0.2±0.2	0.2±0.2	0.2±0.2§§	3.1±1.2§	0.2±0.2§	0.2±0.2
CA3	Withdrawal	0.2±0.1	0.2±0.1	2.2±0.5*‡‡	0.8±0.1	2.1±1.8	0.2±0.1
	Challenge	0.2±0.1	0.2±0.1	0.8±0.1‡‡	2.2±0.1	0.2±0.1	0.2±0.1
DG	Withdrawal	3.5±0.5	0.5±0.2	12.1±1.5	3.5±0.2	4.1±0.8	2.5±0.2
	Challenge	3.5±0.6	3.1±0.2	1.2±1.1§§	3.5±0.2	0.8±0.8§	3.5±0.2
Amygdala							
BLA	Withdrawal	4.1±0.7	4.1±0.2	12.1±1.5*#‡‡	4.2±0.3	10.1±1.1*#‡‡	7.4±2.5*#
	Challenge	7.2±0.7	5.1±0.3	4.3±0.6§§‡‡	8.2±1.1§§	5.3±1.1§§	4.4±1.5
CeA	Withdrawal	3.3±0.5	3.1±0.5	11.5±0.8*#‡‡	7.5±3.2*#	11.4±1.1*#‡‡	5.1±1.1
	Challenge	4.1±0.5	13.4±2.1*§§	1.1±0.3§§##	2.5±0.2##	1.2±0.2§§##	4.2±0.4#
VTA	Withdrawal	1.1±0.4	1.5±0.4	11.5±0.8*#‡‡	8.5±0.4*#‡‡	2.5±0.4	3.5±0.6
	Challenge	2.8±0.5	2.4±0.6	0.8±0.5§§	3.1±0.5§§	0.8±0.8	3.2±0.6

mPFC, Medial prefrontal cortex; PrL, prelimbic cortex; IL, infralimbic cortex; Cg1, cingulate anterior cortex; DMS, dorsomedial striatum; DLS, dorsolateral striatum; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; CA1, cornu ammon 1; CA2, cornu ammon 2; CA3, cornu ammon 3; DG, dentate gyrus; BLA, basolateral nuclei of amygdala; CeA, central nuclei of amygdala; VTA, ventral tegmental area.

The data are expressed as means ± S.E.M. and represent the number of pERK immunoreactive cells per $2.5 \times 10^3\ \mu m^2$.

* $p<0.05$, ** $p<0.01$ in relation to saline group; # $p<0.05$, ## $p<0.01$ in relation to control group; ‡ $p<0.05$, ‡‡ $p<0.01$ in relation to its respective sham group; § $p<0.05$, §§ $p<0.01$ in relation to withdrawal period.

(in a specific acupoint manner) the effect of ethanol challenge over pERK expression at the dorsomedial striatum and infralimbic cortex. Furthermore, Pearson correlation test revealed significant and robust correlations between disruption of locomotor sensitization and pERK expression in the infralimbic cortex ($r^2 = 0.70$, $p<0.001$) and dorsomedial striatum ($r^2 = 0.49$, $p=0.015$) (see Fig. 5). Therefore, the expression of ethanol-induced locomotor sensitization recruits ERK signalling in prefrontal cortex and striatum. Interestingly, only electroacupuncture at 2 Hz in the Du-14 (Dazhui) and Du-20 (Baihui) acupoints impaired both ethanol-induced locomotor sensitization and pERK enhancement in infralimbic cortex and dorsomedial striatum. Another marked finding was the pERK expression changes induced by electroacupuncture in several encephalic nuclei, in that ethanol did not promote any changes. Curiously, these changes occurred only in the second experiment, during the withdrawal period (See Table 2). Ea1_100

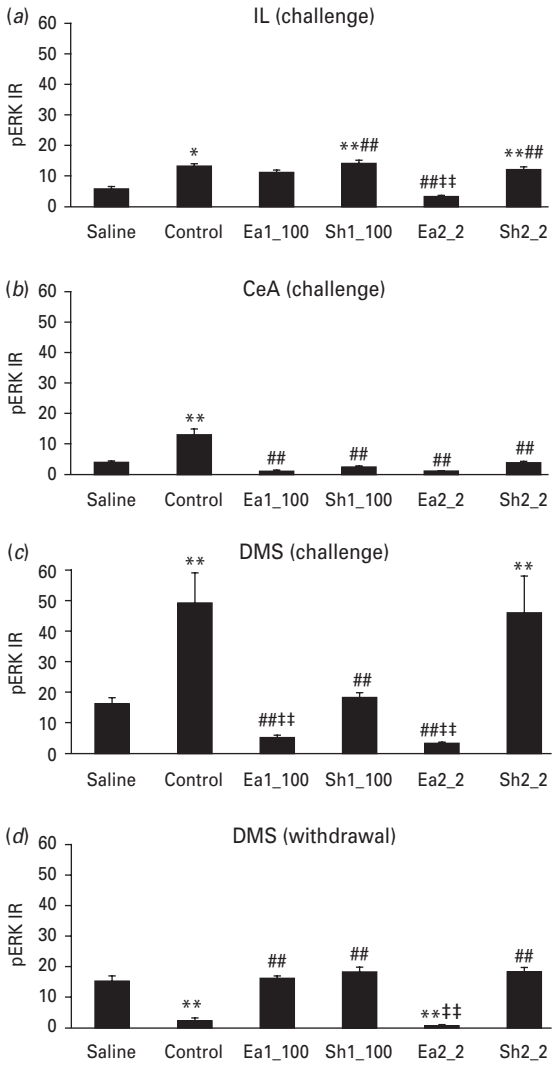

Fig. 3. pERK immunoreactivity (IR) in infralimbic (IL), central nucleus of amygdala (CeA) after ethanol challenge, as well as, in the dorsomedial striatum (DMS) in both withdrawal and after ethanol challenge. The data were expressed as mean ± s.e.m. and represent the number of cells immunoreactive for pERK in a 2.5×10^3 μm^2. * $p < 0.05$, ** $p < 0.01$ in relation to saline group, during ethanol withdrawal. ## $p < 0.01$ in relation to control group. †† $p < 0.01$ in relation to their respective sham groups.

and Ea2_2 (but not their respective sham groups) increased pERK expression in the CA1 and CA2 subfield of the hippocampus. A similar increase was seen in dentate gyrus for Ea1_100. Furthermore, Ea1_100 and Ea2_2 increased pERK in the basolateral and central nucleus of amygdala, respectively. Finally, Ea1_100 also increased pERK expression in the dorsolateral striatum. Besides the lack of difference between saline_wd and control_wd groups in these nuclei during the ethanol withdrawal period, it is possible that these changes induced by electroacupuncture could be functional consequences during ethanol withdrawal.

Discussion

The present study showed that ethanol-induced locomotor sensitization is correlated to enhancement of pERK immunoreactivity in the prefrontal cortex, dorsomedial striatum and central nuclei of amygdala, as well as to decreases in pERK expression in the NAcC. These changes emerged from ethanol challenge, because ethanol-withdrawal mice had only a decrease of pERK in the dorsomedial striatum.

Electroacupuncture provided at 100 Hz in the ST-36 (Zusanli) and PC-6 (Neiguan) disrupted the locomotor sensitization induced by ethanol. On the other hand, only electroacupuncture at 2 Hz in the Du-14 (Dazhui) and Du-20 (Baihui) acupoints reversed the effects of ethanol over the locomotor sensitization and pERK expression in infralimbic and dorsomedial striatum. It is well established that acupuncture minimizes several behavioural and neurochemical changes induced by ethanol (Dos Santos *et al.* 2009; Kim *et al.* 2005; Lee *et al.* 2008; Yang *et al.* 2010; Yoon *et al.* 2004; Zhao *et al.* 2006).

Regarding ethanol withdrawal, a randomized, placebo-controlled inpatient study revealed that acupuncture minimizes alcohol-withdrawal symptoms (Karst *et al.* 2002). Moreover, acupuncture at Shenmen (HT7) acupoint minimizes the decrease of accumbal dopamine release in ethanol-withdrawn rats (Lee *et al.* 2008; Zhao *et al.* 2006). Similarly, acupuncture at Zusanli (ST-36) or Sanyinjiao (SP6) acupoints (both stimulated in the Ea1 groups) inhibits alcohol-withdrawal symptoms and c-Fos immunoreactivity of rats undergoing ethanol injections (Kim *et al.* 2005). Here, none of the electroacupuncture procedures were able to minimize the pERK changes induced by ethanol withdrawal. Therefore, despite the well established beneficial effects of acupuncture over ethanol withdrawal, our results suggest that these effects are unrelated to ERK signalling.

Similarly to ethanol withdrawal, there are several evidences suggesting that electroacupuncture minimizes some behavioural and neurochemical changes induced by ethanol challenge. In a clinical trial reported by Kim and colleagues, alcoholics given needle acupuncture at Zhubin acupoint (KI9) exhibited an expressive reduction in alcohol craving compared to control groups (Kim *et al.* 2005). From a neurobiological point of view, a previous study from our laboratory showed that electroacupuncture

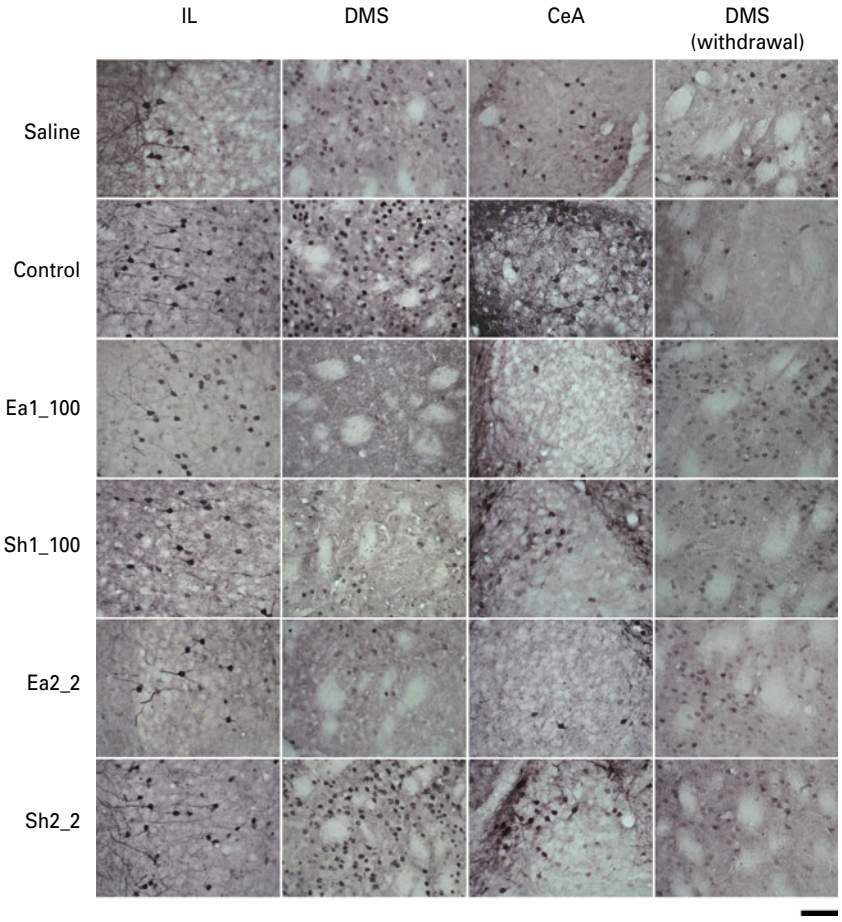

Fig. 4. Ilustrative photomicrography of pERK immunoreactivity. Scale bar, 25 μm. IL, Infralimbic cortex; DMS, dorsomedial striatum; CeA, central nuclei of amygdala.

blocked the decrease of *homer1A* mRNA expression triggered by ethanol in the acquisition (striatum and prefrontal cortex), expression (hippocampus), and in the maintenance (hippocampus and prefrontal cortex) of locomotor sensitization induced by ethanol (Dos Santos *et al.* 2009). Moreover, acupuncture inhibits GABA neuron activity in the ventral tegmental area and reduces ethanol self-administration (Yang *et al.* 2010), and also prevents the increase of dopamine in the NAc induced by ethanol challenge in withdrawn rats chronically exposed to ethanol (Zhao *et al.* 2006).

Here, we provided an additional neurochemical mechanism related to the behavioural effects of electroacupuncture in animal models of alcoholism. Electroacupuncture at 2 Hz at the Du-14 (Dazhui) and Du-20 (Baihui) acupoints reversed both locomotor sensitization and pERK increases in dorsomedial striatum and infralimbic cortex that follows ethanol challenge. The dorsomedial striatum is important for the formation and processing of action–outcome associations (Balleine *et al.* 2007; O'Doherty *et al.* 2004). Thus, the increases of pERK seen after ethanol challenge could represent sensitization mechanisms of the goal-directed behaviours linked to ethanol. Interestingly, similar features have been described for locomotor sensitization induced by psychostimulants (Corbillé *et al.* 2007; Pascoli *et al.* 2011; Valjent *et al.* 2010). With this point of view, the behavioural effects of electroacupuncture at low frequency in Du-14 (Dazhui) and Du-20 (Baihui) could be due, at least in part, to the blockade of pERK signalling in the dorsomedial striatum and, as consequence, to goal-directed behaviours. On the other hand, the medial prefrontal cortex plays a crucial role in the cognitive functions linked to drug addiction (George & Koob, 2010). It has been described that ERK signalling in the prefrontal cortex is necessary for the consolidation and recall of recent spatial memory in the water maze (Leon *et al.* 2010), for long-term retention of recognition memory (Nagai *et al.* 2007) and for trace fear

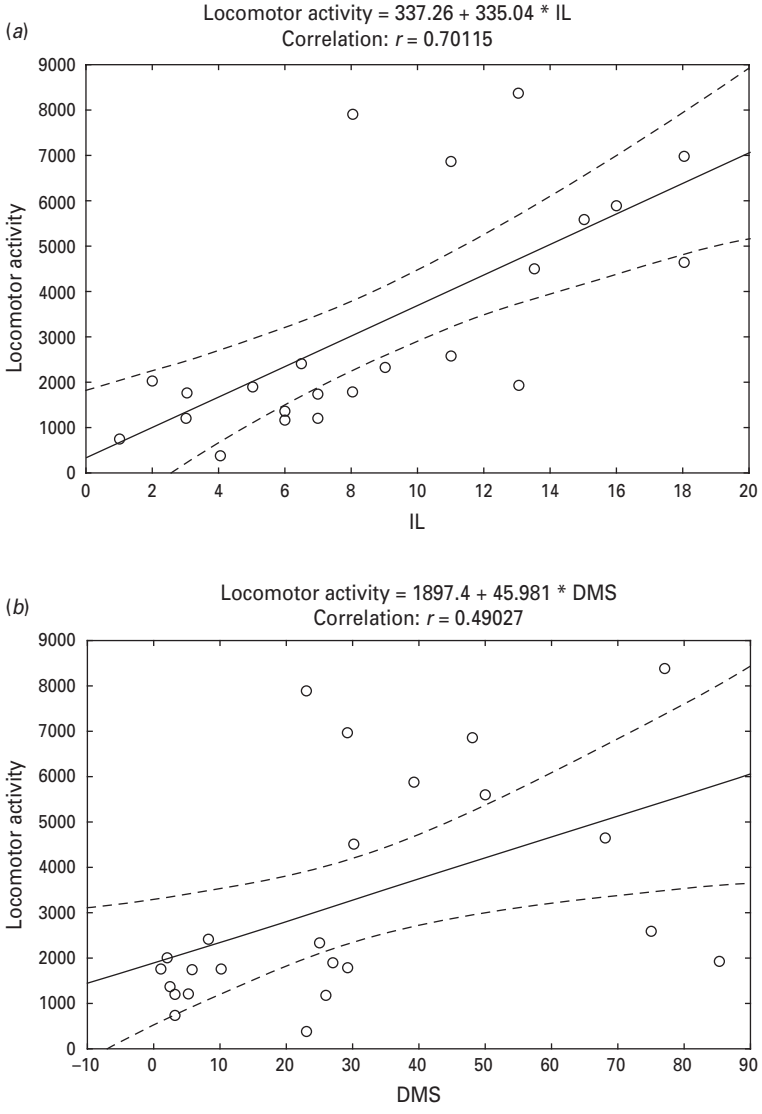

Fig. 5. Correlation between pERK immunoreactivity in (*a*) infralimbic cortex (IL) and (*b*) dorsomedial striatum (DMS) to locomotor activity after ethanol challenge, according to Pearson's test.

conditioning (Runyan & Dash, 2004; Runyan *et al.* 2004). Moreover, pERK expression in the ventral medial prefrontal cortex modulates the incubation of cocaine craving in instrumental learning (Koya *et al.* 2009). Therefore, we hypothesized that the increase of pERK seen after ethanol challenge could be due to recall of contextual memory linked to ethanol induced locomotor sensitization. This hypothesis is in accord with Marin *et al.* (2009), who revealed that increases in pERK expression which follow locomotor sensitization induced by cocaine are context-specific. Given that electroacupuncture at low frequency in Du-14 (Dazhui) and Du-20 (Baihui) disrupted pERK expression induced by ethanol challenge in the infralimbic cortex, its behavioural effects could be related, at least in part, to the impairment of the retrieval mechanisms linked to locomotor sensitization induced by ethanol.

An interesting characteristic of electroacupuncture is its differential effect when the stimuli are provided at a low or high frequency. At a low frequency electroacupuncture increases β-endorphin and enkephalin, while at a high frequency, it increases dynorphin. These peptides have distinct affinity to opioid receptors. β-endorphin and enkephalin interact more specifically with the MOR, while dynorphin has

higher affinity with the KOR (Di Chiara & Imperato, 1988; Spanagel, 1990*a*,*b*). Hence, MOR signalling plays a major role for Du-14 (Dazhui) and Du-20 (Baihui) acupoint stimulation, confirming the already established role of MOR signalling in locomotor sensitization induced by ethanol (Pastor & Aragon, 2006). Conversely, the encountered effects of ST-36 (Zusanli) and PC-6 (Neiguan) acupoints could be associated, at least in part, with KOR signalling. Curiously, although KOR signalling plays a pivotal role in ethanol addiction (Wee & Koob, 2010), there was no evidence for it in the locomotor sensitization induced by ethanol. Here, we provide indirect evidence that supports the participation of KOR signalling in the locomotor sensitization induced by ethanol.

Finally, electroacupuncture changed pERK immunoreactivity (in a specific acupoint stimulation) in some encephalic nuclei in which the control group did not differ from the saline group. Interestingly, this effect occurred only in ethanol-abstinent mice. Both Ea1_100 and Ea2_2 increased pERK in basolateral amygdala, and CA1 and CA2 subfields of the hippocampus. Moreover, only Ea1_100 increased pERK in dorsolateral and dentate gyrus. Given the crucial role of these structures in cognition and emotionality (Lamprecht & LeDoux, 2004; McEwen, 2007), we cannot rule out the influence of pERK signalling in these nuclei over the behavioral effects of electroacupuncture.

To summarize, electroacupuncture inhibits the maintenance of ethanol-induced locomotor sensitization and the pERK changes induced by ethanol challenge. These effects were dependent upon acupoints and stimulation frequencies, as well as, the encephalic nuclei evaluated.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

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Statement of Interest

None.

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