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ARTICLE

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Regionally selective activation and differential regulation of ERK, JNK and p38 MAP kinase signalling pathway by protein kinase C in mood modulation

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Abstract

A growing body of evidence indicates that the extracellular signal-regulated kinase (ERK) pathway may participate in the neuronal modulation of depression. p38MAPK and c-Jun-*N*-terminal kinase/stress-activated protein kinase (JNK/SAPK) also belong to the MAPK family which mainly function as mediators of cellular stresses. Since increasing evidence implicates stress as an important factor in vulnerability to depressive illnesses, the involvement of ERK, JNK and p38MAPK pathways in the modulation of mood was investigated in the forced swim test (FST) and tail suspension test (TST). The effect produced by a single acute session of FST and TST on hippocampal and cortical MAPK expression and phosphorylation was investigated by immunoblotting experiments. In the hippocampus of animals exposed to FST and TST, an intensive, PKC-dependent, ERK1, ERK2, JNK, and p38MAPK phosphorylation was observed. In the frontal cortex, the FST and TST produced a PKC-dependent increase of ERK2 and p38MAPK phosphorylation, a PKC-independent activation of JNK and cAMP response element-binding protein (CREB) whereas any involvement of ERK1 was detected. The PKC blocker calphostin C (0.05–0.1 μg i.c.v.), the MEK inhibitor U0126 (10–20 μg i.c.v.), the p38MAPK inhibitor SB203580 (5–20 μg i.c.v.) and the JNK inhibitor II (0.5–5 μg i.c.v.), produced antidepressant-like behaviour without altering locomotor activity. These results illustrate a differentially mediated activation of MAPK in hippocampus and frontal cortex of animals exposed to behavioural despair paradigms. An antidepressant-like phenotype produced by acute blockade of MAPK signalling was also demonstrated.

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Introduction

Mitogen-activated protein kinases (MAPK) are members of a superfamily of serine/threonine protein kinases extensively distributed throughout the central nervous system that play a crucial role in transducing signals to the nucleus, and thereby regulate the genes involved in wide variety of cellular processes, including cell proliferation, differentiation, apoptosis, and synaptic plasticity (Johnson & Lapadat, 2002; Robinson & Cobb, 1997). Several MAPK cascades have been characterized, of which the best studied MAPKs are the extracellular signal-regulated kinases ERK1 and 2 (also known as p44 and p42 MAPK, respectively). When activated, the phosphorylation state of the ERK1/2, phospho-ERK1/2 (pERK1/2), primarily regulates neuronal growth, differentiation and apoptosis (Houslay & Kolch, 2000; Sweatt, 2001). Recently, the role of the ERK pathway in the molecular mechanism of depression has been increasing, and a growing body of evidence indicates that the ERK pathway may participate in the neuronal modulation of depression (Fumagalli *et al.* 2005; Todorovic *et al.* 2009). However, there is very little direct evidence for the role of ERK pathway in depression and the results are not consistent. For example, Duman *et al.* (2007) demonstrated that inhibition of the ERK pathway produces a depressive-like response, but Einat *et al.*

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(2003) observed an antidepressant-like effect produced by ERK inhibition. The role of ERK pathway in depression still awaits further investigation.

p38MAPK and c-Jun-*N*-terminal kinase/stress-activated protein kinase (JNK/SAPK) also belong to the MAPK family which mainly function as mediators of cellular stresses such inflammation and apoptosis. JNK is a stress-activated kinase that plays a critical role in the regulation of T-lymphocyte differentiation and cytokine production (Dong *et al.* 2002). Inflammatory cytokines, brain injury, and ischaemic insult induce activation of JNK (Nishima *et al.* 1997; Schroeter *et al.* 2003; Ward & Hagg, 2000). p38MAPK is another stress-activated kinase that plays an important role in the regulation of cytokine production in the immune system (Dong *et al.* 2002). In the brain, p38MAPK is activated in microglia in response to ischaemic insults and may play a role in the microglial response to stress (Walton *et al.* 1998). Over-activation of these stress-activated kinases is known to cause neuronal degeneration and impairment of the function of the central nervous system (Nishima *et al.* 1997; Schroeter *et al.* 2003). Physiologically stressful stimuli, including seizure induction (Murray *et al.* 1998), ischaemic insult (Alessandrini *et al.* 1999), visceral pain (Gioia *et al.* 2001), and electroconvulsive shock (Bhat *et al.* 1998; Oh *et al.* 1999) have also been shown to rapidly activate MAPKs in various brain regions. Furthermore, increasing evidence implicates stress as an important factor in the vulnerability to depressive and other mental illnesses (Garcia 2002; Kendler *et al.* 2001; Post 1992).

As a step towards better understanding of the possible involvement of MAPK in mood disorders, we further investigated the ERK pathway and we examined JNK and p38MAPK catalytic activity and protein expression in hippocampus and frontal cortex of mice exposed to the forced swim test (FST) and tail suspension test (TST), animal models which emulate the behavioural despair paradigm of depression. In this study we also tested the hypothesis that it might be possible to modulate behaviour in antidepressant-responsive behavioural paradigms by directly modifying the function of the MAPK pathway by using pharmacological inhibitors of ERK, JNK and p38MAPK. Finally, we investigated the contribution of protein kinase C (PKC) as upstream MAPK activator to the modulation of mood.

Materials and methods

Animals

Male Swiss albino mice (20–22 g) from the Morini (San Polo d'Enza, Italy) breeding farm were used. Mice were housed 10 per cage (26×41 cm). The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet with tap water available *ad libitum* and maintained at 23 ± 1 °C on a 12-h light/dark cycle (lights on 07:00 hours). All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Intracerebroventricular (i.c.v.) injection technique

I.c.v. administration was performed under ether anaesthesia as described previously (Galeotti *et al.* 2003).

FST

The FST used was the same as described by Porsolt *et al.* (1977). Briefly, mice were placed individually into glass cylinders (height 25 cm, diameter 10 cm) containing 12 cm of water maintained at 22–23 °C and left there for 6 min. A mouse was judged to be immobile when it floated in the water, in an upright position, and made only small movements to keep its head above water. The duration of immobility was recorded during the last 4 min of the 6-min test. A decrease in the duration of immobility is indicative of an antidepressant-like effect. There were 12–15 mice per group tested.

TST

A piece of tape was adhered to the upper middle of the tail of each animal, creating a flap with the overlap of tape. Mice were suspended from a plastic rod mounted 50 cm above the surface by fastening the tail to the rod with adhesive tape. The duration of the test was 6 min and immobility was measured during the last 4 min to facilitate comparison with the FST. Immobility was defined as the absence of any limb or body movements, except those caused by respiration. There were 12–15 mice per group tested.

Locomotor activity

Locomotor activity was evaluated by using the hole-board test. The apparatus consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4×4 in an equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed to move about freely for a period of 10 min each. Two photobeams, crossing the plane from mid-point to mid-point of opposing sides, thus dividing the plane into four equal quadrants,

automatically signalled the movement of the animal (in 5-min counts) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (in 5-min counts) the exploration of the holes (exploratory activity) by the mice; 12–15 mice per group were tested.

Preparation of membranes

Brain areas required to conduct Western blotting experiments were collected immediately after the end of the FST and TST. Brains were dissected to separate specific areas. The hippocampus and frontal cortex were homogenized in an homogenization buffer containing 25 mM Tris–HCl (pH 7.5), 25 mM NaCl, 5 mM EGTA, 2.5 mM EDTA, 2 mM sodium pyrophosphate (NaPP), 4 mM *p*-nitrophenylphosphate (PNFF), 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin, 50 μg/ml aprotinin, 0.1% SDS. The homogenate was centrifuged at 9000 ***g*** for 15 min at 4 °C, the low-speed pellet was discarded and the supernatant (whole proteins) was stored at −80 °C. To obtain the membrane and cytosol fractions, the supernatant was centrifuged at 100 000 ***g*** for 60 min at 4 °C. The resulting supernatant was the cytosol fraction, and the pellet was resuspended in the homogenizing buffer containing 0.2% (w/v) Triton X-100. The homogenate was kept at 4 °C for 60 min with occasional stirring and then centrifuged at 100 000 ***g*** for 60 min at 4 °C. The resulting supernatant was used as the membrane fraction. Protein concentration was quantified using Bradford's method (protein assay kit, Bio-Rad Laboratories, Italy).

Western blot analysis

Animals were divided into five groups: (1) naive control mice, (2) mice that performed the FST, (3) mice that performed the TST, (4) mice treated with the PKC-blocker calphostin C (0.1 μg per mouse), (5) mice pre-treated with the PKC-blocker calphostin C 60 min before performing FST or TST. Membrane homogenates (10–50 μg) made from hippocampal and frontal cortex regions of the five groups were separated on 10% SDS–PAGE and transferred onto nitrocellulose membranes (110 min at 120 V) using standard procedures. Membranes were blocked in PBST (PBS containing 0.1% Tween) containing 5% non-fat dry milk for 120 min. Following washing, blots were incubated overnight at 4 °C with specific antibodies against PKCε (1:800), PKCε phosphorylated on Ser^{729} (p-PKCε, 1:750; Santa Cruz Biothechnology Inc., USA), ERK1/2 (1:500), ERK1/2 phosphorylated on Thr^{202}/Tyr^{204} (pERK1/2, 1:500), SAPK/JNK (1:750), SAPK/JNK phosphorylated on Thr^{183}/Tyr^{185} (p-SAPK/JNK, 1:750), p38MAPK (1:500), p38MAPK phosphorylated on Thr^{180}/Tyr^{182} (p-p38MAPK, 1:250), CREB (1:500), CREB phosphorylated on Ser^{133} (p-CREB, 1:500) (Cell Signalling Technology). After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antisera (1:10 000) and left for 1 h at room temperature. Blots were then extensively washed and developed using an enhanced chemiluminescence detection system (Pierce, Italy). Exposition and developing time used was standardized for all the blots. Densitometric analysis of scanned images was performed on a Macintosh iMac computer using the public domain NIH Image program. Measurements in control samples were assigned a relative value of 100%. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control.

Drug administration

D-amphetamine hydrochloride (De Angeli, Italy) and amitriptyline (Sigma, Italy) were dissolved in isotonic (0.9% NaCl) saline solution, calphostin C (Calbiochem, Italy) in 0.5% DMSO, U0126, SB203580, JNK inhibitor II (Calbiochem) in 20% DMSO. Doses and administration schedule for calphostin C and amitriptyline were chosen on the basis of time-course and dose–response curves performed in our laboratory.

U0126 (5–20 μg i.c.v. per mouse), SB203580 (0.1–20 μg i.c.v. per mouse), JNK inhibitor II (0.01–5 μg i.c.v. per mouse), and calphostin C (0.001–0.1 μg i.c.v. per mouse) were administered 60 min before the behavioural tests, whereas amitriptyline (10 mg/kg i.p.) was injected 30 min before the behavioural tests.

In Western blotting experiments a dose of calphostin C of 0.2 μg i.c.v. per mouse was used.

D-amphetamine (2 mg/kg i.p.), was administered 15 min before the evaluation of the locomotor activity (hole-board test).

Statistical analysis

All experimental results are given as the mean ± S.E.M. Analysis of variance (ANOVA) followed by Bonferroni's/Dunn's *post-hoc* test was used for statistical analysis.

Results

Increased phosphorylation of PKCε isoform by acute FST and TST

Frontal cortex and hippocampus of mice were examined for the protein expression and phosphorylation

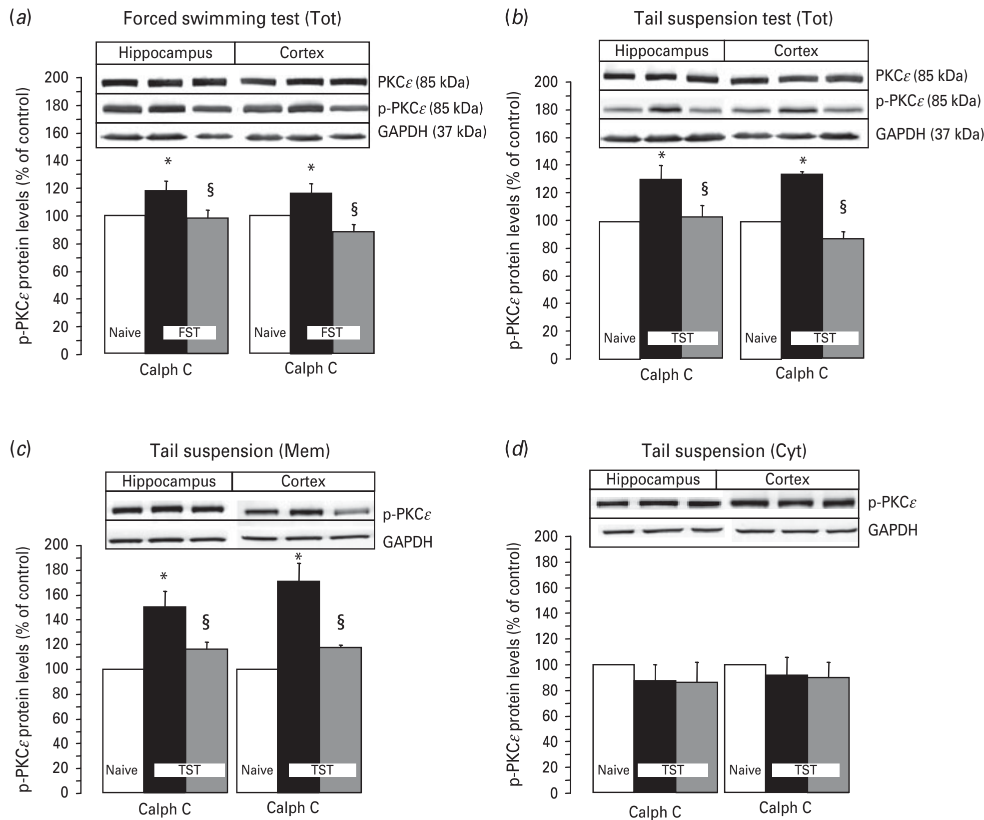

Fig. 1. Increased phosphorylation of PKCε by the forced swim test (FST) and tail suspension test (TST). Protein levels were detected in the mouse hippocampus and frontal cortex using immunoblotting technique. The columns represent the densitometric quantitation of immunoreactive p-PKCε expressed relative to control. Data are expressed as mean$\pm$S.E.M. of band intensities. Representative immunoblots are reported in each panel. (*a*) FST increased PKCε phosphorylation in the total protein preparation (Tot) without affecting PKCε expression. (*b*) TST increased p-PKCε levels in the total protein preparation (Tot) and (*c*) membrane (Mem) fraction, whereas in (*d*) the cytosol (Cyt) fraction any variation was detected. Calphostin C (Calph C) decreased the phosphorylation of PKCε up to control values. * $p<0.05$ compared to control group; § $p<0.05$ compared to FST/TST exposed mice.

level of PKCε. The protein levels were detected in the whole protein preparation (Fig. 1*a*, *b*), in the membrane (Fig. 1*c*) and cytosol (Fig. 1*d*) fractions. FST (Fig. 1*a*) and TST (Fig. 1*b*) significantly increased phosphorylation of PKCε in the hippocampus (FST: $F_{1,10}=4.961$, $p<0.001$; TST: $F_{2,12}=5.843$, $p<0.001$), and frontal cortex (FST: $F_{1,10}=4.288$, $p<0.001$; TST: $F_{2,12}=5.335$, $p<0.001$), compared to naive mice (Fig. 1*a*, *b*). This effect was prevented by pre-treatment with the PKC blocker calphostin C (Fig. 1*b*). A more evident increase of the phosphorylation of PKCε was observed in the membrane fraction (Fig. 1*c*) (hippocampus: $F_{2,12}=15.536$, $p<0.001$; frontal cortex: $F_{2,12}=18.196$, $p<0.0001$) whereas in the cytosol fraction the levels of p-PKCε were similar to the control group (Fig. 1*d*). Calphostin C treatment reduced the activation of PKCε isoform as demonstrated by the decrease of p-PKCε levels up to values corresponding to the control (Fig. 1*c*). Similar results were obtained in the FST (Fig. 1*a*). PKCε protein expression was unchanged after the acute session of FST (Fig. 1*a*) and TST (Fig. 1*b*).

Regionally selective increased phosphorylation of ERK1 and ERK2 isoforms by acute FST and TST

FST largely increased the phosphorylation of ERK1/2 in the hippocampus ($F_{1,14}=9.471$, $p<0.001$) and, to a more modest extent, in the frontal cortex ($F_{1,10}=9.273$, $p<0.001$) (Fig. 2*a*). TST experiments produced similar results showing increased ERK1/2 phosphorylation in the hippocampus ($F_{2,15}=8.772$, $p<0.001$) and frontal cortex ($F_{2,15}=5.264$, $p<0.001$), an effect that was prevented by pre-treatment with the PKC blocker calphostin C with an analogous profile in both TST

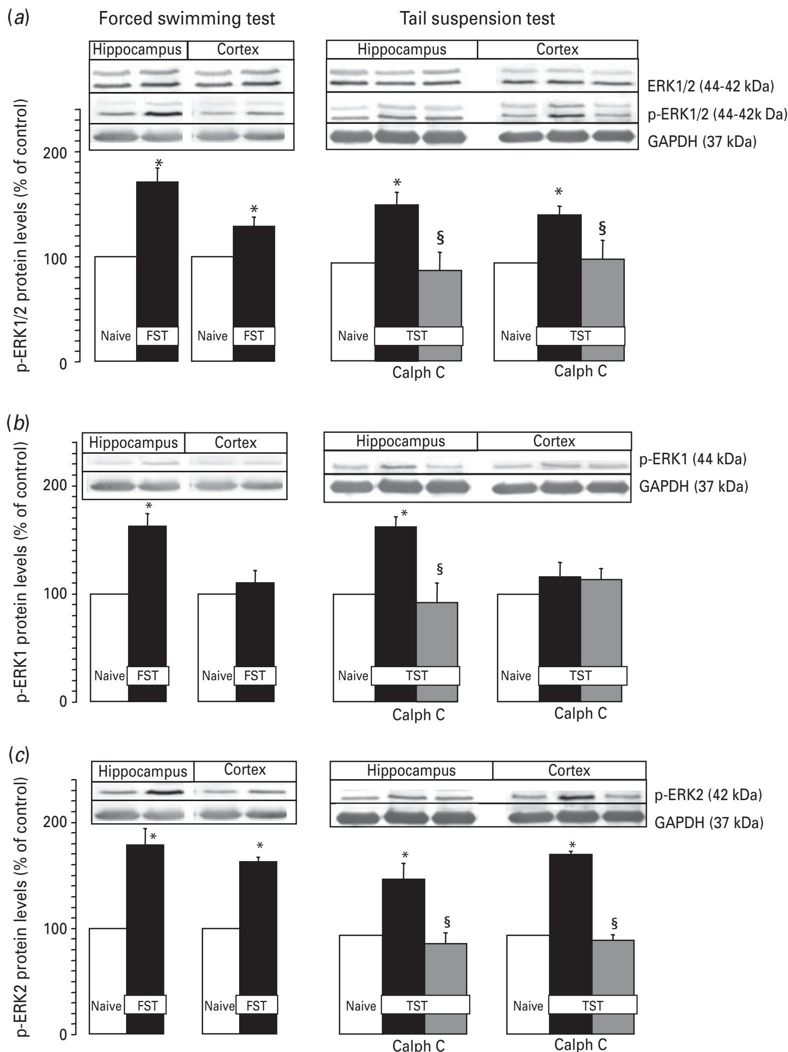

Fig. 2. Elevation of ERK1/2 phosphorylation in the hippocampus and frontal cortex of mice exposed to an acute session of forced swim test (FST) and tail suspension test (TST). The columns represent the densitometric quantitation of immunoreactive phosphorylated protein expressed relative to control. Data are expressed as mean ± S.E.M. of band intensities. Representative immunoblots are reported in each panel. (*a*) FST and TST increased p-ERK1/2 levels without modifying expression of ERK1/2, (*b*) elevated the activation of ERK1 in the hippocampus but not in the frontal cortex and (*c*) increased the levels of p-ERK2 in both cerebral areas. Calphostin C (Calph C, 0.2 μg i.c.v. per mouse) prevented the activation of ERK1/2, ERK1 and ERK2. GADPH was used as loading control. * $p<0.05$ compared to control group; § $p<0.05$ compared to TST-exposed mice.

(Fig. 2*a*) and FST (hippocampus 92.3 ± 6.4, cortex 95.1 ± 5.8). No modification of the ERK1/2 protein expression was observed. A representative immunoblot showing the lack of any modification of ERK1/2 protein levels induced by FST and TST in the mouse hippocampus and frontal cortex is shown in Fig. 2*a* (top).

The activation of ERK1 and ERK2 by behavioural despair paradigms was also detected in order to investigate the role of each ERK isoform. In the hippocampus, FST produced a robust increase of pERK1 ($F_{1,14}=9.486$, $p<0.001$; Fig. 2*b*) and pERK2 ($F_{1,12}=8.991$, $p<0.001$; Fig. 2*c*). Similar results were obtained after TST where a PKC-dependent increase of pERK1 ($F_{2,12}=5.336$, $p<0.001$; Fig. 2*b*) and pERK2 ($F_{2,12}=3.989$, $p<0.001$; Fig. 2*c*) was observed. Densitometric analysis revealed that the administration of the PKC blocker calphostin C prevented the phosphorylation of both ERK isoforms. In the frontal cortex, an increase of pERK2 levels to a similar extent of that observed in the hippocampus was detected in the brain of mice exposed to FST ($F_{1,12}=7.846$, $p<0.001$; Fig. 2*c*) or to TST. Pre-treatment with calphostin C

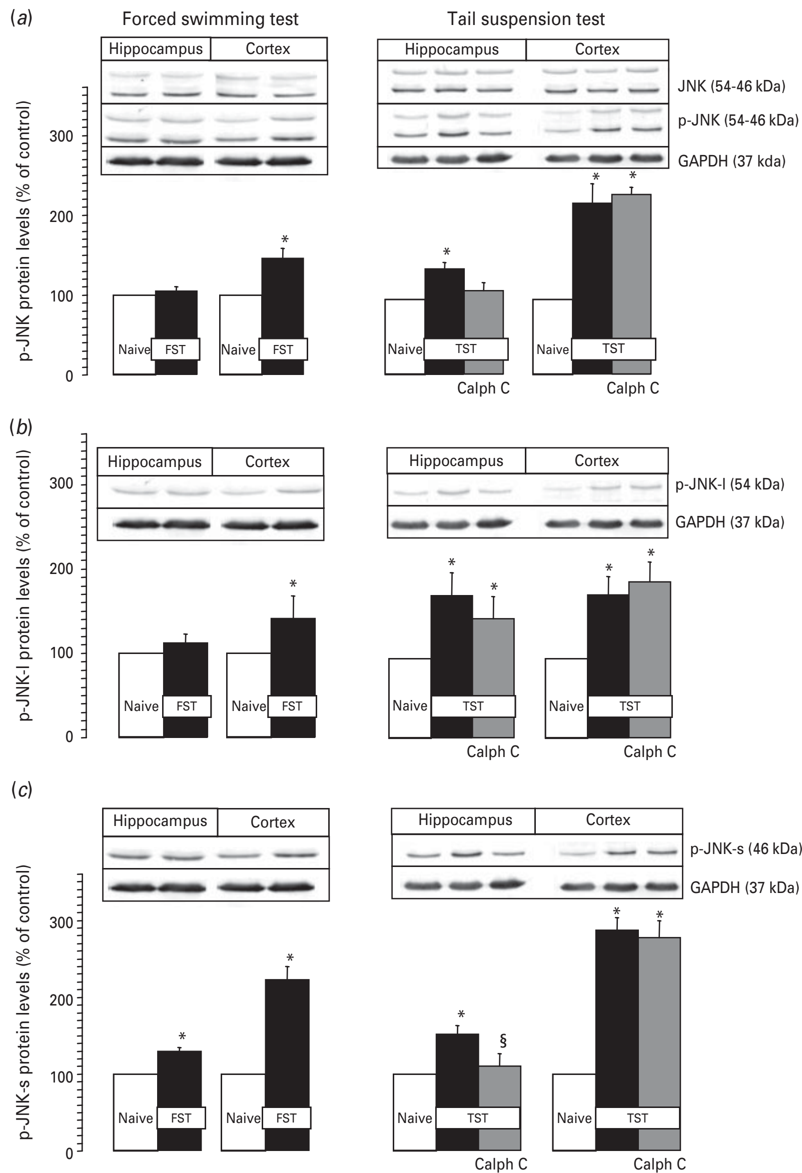

Fig. 3. Elevation of JNK phosphorylation in the hippocampus and frontal cortex of mice exposed to an acute session of forced swim test (FST) and tail suspension test (TST). The columns represent the densitometric quantitation of immunoreactive phosphorylated protein expressed relative to control. Data are expressed as mean ± S.E.M. of band intensities. Representative immunoblots are reported in each panel. (*a*) FST and TST increased p-JNK levels without modifying the expression of JNK, (*b*) elevated the activation of the JNK long form (JNK-l), and (*c*) short form (JNK-s). Calphostin C (Calph C) was administered at the dose of 0.2 μg i.c.v. per mouse 60 min before the test. GAPDH was used as loading control. * $p<0.05$ compared to control group; § $p<0.05$ compared to TST-exposed mice.

abolished this effect in the TST ($F_{2,12}=4.020$, $p<0.001$; Fig. 2*c*) and FST ($F_{2,12}=3.981$, $p<0.001$). Conversely, no activation of ERK1 in the frontal cortex after either FST (Fig. 2*c*) or TST (Fig. 2*c*) was observed.

Immunoblots were re-probed for a protein considered not to be regulated as GAPDH and no significant density difference was revealed for this protein between samples (Fig. 2*a*–*c*).

FST and TST elicit large cortical PKC-independent increase in phosphorylation of JNK

Conversely to ERK1/2, FST increased phosphorylation of JNK in the frontal cortex ($F_{1,14}=8.024$, $p<0.001$) whereas no activation was detected in the hippocampus (Fig. 3*a*). Similarly to FST, an acute session of TST produced a large increase in JNK phosphorylation

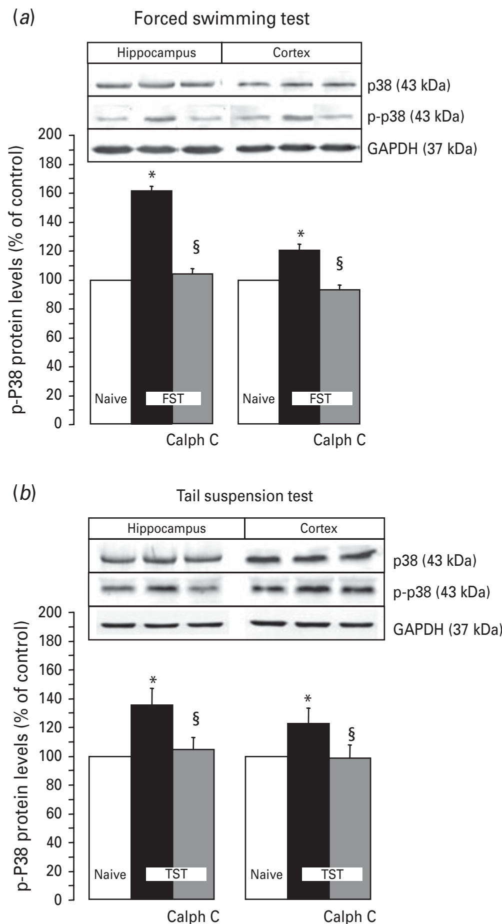

Fig. 4. Elevation of p38MAPK activation in the hippocampus and frontal cortex of mice exposed to an acute session of forced swim test (FST) and tail suspension test (TST). The columns represent the densitometric quantitation of immunoreactive phosphorylated protein expressed relative to control. Data are expressed as mean ± S.E.M. of band intensities. Representative immunoblots are reported in each panel. (*a*) FST and (*b*) TST increased p-p38MAPK levels without modifying the expression of p38MAPK. Calphostin C (Calph C) was administered at the dose of 0.2 μg i.c.v. per mouse 60 min before the test. GAPDH was used as loading control. * $p<0.05$ compared to control group; § $p<0.05$ compared to TST-exposed mice.

in the frontal cortex ($F_{2,15}=20.962$, $p<0.001$) and, conversely to FST, a p-JNK increase to a much smaller extent in the hippocampus ($F_{2,15}=8.028$ $p<0.001$) (Fig. 3*a*). The TST (Fig. 3*a*) and FST-induced (FST: 149.2 ± 9.3; FST + calph C: 154.8 ± 8.6) effect was PKC-independent since calphostin C was unable to modify the levels of p-JNK in the frontal cortex and hippocampus. In contrast, JNK levels were not modified by either the FST or TST (Fig. 3*a*). Representative immunoblots of changes in p-JNK after FST and TST are shown in Fig. 3*a* (top).

The analysis of the two main isoforms of JNK, the long (JNK-l; 54 kDa) and the short (JNK-s; 46 kDa) isoforms, showed a different influence of behavioural despair paradigms on activation of JNK isoforms. Specifically, FST ($F_{1,14}=6.852$, $p<0.01$) and TST ($F_{2,15}=6.570$, $p<0.001$) induced elevation of activated JNK-l in the frontal cortex (Fig. 3*b*). A regionally selective JNK-l modulation was detected since the influence on JNK-l activity in the hippocampus was absent (FST) or significant (TST) ($F_{2,15}=4.821$, $p<0.01$) (Fig. 3*b*). Pre-treatment with calphostin C did not prevent JNK-l activation (Fig. 3*b*). Analogous to its effect on JNK-l, but much more markedly, FST ($F_{1,14}=6.340$, $p<0.001$) and TST ($F_{2,15}=9.587$, $p<0.001$) increased phosphorylation of JNK-s in the frontal cortex in a PKC-independent manner (Fig. 3*c*). In contrast to JNK-l, elevation of activated JNK-s was also detected in the hippocampus after both FST ($F_{1,13}=2.484$, $p<0.05$) and TST ($F_{2,15}=5.837$, $p<0.01$), even if to a lesser extent (Fig. 3*c*). This effect was PKC-dependent since it was prevented by pre-treatment with calphostin C after exposure to TST (Fig. 3*c*) and FST (94.2 ± 6.3). Representative immunoblots of changes in p-JNK-l (Fig. 3*b*, top) and p-JNK-s (Fig. 3*c*, top) after FST and TST are illustrated.

Immunoblots were re-probed for a protein considered not to be regulated as GAPDH and no significant density difference was revealed for this protein between samples (Fig. 3*a*–*c*).

Effect of acute FST and TST on p38MAPK activation

Similarly to its effect on ERK1/2 and in contrast to the effect on JNK, FST ($F_{1,10}=6.042$, $p<0.01$) and TST ($F_{2,15}=5.372$, $p<0.01$) increased phosphorylation of p38MAPK in the hippocampus that was prevented by the PKC blocker calphostin C (Fig. 4*a*, *b*). A more modest elevation of the activated p38MAPK was also detected in the frontal cortex after FST ($F_{1,13}=2.991$, $p<0.05$) and TST ($F_{1,13}=2.676$, $p<0.05$) in a PKC-dependent manner (Fig. 4*a*, *b*).

Representative immunoblots of changes in p-p38MAPK after FST (Fig. 4*a*, top) and TST (Fig. 4*b*, top) are shown.

Immunoblots were re-probed for a protein considered not to be regulated as GAPDH and no significant density difference was revealed for this protein between samples (Fig. 4*a*, *b*).

Effect of acute TST on CREB and p-CREB levels

While not significant, we observed a trend towards decreased CREB and CREB phosphorylation in the

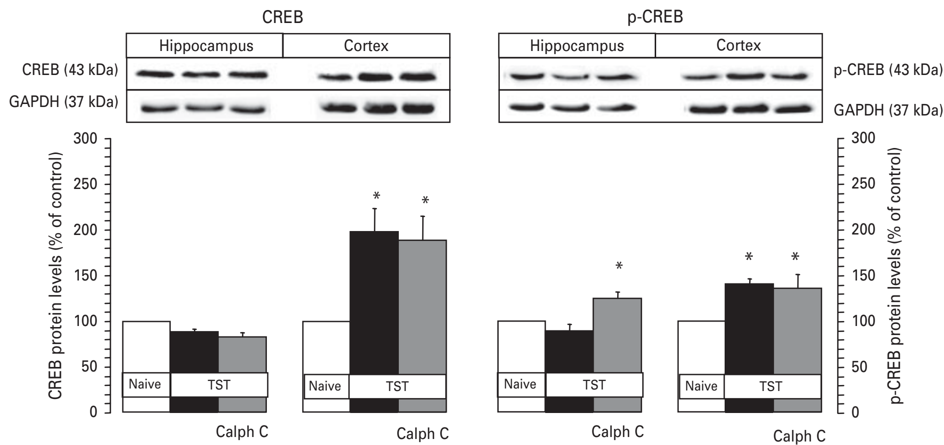

Fig. 5. Evaluation of CREB and p-CREB levels in the hippocampus and frontal cortex of mice exposed to an acute session of the tail suspension test (TST). The columns represent the densitometric quantitation of immunoreactive protein expressed relative to control. Data are expressed as mean ± S.E.M. of band intensities. Representative immunoblots are reported in each panel. Calphostin C (Calph C) was administered at the dose of 0.2 μg i.c.v. per mouse 60 min before the test. GADPH was used as loading control. * $p < 0.05$ compared to control group.

hippocampus following TST. Pre-treament with the PKC blocker calphostin C increased the p-CREB levels ($F_{1,8} = 2.102$, $p < 0.05$) (Fig. 5).

In the frontal cortex we observed an increase of CREB ($F_{1,8} = 3.200$, $p < 0.05$) and p-CREB ($F_{1,8} = 8.030$, $p < 0.01$) levels that were unmodified by pre-trement with calphostin C (Fig. 5).

Representative immunoblots of changes in CREB and p-CREB after TST are shown in the top part of Fig. 5.

Immunoblots were re-probed for a protein considered not to be regulated as GAPDH and no significant density difference was revealed for this protein between samples (Fig. 5).

Effect of blockade of PKC-MAPK pathway in the mouse behavioural despair paradigms

The administration of calphostin C decreased the immobility time values in the mouse TST. Calphostin C, at 0.001 μg i.c.v. per mouse, was devoid of any effect, whereas the doses of 0.01 and 0.02 μg per mouse decreased the immobility time without reaching statistical significance. ANOVA on immobility times revealed a significant group effect ($F_{6,65} = 12.556$, $p < 0.0001$). Treatment with either 0.05 μg i.c.v. ($p < 0.05$) or 0.1 μg i.c.v. ($p < 0.01$) per mouse showed a statistically significant antidepressant-like effect (Fig. 6*a*). Similarly, the administration of the MEK blocker U0126 produced an antidepressant-like effect ($F_{4,45} = 14.989$, $p < 0.0001$). The dose of 5 μg i.c.v. per mouse was ineffective whereas statistical significance was reached at 10 μg i.c.v. ($p < 0.01$) and 20 μg i.c.v. ($p < 0.0001$) per mouse (Fig. 6*b*). The p38MAPK inhibitor SB203580 dose-dependently reduced immobility time ($F_{4,45} = 7.437$, $p < 0.0001$) at the doses of 5 μg ($p < 0.05$), 10 or 20 μg ($p < 0.01$) per mouse (Fig. 6*c*). A reduction in immobility was also produced by the JNK blocker JNK inhibitor II ($F_{5,50} = 7.324$, $p < 0.0001$) in a dose-dependent manner. A statistically significant effect was reached at the doses of 0.5, 1 and 5 μg i.c.v. per mouse (Fig. 6*d*). The intensity of the calphostin C, U0126, SB203580 and JNK inhibitor II antidepressant-like effect was comparable to that produced by amitriptyline (10 mg/kg s.c.), used as reference drug.

Similar results were obtained in the FST (data not shown).

Effect of MAPK modulators on mouse locomotor behaviour

The spontaneous mobility (Fig. 7*a*) and exploratory activity (Fig. 7*b*) of mice treated with the highest effective doses of U0126 (10 μg i.c.v. per mouse), SB203580 (10 μg i.c.v. per mouse) and JNK inhibitor II (1 μg i.c.v. per mouse) were unmodified compared to the control group. In the same experimental conditions D-amphetamine, used as positive control, significantly increased both spontaneous mobility ($F_{4,66} = 4.334$) and exploratory activity ($F_{4,66} = 4.011$) (Fig. 7*a*, *b*).

Discussion

We studied the involvement of the MAPK pathway in two behavioural despair paradigms, the FST and TST, providing behavioural, biochemical and

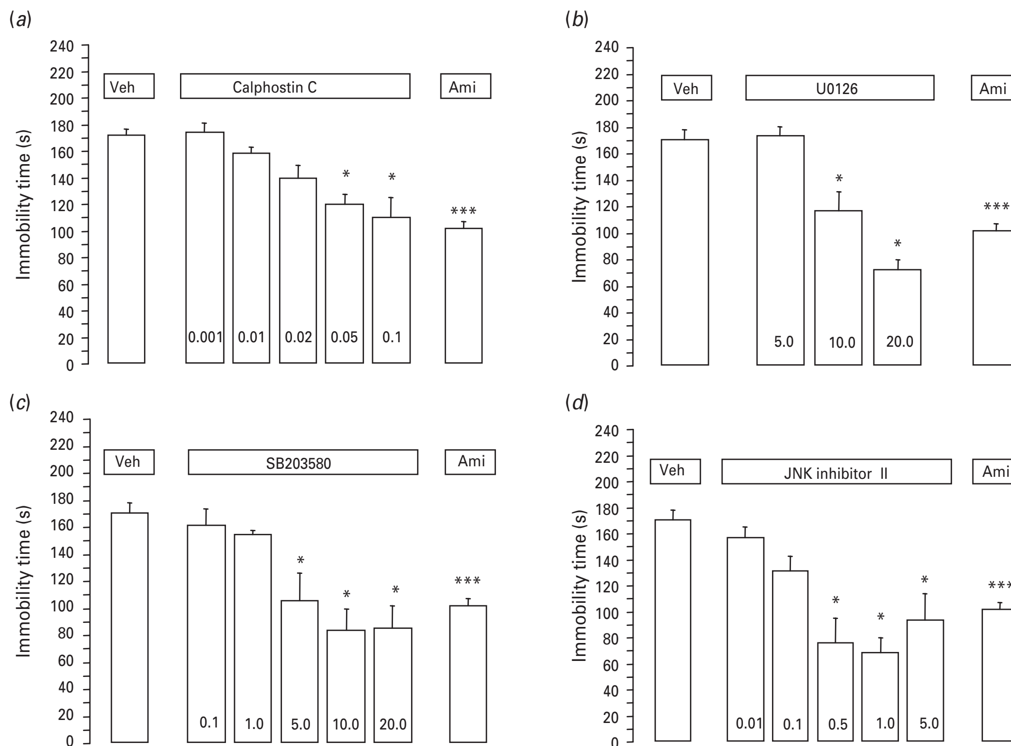

Fig. 6. Antidepressant-like effect produced by pharmacological blockade of PKC and MAPK activity. Decrease in immobility time in the mouse tail suspension test by (*a*) the PKC blocker calphostin C (Calph C), (*b*) the MEK inhibitor U0126, (*c*) the p38MAPK inhibitor SB203580 and (*d*) the JNK blocker JNK inhibitor II. Doses of all inhibitors are expressed as μg i.c.v. per mouse. Vertical lines represent S.E.M. * $p<0.05$, *** $p<0.001$ compared to vehicle-treated mice. Veh, Vehicle; Ami: amitriptyline.

neuroanatomical evidence for a role of the MAPK pathway in the induction of a depressive-like condition. FST and TST (Cryan *et al.* 2005) are widely used to detect antidepressant-like activities that are sensitive to acute drug administration. A single i.c.v. administration of the specific MEK inhibitor U0126 produced an antidepressant-like response of intensity comparable to that induced by amitriptyline, taken as antidepressant reference drug. This antidepressant phenotype appeared at doses unable to modify spontaneous mobility. An unspecific effect of the treatment can be excluded since U0126 blocks only ERK1/2 without affecting p38, JNK or other MAP kinase pathways (Favata *et al.* 1998). These data give a first indication for an ERK-mediated depressant-like activity. Since blockade of ERK produces an antidepressant-like phenotype, we believe that activation of ERK might be responsible for a depressive condition.

A further demonstration arises from biochemical results, which established that an acute 6-min session of FST and TST robustly elevated the phosphorylation of ERK1/2 in the hippocampus and, to a smaller extent, in the frontal cortex. ERK1/2 is extensively distributed throughout the central nervous system and prominently found in the hippocampus and prefrontal cortex (Flood *et al.* 1998), brain regions most likely to be implicated in response to depression. There are morphological and functional alterations in both brain regions in humans with major depression, such as reduced hippocampal volume (Bremner 2002), and altered metabolism in the prefrontal cortex (Drevets *et al.* 1997). A region-specific activation of the kinase was more evident by analysing the influence of a FST or TST session on ERK1 and ERK2 isoforms. ERK1 was activated in the hippocampus, but no modification in p-ERK1 levels was revealed in the frontal cortex. Conversely, an increase in the phosphorylation of ERK2 of similar intensity was observed in both hippocampus and frontal cortex. This stronger modulation of ERK1 activity in the hippocampus allowed us to hypothesize that hippocampal ERK1 phosphorylation might have a prominent role in the induction of depressant-like behaviour. The presence of an antidepressant phenotype demonstrated by ERK1 knockouts that is further potentiated

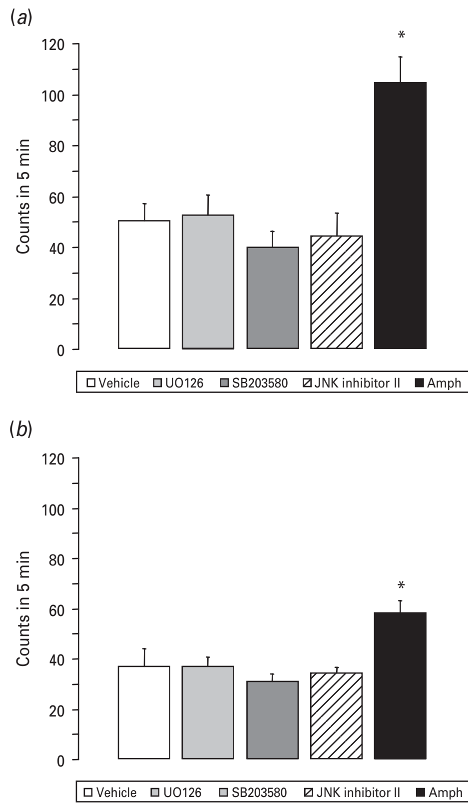

Fig. 7. Lack of influence on mouse locomotor activity by MAPK blockers. The absence of any alteration of (*a*) spontaneous mobility and (*b*) exploratory activity by the MEK inhibitor U0126, the p38MAPK inhibitor SB203580, and the JNK blocker JNK inhibitor II was observed compared to vehicle-treated mice. Amphetamine (Amph) was used as positive control. Vertical lines represent S.E.M. * $p<0.05$ compared to control group.

by intrahippocampal administration of U0126 (Tronson *et al.* 2008), is in agreement with this hypothesis. Furthermore, it has been recently reported that stress-induced depressive-like behaviours were correlated with an increase in hippocampal p-ERK1. Chronic treatment with desipramine prevented this depressant-like behaviour and p-ERK1 increase, suggesting that alterations in the MAPK/ERK transduction pathway might promote depressive-like behaviour (Bravo *et al.* 2009).

Some of the main upstream regulators of ERK activity include the mitogen-activated and extracellular signal-regulated kinase (MEK), cAMP-dependent protein kinase (PKA), and protein kinase C (PKC) (Pouyssegur *et al.* 2002). MEK directly phosphorylates and activates ERK (Crews & Erikson, 1992), whereas PKA and PKC regulate ERK activity by indirect, MEK-dependent (Yuan *et al.* 2002) or MEK-independent (Grammer & Blenis, 1997; Kinkl *et al.* 2001) mechanisms. Similarly, extensive intracellular cross-talk between hippocampal PKA, PKC and MEK converging at ERK was shown in hippocampal slices (Robertson *et al.* 1999) and *in vivo* (Ahi *et al.* 2004). Among the upstream regulators of ERK activity, PKC appears to be one of the most interesting. There is mounting evidence suggesting that elevated phosphoinositide-protein kinase C (PI-PKC) signal transduction pathway may be a pathophysiological feature of bipolar disorder (Friedman *et al.* 1993; Wang *et al.* 1999) and major depressive disorders (Karege *et al.* 1996; Pandey *et al.* 1998). In agreement with the suggested role of PKC in depression, we observed an antidepressant-like behaviour by the selective PKC blocker calphostin C that was not accompanied by any modification of spontaneous mobility and locomotor activity of mice, a potential confound for the evaluation of a depressant/antidepressant effect. Additionally, an acute session of FST or TST increased the phosphorylation in the hippocampus and frontal cortex of PKCε, an isoform highly detected in the central nervous system. In particular, the translocation of PKC enzymes from the cytosol to the synaptic membrane is thought to be necessary for their activation (Nishizuka, 1992). We discovered a lack of increase in the phosphorylation of PKCε in the cytosol compared to the high phosphorylated form levels observed in the membrane fraction. The prevention of the PKCε phosphorylation in the whole protein preparation as well as in the membrane fraction by pretreatment with calphostin C, further supports the hypothesis of a PKC hyperactivity in depression emerging from behavioural results.

Next, the potential involvement of PKC as upstream modulator of ERK activity was investigated. Pretreatment with calphostin C prevented the phosphorylation of ERK1 and ERK2 induced by FST and TST in the hippocampus and frontal cortex, suggesting that PKC activation might contribute to the ERK activation observed in animals with a depressant-like behaviour.

JNK/SAPK and p38MAPK belong to the MAPK family and mainly function as mediators of cellular stress. Since increasing evidence implicates stress as an important factor in the vulnerability to depression (Kendler *et al.* 2001), the role of p38MAPK and JNK in the induction and modulation of a depressant-like condition was investigated. Inhibition of the activity of JNK and p38MAPK following i.c.v. administration of specific blockers (JNK inhibitor II and SB203580, respectively) produced an antidepressant-like behaviour. This effect was comparable to that produced by

amitriptyline and occurred at doses unable to modify spontaneous mobility or alter locomotor activity. JNK and p38MAPK pathways appear to participate to the induction of a depressant-like behaviour, suggesting their potential involvement in mood modulation. FST or TST, similarly to that observed for ERK activity, elevated the phosphorylation of p38MAPK in the hippocampus and frontal cortex with a prominent effect on the hippocampus. This increased p38MAPK phosphorylation is prevented by blockade of PKC activity, suggesting PKC as an upstream modulator of p38MAPK pathway in the induction of a depressant-like phenotype. An elevation of JNK activity was also detected following an acute FST or TST session, but with a different profile. Conversely to ERK and p38MAPK pathways, we detected a profound increase in phosphorylation of JNK in the frontal cortex compared to the significant, but much lower intensity, elevation of JNK activity in the hippocampus. Furthermore, the hippocampal increase of p-JNK levels was observed after exposure to TST, but not FST. There are three *JNK* genes (*JNK1*, *JNK2*, *JNK3*): *JNK1* and *JNK2* are ubiquitously expressed while *JNK3* is restricted to brain, heart and testes. Each *JNK* is expressed as a short form (JNK-s, 46 kDa) and a long form (JNK-l, 56 kDa) (Pulverer *et al.* 1991). We studied the long (JNK-l) and short (JNK-s) isoforms of JNK since all three JNK isoforms (JNK1, 2, 3) might potentially be involved in depressive disorders. Densitometric analysis of the activation of the short and long JNK forms showed a significant increase of both isoforms after exposure to TST and modest activation of the short form after FST in the hippocampus. This increased phosphorylation was prevented by the PKC-blocker calophostin C, showing a hippocampal JNK activation profile similar to ERK and p38MAPK pathways. In the frontal cortex there was a marked increase in activation of the short form and, to a lesser extent, of the long form of JNK, an effect that was PKC-insensitive. A regionally selective differential upstream modulation of JNK activity following exposure to depressant tasks emerged from these data. A higher sensitivity to TST than to FST emerged, suggesting that these two behavioural tasks produced similar, but not identical, cerebral cellular events. JNK represents a MAPK highly involved in the response to stress stimuli (Johnson & Nakamura, 2007). This feature is also demonstrated by the profound increase in JNK activity in different brain areas following exposure to stress paradigms (Liu *et al.* 2004). Taking into consideration that repeated or longer sessions of FST are also used as stress paradigms, we can hypothesize that exposure to an acute 6-min session of FST or TST might produce not only depressant-like behaviour, but also cellular modifications involved in the response to a stress condition. We cannot exclude that the PKC-independent cortical JNK activation mainly represents a stress response to FST or TST.

To further clarify the relevance of the stress-induced cellular modifications after an acute FST or TST session for the results obtained, we detected the levels of CREB and p-CREB in the hippocampus and frontal cortex. Variation of CREB and p-CREB contents showed opposite profiles when detected after exposure to stress or in a depressive condition. Stress exposure leads to alterations in CREB protein activation in a variety of brain regions. In particular, acute stressors, such as swim stress, footshock, restraint stress, and social stress, increase CREB activity whereas chronic stressors and anxiety states decrease CREB activity in the hippocampus and frontal cortex (Briand & Blendy, 2010). An involvement of CREB in the pathophysiology of depressive disorders has been postulated since antidepressant drugs and electroconvulsive shock up-regulate cerebral CREB. Increasing CREB levels in rodent models (Blendy, 2006) and over-expression of CREB in hippocampus results in antidepressant-like behaviour (Chen *et al*. 2001). The protein and mRNA expression of CREB is also reduced in the post-mortem brain of depressed patients (Dwivedi *et al*. 2003; Yamada *et al.* 2003), and in human fibroblasts (Manier *et al.* 2002) and neutrophils (Ren *et al.* 2011) of patients with major depression. While not significant, we see a trend towards decreased CREB and CREB phosphorylation in the hippocampus following TST. Pre-treament with the PKC blocker calphostin C increased p-CREB levels, in accord with the effects produced by antidepressant drugs (Blendy, 2006; Mombereau *et al.* 2010). Conversely, in the frontal cortex a PKC-insensitive increase in CREB and pCREB levels was detected, suggesting a prevalent involvement of a stress-induced response for the cortical PKC-independent effects.

The present results illustrate a regionally selective and differentially PKC-modulated activation of MAPK in hippocampus and frontal cortex of animals exposed to behavioural despair paradigms. We also demonstrate that acute pharmacological blockade of MAPK signalling produces an antidepressant-like phenotype. These findings identify hippocampal and cortical MAPKs as critical targets in depressive-like tasks and should facilitate comprehension of the aetiopathology of mood disorders and help in developing new therapeutical strategies.

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Statement of Interest

None.

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