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Fluoxetine increases the activity of the ERK-CREB signal system and alleviates the depressive-like behavior in rats exposed to chronic forced swim stress

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ABSTRACT

Our previous research indicates that the extracellular signal-regulated kinase (ERK)-cyclic AMP-responsive-element-binding protein (CREB) signal system may be involved in the molecular mechanism of depression. The present study further investigated the effect of antidepressant fluoxetine on the ERK-CREB signal system and the depressive-like behaviors in rats. Fluoxetine was administrated to either naive rats or stressed rats for 21 days. The results showed that chronic forced swim stress induced depressive-like behaviors and decreased the levels of P-ERK2, P-CREB, ERK1/2 and CREB in hippocampus and prefrontal cortex. Fluoxetine alleviated the depressive-like behaviors and reversed the disruptions of the P-ERK2 and P-CREB in stressed rats. Fluoxetine also exerted mood-elevating effect and increased the levels of the P-ERK2 and P-CREB in naive rats. These results suggest that the ERK-CREB signal system may be the targets of the antidepressant action of fluoxetine and participate in the neuronal mechanism of depression.

Introduction

Our previous study demonstrated that the extracellular signal-regulated kinase (ERK) signal pathway participated in stress response and correlated with the stress-induced depressive-like behaviors (Qi et al., 2006), which suggests that the ERK signal pathway may participate in the molecular mechanism of depression. The present study was to further investigate the effect of the antidepressant fluoxetine on the ERK signal system which could lead to better understanding the signalization cascade and novel therapeutic targets in depression.

Fluoxetine is a widely used antidepressant, which acts via inhibiting 5-hydroxytraptamine (5-HT) reuptake in the central nervous system (Cipriani et al., 2005; Lee et al., 2001). Novel theories propose that signal pathway related to synaptic plasticity may be the mechanism of antidepressant action and the pathophysiology of depression (Einat et al., 2003; Malberg et al., 2000; Manji et al., 2001). ERK is the most-studied member of the mitogen-activated protein kinase (MAPK) family, and the ERK pathway is the major convergence point in all signal pathways, regulating cellular growth and differentiation and neuronal plasticity (Chen et al., 2001; Schafe et al., 2000; Zhu et al., 2002; Sweatt, 2001). At present, the vital role of the ERK signal system in the regulations of emotional responses increasingly becomes the focus of much research. ERK1 and ERK2 are prominently found in hippocampus and prefrontal cortex (Flood et al., 1998; Ortiz et al., 1995), which are brain regions most likely to be implicated in stress response and depression (Gruen et al., 1995). Recent research indicates that brain-derived neurotrophic factor can ameliorate depression via activating the ERK pathway (Shirayama et al., 2002), and the 5-HT treatment can induce ERK phosphorylation (phospho-ERK1/2, P-ERK1/2) (Mattson et al., 2004; Cipriani et al., 2005; Lee et al., 2001). These data suggest that the ERK pathway may be the potential target of antidepressant and participate in the molecular mechanism of depression.

Cyclic AMP-responsive-element-binding protein (CREB) is a transcription factor and downstream target of ERK pathway (Xing, 1996). When the ERK activation is depressed, the CREB-dependent plasticity will be disrupted (Hardingham et al., 2001; Davis et al., 2000; Ying et al., 2002). Similarly, CREB phosphorylation (phospho-CREB, P-CREB) is essential for the induction of the ERK-dependent plasticity (Davis et al., 2000; Ying et al., 2002). ERK and CREB have emerged as critical points of convergence in signaling pathways regulating neuronal plasticity.

In order to further confirm the role of the ERK-CREB signal system in the molecular mechanism of depression and explore the potential target of antidepressant, the present study was to investigate the effect of fluoxetine on the ERK-CREB signal system and the depressive-like behaviors in stressed rats. In the experiment, forced swim stress, a putative animal model of depression with good face validity and the highest degree of pharmacological predictive validity (Porsolt et al., 1978; Lucki, 1997; Qi et al., 2006), was applied. A series of behavioral tests, including open field test, saccharin preference test and elevated

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plus maze test, were used to assess emotionality, locomotor activity, anhedonia of depression, as well as anxiety.

Methods and materials

Animals

Male Sprague-Dawley (250-275 g) rats were obtained from Wei Tong Li Hua Lab Animal Center (Beijing, China). All rats were housed individually. They were acclimated to 3 min of handling once a day for 7 days before being used in experiment. Rats were maintained on a 12-h light/dark cycle (lights on at 08:00 h) with free access to food and water except for the saccharin preference test time. The experiment procedures were approved by the International Review Board of the Institute of Psychology, Chinese Academy of Science, and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Forced swim stress and drug administration

After initial handling, rats were randomly assigned to one of four groups and then underwent 21 days of one of four different treatments. The first group (Con — controls, $n=10$) received a daily intraperitoneal (i.p.) injection of 1 ml/kg saline (0.9% NaCl) for 21 days. The second group (FSS — forced swim stress, $n=9$) received a daily i.p. injection of saline immediately after undergoing one trial per day of forced swim stress for 21 days. The third group (Con-Flu — fluoxetine, $n=8$) received a daily i.p. injection of fluoxetine (Sigma-Aldrich, St Louis, MO, USA) for 21 days, 10 mg/kg diluted in saline. The fourth group (FSS-Flu — forced swim stress exposed and fluoxetine treated, $n=8$) received an i.p. injection of fluoxetine immediately after forced swim stress as did the second group. The dosage of 10 mg/kg and the time schedule of 21 days for fluoxetine have been reported to show antidepressant action in a previous work (Svenningsson et al., 2002; De La Garza et al., 2002; Grippo et al., 2006; Rygula et al., 2006).

The forced swim stress was performed between 08:00 h and 10:00 h in a stainless steel tank (2.0 ml × 1.0 mW × 1.5 mH) filled with 30 cm depth of water (10±0.5 °C). Each rat was forced to swim individually for 5 min once a day for 21 consecutive days.

Behavioral tests

Open field test

The test was performed between 08:00 and 12:00 h. The apparatus was a circular arena, 180 cm in diameter with 50 cm wall. The test room had a dim illumination (40 W) in order to decrease the averseness of the test (Sarkisova and Kulikov, 2006; Qi et al., 2006). One rat was placed in the center of the field and the following variables were recorded for 5 min and analyzed by a computer-based system (EthoVision, Noldus Information Technology b.v., Netherlands): distance traveled, freezing time, and the number of the fecal boli defecated during open field exploration were counted (Hall, 1934). The open field was cleaned each time after testing a rat.

Elevated plus maze test

The test was performed between 08:10 and 12:10 h immediately after the open field test. When the open field test was finished, the same rat was immediately placed into the elevated plus maze. The maze procedures were run as previously described (Sarkisova and Kulikov, 2006; Qi et al., 2006). Briefly, the apparatus consisted of two opposite open arms (50.8 cm × 10.2 cm × 1.3 cm) and two opposite closed arms (50.8 cm × 10.2 cm × 40.6 cm). The arms were connected by a central square (10.2 cm × 10.2 cm). The apparatus was 72.4 cm above floor and exposed to dim illumination. An animal was placed in the center of the maze facing a closed arm. The behavior was recorded for 5 min and analyzed by a computer-based system (MED-VPM-RS, Med Associate Inc, USA).The number of exploration in open arms and closed arms, the number of entries into the open arms and the closed arms, and the time spent in open arms, closed arms, and the central area were assessed. An individual exploration was recorded when the animal entered the arm with at least two front paws and half of its body, and an individual entry was recorded when the whole body of an animal entered the arm. The shorter the time spent in the open arms, the higher is the anxiety and vice versa (Ho et al., 2002; Mechan et al., 2002). After each trial, the apparatus was cleaned with 30% ethanol solution.

Saccharin preference test

On the last-stressed day, rats were deprived of water (from 20:00 h). From the next day on, rats were given a 3-h window saccharin preference test (14:00-17:00 h) once a day for 4 days. The rats were given two bottles, one containing water and the other containing 1% sodium saccharin solution. The amount of each liquid intake was determined by weighing the bottles before and after the 3-h window. The saccharin solution intake, the water intake and the total liquid intake in 4 days were computed. Reduced consumption of sweet solutions (sucrose, saccharin) by chronic mild stress rats is a measure of anhedonia (Cryan et al., 2002). The saccharin preference was calculated as the ratio of the saccharin solution intake to total liquid intake. The positions of the bottles on the cages were changed every day. At the end of the preference test, rats were given free access to water.

Western blot

The antibodies of ERK1/2 primary antibody (rabbit monoclonal), P-ERK1/2 primary antibody (Thr202/Tyr204, mouse monoclonal), CREB primary antibody (rabbit monoclonal), P-CREB primary antibody (Ser133, mouse monoclonal), β-Actin primary antibody (mouse monoclonal), horseradish-peroxidase-labeled goat antirabbit secondary antibody IgG and horseradish-peroxidase-labeled goat antimouse secondary antibody IgG were obtained from Sigma-Aldrich, St Louis, MO, USA. 0.2 μm nitrocellulose filter (NC), polyacrylamide and Buffer were also from Sigma-Aldrich, St Louis, MO, USA. Bicinchoninic acid assay kit (BCA) and enhanced chemiluminescence (ECL) reagent were obtained from Pierce Biotechnology, Rockford, IL, USA. Gel Doc™ XR System and Quantity One® 1-D analysis software were purchased from Bio-Rad, Hercules, CA, USA.

All rats were decapitated after behavioral tests, and brains were rapidly removed on ice. The brain was placed in a stainless steel brain matrix, and the prefrontal cortex and hippocampus were dissected bilaterally according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). The prefrontal cortex included the area between 1 and 4 mm posterior to the front pole of the cortex, and between 0 and 3 mm from the midline. The hippocampus has clear boundaries with other structures and the whole body of hippocampus which includes both the dorsal and the ventral parts was dissected from the brain. All tissues were placed into liquid nitrogen to be frozen. Tissues were homogenized in 20 volumes of Buffer (PH 7.5, containing 50 mM Tris-Cl, 2 mM EDTA, 2 mM EGTA, 0.05 mM okadaic acid, 1 μM sodium vanidate, 5 μg/ml pepstatinA and 0.5% Nonidet P-40). Protein content of lysates was determined using BCA assay kits. Lysates were mixed with 5× sodium dodecyl sulfate (SDS) to prepare for certain concentration of sample solutions. All the sample solutions were stored at -80 °C for use.

Proteins were separated by SDS-PAGE on 10% polyacrylamide gels. Then proteins were moved to NC by electrophoretic transfer. Blots were incubated in blocking buffer (10% nonfat dry milk powder in Tris-buffered saline containing 0.5% Tween-20, TTBS) for 1 h at room temperature (RT), washed 10 min ×3 in TTBS. Blots were incubated with P-ERK1/2 primary antibody overnight at 4 °C and then washed 10 min ×3 in TTBS. Blots were incubated with horseradish-peroxidase-labeled goat antimouse secondary antibody IgG for 1 h at RT, washed

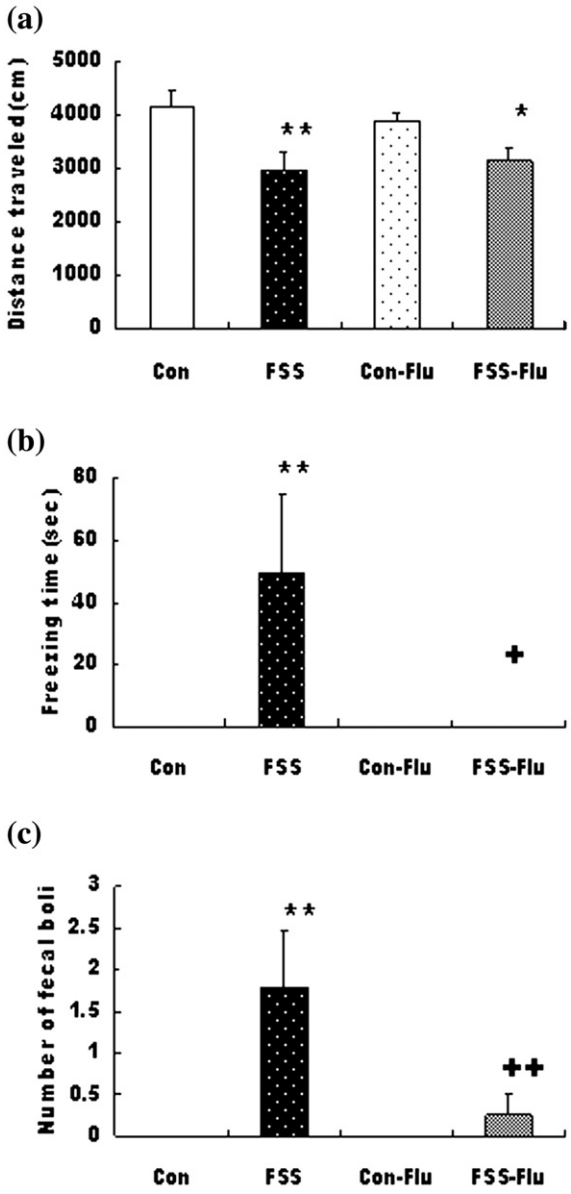

Fig. 1. The effect of fluoxetine on distance traveled (a), freezing time (b), and number of fecal boli (c) in open field in rats. Data expressed as mean+SEM, n=8-10 per group. *P<0.05, **P<0.01 *vs* Con, +P<0.05, ++P<0.01 *vs* FSS.

10 min ×3 in TTBS, treated with ECL reagents and exposed to film. Then blots were stripped of antibodies by incubation for 10 min at 50 °C with stripping buffer, re-blocked, washed, incubated for 3 h at RT with ERK1/2 primary antibody which recognized total antigen protein, incubated with horseradish-peroxidase-labeled goat antirabbit secondary antibody IgG for 1 h at RT, then the total protein of ERK1/2 was visualized by treatment with ECL reagent and exposure to film. Repeat the above procedures, and the antigen of β-Actin was visualized to film by binding to the proper primary antibody (β-Actin primary antibody) and secondary antibody (horseradish-peroxidase-labeled goat antimouse secondary antibody IgG). The P-CREB, CREB and their corresponding β-Actin were determined in a different NC using the same procedures.

Immunoblots were analyzed using Quantity One® 1-D analysis software. For each blot of P-ERK1/2, ERK1/2, P-CREB and CREB, the relative protein level was calculated from the ratio of the absorbance of P-ERK1/2, ERK1/2, P-CREB and CREB to their corresponding β-Actin to correct for small difference in protein loading.

Statistical analysis

Differences among groups were examined using one-way ANOVA, followed by LSD post hoc test (two-tailed). The statistical analysis was performed using SPSS 11.5 for Windows. Data were presented as means+SEM. $P \leq 0.05$ was the accepted level of significance.

Results

Open field test

The open field test has been used widely to assess emotionality and locomotor performance (Ramos and Mormede, 1997; Prut and Belzung, 2003). The behavior in the peripheral zone and along the

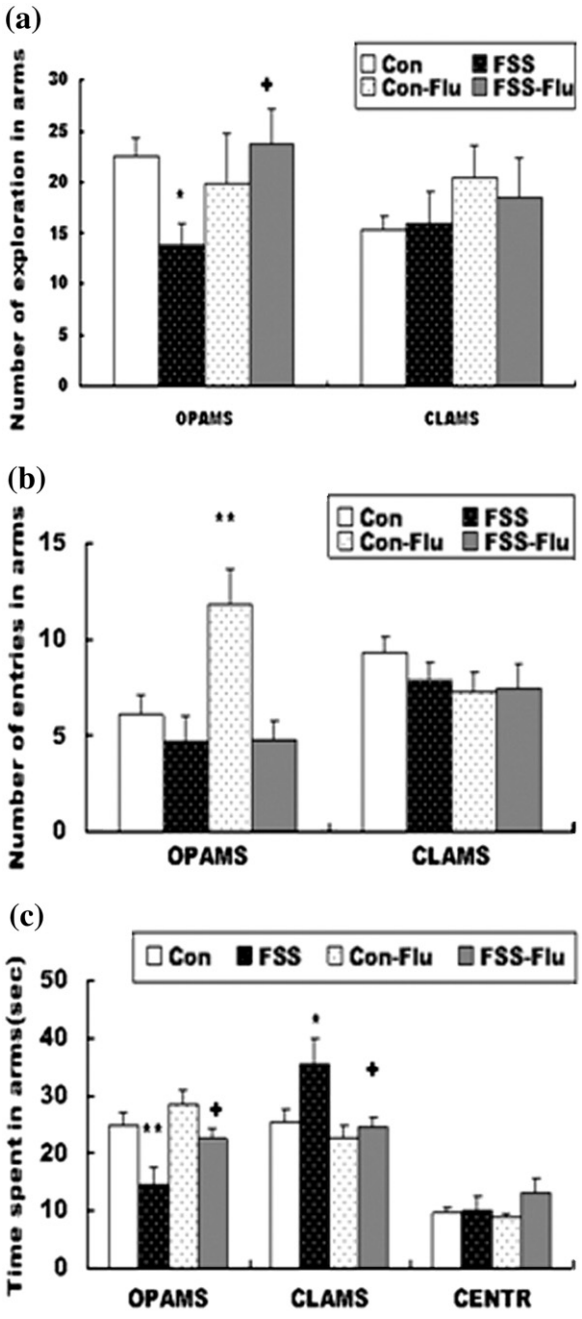

Fig. 2. The effect of fluoxetine on number of exploration in arms (a), number of entries into arms (b), and time spent in arms (c) in elevated plus maze in rats. OPAMS, open arms; CLAMS, closed arms; CENTR, central area. Data expressed as mean+SEM, n=8-10 per group. *P<0.05, **P<0.01 *vs* Con, +P<0.05 *vs* FSS.

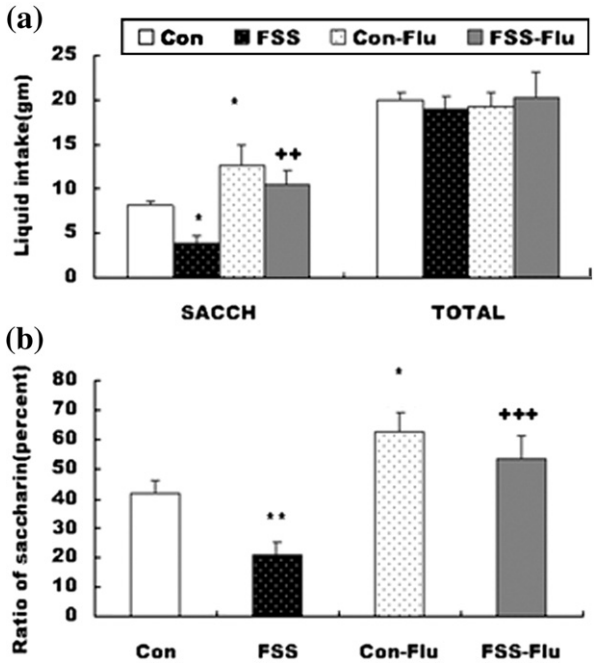

Fig. 3. The effect of fluoxetine on liquid intake (a) and ratio of saccharin (b) of rats. SACCH, saccharin solution; TOTAL, total liquid. Data expressed as mean+SEM, n=8-10 per group. *P<0.05, **P<0.01 *vs* Con, ++P<0.01, +++P<0.001 *vs* FSS.

walls of the field is thought to reflect general activity (Belzung and Griebel, 2001; Prut and Belzung, 2003). A one-way ANOVA indicated significant differences among groups in the distance traveled [$F_{(3,31)}$= 4.37, P<0.01]. A further post hoc test revealed that the distance traveled was significantly decreased in FSS and FSS-Flu rats compared to Con rats (P<0.01 and P<0.05, respectively), and there were no significant differences in the distance traveled between FSS rats and FSS-Flu rats, suggesting that fluoxetine did not prevent the stress-induced decreased locomotor activity (Fig. 1a). A one-way ANOVA on the freezing time indicated significant differences among groups [$F_{(3,31)}$=3.76, P<0.05]. Post hoc test revealed that the freezing time was significantly increased in FSS rats when compared to Con (P<0.01) and FSS-Flu animals (P<0.05), which indicates that fluoxetine alleviated the stress-induced increase of freezing behavior (Fig. 1b). A one-way ANOVA indicated significant differences among groups in the number of boli of defecation [$F_{(3,31)}$=5.21, P<0.01]. Post hoc test revealed that the FSS rats had increased number of boli of defecation compared to Con and FSS-Flu animals (P<0.01, either), which is indicative of decreased irritability in stressed rats following fluoxetine treatment (Fig. 1c).

Elevated plus maze test

The elevated plus maze is a commonly used test of anxiety in mice and rats (Handley and Mithani, 1984; Espejo, 1997; Wall and Messier, 2001). The test is based on the conflict between the drive to explore, and the innate avoidance of a novel and an open space (Montgomery, 1955; Treit, 1985). The reduced number of entries into the open arms and the decreased time spent in the open arms are used to assess a state of anxiety (Cruz et al., 1994; Mechan et al., 2002). A one-way ANOVA indicated significant differences among groups in the number of exploration in open arms [$F_{(3,31)}$=2.10, P<0.05]. A further post hoc test revealed that the number of exploration in open arms was significantly decreased in FSS group when compared to Con and FSS-Flu groups (P<0.05, either), suggesting that fluoxetine significantly reversed the stress-induced decrease of exploratory activity in open arms (Fig. 2a). One-way ANOVA indicated that the number of entries into open arms significantly differed among groups [$F_{(3,31)}$=6.45, P<0.01]. Post hoc test revealed that the Con-Flu group had increased number of entries into the open arms compared to Con group

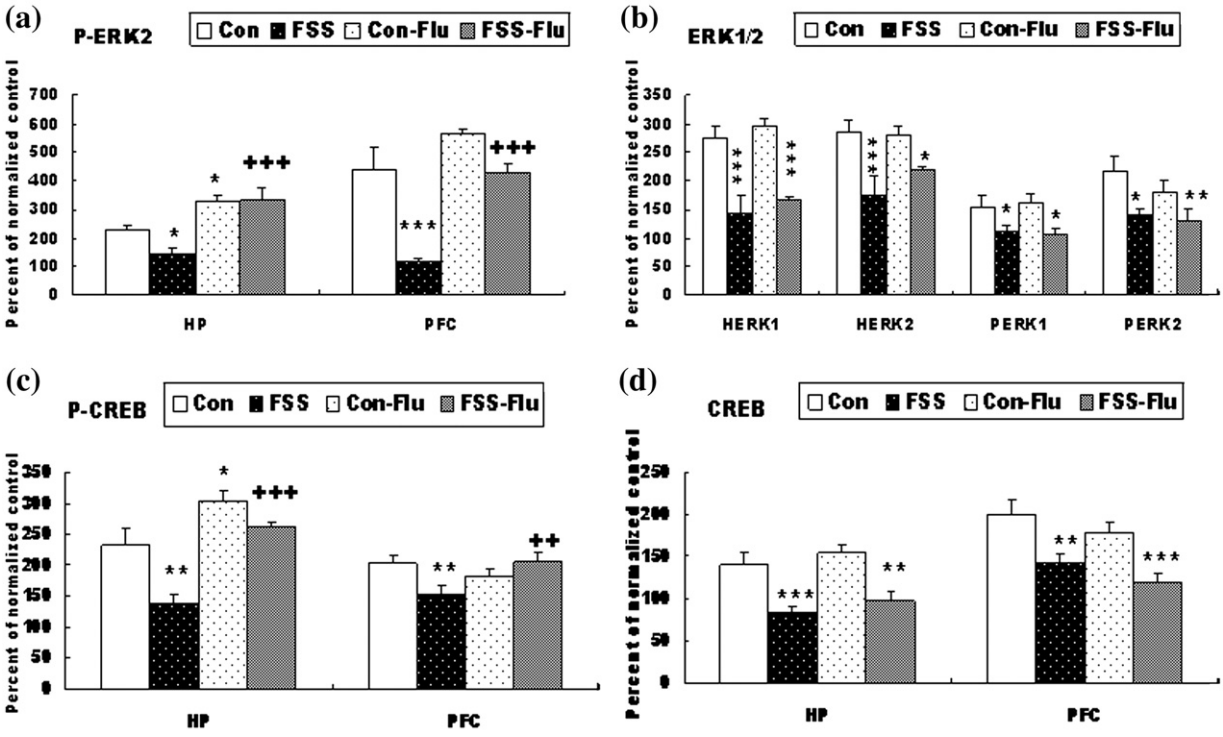

Fig. 4. The effect of fluoxetine on expression of P-ERK2 (a), ERK1 and ERK2 (b), P-CREB (c), and CREB (d) in hippocampus and prefrontal cortex of rats. HP, hippocampus; PFC, prefrontal cortex; HERK1, ERK1 in the hippocampus; HERK2, ERK2 in the hippocampus; PERK1, ERK1 in the prefrontal cortex; PERK2, ERK2 in the prefrontal cortex. Data expressed as mean+SEM, n=8-10 per group. *P<0.05, **P<0.01, ***P<0.001 *vs* Con, ++P<0.01, +++P<0.001 *vs* FSS.

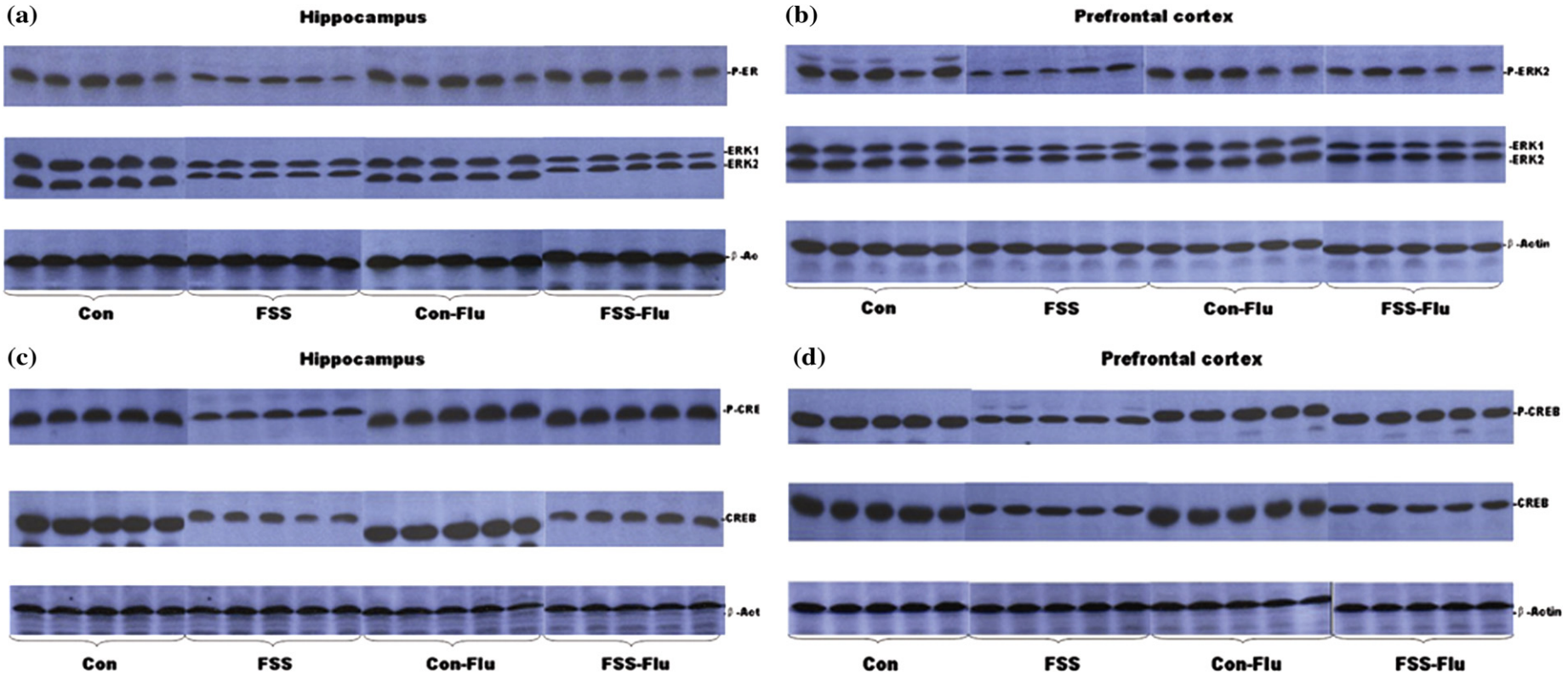

Fig. 5. Representative western blots of P-ERK2, ERK1, ERK2, P-CREB, CREB and their corresponding normalized control β-Actin in hippocampus and prefrontal cortex.

($P<0.01$), indicating that fluoxetine increased the entries into the open arms in normal rats (Fig. 2b). One-way ANOVA on the time spent in arms indicated that the time spent in open arms and the time spent in closed arms both significantly differed among groups [$F_{(3,31)}=5.94$, $P<0.01$, and $F_{(3,31)}=3.79$, $P<0.05$, respectively]. Post hoc tests revealed that the FSS rats spent shorter time in open arms and longer time in closed arms than did Con ($P<0.01$ and $P<0.05$, respectively) and FSS-Flu animals ($P<0.05$, either), which indicates that fluoxetine increased the exploratory activity in open arms and decreased the exploratory activity in closed arms in stressed rats (Fig. 2c). The number of entries into closed arms, the number of exploration in closed arms, and the time spent in central area did not differ among groups.

Saccharin preference test

The reinforcing properties of saccharin solution are used as an index of hedonic alterations, and the reduced consumption of sweet solutions is a measure of anhedonia (Cryan et al., 2002).One-way ANOVA indicated that both the saccharin solution intake and the saccharin preference significantly differed among groups [$F_{(3,31)}=7.36$, $P<0.001$, and $F_{(3,31)}=9.88$, $P<0.001$, respectively]. Post hoc tests revealed that the FSS rats exhibited decreased saccharin solution intake and decreased saccharin preference when compared to Con ($P<0.05$ and $P<0.01$, respectively) and FSS-Flu groups ($P<0.01$ and $P<0.001$, respectively), and Con-Flu rats had increased saccharin solution intake and increased saccharin preference compared to Con rats ($P<0.05$, either), which is indicative of a higher sensitivity to the rewarding effect of saccharin solution after fluoxetine treatment whether in stressed rats or in normal rats (Figs. 3a and b). The total liquid intake did not differ among groups.

Expression of P-ERK2 and ERK1/2 in the hippocampus and prefrontal cortex

P-ERK1 and P-ERK2 changed in parallel, but in short exposure, the signals of the P-ERK1 were too weak to be detected, so only the P-ERK2 signals were quantified.

One-way ANOVA indicated that the P-ERK2 level significantly differed among groups both in the hippocampus and in the prefrontal cortex [$F_{(3,31)}=9.72$, $P<0.001$, and $F_{(3,31)}=16.51$, $P<0.001$, respectively]. Post hoc tests revealed that the level of P-ERK2 in the hippocampus and prefrontal cortex was significantly decreased in FSS rats compared to Con ($P<0.05$ and $P<0.001$, respectively) and FSS-Flu animals ($P<0.001$, either), suggesting that fluoxetine had a reversing effect on the stress-induced decreased P-ERK2 level both in the hippocampus and in the prefrontal cortex. Post hoc test also revealed that the Con-Flu group had increased hippocampal level of P-ERK2 compared to Con group ($P<0.05$), indicating that fluoxetine increased the hippocampal level of P-ERK2 in normal rats (Figs. 4a, 5a and b).

For the levels of ERK1 and ERK2, one-way ANOVA indicated significant differences among groups both in hippocampus [$F_{(3,31)}=13.62$, $P<0.001$, and $F_{(3,31)}=5.33$, $P<0.01$, respectively] and in prefrontal cortex [$F_{(3,31)}=3.54$, $P<0.05$, and $F_{(3,31)}=3.45$, $P<0.05$, respectively]. Post hoc tests revealed that the ERK1 and ERK2 in both the hippocampus and the prefrontal cortex were significantly decreased in FSS group compared to Con group (hippocampus, $P<0.001$, either; prefrontal cortex, $P<0.05$, either), and there were no significant differences in the levels of ERK1 and ERK2 in the hippocampus and prefrontal cortex between FSS-Flu group and FSS group, suggesting that forced swim stress decreased the levels of ERK1 and ERK2 both in the hippocampus and in the prefrontal cortex, and fluoxetine had no reversing effect on the stress-induced decrease of ERK1 and ERK2. Post hoc tests also revealed that there were no significant differences in the levels of ERK1 and ERK2 between Con-Flu group and Con group, indicating that fluoxetine exerted no effect on the ERK1 and ERK2 in normal rats (Figs. 4b, 5a and b).

Expression of P-CREB and CREB in the hippocampus and prefrontal cortex

One-way ANOVA indicated that the P-CREB level significantly differed among groups both in hippocampus and in prefrontal cortex [$F_{(3,31)}=11.73$, $P<0.001$, and $F_{(3,31)}=3.59$, $P<0.05$, respectively]. Post hoc tests revealed that the P-CREB in the hippocampus and prefrontal cortex was significantly decreased in FSS group compared to Con ($P<0.01$, either) and FSS-Flu groups ($P<0.001$ and $P<0.01$, respectively), suggesting that fluoxetine had a reversing effect on the decreased P-CREB level both in the hippocampus and in the prefrontal cortex in stressed rats. Post hoc test also revealed that Con-Flu group had increased hippocampal P-CREB level compared to Con group ($P<0.05$), indicating that fluoxetine increased the hippocampal level of P-CREB in normal rats (Figs. 4c, 5c and d).

One-way ANOVA indicated that the CREB level significantly differed among groups both in hippocampus and in prefrontal cortex [$F_{(3,31)}=9.23$, $P<0.001$, and $F_{(3,31)}=7.10$, $P<0.001$, respectively]. Post hoc tests revealed that the level of CREB in the hippocampus and prefrontal cortex was significantly decreased in FSS group compared to Con group ($P<0.001$ and $P<0.01$, respectively), and there were no significant differences in the level of CREB in the hippocampus and prefrontal cortex between FSS group and FSS-Flu group, indicating forced swim stress decreased CREB level both in hippocampus and in prefrontal cortex, but fluoxetine had no reversing effect on the stress-induced decrease of CREB. Post hoc tests also revealed that there were no significant differences in the level of CREB between Con-Flu group and Con group, suggesting that fluoxetine had no effect on the CREB in normal rats (Figs. 4d, 5c and d).

Discussion

The results of the present study demonstrated that chronic forced swim stress induced depressive-like behaviors in rats, and fluoxetine reversed almost all the behavioral alterations observed in the model. FSS-Flu rats compared to FSS rats exhibited shorter freezing time and decreased number of fecal boli in the open field, which is indicative of decreased freezing behavior and decreased irritability (Ramos and Mormede, 1997; Prut and Belzung, 2003); more exploration in open arms, longer time spent in open arms and shorter time spent in closed arms in the elevated plus maze, which indicates decreased anxiety (Ho et al., 2002; Mechan et al., 2002); more saccharin solution intake and higher saccharin preference, which suggests decreased anhedonia (Cryan et al., 2002). Our results are in concordance with data from other studies which demonstrate that chronic fluoxetine treatment counteracts stress-induced freezing behavior and defecation, and reverses chronic mild stress-induced anhedonia (Zhang et al., 2000; Grippo et al., 2006). However, differing with a previous report that fluoxetine counteracts deficits in locomotor activity induced by chronic social stress (Rygula et al., 2006), the present study observed that the distance traveled in the open field was still lower in FSS-Flu rats than that in control rats. Possible reasons for the discrepancy are not apparent, but may be due to the category of stress applied and other experimental procedures. In comparison with control animals, Con-Flu rats exhibited increased number of entries into the open arms, increased saccharin solution intake and higher saccharin preference, which indicates that fluoxetine exerts mood-elevating effect on the naive animals. The present study demonstrated that the depressive-like behaviors were accompanied with the anxiety-like behaviors in stressed rats, and fluoxetine could reverse these two categories of behavioral deficits. Our results confirmed previous clinical observations, which indicate that a majority of patients suffering from major depression also have comorbid anxiety (Zimmerman et al., 2000), and there is significant efficacy for fluoxetine in treatment of depression with comorbid anxiety (Argyropoulos et al., 2000; Nash and Hack, 2002).

The present study demonstrated that chronic forced swim stress decreased the expression of P-ERK2, ERK1 and ERK2 in the hippocampus

and prefrontal cortex in rats; fluoxetine reversed the stress-induced disruption of the P-ERK2, which is indicated by the increased level of the P-ERK2 in the hippocampus and prefrontal cortex in FSS-Flu group compared to FSS group, but exhibited no effect on the stress-induced decrease of the ERK1 and ERK2; fluoxetine increased the hippocampal P-ERK2 but had no effect on the ERK1 and ERK2 in the hippocampus and prefrontal cortex in naive rats. Our results are in accordance with previous reports which indicate decreased P-ERK2 in stressed animals (Qi et al., 2006; Meller et al., 2003), and decreased P-ERK2 and ERK1/2 in depressed rats and depressed human beings (Feng et al., 2003; Dwivedi et al., 2001). Although recent documents indicate that lithium and valproate, two medications largely used for the treatment of bipolar disorder illness, stimulate the ERK pathway (Einat et al., 2003), the effect exerted by fluoxetine on the ERK signal system in brain has been poorly documented. To our knowledge, there is only one study so far evaluating this issue, which demonstrates that fluoxetine markedly and significantly increases the protein levels of ERK1 and ERK2 in prefrontal cortex but not in hippocampus, and has no effect on the level of P-ERK2 in naive rats (Tiraboschi et al., 2004). Differing with their results, the present study indicates that fluoxetine significantly increases the hippocampal P-ERK2 level, but has no effect on the protein levels of ERK1 and ERK2 both in the hippocampus and in the prefrontal cortex in naive rats. The discrepancy needs further investigations. It is notable that there is no study to date to investigate the effect of fluoxetine on the stress-induced alterations in ERK pathway, and the present study demonstrates for the first time that fluoxetine reverses the stress-induced decrease of P-ERK2 in the hippocampus and prefrontal cortex.

As the results of the present study demonstrated, fluoxetine reversed the disruption of P-CREB in the hippocampus and prefrontal cortex in stressed rats and increased the P-CREB in the hippocampus in naive rats, but exhibited no effect on the protein level of CREB whether in stressed rats or in naive rats. Several lines of evidence showed recently that the P-CREB is decreased in stressed animals and in depressed human beings (Xu et al., 2006; Laifenfeld et al., 2005; Yamada et al., 2003), and fluoxetine increases the brain level of P-CREB in naive rats (Tiraboschi et al., 2004; Dowlatshahi et al., 1998; Thome et al., 2000), however, little is known about the effect of fluoxetine on the stress-induced P-CREB reduction. The present study is consistent with one recent published literature which indicates that fluoxetine reverses the decreased activation of CREB in the brain induced by stress (Laifenfeld et al., 2005). Tiraboschi et al. recently reported that fluoxetine increases the phosphorylation of CREB, without affecting the CREB protein level in naive rats (Tiraboschi et al., 2004), which is in accordance with our results. Moreover, we also observed that fluoxetine is lack of effect on the stress-induced decrease of CREB level. An interesting finding of the present study is the selective increase of the P-ERK2 and P-CREB induced by fluoxetine in rats. It thus appears that the major effect of fluoxetine is to activate the ERK and CREB to increase the levels of P-ERK2 and P-CREB, but not to increase the ERK and CREB expression.

The present study demonstrated that fluoxetine increased the activity of the ERK-CREB signal system. The ERK/MAPK cascade is among the major pathways leading to the phosphorylation of CREB and modulation of transcriptional activity, mainly in response to stress-induced signaling (Ginty et al., 1994; Reusch et al., 1994). Several lines of evidence showed recently that the ERK activation is necessary to induce sustained and neuronal activity-dependent CREB phosphorylation (West et al., 2002). Our finding that the increase of CREB phosphorylation by fluoxetine is accompanied by the upregulation of ERK activation may suggest a role of the ERK pathway in the increase of CREB phosphorylation in the chronic action of fluoxetine, although these data are not sufficient to restrict the induction of P-CREB to this pathway. It could be envisaged that additional pathways are involved in this mechanism.

The present study demonstrated that stress disrupted the activity of the ERK-CREB signal system and induced depressive-like behavior, and fluoxetine reversed the stress-induced disruption of the activity of the ERK-CREB signal system and alleviated the depressive-like behavior. These results strongly support the assumption that the ERK-CREB signal system participates in mediating stress response, as well as the effective pharmacotherapy of depression. It is intriguing to speculate, therefore, that the initial reduction in activities of the ERK-CREB signal system consequent to stress exposure, and their subsequent normalization by fluoxetine, may represent a mechanism of action by which fluoxetine reverses the stress-induced depressive-like behaviors, and a neuronal mechanism of depression in which the ERK-CREB signal system is involved. Recent studies indicate that fluoxetine reverses several kinds of alterations in neuronal plasticity induced by stress, for example, fluoxetine reverses stress-induced decreased cell proliferation in dentate gyrus of hippocampus (Lee et al., 2001), numerical decrease of hippocampal astrocytes (Czeh et al., 2006), and impairment of long-term potentiation formation in hippocampal/prefrontal circuits (Rocher et al., 2004). ERK and CREB are critical points of convergence in the signaling pathways regulating neuronal plasticity, which raises the possible mechanism that by reversing the disrupted activities of the ERK-CREB signal system, fluoxetine reverses the alterations in neuronal plasticity and the functional deficits of entire neuronal circuits, and ultimately reverses stress-induced depressive-like behaviors.

In summary, the present study identified the disrupted activities of both the ERK and the CREB in hippocampus and prefrontal cortex in rats that displayed depressive-like behaviors after receiving chronic forced swim stress. The reduction in the activities of the ERK and CREB in both the regions and the depressive-like behaviors exhibited in stressed rats were reversed by chronic fluoxetine treatment. Basing on the results, the present study speculated that the ERK-CREB signal system may be involved in mediating stress response, the mechanism that fluoxetine exerts antidepressant actions, and the neuronal mechanism of depression. Further studies are needed to examine the role of the ERK-CREB system in depression, and to determine whether there is a causal relationship between the disruption of the ERK-CREB system and depression.

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References

Argyropoulos, S.V., Sandford, J.J., Nutt, D.J., 2000. The psychobiology of anxiolytic drugs. Part 2: pharmacological treatments of anxiety. Pharmacol. Ther. 88, 213–227.
Belzung, C., Griebel, G., 2001. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav. Brain Res. 125, 141–149.
Chen, Z., Gibson, T.B., Robinson, F., Silvestro, L., Pearson, G., Xu, B., Wright, A., Vanderbilt, C., Cobb, M.H., 2001. MAP kinases. Chem. Rev. 101, 2449–2476.
Cipriani, A., Brambilla, P., Furukawa, T., Geddes, J., Gregis, M., Hotopf, M., Malvini, L., Barbui, C., 2005. Fluoxetine versus other types of pharmacotherapy for depression. Cochrane. Database Syst. Rev. 4 CD004185.
Cruz, A.P.M., Frei, F., Graeff, F.G., 1994. Ethopharmacological analysis of rat behavior on the elevated plus-maze. Pharmacol. Biochem. Behav. 49, 171–176.
Cryan, J.F., Markou, A., Lucki, I., 2002. Assessing antidepressant activity in rodents: recent developments and future needs. Trends. Pharmacol. Sci. 23, 238–245.
Czeh, B., Simon, M., Schmelting, B., Hiemke, C., Fuchs, E., 2006. Astroglial plasticity in the hippocampus is affected by chronic psychosocial stress and concomitant fluoxetine treatment. Neuropsychopharmacology 31, 1616–1626.
Davis, S., Vanhoutte, P., Pages, C., Caboche, J., Laroche, S., 2000. The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyms in vivo. J. Neurosci. 20, 4563–4572.
De La Garza II, R., Jentsch, J.D., Verrico, C.D., Roth, R.H., 2002. Adaptation of monoaminergic responses to phencyclidine in nucleus accumbens and prefrontal cortex following repeated treatment with fluoxetine or imipramine. Brain Res. 958, 20–27.
Dowlatshahi, D., MacQueen, G.M., Wang, J.F., Young, L.T., 1998. Increased temporal cortex CREB concentrations and antidepressant treatment in major depression. Lancet 352, 1754–1755.

Dwivedi, Y., Rizavi, H.S., Roberts, R.C., Conley, R.C., Tamminga, C.A., Pandey, G.N., 2001. Reduced activation and expression of ERK1/2 MAP kinase in the post-mortem brain of depressed suicide subjects. J. Neurochem. 77, 916–928.
Einat, H., Yuan, P.X., Gould, T.D., Li, J.L., Du, J.H., Zhang, L., Manji, H.K., Chen, G., 2003. The role of the extracellular signal-regulated kinase signaling pathway in mood modulation. J. Neuroscie. 23, 7311–7316.
Espejo, E.F., 1997. Effects of weekly or daily exposure to the elevated plus-maze in male mice. Behav. Brain Res. 87, 233–238.
Feng, P.F., Guan, Z.W., Yang, X.P., Fang, J.D., 2003. Impairments of ERK signal transduction in the brain in a rat model of depression induced by neonatal exposure of clomipramine. Brain Res. 991, 195–205.
Flood, D.G., Finn, J.P., Walton, K.M., Dionne, C.A., Contreras, P.C., Miller, M.S., Bhat, R.V., 1998. Immunolocalization of the mitogen-activated protein kinases p42MAPK and JNK1, and their regulatory kinases MEK1 and MEK4, in adult rat central nervous system. J. Comp. Neurol. 398, 373–392.
Ginty, D.D., Bonni, A., Greenberg, M.E., 1994. Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-*fos* transcription via phosphorylation of CREB. Cell 77, 713–725.
Grippo, A.J., Beltz, T.G., Weiss, R.M., Johnson, A.K., 2006. The effects of chronic fluoxetine treatment on chronic mild stress-induced cardiovascular changes and anhedonia. Biol. Psychiatry 59, 309–316.
Gruen, R.J., Wenberg, K., Elahi, R., Friedhoff, A.J., 1995. Alterations in GABAA receptor binding in the prefrontal cortex following exposure to chronic stress. Brain Res. 684, 112–114.
Hall, C.S., 1934. Emotional behavior in the rat. Part I: defecation and urination as measures of individual differences in emotionality. J. Comp. Psychol. 18, 385–403.
Handley, S.L., Mithani, S., 1984. Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. Naunyn-Schmiedeberg's. Arch. Pharmacol. 327, 1–5.
Hardingham, G.E., Arnold, F.J., Bading, H., 2001. A calcium microdomain near NMDA receptors: on switch for ERK dependent synapse-to-nucleus communication. Nat. Neurosci. 4, 565–566.
Ho, Y.J., Eichendorff, J., Schwarting, R.K., 2002. Individual response profiles of male Wistar rats in animal models for anxiety and depression. Behav. Brain Res. 136, 1–12.
Laifenfeld, D., Karry, R., Grauer, E., Klein, E., Ben-Shachar, D., 2005. Antidepressants and prolonged stress in rats modulate CAM-L1, laminin, and pCREB, implicated in neuronal plasticity. Neurobiol. Dis. 20, 432–441.
Lee, J.H., Kim, J.W., Yim, S.V., Kim, M.J., Kim, S.A., Kim, Y.J., Kim, C.J., Chung, J.H., 2001. Fluoxetine enhances cell proliferation and prevents apoptosis in dentate gyrus of maternally separated rats. Mol. Psychiatry 610, 725–728.
Lucki, I., 1997. The forced swim test as a model for core and component behavioral effects of antidepressant drugs. Behav. Pharmacol. 8, 523–532.
Malberg, J., Eisch, A., Nestler, E., Duman, R.S., 2000. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J. Neurosci. 20, 9104–9110.
Manji, H.K., Drevets, W.C., Charney, D.S., 2001. The cellular neurobiology of depression. Nat. Med. 7, 541–547.
Mattson, M.P., Maudsley, S., Martin, B., 2004. A neural signaling triumvirate that influences ageing and age-related disease: insulin/IGF-1, BDNF and serotonin. Ageing Res. Rev. 3, 445–464.
Mechan, A.O., Moran, P.M., Elliott, J.M., Young, A.J., Joseph, M.H., Green, A.R., 2002. A comparison between Dark Agouti and Sprague–Dawley rats in their behaviour on the elevated plus-maze, open field apparatus an activity meters, and their response to diazepam. Psychopharmacology (Berl) 159, 188–195.
Meller, E., Shen, C., Nikolao, T.A., Jensen, C., Tsimberg, Y., Chen, J., Gruen, R.J., 2003. Region-specific effects of acute and repeated restraint stress on the phosphorylation of mitogen-activated protein kinases. Brain Res. 979, 57–64.
Montgomery, K.C., 1955. The relation between fear induced by novel stimulation and exploratory behavior. J. Comp. Physiol. Psychol. 48, 254–260.
Nash, L.T., Hack, S., 2002. The pharmacological treatment of anxiety disorders in children and adolescents. Expert. Opin. Pharmacother. 3, 555–571.
Ortiz, J., Harris, H.W., Guitart, X., Terwilliger, R.Z., Haycock, J.W., Nestler, E.J., 1995. Extracellular signal-regulated protein kinases (ERKs) and ERK kinase (MEK) in brain: regional distribution and regulation by chronic morphine. J. Neurosci. 15, 1285–1297.
Paxinos, G., Watson, C.R., 1998. The Rat Brain in Stereotaxic Coordinates. Academic Press, New York.
Porsolt, R.D., Anton, G., Blavet, N., Jalfre, M., 1978. Behavioral despair in rats: a new model sensitive to antidepressant treatments. Eur. J. Pharmacol. 47, 379–391.
Prut, L., Belzung, C., 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur. J. Pharmacol. 463, 3–33.
Qi, X.L., Lin, W.J., Li, J.F., Pan, Y.Q., Wang, W.W., 2006. The depressive-like behaviors are correlated with decreased phosphorylation of mitogen-activated protein kinases in rat brain following chronic forced swim stress. Behav. Brain Res. 175, 233–240.
Ramos, A., Mormede, P., 1997. Stress and emotionality: a multidimensional and genetic approach. Neurosci. Biobehav. Rev. 22, 33–57.
Reusch, J.E., Hsieh, P., Klemm, D., Hoeffler, J., Draznin, B., 1994. Insulin inhibits dephosphorylation of adenosine 30,50-monophosphate response element-binding protein/activating transcription factor-1: effect on nuclear phosphoserine phosphatase-2a. Endocrinology 135, 2418–2422.
Rocher, C., Spedding, M., Munoz, C., Jay, T.M., 2004. Acute stress-induced changes in hippocampal/prefrontal circuits in rats: effects of antidepressants. Cereb. Cortex 14, 224–229.
Rygula, R., Abumaria, N., Domenici, E., Hiemke, C., Fuchs, E., 2006. Effects of fluoxetine on behavioral deficits evoked by chronic social stress in rats. Behav. Brain Res. 174, 188–192.
Sarkisova, K.Y., Kulikov, M.A., 2006. Behavioral characteristics of WAG/Rij rats susceptible and non-susceptible to audiogenic seizures. Behav. Brain Res. 166, 9–18.
Schafe, G.E., Atkins, C.M., Swank, M.W., Bauer, E.P., Sweatt, J.D., LeDoux, J.E., 2000. Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of Pavlovian fear conditioning. J. Neurosci. 20, 8177–8187.
Shirayama, Y., Chen, A.C., Nakagawa, S., Russell, D.S., Duman, R.S., 2002. Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J. Neurosci. 22, 3251–3261.
Svenningsson, P., Tzavara, E.T., Witkin, J.M., Fienberg, A.A., Nomikos, G.G., Greengard, P., 2002. Involvement of striatal and extrastriatal DARPP-32 in biochemical and behavioral effects of fluoxetine (Prozac). P.N.A.S. 99, 3182–3187.
Sweatt, J.D., 2001. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. J. Neurochem. 76, 1–10.
Thome, J., Sakai, N., Shin, K., Steffen, C., Zhang, Y.J., Impey, S., Storm, D., Duman, R.S., 2000. cAMP response element-mediated gene transcription is upregulated by chronic antidepressant treatment. J. Neurosci. 20, 4030–4036.
Tiraboschi, E., Tardito, D., Kasahara, J., Moraschi, S., Pruneri, P., Gennarelli, M., Racagni, G., Popoli, M., 2004. Selective phosphorylation of nuclear CREB by fluoxetine is linked to activation of CaM kinase IV and MAP kinase cascades. Neuropsychopharmacology. 29, 1831–1840.
Treit, D., 1985. Animal models for the study of anti-anxiety agents: a review. Neurosci. Biobehav. Rev. 9, 203–222.
Wall, P.M., Messier, C., 2001. Methodological and conceptual issues in the use of the elevated plus-maze as a psychological measurement instrument of animal anxiety-like behavior. Neurosci. Biobehav. Rev. 25, 275–286.
West, A.E., Griffith, E.C., Greenberg, M.E., 2002. Regulation of transcription factors by neuronal activity. Nat. Rev. 3, 921–931.
Xing, J., 1996. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 273, 959–963.
Xu, Y., Ku, B.S., Tie, L., Yao, H.Y., Jiang, W.G., Ma, X., Li, X.J., 2006. Curcumin reverses the effects of chronic stress on behavior, the HPA axis, BDNF expression and phosphorylation of CREB. Brain. Res. 1122, 56–64.
Yamada, S., Yamamoto, H., Qzawa, H., Riederer, P., Saito, T., 2003. Reduced phosphorylation of cyclic AMP-responsive element binding protein in the postmortem orbitofrontal cortex of patients with major depressive disorder. J. Neural. Transm. 110, 671–680.
Ying, S.W., Futter, M., Rosenblum, K., Webber, M.J., Hunt, S.P., Bliss, T.V.P., Bramham, C.R., 2002. Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of *Arc* synthesis. J. Neurosci. 22, 1532–1540.
Zhang, Y., Raap, D.K., Garcia, F., Serres, F., Ma, Q., Battaglia, G., Van de Kar, L.D., 2000. Long-term fluoxetine produces behavioral anxiolytic effects without inhibiting neuroendocrine responses to conditioned stress in rats. Brain. Res. 855, 58–66.
Zhu, J.J., Qin, Y., Zhao, M., Van Aelst, L., Malinow, R., 2002. Ras and Rap control AMPA receptor trafficking during synaptic plasticity. Cell. 110, 443–455.
Zimmerman, M., McDermut, W., Mattia, J.I., 2000. Frequency of anxiety disorders in psychiatric outpatients with major depressive disorder. Am. J. Psychiatr. 157, 1337–1340.