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A common mechanism of action of the selective serotonin reuptake inhibitors citalopram and fluoxetine: Reversal of chronic psychosocial stress-induced increase in CRE/CREB-directed gene transcription in transgenic reporter gene mice

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ABSTRACT

The transcription factor CREB regulates adaptive responses like memory consolidation, addiction, and synaptic refinement. Recently, chronic psychosocial stress as animal model of depression has been shown to stimulate CREB transcriptional activity in the brain; this stimulation was prevented by treatment with the antidepressant imipramine, which inhibits both noradrenaline and serotonin reuptake. However, it was unknown whether the selective inhibition of serotonin reuptake is sufficient for inhibition of stress-induced CREB activation, as it is for the clinical antidepressant effect. Therefore, the effect of two selective serotonin reuptake inhibitors (SSRIs), citalopram and fluoxetine, was examined in this study. Transgenic CRE-luciferase reporter gene mice were used to monitor gene transcription directed by the CREB DNA binding site (CRE) in vivo. Chronic psychosocial stress for 25 days stimulated CRE/CREB-directed luciferase expression in the hippocampus and other brain regions. When applied alone to non-stressed mice, citalopram caused a transient increase after 24 h that was lost after 21 days of treatment, whereas fluoxetine had no effect after 24 h and produced an inhibition in the pons and hypothalamus after 21 days of treatment. However, both citalopram and fluoxetine treatment completely abolished the increase in CRE/CREB-directed transcription induced by chronic psychosocial stress. As indicated by Western blots, the changes in CRE/CREB-directed transcription were accompanied by corresponding changes in the phosphorylation of CREB at serine-119. These results further emphasize the role of CREB in stress-induced gene expression and suggest furthermore that inhibition of stress-induced CREB activity may be a common mechanism of action of SSRIs underlying their antidepressive effect.

1. Introduction

The transcription factor CREB has been implicated in the mechanism of action of antidepressant drugs. CREB is thought to mediate some of the changes in gene expression that are induced by chronic treatment with these drugs and that may underlie their antidepressant effect, as chronic, not acute, treatment is required for their beneficial clinical effect in depressive patients (Tardito et al., 2006). Many investigations studied the effect of antidepressant drugs on CREB expression in the brain and the phosphorylation of CREB at serine-119 (in CREB-327, corresponding to serine-133 in CREB-341) (Kuipers et al., 2006; Tardito et al., 2006; Thome et al., 2000; Tiraboschi et al., 2004). While this phosphorylation allows the recruitment of the CREB coactivator CBP (Shaywitz and Greenberg, 1999) and is thus required for CREB activation, it is not sufficient for CREB transcriptional activity (Boer et al., 2007b; Ravnskjaer et al., 2007; Schwaninger et al., 1995a). Those investigations are therefore difficult to interpret with respect to gene activation by CREB. Furthermore, antidepressant drugs are well known to produce no mood-elevating or euphoric effect in healthy individuals but to correct the mood disorder in patients that suffer from depression (Nelson, 1999). This questions the significance of studies performed in normal subjects. Although the etiology of depression is still unknown, it is generally accepted that repeated stressful events can precipitate the disease (Nestler et al., 2002). Chronic psychosocial stress in mice and other species has been shown to produce symptoms typically found in depressive patients (Fuchs and Flugge, 2002; Kudryavtseva et al., 1991; Rygula et al., 2005). Noteworthy, a recent study used for the first time chronic psychosocial stress in mice as an animal model of depression and studied the effect of an antidepressant drug on CREB activity in the brain as revealed by gene expression directed by the CREB DNA binding site, the cAMP response element (CRE), in CRE-luciferase transgenic reporter gene mice (Boer et al., 2007a). It was found that chronic psychosocial stress stimulates CREB activity in the brain and that chronic treatment with imipramine prevented the stress-induced increase in CREB activity. This study further emphasizes the role of CREB in the mechanism of action of antidepressant drugs.

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Imipramine is a tricyclic antidepressant and was one of the first antidepressant drugs that was introduced into the clinic (Nestler et al., 2002). It inhibits the neuronal reuptake transporters of serotonin and noradrenaline with almost similar potency (Owens et al., 1997). The extraneuronal concentrations of serotonin and noradrenaline increase early after initiation of therapy and produce under continued treatment with a delay of several weeks a new pattern of gene expression and the clinical antidepressant effect (Tardito et al., 2006). Today, in the treatment of depression drugs that are increasingly used selectively block the neuronal reuptake of serotonin. These selective serotonin reuptake inhibitors (SSRIs) are as, or almost as, effective as the traditional tricyclic antidepressant drugs, but are, in general, better tolerated due to less severe side effects (Turner et al., 2008; Westenberg and Sandner, 2006). However, it was unknown whether the selective inhibition of the neuronal reuptake of serotonin is sufficient to prevent the stress-induced increase in CREB activity as does the nonselective inhibition of both serotonin and noradrenaline reuptake by imipramine. Therefore, the effect of two SSRIs, citalopram and fluoxetine, was investigated in the present study.

2. Materials and methods

2.1. Animal housing conditions

The generation and characterization of the transgenic CRE-Luc mice used in this study has been described previously. The transgene in these mice consists of the luciferase reporter gene under control of four copies of the CRE from the rat somatostatin promoter and a truncated thymidine kinase promoter. Animals were kept on a 12-h light–dark cycle. Food pellets and water were available ad libitum. For acute and chronic drug treatment mice were single-housed in plastic cages (28 cm $L \times 16$ cm $W \times 13$ cm H). All animal studies were conducted according to the National Institutes of Health's Guidelines for Care and Use of Experimental Animals and were approved by the Committee on Animal Care and Use of the local institution and state.

2.2. Stress model

The model of chronic psychosocial stress in mice has been described before. Briefly, two male transgenic mice (12–15 weeks old) were housed in a cage separated by a perforated plastic partition. During 25 days the partition was removed every day for 10 min resulting in daily social conflicts. The daily interactions were recorded by two independent observers. After 3–5 days one mouse developed a dominant aggressive behaviour whereas the other became subordinate. Chasing and biting behaviour was defined as dominant whereas flight, freezing and vocalization indicated the subordinate posture and thus social stress. Non-stressed mice from the same litter kept separately served as controls. On day 26, 14 h after the last stress exposure, stressed and control mice were decapitated and brains were dissected.

As repeated stress with social defeat has been implicated in the development of depression, the effect of chronic psychosocial stress with and without SSRI treatment on CRE/CREB-directed gene transcription was investigated. The effect of chronic psychosocial stress on CRE activity using the sensory contact model has been reported previously (Boer et al., 2007a). In this model, the subordinate mouse is chronically stressed by the unavoidable sensory contact with the dominant conspecific. The dominant and subordinate behaviours as well as stress-induced weight loss of the subordinate animal were similar as has been reported before (data not shown) (Boer et al., 2007a).

2.3. Citalopram and fluoxetine treatment

Acute treatment: 25 mg/kg citalopram (Lundbeck, Denmark) was applied i.p. at 0900 and 1800. 10 mg/kg fluoxetine was applied i.p. at 0900. Mice were decapitated the following day at 0900 and brains were taken out for luciferase assay. Chronic treatment: 25 mg/kg citalopram was applied i.p. for 21 days at 0900 and 1800. 10 mg/kg fluoxetine was applied for 21 days at 0900. The mice were decapitated on day 22 of the treatment at 0900 and brains were dissected. Chronic treatment in stressed mice: mice were exposed to chronic psychosocial stress. The submissive animal was treated from day 5 to day 25 with citalopram or fluoxetine (as described above) while the daily stress procedure continued. On day 26, 14 h after the last stress exposure, the mice were decapitated and brains were taken out. Within all treatments, controls from the same litter received the solvent only. Serum concentrations of the drugs were determined in trunk blood.

2.4. Dissection of brain regions and luciferase assay

Transgenic mice were decapitated, the brains were removed and rinsed with ice-cold PBS. Defined regions were dissected in the following order: Olfactory bulb, cerebellum, pons, frontal part of the brain anterior to the optic chiasm containing parts of the prefrontal cortex and the striatum (PFC), colliculi, hypothalamus, cortex, and hippocampus. Pieces of tissue were frozen in liquid nitrogen and stored at −80 °C. The rationale for examining these regions was to study whether effects of psychosocial stress and/or antidepressant treatment are similar or different in diverse brain regions.

For the luciferase assay, the tissue was homogenized in potassium phosphate buffer (0.1 M K_2HPO_4, 0.1 M KH_2PO_4, pH 7.8) supplemented with 1 mM DTT, 4 mM EGTA, 4 mM EDTA, 0.7 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml pepstatin and 5 µg/ml aprotinin (all from Sigma), and subjected to three cycles of freeze–thawing. After centrifugation, 50 µl of supernatants was mixed with 370 µl assay buffer containing 16.5 mM potassium phosphate, 20 mM glycylglycine, 12 mM $MgSO_4$, 3.2 mM EDTA, 1 mM DTT, and 2.2 mM ATP (Sigma), and luciferase activity was measured by adding the substrate luciferin (Promega, Mannheim, Germany) in glycylglycine buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 10 mM DTT) with an automatic dispenser (Autolumat 953, Berthold, Bad Wildbach, Germany). Measured relative light units were normalized to protein content determined by Bradford (Boer et al., 2007a).

2.5. Western blot in brain homogenates

Transgenic mice were decapitated, the brains were removed and immediately frozen in liquid nitrogen. Frozen tissue was pulverized under liquid nitrogen. Powder was transferred to boiling SDS sample buffer and boiled for 5 min. Tissue lysate proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. CREB and phospho-CREB (phospho-Ser-119 in CREB-327 / phospho-Ser-133 in CREB-341) were immunostained with specific antibodies (NewEngland Biolabs, Frankfurt a.M., Germany) and respective peroxidase-labeled secondary antibodies. Immunoreactivity was detected using the ECL reaction (Amersham Pharmacia, Germany).

Specific bands were quantified densitometrically and the ratio between the intensity of phospho-CREB and CREB from the same homogenate was calculated.

2.6. Determination of citalopram and fluoxetine

Citalopram, fluoxetine and their pharmacologically active metabolites norfluoxetine and demethylcitalopram were determined in blood plasma using a high-performance liquid chromatography (HPLC) method with column switching and spectrophotometric detection, as described previously for citalopram (Rygula et al., 2005). Serum (0.1 ml) was injected into the HPLC system. For online sample clean-up on a column (10 × 4.0 mm i.d.) filled with LiChrospher CN material of 20 µm particle size (MZ-Analysentechnik, Mainz, Germany), the column was washed with deionized water containing 8% (v/v) acetonitrile to remove

proteins and other interfering compounds. Drugs were eluted and separated on LiChrospher CN material (5 µm; column size 250×4.6 mm i.d., MZ-Analysentechnik) using phosphate buffer (8 mM, pH 6.4) containing 500 ml/l acetonitrile, and were quantified by ultraviolet (UV) spectroscopy at 210 nm. HPLC analysis was completed within 20 min. Each analytical series included at least two control samples containing low (50 ng/ml) or high (400 ng/ml) concentrations of citalopram, fluoxetine, demethylcitalopram and norfluoxetine, respectively. There was a linear correlation between drug concentration and UV signal from 10 ng/ml to at least 600 ng/ml (FLX) and 5 ng/ml to 250 ng/ml (CIT). The limit of quantification was 10 ng/ml (FLX) and 3 ng/ml (CIT). Intra- and interassay reproducibility of quality control samples was within 10% (CIT)–15% (FLX).

2.7. Statistics

The analysis of data for luciferase activity in brain regions was performed by two-factorial ANOVA (region vs. treatment) followed by Student's *t*-test for unpaired samples. Western blot data were analyzed by one-factorial ANOVA, followed by Student's *t*-test for unpaired samples. All analyses were performed using the software STATISTICA 7.1, statsoft, Tulsa, USA.

3. Results

3.1. Acute and chronic treatment of transgenic mice with the SSRIs citalopram and fluoxetine

To study the effect of SSRIs on CRE/CREB-directed gene transcription in vivo, CRE-luciferase transgenic mice were treated with citalopram or fluoxetine. The acute treatment (24 h) with citalopram stimulated CRE-directed gene expression throughout the brain (Table 1). Luciferase reporter gene expression was significantly elevated in the bulbus, cerebellum, pons, colliculi, PFC, and cortex (Table 1). In the hippocampus, values only approached significance ($P=0.061$) (Table 1). However, the stimulation by citalopram was transient. The chronic treatment (21 days) with citalopram did not result in significant changes in luciferase activity in any brain region dissected (Table 1). At the end of the chronic treatment (21 days) with citalopram the plasma levels were 356 ± 65 ng/ml for citalopram and 305 ± 34 ng/ml for its N-desmethyl metabolite.

The acute treatment (24 h) with fluoxetine did not significantly change luciferase expression in the brain, although there was a slight tendency towards a decrease (Table 2). In contrast, chronic treatment (21 days) with fluoxetine significantly reduced luciferase expression in the pons and hypothalamus (Table 2). At the end of the chronic treatment (21 days) with fluoxetine the plasma levels were 570 ± 61 ng/ml for fluoxetine and 823 ± 69 ng/ml for its N-desmethyl metabolite.

Table 1
Luciferase activity in citalopram-treated CRE-Luc mice.

	Acute treatment (24 h)		Chronic treatment (21 days)	
	Control	CIT	Control	CIT
Bulbus	100 ± 9.74	167.37 ± 28.82[a]	100 ± 10.74	139.13 ± 20.74
Cerebellum	100 ± 8.86	188.94 ± 31.06[a]	100 ± 8.28	111.18 ± 7.98
Pons	100 ± 10.14	162.57 ± 25.66[a]	100 ± 17.80	93.92 ± 10.41
Colliculi	100 ± 8.04	152.95 ± 19.06[a]	100 ± 16.79	126.69 ± 29.81
PFC	100 ± 10.73	194.83 ± 25.67[b]	100 ± 8.30	88.61 ± 9.19
Hypothalamus	100 ± 11.59	123.44 ± 15.35	100 ± 18.22	111.89 ± 7.73
Hippocampus	100 ± 10.18	190.65 ± 40.86[c]	100 ± 10.10	110.91 ± 9.16
Cortex	100 ± 10.14	213.27 ± 24.56[b]	100 ± 12.40	100.95 ± 8.26

Acute and chronic treatments with citalopram were performed in CRE-Luc mice that were not stressed. CIT: citalopram. PFC: prefrontal cortex. Values are means and expressed as percentage of the mean value in controls (solvent-treated littermates) (±SEM) from 8 (acute) and 10 (chronic) animals per group. Chronic treatment decreased luciferase activity significantly in the pons and hypothalamus. [a]$P<0.05$; [b]$P<0.01$; [c]$P=0.061$ as determined by two-way ANOVA followed by Student's *t*-test for unpaired samples.

Table 2
Luciferase activity in fluoxetine-treated CRE-Luc mice.

	Acute treatment (24 h)		Chronic treatment (21 days)	
	Control	FLX	Control	FLX
Bulbus	100 ± 17.97	84.47 ± 15.23	100 ± 15.46	79.52 ± 13.80
Cerebellum	100 ± 16.82	83.35 ± 14.79	100 ± 5.91	89.95 ± 9.72
Pons	100 ± 14.63	80.86 ± 13.74	100 ± 7.47	74.22 ± 8.85[a]
Colliculi	100 ± 13.71	88.15 ± 15.23	100 ± 17.89	73.24 ± 18.04
PFC	100 ± 17.34	87.94 ± 16.64	100 ± 15.04	109.78 ± 17.14
Hypothalamus	100 ± 18.5	83.34 ± 13.84	100 ± 6.51	53.79 ± 11.64[b]
Hippocampus	100 ± 18.19	87.09 ± 15.88	100 ± 9.60	78.03 ± 12.48
Cortex	100 ± 15.27	86.61 ± 15.53	100 ± 9.40	76.99 ± 8.78

Acute and chronic treatments with FLX were performed in CRE-Luc mice that were not stressed. FLX: fluoxetine. PFC: prefrontal cortex. Values are means and expressed as percentage of the mean value in controls (solvent-treated littermates) (±SEM) from 10 (acute) and 9 (chronic) animals per group. Chronic treatment decreased luciferase activity significantly in the pons and hypothalamus. [a]$P<0.05$; [b]$P<0.01$ as determined by two-way ANOVA followed by Student's *t*-test for unpaired samples.

3.2. Chronic psychosocial stress and SSRI treatment

Consistent with previous data (Boer et al., 2007a), chronic psychosocial stress stimulated CRE-directed gene expression in the brain with significant increases in the hippocampus and colliculi (Fig. 1). Luciferase reporter gene expression was significantly elevated also in the hypothalamus (Fig. 1). In the PFC, values approached significance ($P=0.061$) (Fig. 1). Although they did in the previous study (Boer et al., 2007a), the increases in the cerebellum and pons did not reach statistical significance (Fig. 1) and probably for methodical reasons may have escaped detection. To investigate the effect of SSRI treatment, the subordinate mice were treated for 21 days with citalopram or fluoxetine while the daily social stress exposure continued. Under these conditions luciferase activity in all brain regions tested did not differ significantly from control levels (Fig. 2), indicating that both, citalopram and fluoxetine, normalized luciferase expression in the stressed animals. Thus, the chronic treatment with citalopram or fluoxetine completely abolished the stimulating effect of chronic psychosocial stress on CRE/CREB-directed gene transcription and resulted in levels similar to control.

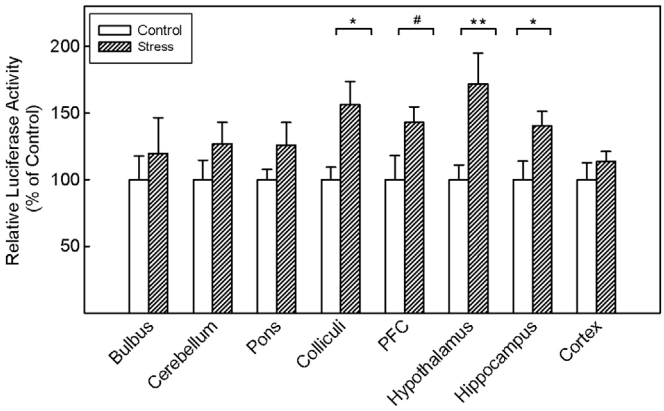

Fig. 1. Effect of chronic psychosocial stress on CRE/CREB-directed luciferase expression. Male CRE-Luc mice were either not treated (black bars) or exposed to psychosocial stress for 25 days (white bars). Luciferase activity was measured in homogenates of different brain regions, normalized to protein contents and expressed as percentage of the mean value in controls (unstressed littermates) (means ± SEM from 6 animals). Stress increased luciferase activity in all brain regions reaching statistical significance in colliculi, hypothalamus and hippocampus. In PFC differences were marginally significant. *$P<0.05$; **$P<0.01$; #$P=0.061$ as determined by two-way ANOVA followed by Student's *t*-test. PFC: prefrontal cortex.

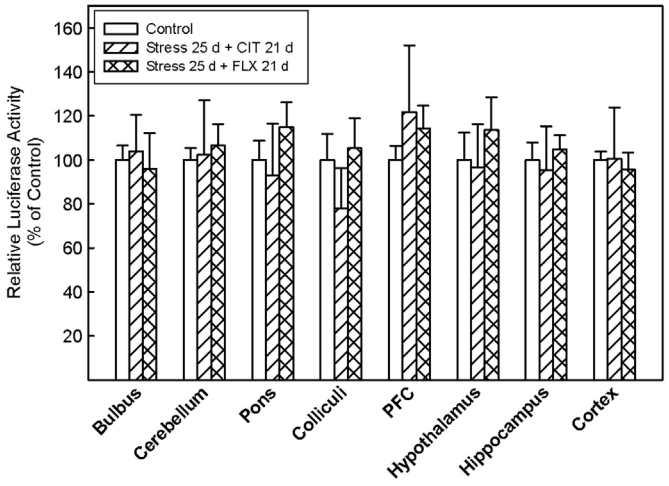

Fig. 2. Effect of chronic psychosocial stress on CRE/CREB-directed luciferase expression in mice that were treated with citalopram or fluoxetine. Male CRE-Luc mice were either solvent-treated (black bars) or exposed to chronic psychosocial stress for 25 days and treated with citalopram (CIT, white bars) or fluoxetine (FLX, hatched bars) for 21 days (from days 5 to 25 of psychosocial stress) while the daily stress exposure continued. Luciferase activity was measured in homogenates of different brain regions, normalized to protein contents and expressed as percentage of the mean value in controls (solvent-treated littermates) (means ± SEM from 9 animals). CIT and FLX reversed the stress-induced increase in luciferase activity resulting in levels close to control in each brain region. PFC: prefrontal cortex.

3.3. Western blot analysis of CREB and phospho-CREB in chronically stressed and SSRI-treated mice

The phosphorylation of CREB at Ser-119 (CREB-327; corresponds to Ser-133 in CREB-341) is required for stimulus-induced CREB transcriptional activity (Shaywitz and Greenberg, 1999). We thus examined CREB phosphorylation in the brains of mice subjected to the various treatments. The Western blots were performed using whole brain homogenates, since a previous study had shown that chronic psychosocial stress, similar to the increase in CRE-directed transcription, induces a widespread uniform increase in CREB phosphorylation

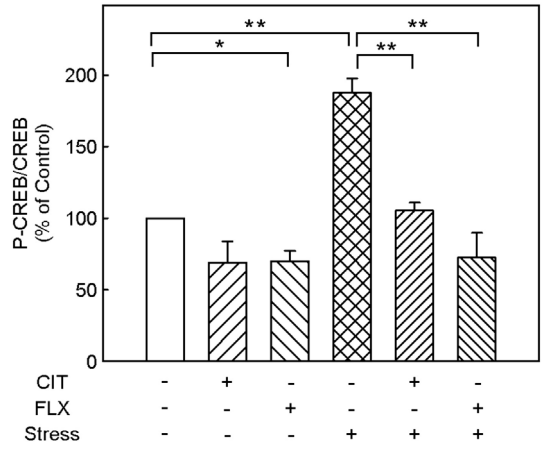

Fig. 3. Effect of citalopram and fluoxetine on CREB phosphorylation in mice that were or were not exposed to chronic psychosocial stress. Male CRE-Luc mice were treated for 21 days with citalopram (CIT) or fluoxetine (FLX) or exposed to chronic psychosocial stress with or without concurrent CIT or FLX treatment for 21 days. Homogenates of whole brains were subjected to immunoblotting using specific CREB and phospho-CREB antibodies. The intensity of specific bands was determined densitometrically and ratios between CREB and phospho-CREB (P-CREB) were calculated. Solvent-treated or untreated littermates served as controls. Values are means ± SEM from 6 animals for stress, 3 animals for CIT with or without stress, and 4 animals for FLX with or without stress. Significant difference: $^{*}P<0.05$; $^{**}P<0.01$; as determined by Student's *t*-test for unpaired samples.

when various brain regions were examined (Boer et al., 2007a). The therefrom calculated mean increase in CREB phosphorylation over all regions corresponded exactly with the increase in CREB phosphorylation measured in the whole brain homogenates (Boer et al., 2007a). Whole brain measurements of CREB phosphorylation are thus a reliable indicator of the effect of chronic psychosocial stress and antidepressant treatment (Boer et al., 2007a). The acute treatment (24 h) of mice with citalopram increased the phosphorylation of CREB to 167 ± 14.7% of controls ($P<0.05$), whereas the chronic treatment (21 days) with citalopram had no effect (Fig. 3). Fluoxetine had no effect after acute treatment (24 h) (data not shown) but decreased the phosphorylation of CREB after chronic treatment (21 days) (Fig. 3). Neither treatment altered the expression level of total CREB (not shown). Chronic psychosocial stress induced an increase in CREB phosphorylation (Fig. 3), as has been reported previously (Boer et al., 2007a). The stress-induced increase in CREB phosphorylation was completely reversed by concurrent citalopram or fluoxetine treatment (Fig. 3). Thus, the changes in the phosphorylation of CREB paralleled those in CRE/CREB-directed gene transcription under chronic psychosocial stress, antidepressant treatment, or both.

4. Discussion

The monoamine hypothesis of depression states that depression is characterized by reduced levels of the monoamines noradrenaline and serotonin in the brain (Nestler et al., 2002). The best known mechanism of antidepressant drug action is the inhibition of neuronal reuptake, by which the extraneuronal monoamine concentrations are increased and thus noradrenergic and serotonergic neurotransmission is stimulated. From this early biochemical change, the clinical antidepressant effect develops gradually over weeks through neuronal adaptation that, at the molecular level, may be based on a new pattern of gene expression (Alfonso et al., 2005; Pittenger and Duman, 2008; Tardito et al., 2006). The notion that CREB may be one of the transcription factors that mediate these changes in gene expression was strongly supported by a recent report demonstrating for the first time that chronic psychosocial stress as an animal model of depression stimulates CREB activity in the brain in vivo and that this activation of CREB is reversed by treatment with the antidepressant imipramine (Boer et al., 2007a). Clinical experience has shown that compounds which inhibit serotonin reuptake with high selectivity are similarly effective as traditional tricyclic antidepressants like imipramine, which inhibits both noradrenaline and serotonin reuptake (Owens et al., 1997). The present study now demonstrates that two SSRIs, citalopram and fluoxetine, also reverse the stimulation of CRE/CREB-directed gene transcription by chronic psychosocial stress in vivo, suggesting a common CREB regulatory mechanism of action of these widely used antidepressant drugs.

Citalopram and fluoxetine produced somewhat different effects when applied alone to non-stressed animals. While citalopram caused a transient stimulation of CRE/CREB-directed luciferase expression by acute treatment that was lost under chronic treatment, fluoxetine had no effect after acute treatment and induced an inhibition in the pons and hypothalamus after chronic treatment. The reasons for these divergent effects are not obvious but may be explained by differences in pharmacodynamics and pharmacokinetics. The K_i values for the inhibition of noradrenaline and serotonin reuptake are 30,285 nM and 8.9 nM for citalopram (3400-fold preference for serotonin reuptake inhibition) and 2190 nM and 20 nM for fluoxetine, respectively (109-fold preference for serotonin reuptake inhibition) (Owens et al., 1997). A similar relative potency and selectivity has been shown also in vivo. Behavioural tests indicate that citalopram and fluoxetine potently inhibit serotonin reuptake in vivo but have no effect on noradrenaline reuptake in vivo, even at the highest doses used (36 and 10 mg/kg i.p., respectively) (Hyttel, 1994). Using the in vitro microdialysis technique, citalopram has been shown to increase the

extracellular concentration of serotonin, but not noradrenaline, in the prefrontal cortex of mice and rats (Kobayashi et al., 2008; Bymaster et al., 2002). Fluoxetine too increases the extracellular concentrations of serotonin, although at high fluoxetine doses (e.g. 30 mg/kg) some (Kobayashi et al., 2008; Bymaster et al., 2002) but not other microdialysis studies (Beyer et al., 2002; Amargos-Bosch et al., 2005) report an increase also of noradrenaline concentrations which, however, may be indirectly produced by fluoxetine (Bymaster et al., 2002). Citalopram is thus the more potent and more selective drug. The transient stimulation of CRE/CREB-directed transcription at the beginning of the treatment with citalopram may therefore be induced by the acute increase in serotonergic neurotransmission whereas fluoxetine may miss this effect due to its lower potency or a different time–course of its concentration in the brain at the beginning of the treatment. The inhibition after chronic fluoxetine treatment (21 days) observed in the present study is somewhat reminiscent of the one reported after chronic imipramine treatment (Boer et al., 2007a). Interestingly, imipramine and fluoxetine, but not citalopram, possess some affinity at 5-HT2a receptors (Owens et al., 1997).

The present study confirms the recent finding (Boer et al., 2007a) that chronic psychosocial stress stimulates CRE/CREB-directed gene transcription in the brain. Increases were found particularly in the hippocampus, colliculi, hypothalamus, and cortical areas. These brain regions are well known to be affected by chronic psychosocial stress (Alfonso et al., 2005; Brandao et al., 2003; Fuchs and Flugge, 2002; Post, 1992) with upregulation of CRE-carrying genes, like the corticotropin-releasing hormone gene in the hypothalamus (de Kloet et al., 1990), the c-fos gene in the hippocampus, colliculi, hypothalamus, and cortical areas (Matsuda et al., 1996), and the brain-derived neurotrophic factor (BDNF) gene in the hippocampus (Pardon et al., 2005). Chronic psychosocial stress in mice, rats, and tree shrews (Fuchs and Flugge, 2002; Kudryavtseva et al., 1991; Rygula et al., 2005) serves as an animal model of depression in which male animals are stressed by a dominant conspecific to induce emotional despair rather than physical discomfort. Chronic psychosocial stress thereby differs from other types of stress, which consequently may produce different effects. As suggested by reduced CREB phosphorylation, CREB activity may be reduced in the hippocampus by a mild physical stressor (foot shocks for 21 days) (Kuipers et al., 2006) and in the frontal cortex, hippocampus, and striatum by variable unpredictable stress (foot shock, cold, restraint) (Laifenfeld et al., 2004). Likewise, BDNF is downregulated in rat hippocampus by some stressors (Nair et al., 2007). The confirmation in the present study that chronic psychosocial stress stimulates CRE/CREB-directed gene transcription further emphasizes a role for CREB in stress-regulated gene expression at the base of depressive disorders.

Antidepressant drugs like fluoxetine may produce some effects in normal human subjects such as improvement in memory in elderly patients with mild cognitive impairment (Mowla et al., 2007) or reduction in negative affective experience as personality trait (Knutson et al., 1998). However, no mood-elevating effect has been found in normal volunteers (Wilson et al., 2002; Gelfin et al., 1998; Knutson et al., 1998). The fact that antidepressant drugs possess no mood-elevating effect in healthy individuals but correct mood disturbances in depression could indicate that antidepressant drug actions that underlie their clinical effects unfold only in depression or under conditions which represent relevant aspects of depression. Thus, the many conflicting results that have been reported from studies on antidepressants and CREB (Tardito et al., 2006) may be meaningless with respect to the drugs' antidepressant effect, because they did not use models of depression. Also in the present study, citalopram and fluoxetine produced somewhat different effects when they were applied alone to normal, non-stressed animals, particularly at the beginning of the treatment. However, both citalopram and fluoxetine nevertheless completely abolished the increase in CRE/CREB-directed gene transcription induced by chronic psychosocial stress indicating that this effect is caused by serotonin reuptake inhibition and, furthermore, that inhibition of serotonin reuptake is sufficient to produce this effect. Inhibition of serotonin reuptake is sufficient also for the clinical antidepressant effect demonstrated by the widespread clinical use of SSRIs. The results of the present study thus suggest inhibition of stress-induced CREB activity as a common molecular mechanism of action of SSRIs underlying their antidepressant effect.

A great number of signalling pathways are now known to converge on CREB (Shaywitz and Greenberg, 1999; Tardito et al., 2006). Chronic psychosocial stress has recently been shown to stimulate the phosphorylation of CREB at serine-119 (in CREB-327) and this phosphorylation was inhibited by imipramine treatment (Boer et al., 2007a). The present study confirms the phosphorylation of CREB by chronic psychosocial stress and shows furthermore that citalopram and fluoxetine, like imipramine, suppress this phosphorylation. This common effect of citalopram and fluoxetine may be considered a stress-specific effect, since chronic treatment with fluoxetine, but not citalopram, produced a significant only partial inhibition of CREB phosphorylation in non-stressed animals and also different effects on CREB phosphorylation were caused by citalopram and fluoxetine after the acute treatment of non-stressed mice. Many signalling pathways including cAMP- and calcium-induced signalling pathways lead to the phosphorylation of CREB at serine-119, which allows CREB the recruitment of the essential coactivator CBP (Shaywitz and Greenberg, 1999). However, signalling pathways regulate CREB activity at multiple additional levels. Signalling cascades, including those that depend on the calcium-/calmodulin-dependent protein phosphatase calcineurin or the dual-leucine-zipper-bearing kinase, control the transcriptional activity of CBP after its recruitment by phospho-CREB (Oetjen et al., 2005, 2006; Schwaninger et al., 1993, 1995a) or govern the nuclear translocation and binding to the CREB leucine zipper domain of another essential CREB coactivator, TORC (Conkright et al., 2003; Ravnskjaer et al., 2007). The corresponding changes in phospho-CREB and luciferase reporter gene expression, as observed in the present study, indicate that chronic psychosocial stress and antidepressant treatment regulate CRE-directed transcription at the level of the phosphorylation of CREB.

Different ideas have been put forward as to the question whether the action of CREB is pro-depressive or antidepressive. Major points in favour of the latter view are: i) chronic treatment with some antidepressants increased the phosphorylation of CREB in certain brain regions (Thome et al., 2000; Tiraboschi et al., 2004), and ii) virus-mediated overexpression of CREB in the hippocampus produced antidepressant-like effects in acute behavioural tests (Chen et al., 2001). In contrast, in favour of the former view argue the following major points: i) chronic treatment with antidepressants inhibits adrenoceptor-associated adenylate cyclase activity (Vetulani and Sulser, 1975), ii) chronic treatment with antidepressants reduces the expression of CRE-containing genes like the genes encoding tyrosine hydroxylase (Nestler et al., 1990), corticotropin-releasing hormone (Brady et al., 1991), and beta-adrenoceptor (Banerjee et al., 1977; Meyerson et al., 1980), iii) chronic treatment with some antidepressants including citalopram decreased the phosphorylation of CREB in certain brain regions (Kuipers et al., 2006; Manier et al., 2002), iv) antidepressants inhibit the membrane depolarization-induced activation of CRE/CREB-directed gene transcription in cultured cells (Schwaninger et al., 1995b), v) virus-mediated overexpression of CREB in the nucleus accumbens increased immobility in the forced swim test (Pliakas et al., 2001), and vi) CREB-deficient ($CREB^{\alpha\Delta}$ mutant) mice demonstrate an antidepressant phenotype in the tail suspension test and forced swim test (Gur et al., 2007). In addition to methodological problems in interpreting these partly conflicting findings (as discussed above and more extensively in Tardito et al. (2006), the action of CREB may not be as uniform as to fit into a simple pro-depressive/antidepressive scheme. The outcome of CREB action may rather depend on the duration of its action, the brain region, and the particular environmental conditions. Noteworthy, not all patients benefit from antidepressant treatment (Nelson, 1999; Nestler et al., 2002). Our present and previous (Boer

et al., 2007a) studies are the first ones to investigate CREB activity in vivo under chronic psychosocial stress and antidepressant treatment. The results obtained speak in favour of CREB inhibition being part of antidepressant action.

In conclusion, using transgenic CRE-luciferase reporter gene mice to monitor CREB activity in vivo, the present study confirms that chronic psychosocial stress, as a model of depression, stimulates CRE/CREB-directed gene transcription in the hippocampus and other brain regions; it now shows that two SSRIs, citalopram and fluoxetine, reverse this stress-induced increase in CRE-directed transcription to control levels, suggesting inhibition of stress-induced CREB activity as a common mechanism of action of SSRIs underlying their antidepressant effect.

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