Pharmacological Reports 71 (2019) 753–761

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

The influence of CaMKII and ERK phosphorylation on BDNF changes observed in mice selectively devoid of CREB in serotonergic or noradrenergic neurons

Katarzyna Rafa-Zabłocka, Grzegorz Kreiner*, Monika Bagińska, Irena Nalepa

Department of Brain Biochemistry, Maj Institute of Pharmacology, Polish Academy of Sciences, Kraków, Poland

ARTICLE INFO

Article history:
Received 12 March 2019
Received in revised form 28 March 2019
Accepted 8 April 2019
Available online 9 April 2019

Keywords:
BDNF
CREB
Desipramine
Fluoxetine
Noradrenergic system
Serotonergic system

ABSTRACT

Background: The transcription factor CREB and the neurotrophin BDNF are important mood regulators due to their profound role in controlling the neuronal plasticity. Our previously published results from transgenic mice functionally lacking CREB in chosen neural populations have shown that BDNF upregulation evoked by chronic treatment with fluoxetine seems to be dependent on CREB residing exclusively in serotonergic neurons. To further elucidate this observation, we focused on the representative signaling cascades engaged in the regulation of BDNF production.

Methods: The study was carried out on mice lacking CREB in noradrenergic ($Creb1^{DBHCre}$) or serotonergic ($Creb1^{TPH2CreERT2}$) neurons in CREM deficient background. Animals received fluoxetine (10 mg/kg, *ip*) or desipramine (20 mg/kg, *ip*) for 21 days. The expression of following proteins and their phosphorylated forms was assessed by Western blot: CREB, BDNF, CaMKIIα, ERK1/2.

Results: We showed that consistent with previously observed BDNF upregulation, chronic treatment with fluoxetine causes an increase in the pool of active CaMKIIα in w/t males, while in $Creb1^{TPH2CreERT2}$ mutants, this effect ceased along with the observed decrease in ERK1/2 phosphorylation. These effects were region- and sex-specific. We did not observe a similar pattern of changes regarding the levels of BDNF expression and the CaMKIIα, ERK1/2 kinases in $Creb1^{DBHCre}$ mice exposed to desipramine. However, sex-dependent changes in the regulation of CaMKIIα and ERK1/2 activity were also observed.

Conclusions: Our study highlights the pivotal role of CREB in response to antidepressants, emphasizing different sex-dependent vulnerabilities to particular drugs and the important impact of CREM on the effects of CREB deletion.

Introduction

Among many putative molecular pathways that contribute to depression and modulate antidepressant treatment, the transcription factor CREB (cyclic AMP response element binding protein) and the neurotrophin BDNF (brain-derived neurotrophic factor) are regulated both in animal models of depression and humans suffering from this illness [1]. Moreover, both molecules are regarded as important mood regulators due to their profound role in controlling the expression of a variety of genes (CREB) and neuronal plasticity [2]. Since the expression of BDNF may be regulated by CREB, many studies have shown a CREB-dependent positive regulation of BDNF expression after antidepressant treatment [3] and a negative influence observed postmortem in patients with depression [4]. However, in animal transgenic models lacking CREB, this relationship is complex, and the data remain inconclusive [5]. Notably, in all these experiments, the researchers neglected the role of cyclic AMP response element modulator (CREM), which is another transcription factor that possibly compensates for the lack of CREB in KO models [6]. Our previous results from transgenic mice lacking both CREB and CREM

Abbreviations: BDNF, brain derived neurotrophic factor; BCA, bicinchoninic acid; BSA, bovine serum albumin; CaMKII, Ca2+/calmodulin-dependent protein kinase II; CREB, cyclic AMP response element binding protein; CREM, cyclic AMP response element modulator; DBH, dopamine β-hydroxylase; DMI, desipramine; ERK, extracellular signal-regulated kinase; ER, estrogen receptor; ERT2, mutant estrogen ligand-binding domain; FLX, fluoxetine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIP, hippocampus; KO, knock out; LC, locus coeruleus; MAP, mitogen-activated protein kinase; PFC, prefrontal cortex; RIPA, radioimmunoprecipitation assay buffer; RN, raphe nuclei; SAL, saline; SSRI, selective serotonin reuptake inhibitor; TPH, tryptophan hydroxylase-2; TrkB, tropomyosin receptor kinase B; TST, tail suspension test.

* Corresponding author.
E-mail address: kreiner@if-pan.krakow.pl (G. Kreiner).

http://dx.doi.org/10.1016/j.pharep.2019.04.008
1734-1140/

in the noradrenergic system confirmed this important interdependence, showing that the lack of CREB alone is not sufficient for the loss of reactivity to desipramine (a common antidepressant belonging to noradrenergic reuptake inhibitors) in despair behavior evaluated by tail suspension test (TST). Only mice devoid of both CREB and CREM (Creb1^{DBHCre}Crem−/− mice) were resistant to behavioral despair induced by desipramine, while animals lacking CREB alone (Creb1^{DBHCre} mice) were not distinguishable from the controls in this experiment [7]. Differences in single and double mutants were also observed in mice with functional CREB ablation in serotonergic cells, where we additionally suggested that BDNF upregulation evoked by chronic fluoxetine treatment seems to be dependent on the transcription factor CREB residing exclusively in serotonergic neurons [8].

To further elucidate this observation, we focused on the representative signaling cascades engaged in the regulation of BDNF production. *Bdnf* gene expression is regulated by the CREB transcription factor because its promoter region contains the CRE site in its sequence [9]. It has been shown that a postsynaptic increase in the intracellular Ca^{2+} concentration may lead to increased BDNF production by activating CaMKII, which in turn phosphorylates CREB [10]. This interaction is particularly important for synaptic plasticity in neurons. [11]. Furthermore, CREB may also be a target of the MAP kinase cascade Raf/MEK/ERK [12], in which phosphorylated ERK translocates to the cellular nucleus where it directly activates transcription factors [13], leading to the survival of neurons [14]. Moreover, all of these pathways may be activated downstream of BDNF binding to its receptor TrkB, creating a positive feedback loop [10] where BDNF stimulates its own expression *via* different kinases and CREB. Overall, both aforementioned BDNF-dependent signaling pathways contribute to its regulation in depression and subsequent antidepressant treatment [15], influencing cognitive ability and synaptic plasticity [16].

Therefore, the aim of this study was to investigate whether the observed role of CREB in serotonergic neurons of raphe nuclei (RN) for maintaining the BDNF response after fluoxetine is associated with changes in selected signaling pathways related to neuroplasticity, neurogenesis and cell survival by evaluating the protein expression and phosphorylation of CaMKIIα and ERK1/2. Additionally, we performed a thorough parallel analysis of the effects of chronic desipramine treatment on BDNF, pCaMKIIα/CaMKIIα and pERK/ERK expression in mice with selective and functional depletion of CREB in noradrenergic neurons of locus coeruleus (LC) (Creb1^{DBHCre} and Creb1^{DBHCre}Crem−/− transgenic mice). While investigating both transgenic lines lacking CREB in serotonergic or noradrenergic cells, we focused on brain structures (the prefrontal cortex and hippocampus) associated with the pathology of depression and profound innervation by noradrenergic and serotonergic projections.

Material and methods

Mice

All animals used in the experiments were of the C56BL/6 N genetic background. Depletion of CREB in selected neuronal populations was achieved by crossing Creb1$^{fl/fl}$ mice with DBHCre or TPH2CreERT2 mice to remove CREB from noradrenergic (Creb1^{DBHCre}) or serotonergic (Creb1^{TPH2CreERT2}) cells, respectively, as previously described [7,17]. To induce the mutation in Creb1^{TPH2CreERT2} mice, 12-week-old animals were given tamoxifen (Sigma-Aldrich, USA) (2 mg/mouse, *ip* 1x day for 5 consecutive days). Moreover, to restrain compensative effects of CREM upregulation in the absence of CREB [6], a group of animals was crossed with Crem−/− mice (Creb1^{DBHCre}Crem−/− or Creb1^{TPH2CreERT2}Crem−/−). The experiment was performed on male and female animals housed with their wild-type (w/t) (Cre-negative or/and CREM+/+) littermates of the same sex in self-ventilated cages (Allentown, USA) under standard laboratory conditions (12 h light/dark cycle with food and water *ad libitum*). This study was carried out in accordance with the EU Directive 2010/63/EU for animal experiments. All experimental procedures were approved by the Animal Ethical Committee at the Maj Institute of Pharmacology, Polish Academy of Sciences (Permit Number: 1125, issued 11/24/2014).

Study design and drug injections

Twelve-week-old mice with selective depletion of CREB in noradrenergic cells (Creb1^{DBHCre} and Creb1^{DBHCre}Crem−/−) were given desipramine (Sigma-Aldrich, USA) (20 mg/kg, *ip* for 21 days), while 15-week-old mice with selective depletion of CREB in serotonergic cells (Creb1^{TPH2CreERT2} and Creb1^{TPH2CreERT2}Crem−/−) received fluoxetine (Carbosynth, UK) (10 mg/kg, *ip*, for 21 days). Control animals were given 0,9% saline solution. Twenty-four hours after the final drug injection, the animals were sacrificed by cervical dislocation. Brain structures (hippocampus and prefrontal cortex) were collected and rapidly frozen. Tissue was stored at −80 °C for further studies.

Protein isolation

Frozen brain structures were homogenized using TissueLyser II (Qiagen, Netherlands) for 2 × 3 min at 30 Hz in ice-cold RIPA buffer (Sigma-Aldrich, USA) with the addition of protease inhibitor cocktail and phosphatase inhibitor cocktail (1:100 v/v concentration, Sigma-Aldrich, USA). Next, the samples were centrifuged (18,000 ×g at 4°C for 20 min), and supernatants were collected. The protein concentration was assessed using a BCA kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. Briefly, 20 μl of 30× diluted protein was added to 200 μl of BCA working reagent, and after 30 min of incubation at 37°C, the absorbance (A_{562}) of each sample was measured. The protein concentration was calculated using a standard curve based on BSA protein solution (Sigma-Aldrich, USA).

Immunoblotting

Protein samples were mixed with 2x Laemmli buffer and loaded on a precast polyacrylamide gel (Criterion TGX, Bio-Rad, USA) at an amount of 15 μg protein per well. The samples were separated by electrophoresis (90 V for 15 min followed by 180 V for 30 min) and then transferred from the gel to a nitrocellulose membrane (Bio-Rad, USA) using ENDURO^{TM} Semi-Dry Blotter (Labnet, USA) (20 V, 35 min). The membranes were stained with Ponseau S to assess transfer efficiency. After destaining with deionized water, the blots were blocked for 1 h in 5% BSA (Sigma-Aldrich, USA) solution in Tris-Buffered saline with 0,1% Tween-20 (TBST) (BioShop, Canada). Next, the membranes were incubated with a solution of primary antibodies diluted in blocking buffer (4 °C overnight with shaking) followed by incubation with appropriate secondary antibodies diluted in 5% nonfat dry milk in TBST. After each incubation step with the antibodies, the blots were washed 3 × 5 min with TBST buffer. The following primary antibodies were used: anti-phospho-CaMKII (Thr286) (#12716, Cell Signaling, USA), anti-CaMKII (05–532, Millipore, USA), anti-phospho-ERK1/2 (sc-7383, Santa Cruz, USA), anti-ERK1/2 (sc-93, Santa Cruz, USA), anti-phospho-CREB (Ser133) (06–519, Millipore, USA), anti-CREB (ab32515, Abcam, UK), anti-BDNF (ab108319, Abcam, UK), anti-GAPDH (MAB374, Millipore, USA), and anti-calnexin (ADI-SPA-860-F, Enzo Life Sciences, USA). Secondary anti-mouse (PI-2000, Vector, USA) or

anti-rabbit (PI-1000, Vector, USA) antibodies conjugated with horseradish peroxidase were used. Signals were developed with a Clarity Western ECL Substrate (Bio-Rad, USA) and imaged using a PXi system (Syngene, UK). The density of the bands was calculated using MultiGauge 3.0 software (FujiFilm, Japan)

Data analysis

Western blot data were calculated from at least two independent electrophoretic runs. All data were normalized against the control group, *i.e.*, w/t littermates of the same sex receiving saline solution. Statistical significance was assessed by two-way ANOVA, followed by Fisher's least difference *post-hoc* test when applicable using Statistica 12 software (StatSoft, USA). Comparisons with *p*-values less than 0.05 were considered statistically significant.

Results

Selective and functional depletion of CREB in serotonergic neurons attenuates the increase in the pool of active CaMKIIα evoked by chronic fluoxetine treatment in the hippocampus of male mice

In all the experiments, because neither the drugs nor the mutations induced any changes in the total level of the investigated kinases, we presented the results of our research as a ratio of phosphorylated protein *vs.* total protein, which is a widely accepted approach to study functional relevance [18]. The analysis revealed upregulation of the pool of phosphorylated CaMKII in the hippocampus of w/t animals after fluoxetine treatment (2-way ANOVA, genotype effect $F(2,35) = 12.39$, $p < 0.0001$; *post-hoc* test $p < 0.05$ *vs.* w/t + SAL), while depletion of CREB (Creb1^{TPH2CreERT2} transgenic mice) abolished the observed effect, diminishing the ratio of pCaMKIIα/CaMKIIα in mutant mice exposed to this drug (*post-hoc* test $p < 0.001$ *vs.* w/t + FLX) (Fig. 1A). In fact, compared with w/t animals, both Creb1^{TPH2CreERT2} and Creb1^{TPH2CreERT2}Crem−/− mice were characterized by diminished pCaMKIIα/CaMKIIα ratios, and the effect was significant in the hippocampus of male Creb1^{TPH2CreERT2} mice treated with saline only (*post-hoc* test $p<0.01$ *vs.* w/t + SAL). Additional removal of CREM seemed to partly revert the changes observed in the mice that only lacked CREB, but the effect was insignificant.

Fluoxetine treatment induced no changes in the phosphorylation of ERK1 or ERK2 in w/t animals, but selective depletion of CREB in serotonergic cells caused a decrease in pERK1/ERK1 (2-way ANOVA, genotype effect $F(2,36) = 5.93$, $p<0.01$; *post-hoc* test $p<0.01$ *vs.* w/t + FLX) and pERK2/ERK2 (2-way ANOVA, genotype effect $F(2,36) = 4.40$, $p<0.05$; *post-hoc* test $p<0.05$ *vs.* w/t + FLX) after drug administration (Fig. 1B, C). In the prefrontal cortex, only a decrease in the pCaMKIIα/CaMKIIα ratio was observed in fluoxetine-treated male Creb1^{TPH2CreERT2} mice *vs.* w/t mice (2-way ANOVA, genotype effect $F(2,36) = 3.65$, $p<0.05$; *post-hoc* test $p<0.05$) (Fig. 1D–F). All the assessed parameters in Creb1^{TPH2CreERT2}Crem−/− males had a similar pattern of changes as in the w/t animals.

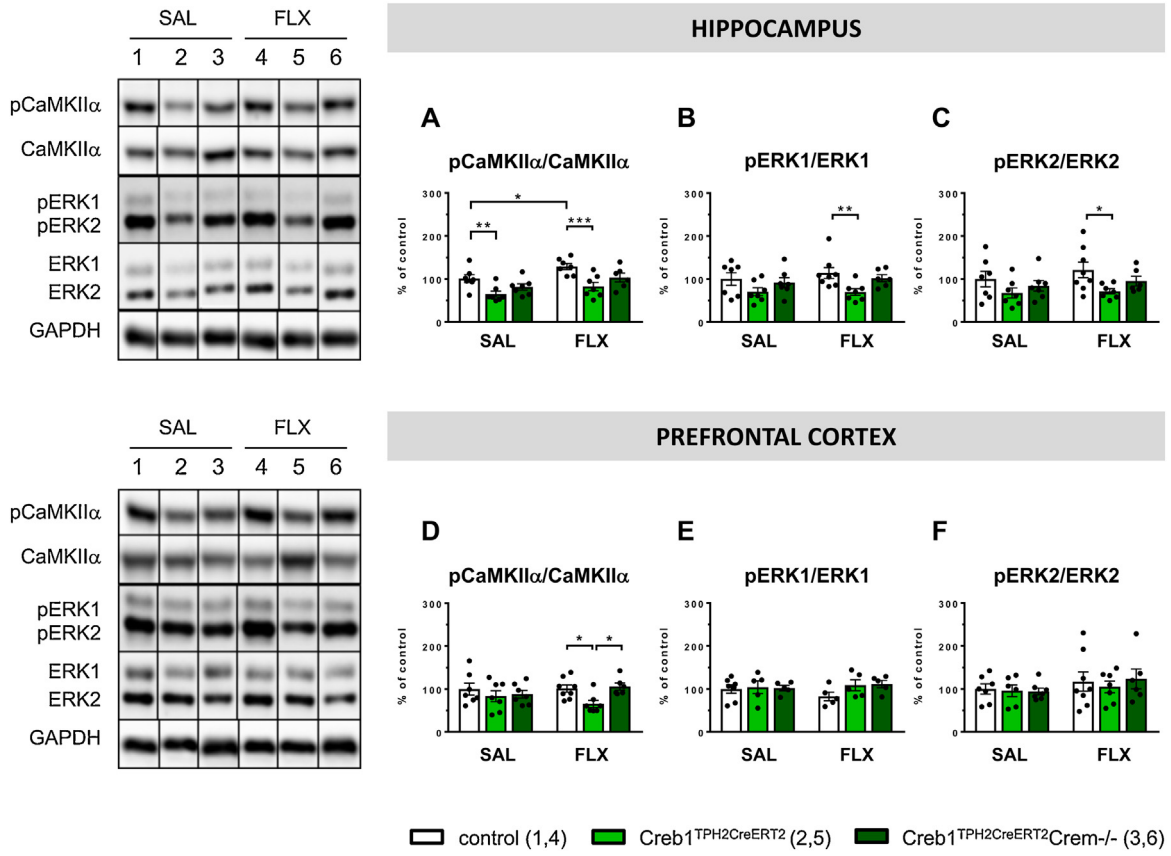

Fig. 1. Immunoblotting studies of CaMKIIα and ERK1/2 phosphorylation in the hippocampus and prefrontal cortex of wild-type, Creb1^{TPH2CreERT2} and Creb1^{TPH2CreERT2}Crem−/− male mice after fluoxetine treatment.Western blot analyses of the effects of fluoxetine administration on the phosphorylation levels of (A, D) CaMKIIα, (B, E) ERK1, and (C, F) ERK2 in the hippocampus (top panel) and prefrontal cortex (bottom panel) of wild-type, Creb1^{TPH2CreERT2}, and Creb1^{TPH2CreERT2}Crem−/− mice. Representative blots show phospho(Thr286) CaMKIIα, CaMKIIα, phospho-ERK1, ERK1, phospho-ERK2, ERK2 and GAPDH in saline-treated (wells 1–3) and fluoxetine-treated (wells 4–6) wild-type (wells 1, 4), Creb1^{TPH2CreERT2} (wells 2, 5) and Creb1^{TPH2CreERT2}Crem−/− (wells 3, 6) mice. Data are presented as the mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; w/t – wild type, SAL – saline, FLX – fluoxetine, HIP – hippocampus, PFC – prefrontal cortex, N = 6–8.

Selective depletion of CREB in serotonergic neurons of female mice increases phosphorylation of ERK1/2 in the prefrontal cortex after chronic fluoxetine treatment

Although the upregulation of BDNF after fluoxetine treatment was observed in the hippocampus of w/t female mice as well [8], here we did not notice any changes in the level of phosphorylation of CaMKII⟨, ERK1 or ERK2 in this structure caused by drug mutation (Fig. 2A–C). The ratio of pCaMKIIα/CaMKIIα in the prefrontal cortex of females did not change after drug treatment (Fig. 2D). However, in the prefrontal cortex, fluoxetine induced an increase in the phosphorylation of ERK1 (2-way ANOVA, genotype effect $F(2,35) = 8.14$, $p<0.01$; *post-hoc* test $p<0.001$ *vs.* w/t + FLX) and ERK2 (2-way ANOVA, genotype effect $F(2,35) = 12.51$, $p < 0.0001$; *post-hoc* test $p < 0.0001$ *vs.* w/t + FLX) in Creb1^{TPH2CreERT2} females (Fig. 2E, F), and this effect was not observed in male mice (Fig. 1E, F).

Chronic desipramine treatment does not influence the level of BDNF protein in the hippocampus or prefrontal cortex of mice

Similar to our previous studies, fluoxetine induced BDNF upregulation in the hippocampus of mice, and CREB deficiency in serotonergic neurons abolished this effect [8]. We investigated whether a similar effect can be observed in mice lacking CREB and CREM in noradrenergic neurons of LC after exposure to desipramine, which is a selective noradrenaline reuptake inhibitor. Therefore, we selectively performed analogous experiments in mice devoid of CREB in noradrenergic neurons (Creb1^{DBHCre} and Creb1^{DBHCre}Crem–/– transgenic mice). However, in this case, the immunoblotting results did not show any impact of chronic desipramine administration on the expression level of BDNF, neither in the hippocampus nor in the prefrontal cortex of mice, regardless of genotype or sex (Fig. 3). We observed only an increase in the BDNF expression level induced by selective depletion of CREB in the noradrenergic system, but only in the prefrontal cortex of female Creb1^{DBHCre} mice with a preserved CREM gene (2-way ANOVA, genotype effect $F(2,35) = 7.20$, $p<0.01$; *post-hoc* test $p<0.01$ *vs.* w/t + SAL).

Selective depletion of CREB in noradrenergic neurons with simultaneous CREM ablation affects the phosphorylation profile of CaMKIIα and ERK1/2 after chronic desipramine treatment in male mice

Although we were not able to confirm the enhancement of BDNF expression after desipramine treatment that has been often reported in other studies [19,20], we investigated whether desipramine causes any differences in the pattern of phosphorylation of the investigated kinases because the lack of BDNF regulation does not exclude the existence of changes at the level of kinases, which might simply be too subtle to evoke further effects. In fact, in the hippocampus, we observed that desipramine decreased the pool of active CaMKIIα only in the Creb1^{DBHCre}Crem–/– male mice (2-way ANOVA, genotype x drug $F(2,47) = 3.90$, $p<0.05$; *post-hoc* $p<0.01$ *vs.* Creb1^{DBHCre}Crem–/– + SAL), while no changes were observed in the w/t or Creb1^{DBHCre} males after drug administration (Fig. 4A).

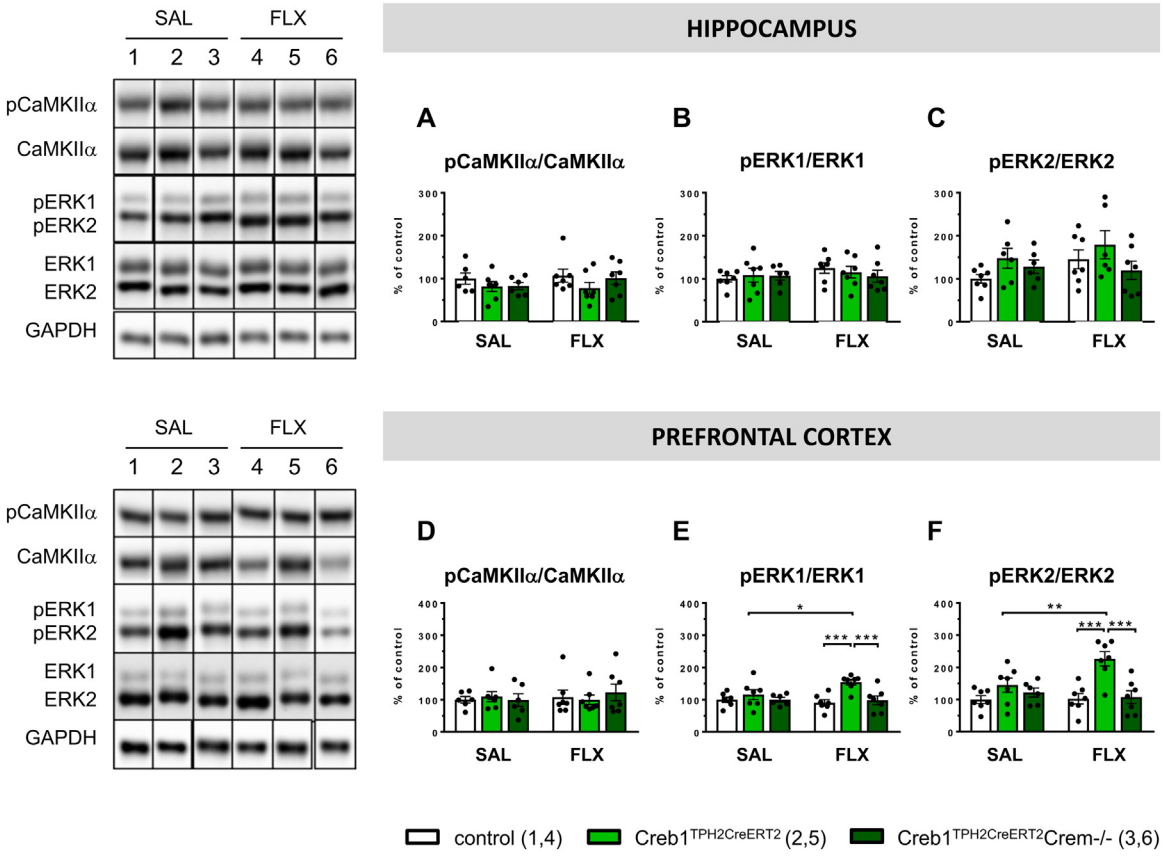

Fig. 2. Immunoblotting studies of CaMKIIα and ERK1/2 phosphorylation in the hippocampus and prefrontal cortex of wild-type, Creb1^{TPH2CreERT2} and Creb1^{TPH2CreERT2}Crem–/– female mice after fluoxetine treatment. Western blot analyses of the effects of fluoxetine administration on the phosphorylation levels of (A, D) CaMKIIα, (B, E) ERK1, and (C, F) ERK2 in the hippocampus (top panel) and prefrontal cortex (bottom panel) of wild-type, Creb1^{TPH2CreERT2}, and Creb1^{TPH2CreERT2}Crem–/– mice. Representative blots show phospho (Thr286)CaMKIIα, CaMKIIα, phospho-ERK1, ERK1, phospho-ERK2, ERK2 and GAPDH in saline-treated (wells 1–3) and fluoxetine-treated (wells 4–6) wild-type (wells 1, 4), Creb1^{TPH2CreERT2} (wells 2, 5) and Creb1^{TPH2CreERT2}Crem–/– (wells 3, 6) mice. Data are presented as the mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; w/t – wild type, SAL – saline, FLX – fluoxetine, HIP – hippocampus, PFC – prefrontal cortex, N = 6–8.

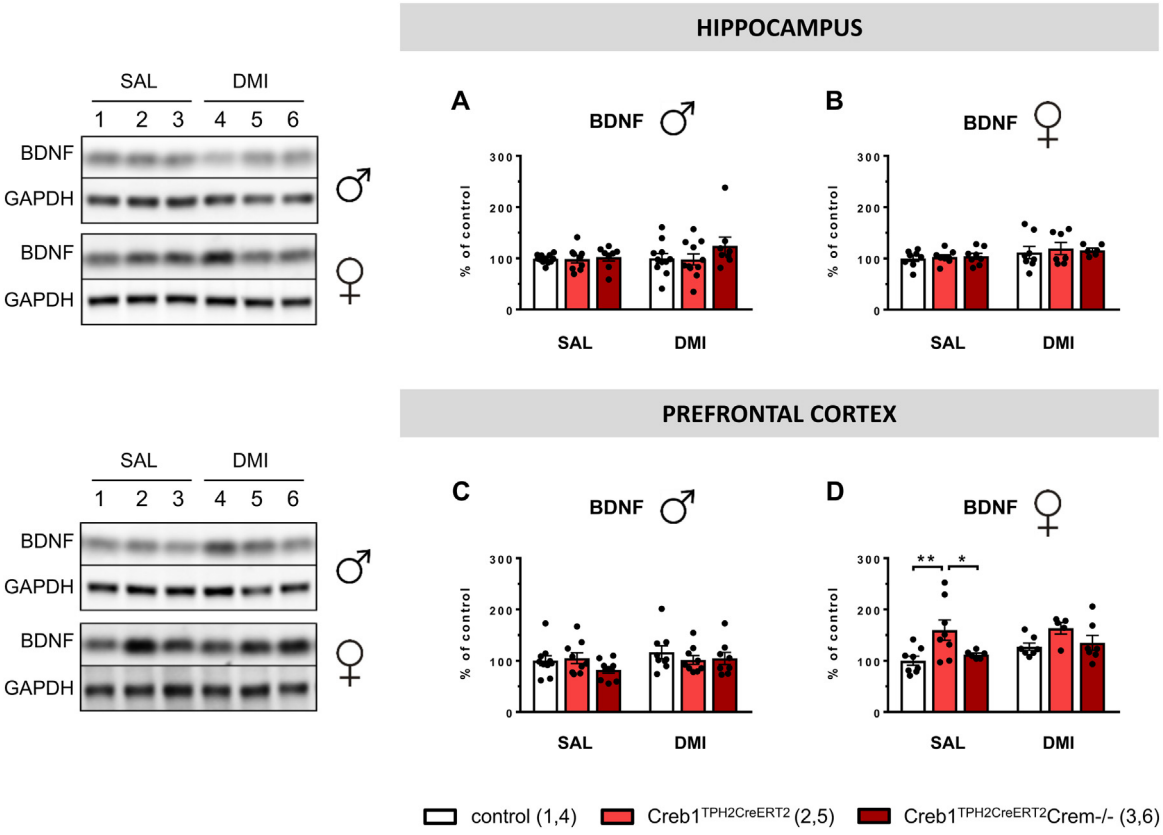

Fig. 3. Lack of a chronic desipramine treatment effect on the BDNF protein expression level in wild-type, Creb1^{DBHCre} and Creb1^{DBHCre}Crem–/– mice.
Western blot analyses of the effects of desipramine administration on the BDNF expression levels in the hippocampus (top panel A, B) and prefrontal cortex (bottom panel C, D) of wild-type, Creb1^{DBHCre} and Creb1^{DBHCre}Crem–/– mice. Representative blots show BDNF and GAPDH in saline-treated (wells 1–3) and desipramine-treated (wells 4–6) wild-type (wells 1, 4), Creb1^{DBHCre} (wells 2, 5) and Creb1^{DBHCre}Crem–/– (wells 3, 6) mice. Data are presented as the mean ± SEM. * $p<0.05$, ** $p<0.01$; w/t – wild type, SAL – saline, DMI – desipramine, HIP – hippocampus, PFC – prefrontal cortex, N = 7–10.

Nevertheless, in the saline-treated groups, we noted lower levels of pCaMKIIα/CaMKIIα in this structure in Creb1^{DBHCre} males compared with the w/t (*post-hoc* $p<0.05$) and Creb1^{DBHCre}Crem–/– ($p<0.01$) groups. Similar to CaMKII, ERK phosphorylation was affected by desipramine only in the Creb1^{DBHCre}Crem–/– mice (Fig. 4B, C), where the drug increased the pool of phosphorylated ERK1 (2-way ANOVA, drug effect $F(1,48) = 6.05$, $p<0.05$) and ERK2 (2-way ANOVA, drug effect $F(1,48) = 6.48$, $p<0.05$) compared with saline-treated double mutants (*post-hoc*: pERK1/ERK1 $p<0.05$; pERK2/ERK2 $p<0.05$). Moreover, the deficiency in both CREB in the noradrenergic system and CREM decreased the level of pERK1/ERK1 in the hippocampus (*post-hoc* $p<0.05$), while in CREB-deficient males only, no changes were observed (Fig. 4B). In the prefrontal cortex, we observed no changes in the phosphorylation of CaMKII and ERK1 caused by the drug or mutation (Fig. 4D, E). However, similar to the hippocampus, desipramine increased the pool of pERK2 in the Creb1^{DBHCre}Crem–/– mice (2-way ANOVA, drug effect $F(1,50) = 7.99$ $p<0.01$; *post-hoc* $p<0.01$ *vs.* Creb1^{DBHCre}Crem–/– + SAL), while no changes were observed in the other groups of animals (Fig. 4F).

Selective depletion of CREB in noradrenergic neurons compared with depletion of both CREB and CREM evoked different effects on the phosphorylation pattern of CaMKIIα and ERK1/2 after chronic desipramine treatment in female mice

ERK1 and ERK2 phosphorylation in the hippocampus of female mice was affected differently by desipramine in mutants depending on whether the genetic background was CREM+/+ or CREM–/– (Fig. 5B, C). The 2-way ANOVA of the pERK1/ERK1 ratio showed significant interaction of genotype x drug $F(2,38) = 4.95$ $p<0.05$, while *post-hoc* test revealed that the phosphorylation of ERK1 in Creb1^{DBHCre}Crem–/– after chronic desipramine treatment is enhanced in comparison to w/t + DMI ($p<0.01$) mice (Fig. 5B). Moreover, similar pattern of changes was observed regarding ERK2 phosphorylation, where the significant effect of interaction was noted (2-way ANOVA, genotype x drug $F(2,38) = 6.14$ $p<0.01$), while *post-hoc* test indicated significant increase of ERK2 phosphorylation after desipramine administration in Creb1^{DBHCre}Crem–/– compared with w/t + DMI ($p<0.01$) and Creb1^{DBHCre} + DMI ($p<0.05$) females (Fig. 5C). Nevertheless, no differences in the phosphorylation of ERK1 and ERK2 were observed between w/t and Creb1^{DBHCre} female mice treated by desipramine, which underlines the discrepancy between the effects of single CREB deletion and additional CREM KO background on the efficacy of this drug.

The pool of active CaMKIIα in the hippocampus of female mice was not affected by the genotype or desipramine (Fig. 5A); however, in the prefrontal cortex, the mutation itself induced an increase in CaMKIIα phosphorylation in the Creb1^{DBHCre}Crem–/– group (2-way ANOVA, genotype effect $F(2,39) = 4.83$ $p<0.05$; *post-hoc* $p<0.01$ *vs.* Creb1^{DBHCre}+SAL), while no drug effects were observed in any of the studied groups of animals (Fig. 5D). Moreover, we observed no changes in ERK1/2 phosphorylation in this structure in females (Fig. 5E, F).

Discussion

The data presented in this paper are a further step in our studies exploiting the pivotal role of CREB residing in serotonergic or

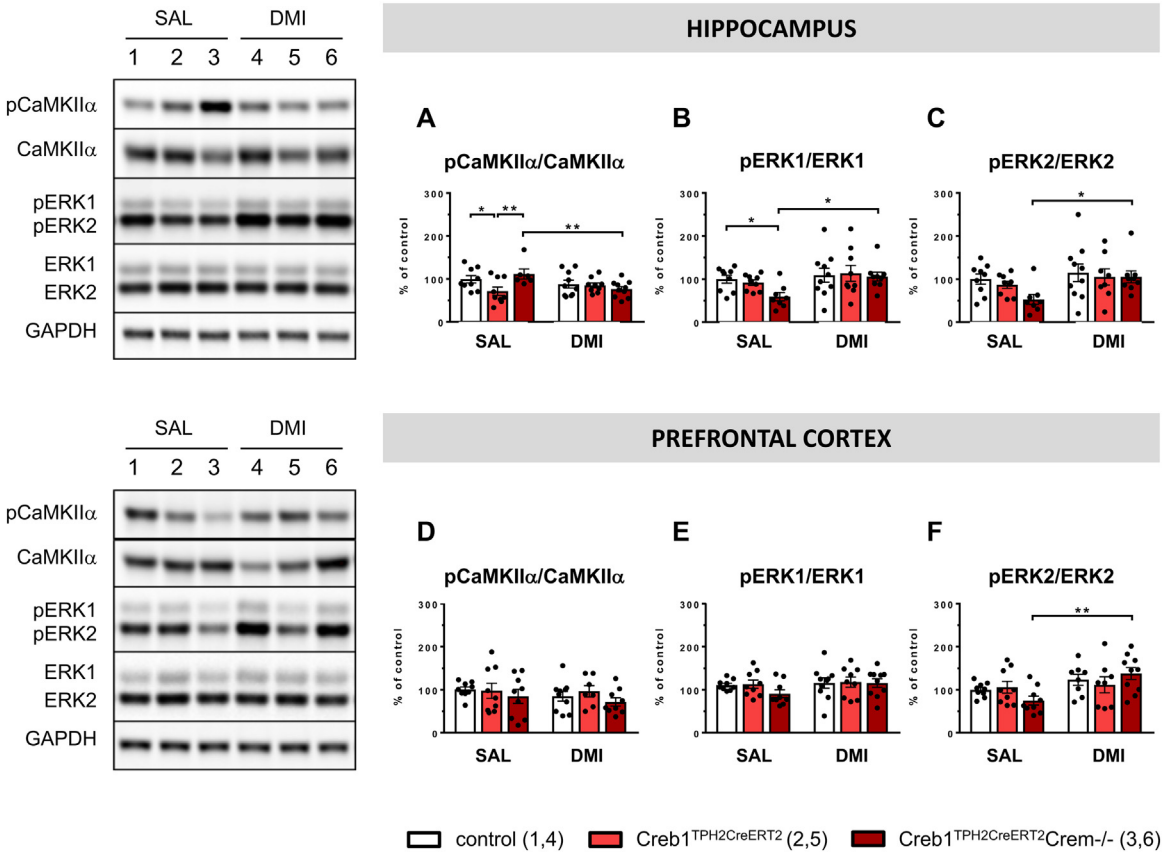

Fig. 4. Immunoblotting studies of CaMKIIα and ERK1/2 phosphorylation in the hippocampus and prefrontal cortex of wild-type, Creb1^{DBHCre} and Creb1^{DBHCre}Crem−/− male mice after desipramine treatment. Western blot analyses of the effects of desipramine administration on the phosphorylation levels of (A, D) CaMKIIα, (B, E) ERK1, and (C, F) ERK2 in the hippocampus (top panel) and prefrontal cortex (bottom panel) of wild-type, Creb1^{DBHCre} and Creb1^{DBHCre}Crem−/− mice. Representative blots show phospho (Thr286)CaMKIIα, CaMKIIα, phospho-ERK1, ERK1, phospho-ERK2, ERK2 and GAPDH in saline-treated (wells 1–3) and desipramine-treated (wells 4–6) wild-type (wells 1, 4), Creb1^{DBHCre} (wells 2, 5) and Creb1^{DBHCre}Crem−/− (wells 3, 6) mice. Data are presented as the mean ± SEM. * $p<0.05$, ** $p<0.01$; w/t – wild type, SAL – saline, DMI – desipramine, HIP – hippocampus, PFC – prefrontal cortex, N = 8–10.

noradrenergic neurons in the mechanism of antidepressant therapies, the protein which is a transcription factor often regarded as a convergence point of transduction pathways involved in depression etiopathology [21]. Previously, we showed that animals with CREB deletion selectively restricted to these two important neuromodulatory systems in depression exhibited different reactivity to antidepressants than w/t littermates, depending on whether the genetic background is CREM+/+ or CREM−/− [7]. Moreover, in the next study, selective CREB deletion restricted to serotonergic cells was shown to counteract BDNF upregulation evoked by chronic fluoxetine administration [8]. However, this effect was not confirmed in parallel experiments with Creb1^{DBHCre} and Creb1^{DBHCre}Crem−/− mice exposed to desipramine (Fig. 3). We assessed the phosphorylation and total level of kinases that are possibly involved in the BDNF-CREB-BDNF positive feedback loop, and CaMKII and ERK1/2 in both transgenic lines were investigated. In this study, our aim was to assess how chronic antidepressant treatment affects two kinases (CaMKIIα and ERK1/2) that act upstream or downstream of BDNF signaling. We narrowed our research to two brain structures, the hippocampus and prefrontal cortex, which have a well-established role in cognitive processes and depression and are also related to alterations in BDNF levels [22].

Our results showed that along with BDNF upregulation, chronic treatment with fluoxetine causes an increase in the pool of active CaMKIIα in w/t males, while Creb1^{TPH2CreERT2} males showed a decrease in the phosphorylation of CaMKIIα and ERK1/2 in the hippocampus after administration of this drug. CaMKIIα is a highly abundant protein in the brain, where it composes 1% of the total protein and up to 2% of the total protein in the hippocampus [23]. CaMKIIα plays a pivotal role in the process of synaptic plasticity after it is activated by Ca^{2+}/calmodulin, which triggers autophosphorylation at Thr286, making this kinase independent of Ca^{2+} stimuli [24]. A number of studies have shown that fluoxetine, similar to other SSRIs such as paroxetine, increases the activity of CaMKIIα, upregulates its protein expression or Thr286 phosphorylation after chronic, but not acute, treatment in the rat brain [25–27]. The interaction is complex and depends on the duration and route of drug administration (intraperitoneal injections *vs.* osmotic pumps), and our results for wild-type males were consistent with previous findings. However, selective ablation of CREB in serotonergic neurons decreased phosphorylation of CaMKIIα along with a simultaneous blockade of BDNF upregulation evoked by fluoxetine. These two effects could be connected, according to recent findings indicating that the inhibition of CaMKIIα in electrically stimulated neurons represses BDNF expression, leading to marked suppression of neurite outgrowth [28], which explains the results observed in the hippocampus of the Creb1^{TPH2CreERT2} male mice. These animals also exhibited diminished phosphorylation of ERK1/2 in the hippocampus after fluoxetine administration, while no changes were detected in the other groups of males. Studies on ERK kinases after the administration of SSRI drugs and antidepressants in general have suggested conflicting results [29]. It has been shown that acute

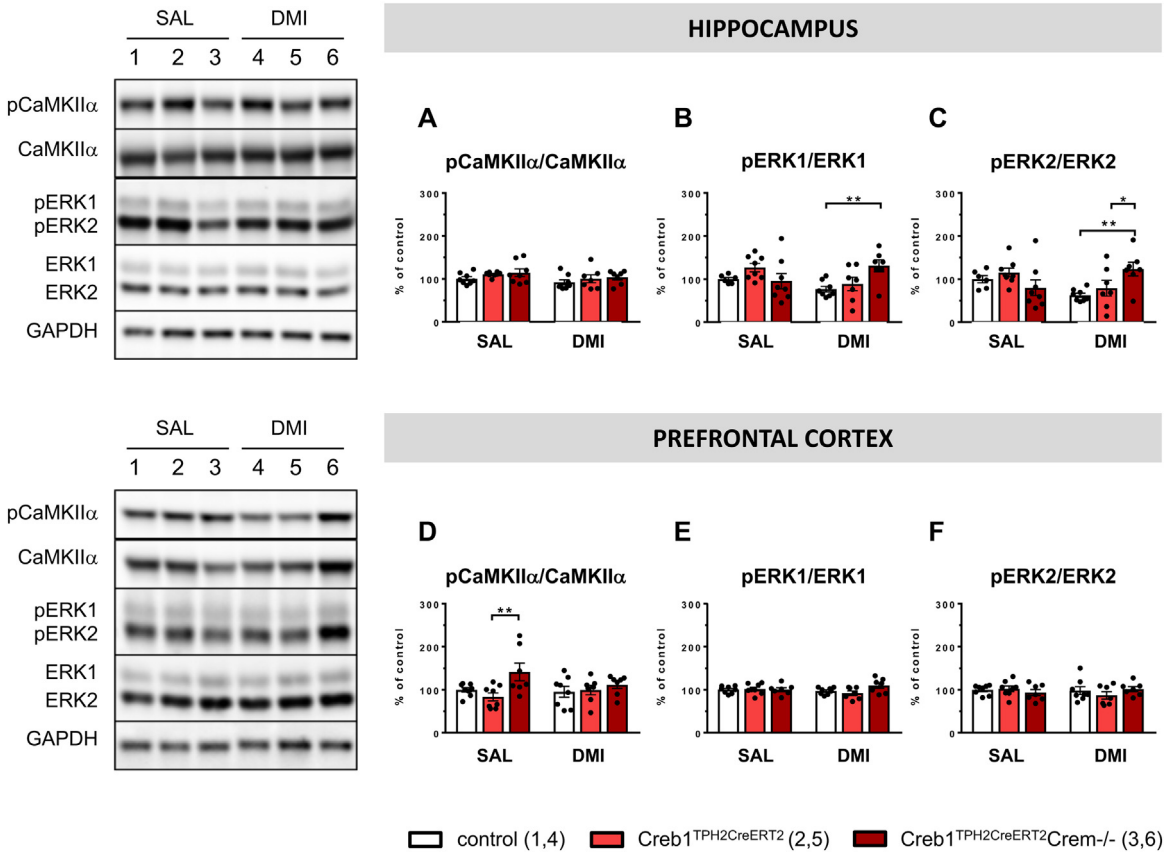

Fig. 5. Immunoblotting studies of CaMKIIα and ERK1/2 phosphorylation in the hippocampus and prefrontal cortex of wild-type, Creb1^{DBHCre} and Creb1^{DBHCre}Crem–/– female mice after desipramine treatment. Western blot analyses of the effects of desipramine administration on the phosphorylation levels of (A, D) CaMKIIα, (B, E) ERK1, and (C, F) ERK2 in the hippocampus (top panel) and the prefrontal cortex (bottom panel) of wild-type, Creb1^{DBHCre} and Creb1^{DBHCre}Crem–/– mice. Representative blots show phospho (Thr286)CaMKIIα, CaMKIIα, phospho-ERK1, ERK1, phospho-ERK2, ERK2 and GAPDH in saline-treated (wells 1–3) and desipramine-treated (wells 4–6) wild-type (wells 1, 4), Creb1^{DBHCre} (wells 2, 5) and Creb1^{DBHCre}Crem–/– (wells 3, 6) mice. Data are presented as the mean ± SEM. * $p<0.05$, ** $p<0.01$; w/t – wild type, SAL – saline, DMI – desipramine, HIP – hippocampus, PFC – prefrontal cortex, N = 6–8.

fluoxetine either does not influence [30] or reduces the phosphorylation of ERK1/2 [31], while 3 weeks of administration of this drug or a different SSRI (escitalopram) decreases the pERK1/2 expression level in the rat prefrontal cortex and hippocampus [30,32]. However, opposite results have also been observed; after 3 weeks of fluoxetine treatment, phosphorylation of both ERK2 and CREB increased in the rat hippocampus [33]. Furthermore, acute application of a MEK inhibitor that prevents the activation of ERK has been shown to render mice irresponsive to antidepressant drugs in a forced swim test [34] and induce a depressive phenotype in Bdnf$^{+/-}$ heterozygous mice. In our studies, no significant impact of fluoxetine on ERKs was observed in wild-type animals, which may be influenced by the use of the C56BL6/N mouse strain, while most of the findings cited above were observed in rats. However, parallel effects, such as a decrease in ERK1/2 phosphorylation and a drug-resistant phenotype in the tail suspension test after fluoxetine administration induced by selective depletion of CREB in serotonergic neurons, as observed previously [7], suggest an important role for ERK1/2 in the mechanism of fluoxetine action, at least in males. Overall, in the current study, we notably observed a concomitant pattern of changes in the CREB-dependent regulation of CaMKIIα and ERK1/2 signaling with the previously observed impact on this mutation on BDNF expression after chronic treatment with fluoxetine [8] in a male cohort of mice.

However, in females, fluoxetine had no impact on any of the investigated kinases in the hippocampus and prefrontal cortex, although we previously reported an increase in BDNF in both brain structures [8]. Moreover, selective depletion of CREB in Creb1^{TPH2CreERT2} mice induced upregulation of pERK1/2 in the female prefrontal cortex, contrary to downregulation of pERK1/2 that was observed in the male hippocampus. These differences may be not surprising considering this particular drug because sex differences in the responsiveness to SSRIs have been observed in many studies and in clinical treatment [35]. Divergent pharmacokinetics and pharmacodynamics between males and females may account for this phenomenon, as females tend to have higher than males plasma levels of norfluoxetine, which is a metabolite of fluoxetine [36]. Moreover, norfluoxetine is an inhibitor of the hepatic P450 isoenzyme CYP3A4, which affects estrogen metabolism [37]. Furthermore, estrogens along with progesterone have been shown to interact with serotonergic transmission, as they modulate the expression of tryptophan hydroxylase 2 in dorsal raphe nuclei [38]. Moreover, an ER agonist has been shown to rescue TPH2-positive cells in the lateral part of dorsal raphe nuclei, which are typically lost in ovariectomized mice [39]. Overall, the serotonergic system and estrogens interact with each other, affecting mood and cognition [40]; however, resolving this question in Creb1^{TPH2CreERT2} mice requires further elucidation. Because the CREB mutation in serotonergic neurons is induced by tamoxifen, which is an ER estrogen receptor antagonist, it could be possible that this pretreatment in females could account for the obtained results. However, only trace amounts of tamoxifen are present in adipose tissue 10 days after cessation of treatment [41], but we waited 21 days before starting

experiments with CreERT2-based transgenic lines; therefore, we assumed that apart from inducing a mutation, the effects of tamoxifen can be neglected.

Surprisingly, in this study, we were not able to confirm the effect of desipramine on BDNF neither in male nor female mice (Fig. 3) that has been observed by others. Assuming the crucial role of BDNF in transducing the effects of antidepressant treatment hypothesized by many researchers [3], the upregulation of this neurotrophic factor was expected after 21 days of treatment with desipramine, which has been previously shown in many studies [20,42]. Furthermore, a 20 mg/kg dose is widely accepted in pharmacological studies as an effective gold standard in tail suspension tests [43]. This was also confirmed in our recent experiments showing the differentiating effects in the reactivity to desipramine between w/t, Creb1^{DBHCre} and Creb1^{DBHCre}Crem−/− mice [7]. Furthermore, there are reports that have not confirmed the effect of desipramine on BDNF expression [44,45] or indicated structure-dependent regulation [46]. Moreover, even among researchers who support the so-called neurotrophic hypothesis of depression, there are reports that contradict this theory, *i.e.*, mice with forebrain deletion of BDNF have not been shown to be associated with a depressive-like phenotype [47]. Notably, data regarding CREB-dependent regulation of BDNF after antidepressant treatment are more robust and corroborative in terms of serotonergic reuptake inhibitors, as these drugs were simply more often studied [48].

Since we did not observe any positive effects on BDNF regulation after chronic treatment with desipramine, it was not surprising that the data regarding the involvement of CaMKIIα and ERK1/2 kinases in the mechanism of this drug action were also not conclusive. However, the important role of CREM in the regulation of the observed effects is notable. Specifically, additional deletion of CREM in the Creb1^{DBHCre}Crem−/− mice had different impacts on changes in the pCaMKIIα/CaMKIIα, pERK1/ERK1 and pERK2/ERK2 ratios after desipramine treatment, particularly in the hippocampus of male mice (Fig. 4A–C and 5 B, C). Although it is difficult to draw direct conclusions, this observation is consistent with the previous one from our behavioral study regarding different effects of regulating the response to desipramine depending on a single CREB or double CREB/CREM deletion [7].

Overall, our study highlights the pivotal role of CREB residing in serotonergic or noradrenergic neurons in response to antidepressant drug treatment, emphasizing the different sex-dependent vulnerabilities to particular drugs and the important impact of CREM on the effects of CREB deletion. Considering that the results obtained in both transgenic lines were the subject of this study and that there was a lack of any profound regulation of the BDNF pathway by desipramine, it seems that future research should focus on exploring the role of CREB in serotonergic neurons of raphe nuclei in the context of other theories of depression (*i.e.*, inflammatory and glutamatergic theories of depression), where the impact of the transcription factor CREB has been recently discussed as a key regulator of the described processes [21,49,50].

Funding

Supported by grant no 2014/13/B/NZ7/02293 (Opus7), financed by the Polish National Science Centre (NCN).

Competing interests

The authors declare that they have no competing interests.

Author contributions

GK designed the study, KRZ performed all Western blot analyses, KRZ and GK performed drug injections and tissue dissections, analyzed the data and wrote the paper, KRZ prepared the figures, MB maintained the transgenic mouse colony and performed genotyping, and IN supervised the study.

References

[1] Nair A, Vaidya VA. Cyclic AMP response element binding protein and brain-derived neurotrophic factor: molecules that modulate our mood? J Biosci 2006;31:423–34.
[2] Ren X, Dwivedi Y, Mondal AC, Pandey GN. Cyclic-AMP response element binding protein (CREB) in the neutrophils of depressed patients. Psychiatry Res 2011;185:108–12.
[3] Bjorkholm C, Monteggia LM. BDNF—a key transducer of antidepressant effects. Neuropharmacology 2016;102:72–9.
[4] Dwivedi Y. Brain-derived neurotrophic factor and suicide pathogenesis. Ann Med 2010;42:87–96.
[5] Blendy JA. The role of CREB in depression and antidepressant treatment. Biol Psychiatry 2006;59:1144–50.
[6] Hummler E, Cole TJ, Blendy JA, Ganss R, Aguzzi A, Schmid W, et al. Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. Proc Natl Acad Sci U S A 1994;91:5647–51.
[7] Rafa-Zablocka K, Kreiner G, Baginska M, Kusmierczyk J, Parlato R, Nalepa I. Transgenic mice lacking CREB and CREM in noradrenergic and serotonergic neurons respond differently to common antidepressants on tail suspension test. Sci Rep 2017;7:13515.
[8] Rafa-Zablocka K, Kreiner G, Baginska M, Nalepa I. Selective depletion of CREB in serotonergic neurons affects the upregulation of brain-derived neurotrophic factor evoked by chronic fluoxetine treatment. Front Neurosci 2018;12.
[9] Ghosh A, Carnahan J, Greenberg ME. Requirement for BDNF in activity-dependent survival of cortical neurons. Science 1994;263:1618–23.
[10] Cunha C, Brambilla R, Thomas KL. A simple role for BDNF in learning and memory? Front Mol Neurosci 2010;3:1.
[11] Leal G, Comprido D, Duarte CB. BDNF-induced local protein synthesis and synaptic plasticity. Neuropharmacology 2014;76(Pt C):639–56.
[12] Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 1996;273:959–63.
[13] Segal RA. Selectivity in neurotrophin signaling: theme and variations. Ann Rev Neurosci. 2003;26:299–330.
[14] Barnabe-Heider F, Miller FD. Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J Neurosci 2003;23:5149–60.
[15] Martinowich K, Manji H, Lu B. New insights into BDNF function in depression and anxiety. Nat Neurosci 2007;10:1089–93.
[16] Vaynman S, Ying Z, Gomez-Pinilla F. Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. Eur J Neurosci 2004;20:2580–90.
[17] Parlato R, Rieker C, Turiault M, Tronche F, Schutz G. Survival of DA neurons is independent of CREM upregulation in absence of CREB. Genesis 2006;44:454–64.
[18] Peck SC. Analysis of protein phosphorylation: methods and strategies for studying kinases and substrates. Plant J 2006;45:512–22.
[19] Nibuya M, Morinobu S, Duman RS. Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. J Neurosci 1995;15:7539–47.
[20] Conti AC, Cryan JF, Dalvi A, Lucki I, Blendy JA. cAMP response element-binding protein is essential for the upregulation of brain-derived neurotrophic factor transcription, but not the behavioral or endocrine responses to antidepressant drugs. J Neurosci 2002;22:3262–8.
[21] Wang HT, Xu JP, Lazarovici P, Quirion RH, Zheng WH. cAMP response element-binding protein (CREB): a possible signaling molecule link in the pathophysiology of schizophrenia. Front Mol Neurosci 2018;11.
[22] Andero R, Choi DC, Ressler KJ. BDNF-TrkB receptor regulation of distributed adult neural plasticity, memory formation, and psychiatric disorders. Prog Mol Biol Trans Sci. 2014;122:169–92.
[23] Swulius MT, Waxham MN. Ca(2+)/calmodulin-dependent protein kinases. Cell Mol Life Sci 2008;65:2637–57.
[24] Hanson PI, Meyer T, Stryer L, Schulman H. Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. Neuron 1994;12:943–56.
[25] Barbiero VS, Giambelli R, Musazzi L, Tiraboschi E, Tardito D, Perez J, et al. Chronic antidepressants induce redistribution and differential activation of alphaCaM kinase II between presynaptic compartments. Neuropsychopharmacology 2007;32:2511–9.
[26] Celano E, Tiraboschi E, Consogno E, D'Urso G, Mbakop MP, Gennarelli M, et al. Selective regulation of presynaptic calcium/calmodulin-dependent protein kinase II by psychotropic drugs. Biol Psychiatry 2003;53:442–9.
[27] Martinez-Turrillas R, Del Rio J, Frechilla D. Neuronal proteins involved in synaptic targeting of AMPA receptors in rat hippocampus by antidepressant drugs. Biochem Biophys Res Commun 2007;353:750–5.
[28] Yan X, Liu J, Ye Z, Huang J, He F, Xiao W, et al. CaMKII-mediated CREB phosphorylation is involved in Ca2+-induced BDNF mRNA transcription and neurite outgrowth promoted by electrical stimulation. PLoS One 2016;11: e0162784.

[29] Duman RS, Voleti B. Signaling pathways underlying the pathophysiology and treatment of depression: novel mechanisms for rapid-acting agents. Trends Neurosci 2012;35:47–56.
[30] Fumagalli F, Molteni R, Calabrese F, Frasca A, Racagni G, Riva MA. Chronic fluoxetine administration inhibits extracellular signal-regulated kinase 1/2 phosphorylation in rat brain. J Neurochem 2005;93:1551–60.
[31] Di Benedetto B, Radecke J, Schmidt MV, Rupprecht R. Acute antidepressant treatment differently modulates ERK/MAPK activation in neurons and astrocytes of the adult mouse prefrontal cortex. Neuroscience 2013;232:161–8.
[32] Alboni S, Benatti C, Capone G, Corsini D, Caggia F, Tascedda F, et al. Time-dependent effects of escitalopram on brain derived neurotrophic factor (BDNF) and neuroplasticity related targets in the central nervous system of rats. Eur J Pharmacol 2010;643:180–7.
[33] Qi X, Lin W, Li J, Li H, Wang W, Wang D, et al. Fluoxetine increases the activity of the ERK-CREB signal system and alleviates the depressive-like behavior in rats exposed to chronic forced swim stress. Neurobiol Dis 2008;31:278–85.
[34] Duman CH, Schlesinger L, Kodama M, Russell DS, Duman RS. A role for MAP kinase signaling in behavioral models of depression and antidepressant treatment. Biol Psychiatry 2007;61:661–70.
[35] Borrow AP, Cameron NM. Estrogenic mediation of serotonergic and neurotrophic systems: implications for female mood disorders. Prog Neuropsychopharmacol Biol Psych 2014;54:13–25.
[36] Bigos KL, Pollock BG, Stankevich BA, Bies RR. Sex differences in the pharmacokinetics and pharmacodynamics of antidepressants: an updated review. J Gend Specif Med 2009;6:522–43.
[37] Thompson DS, Kirshner MA, Klug TL, Kastango KB, Pollock BG. A preliminary study of the effect of fluoxetine treatment on the 2 : 16-alpha-hydroxyestrone ratio in young women. Ther Drug Monit 2003;25:125–8.
[38] Donner N, Handa RJ. Estrogen receptor beta regulates the expression of tryptophan-hydroxylase 2 mRNA within serotonergic neurons of the rat dorsal raphe nuclei. Neuroscience 2009;163:705–18.
[39] Suzuki H, Barros RP, Sugiyama N, Krishnan V, Yaden BC, Kim HJ, et al. Involvement of estrogen receptor beta in maintenance of serotonergic neurons of the dorsal raphe. Mol Psych 2013;18:674–80.
[40] Amin Z, Canli T, Epperson CN. Effect of estrogen-serotonin interactions on mood and cognition. Beh Cog Neurosci Rev 2005;4:43–58.
[41] Ye R, Wang QA, Tao C, Vishvanath L, Shao M, McDonald JG, et al. Impact of tamoxifen on adipocyte lineage tracing: inducer of adipogenesis and prolonged nuclear translocation of Cre recombinase. Mol Metab 2015;4:771–8.
[42] Jacobsen JP, Mork A. The effect of escitalopram, desipramine, electroconvulsive seizures and lithium on brain-derived neurotrophic factor mRNA and protein expression in the rat brain and the correlation to 5-HT and 5-HIAA levels. Brain Res 2004;1024:183–92.
[43] Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 2005;29:571–625.
[44] Vinet J, Carra S, Blom JMC, Brunello N, Barden N, Tascedda F. Chronic treatment with desipramine and fluoxetine modulate BDNF, CaMKK alpha and CaMKK beta mRNA levels in the hippocampus of transgenic mice expressing antisense RNA against the glucocorticoid receptor. Neuropharmacology 2004;47:1062–9.
[45] Kozisek ME, Middlemas D, Bylund DB. The differential regulation of BDNF and TrkB levels in juvenile rats after four days of escitalopram and desipramine treatment. Neuropharmacology 2008;54:251–7.
[46] Balu DT, Hoshaw BA, Malberg JE, Rosenzweig-Lipson S, Schechter LE, Lucki I. Differential regulation of central BDNF protein levels by antidepressant and non-antidepressant drug treatments. Brain Res 2008;1211:37–43.
[47] Monteggia LM, Barrot M, Powell CM, Berton O, Galanis V, Gemelli T, et al. Essential role of brain-derived neurotrophic factor in adult hippocampal function. Proc Natl Acad Sci U S A 2004;101:10827–32.
[48] Castren E, Kojima M. Brain-derived neurotrophic factor in mood disorders and antidepressant treatments. Neurobiol Dis 2017;97:119–26.
[49] Dey A, Giblin PAH. Insights into macrophage heterogeneity and cytokine-induced neuroinflammation in major depressive disorder. Pharmaceuticals 2018;11.
[50] Ortega-Martinez S. A new perspective on the role of the CREB family of transcription factors in memory consolidation via adult hippocampal neurogenesis. Front Mol Neurosci 2015;8.