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CALCINEURIN (PROTEIN PHOSPHATASE 2B) IS INVOLVED IN THE MECHANISMS OF ACTION OF ANTIDEPRESSANTS

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Abstract—Calcineurin (PP2B) is a Ca^{2+}-dependent protein phosphatase enriched in the brain that takes part in intracellular signaling pathways regulating synaptic plasticity and neuronal functions. Calcineurin-dependent pathways are important for complex brain functions such as learning and memory. More recently, they have been suggested to play a role in the processing of emotional information. The aim of this study was to investigate whether calcineurin may be involved in the effect of antidepressants. We first found that chronic antidepressant treatment in mice leads to an increase of calcineurin levels in the hippocampus. We then studied the behavioral and molecular responses to fluoxetine of mice with a genetic overactivation of calcineurin in the hippocampus (constitutively-activated calcineurin transgenic mouse line #98, CN98 mice). We observed that CN98 mice are more sensitive to the behavioral effect of fluoxetine and desipramine tested in the tail suspension test. Moreover, the basal expression of growth factor brain-derived neurotrophic factor and subunit 1 of AMPA glutamate receptor, GluR1, both of which are modified after chronic antidepressant administration, are altered in the hippocampus of CN98 mice. These results suggest that calcineurin-dependent dephosphorylation plays an important role in the mechanisms of action of antidepressants, providing a new starting point for developing improved therapeutic treatments for depression.

Key words: dephosphorylation, FK-506, fluoxetine, BDNF, GluR1, hippocampus.

Major depression is a frequent mood disorder that affects up to 20% of the population worldwide. The primary medical treatment of depression has been through drugs that increase the concentration of biogenic amines (5-HT, norepinephrine and/or dopamine), leading to the biogenic amine theory of depression. Although current antidepressant treatments are efficient, they suffer from a number of side effects that limit their utility, and usually three to four weeks of daily treatment are needed before a therapeutic effect is achieved. Research efforts on depression are currently focusing on downstream effectors of antidepressant activity, since they may represent novel targets for the development of new more robust and rapid antidepressants. So far, the cAMP-dependent-protein-kinase-A (PKA), mitogen-activated protein kinase (MAPK), brain-derived neurotrophic factor (BDNF) and cAMP-regulated-phosphoprotein-of-Mr32,000 (DARPP-32) pathways have been shown to be activated by antidepressant administration. Here, we studied the possible involvement of calcineurin or protein phosphatase 2B (PP2B), a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase enriched in neurons. This heterodimer protein is composed of a catalytic (CNA) and a regulatory subunit (CNB), each having different subtypes that are encoded by different genes or generated by alternative splicing (Rusnak and Mertz, 2000). Because of its high sensitivity to Ca^{2+} (0.1–1 nM) allowing to function as a Ca^{2+} sensor and a regulator of Ca^{2+} homeostasis, calcineurin largely contributes to the shaping of Ca^{2+}-dependent processes such as neuronal activity, signaling, survival, neuroplasticity and cellular resilience (reviewed in Xia and Storm, 2005). Calcineurin functions in concert with another Ser/Thr protein phosphatase, the protein-phosphatase-1 (PP1), as well as cognate protein kinases such as the Ca^{2+}/calmodulin-dependent-protein-kinase (CaMK), the PKA or the protein-kinase-C (PKC) to control the activity of specific targets such as ion channels, neurotransmitter receptors, signaling enzymes, transcription and translation factors (reviewed in Xia and Storm, 2005). Some of these targets are critical cross-talks and major determinants of the balance between kinases and phosphatases. For instance, DARPP-32 and its analog the inhibitor-1 (I-1) are activated by PKA-dependent phosphorylation at Thr34 or Thr35 respectively, and become potent inhibitors of PP1 that itself, controls the dephosphorylation and activity of several downstream substrates (Hemmings et al., 1984). DARPP-32 and I-1 activation is turned down by dephosphorylation by calcineurin (Nishi et al., 1997).

In the balance between protein kinase and protein phosphatase activity (reviewed in Xia and Storm, 2005), which is essential for synaptic plasticity and for the control of neuronal functions, calcineurin has been demonstrated to act as a major negative regulator. Recent studies indicate a possible involvement of calcineurin in affective processing. Deficient calcineurin expression was recently found to be associated with schizophrenia in humans and schizophrenia-like symptoms in a knock-out mouse model (Gerber et al., 2003; Miyakawa et al., 2003), while psycho-

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Abbreviations: BDNF, brain-derived neurotrophic factor; CN98, constitutively-activated calcineurin transgenic mouse line #98; DARPP-32, cAMP-regulated-phosphoprotein-of-Mr32,000; ERK, extracellular signal-regulated kinase; GluR1, subunit 1 of AMPA glutamate receptor; PKA, cAMP-dependent-protein-kinase-A; PKC, protein-kinase-C; PP1, protein-phosphatase-1; SSRI, serotonin-selective re-uptake inhibitor; TST, tail suspension test; WT, wild-type.

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social stress was shown to downregulate forebrain calcineurin expression (Gerges et al., 2003). Furthermore, modulation of DARPP-32 phosphorylation and downstream targets in limbic and cortical regions is a key process in the control of emotional states and the action of psychotropic medications such as antidepressants and psychotomimetics (Svenningsson et al., 2002, 2003).

Calcineurin A is mainly localized in the cerebral cortex, the striatum, the hippocampus, and the cerebellum. On the contrary, there is no calcineurin A in thalamic and hypothalamic neurons (Sola et al., 1999). Many brain regions have been involved in the pathophysiology of depression. Most evidence from animal models and from studies in humans links depression to neuroanatomical, neurochemical and signaling abnormalities in the hippocampus, prefrontal cortex and striatum, regions that express high levels of calcineurin.

Therefore, here we investigated the role of calcineurin in the effect of antidepressants. First, we assessed the effects of chronic treatment with the serotonin-selective re-uptake inhibitor (SSRI) fluoxetine, on calcineurin levels in mouse hippocampus. We then explored if calcineurin inhibition by systemic injection of FK-506 could modify behavioral readouts related to antidepressant effects. Indeed, the immunosuppressive drug FK-506, which can easily cross the blood–brain barrier unlike cyclosporine A (Pong and Zaleska, 2003), binds to the ubiquitous intracellular protein cyclophilin, and calcineurin phosphatase activity is noncompetitively inhibited by the binding of the complex FK-506-cyclophilin. Additionally, we studied mice overexpressing an active form of calcineurin to develop a better understanding of the involvement of calcineurin signaling pathways in antidepressant action.

EXPERIMENTAL PROCEDURES

Animals

Transgenic mice (constitutively-activated calcineurin transgenic mouse line #98, CN98 mice) expressing a constitutively active form of calcineurin Aα subunit restricted to the forebrain and throughout the hippocampus and dentate gyrus were used (Winder et al., 1998). The CN98 mice were maintained on a C57/Bl6 background; breeding and maintenance of the mice as well as the PCR determination of the genotype were performed as described previously (Mansuy et al., 1998; Biala et al., 2005). Mice, bred in INSERM U-513 as described (Biala et al., 2005) from initial breeding couples provided by Dr. I. M. Mansuy (Brain Research Institute, University of Zürich and Swiss Federal Institute of Technology, Zürich, Switzerland), were maintained in standard laboratory conditions (12-h light/dark cycle with light on at 07:30 h; room temperature 21±1 °C), with food and water *ad libitum*. For experiments, both males and females were used and each mouse was tested only once. Behavioral tests were performed by an experimenter blinded to genotype and treatment. All experiments were carried out in accordance with the European Communities Council Directive (86/809/EEC) on the ethical use of animals and approved by the local ethical committee. Every effort was made to minimize the number of animals used and their suffering.

Treatments

All drugs, except FK-506, were dissolved in 0.9% NaCl and injected intraperitoneally (10 ml/kg). Fluoxetine and desipramine were purchased from Sigma (L'Isle d'Abeau Chesnes, France). For dose-response experiments, fluoxetine was administered at 10 and 20 mg/kg and desipramine at 16 and 32 mg/kg. The chronic fluoxetine treatment was administered at 10 mg/kg once daily for 21 days and mice were killed 15 min after the last injection. In a previous study (Svenningsson et al., 2003) it has been shown that fluoxetine exerts its acute central effects (protein phosphorylation, c-fos induction) 15 min post-injection. FK506 (Alexis, Lausen, Switzerland) was injected intraperitoneally (20% EtOH, 20% cremophor EL, 60% saline) at the dose of 5 mg/kg.

Tail suspension test (TST)

Behavioral despair was investigated by tail suspension according to the standard procedure (Steru et al., 1985). Fifteen minutes (for desipramine and fluoxetine experiments) or two hours (for FK-506 experiments) after drug injection, mice were individually suspended by their tail using a paper adhesive tape placed ~1 cm from the tip of the tail in the chamber of a TST apparatus (Bioseb, Chaville, France). Mice were considered immobile when they were completely motionless and immobility (seconds) was mechanically and automatically recorded during the 5-min test period.

In situ hybridization

Tissue preparation was as described previously (Moutsimilli et al., 2005). Brains were rapidly dissected and frozen in isopentane at −30 °C. Ten-micrometer thick sections were cut with a cryostat at −20 °C, thaw-mounted on glass slides, fixed in formaldehyde (4%), washed with PBS, dehydrated in 50% and 70% ethanol, air-dried and stored at −80 °C until use. BDNF specific antisense oligonucleotides (Helios Biosciences, Créteil, France) were labeled with [^{35}S]-dATP, using terminal transferase (Amersham Biosciences, Little Chalfont, UK), to a specific activity of 5×10^8 d.p.m./μg. Sections were covered with 100 μl of hybridization medium (Helios Biosciences) containing $3–5\times10^5$ d.p.m. of labeled probe. Slides were incubated overnight at 53 °C in a humid chamber (50% formamide), washed and exposed to X-ray films (Biomax, Eastman Kodak, Rochester, NY, USA) for 21 days. The films were scanned with a Fujifilm BioImaging Analyzer BAS-5000 and quantification was performed using the NIH image software.

Western blot

Mice were decapitated and brains were immediately removed on ice. Hippocampi were dissected and homogenized in buffer A (50 mM Hepes pH 7,4; 0.5% NP40; 10% glycerol; 137 mM NaCl; 10 mM NaF; 100 mM DTT; Protease Inhibitor Cocktail (Sigma) Phosphatase inhibitor cocktail 1 or 2 (Sigma). For Western blots, 5 μg of total protein was resuspended in Laemmli buffer and loaded in a 12% acryl/bisacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). Prestained Protein Marker Broad Range was from New England Biolabs (Ozyme, St. Quentin en Yvelines, France). Proteins were electrically transferred onto a nitrocellulose sheet. The blot was incubated overnight with anti-subunit 1 of AMPA glutamate receptor (GluR1) (1:1000) or anti-calcineurin Aα (1:1000) antibody (Upstate Biotechnology, Lake Placid, NY, USA). The blot was washed three times, incubated for 1 h with anti-rabbit peroxidase-conjugated IgG (1:10,000, Sigma) and immunoreactivity was visualized with the enhanced chemiluminescence method (ECL plus, Amersham). Densitometric quantification of Western blots was performed by computer analysis of gel images with NIH image. Blots were systematically re-hybridized for one hour with anti-α-tubulin antibody (1:10,000, Sigma) to quantify sample loading.

Data analysis

Results were analyzed with one-, or two-way ANOVA (as appropriate) with the Statistica software. Data represent mean±S.E.M. of the indicated number of animals per group. For *in situ* hybridization, quantification is expressed as ratio to background (white matter) and for Western blot analyses, the results are expressed as ratio to α-tubulin.

RESULTS

Pharmacological inhibition of calcineurin increases immobility in the TST

We first wanted to address the relationship between calcineurin activity and behavioral assessment of despair in mice. To investigate this relationship, we first inhibited calcineurin with the immunosuppressant FK506 in wild-type (WT) mice prior to submitting them in the TST, a validated model of behavioral despair used to test antidepressant efficacy in rodents (Bai et al., 2001; El Yacoubi et al., 2003). We have used the same protocol as described by Pardo et al. (2006), in which the authors show a maximum efficiency on the inhibition of *in vivo* brain calcineurin activity. FK506 (5 mg/kg) administered i.p. 2 h before the TST significantly increased the time spent in immobility and reduced the frequency of escape attempts ($F(1,12)=4.8$, $P<0.05$, one-way ANOVA, Fig. 1a).

Chronic fluoxetine treatment up-regulates calcineurin protein levels in the hippocampus

In a second set of experiments, we directly explored whether calcineurin expression was altered by fluoxetine in WT mice. As fluoxetine requires chronic administration to induce behavioral effects in humans, we administered fluoxetine at the dose of 10 mg/kg daily for 21 days in WT mice. Interestingly, we observed a marked increase of calcineurin protein levels in the hippocampus ($F(1,11)=4.86$ $P<0.05$; one-way ANOVA, Fig. 1b).

Calcineurin overexpression enhanced behavioral responsiveness to antidepressants

As chronic fluoxetine treatment increased calcineurin levels in the hippocampus, in a genetic approach, we studied the CN98 mice, a useful model of forebrain-specific calcineurin overactivation. When subjected to the TST, CN98 and WT spent overall a similar amount of time immobile (Fig. 2a, b). However, when treated acutely with the antidepressants fluoxetine (an SSRI) or desipramine (a tricyclic), CN98 showed a higher sensitivity to the drugs responding to significantly lower doses. Fluoxetine reduced immobility and increased attempts to escape at 10 mg/kg in CN98, while in WT mice 20 mg/kg was needed to obtain a similar response (Fig. 2a). Likewise, desipramine significantly reduced immobility and increased attempts to escape at 16 mg/kg in CN98 mice but only at 32 mg/kg in WT mice (Fig. 2b) (genotype and treatment effect: fluoxetine (10 or 20 mg/kg) $F(1,47)=12.12$, $P<0.01$ and $F(2,47)=24.48$, $P<0.001$; desipramine (16 or 32 mg/kg) $F(1,54)=16.08$, $P<0.001$ and $F(2,54)=11.84$, $P<0.001$, two-way ANOVA). To ensure that the enhanced responsiveness of CN98 mice to fluoxetine and desipramine was due to the antidepressant property of the drugs and not to a change in locomotor activity, we measured locomotion after drug administration. Both fluoxetine (10 mg/kg) and desipramine (16 mg/kg) similarly reduced ambulations in WT and CN98 mice (data not shown).

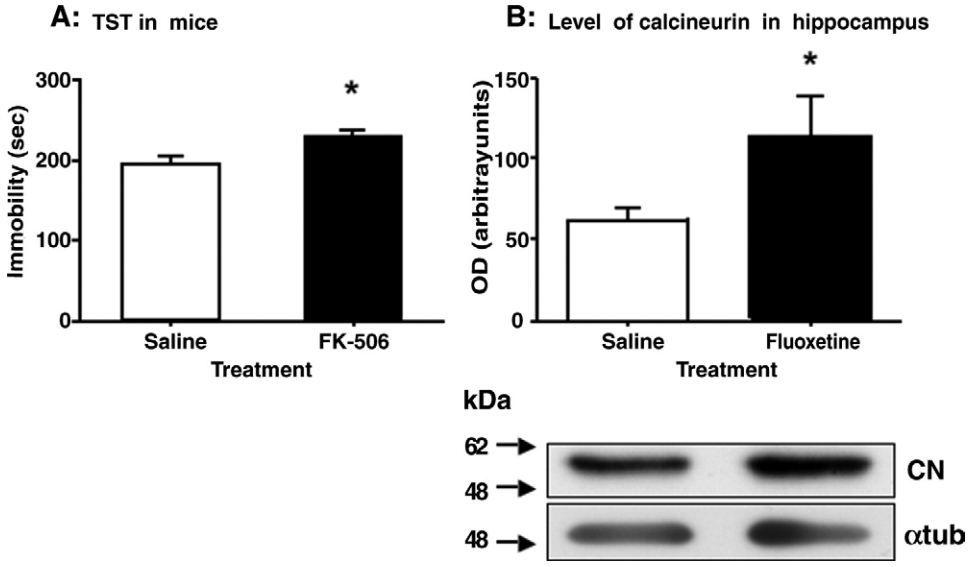

Fig. 1. (a) Pharmacological inhibition of calcineurin increases immobility in the TST. Effects of the calcineurin inhibitor FK506 on time spent in immobility in TST; $n=8–12$ animals per group. * $P<0.05$ for animals treated with the inhibitor versus animals treated with vehicle (one-way ANOVA). (b) Chronic fluoxetine administration increases the level of calcineurin in the hippocampus. Quantification graph showing the level of calcineurin Aα in hippocampal extracts from mice injected chronically with saline or fluoxetine; $n=7$ animals per group. * $P<0.05$ for animals treated with the antidepressant versus animals treated with vehicle (one-way ANOVA). Below, a representative Western blot experiment is shown, expected molecular weights are of 58 kDa for calcineurin Aα and 50 kDa for α-tubulin.

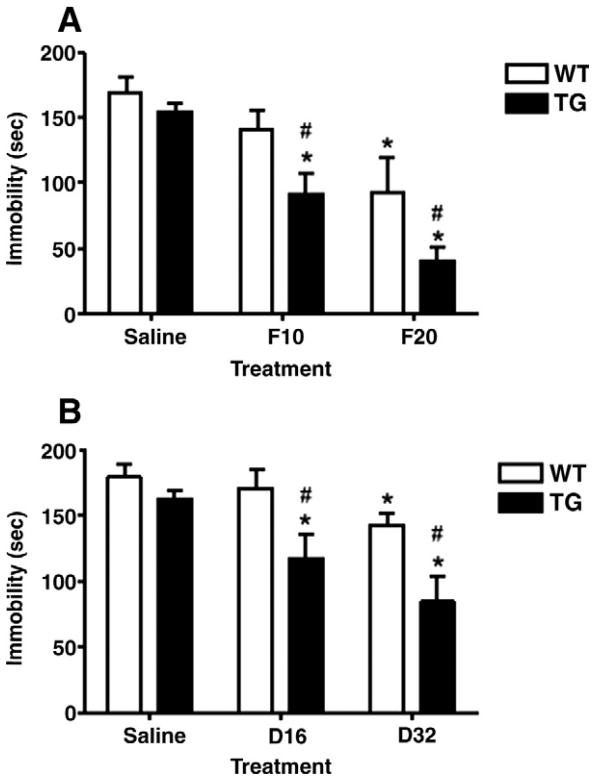

Fig. 2. CN98 mice are more sensitive to the antidepressant-like effect of fluoxetine and desipramine (a, b). Time spent in immobility in TST for WT and CN98 mice after an acute i.p. injection of (a) 10 or 20 mg/kg fluoxetine (n=6–9 animals per group) or (b) 16 or 32 mg/kg desipramine (n=6–14 animals per group). * $P<0.05$ for animals treated with the antidepressant versus animals of the same genotype treated with vehicle, # $P<0.05$ for WT versus CN98 animals for each antidepressant dose (two-way ANOVA).

CN98 mice exhibited altered basal levels of GluR1and BDNF in the hippocampus

To investigate further if calcineurin overactivation is a feature of chronic antidepressant administration, we explored how different markers known to be altered following antidepressant treatments may be modified in CN98 mice. We measured the levels of the glutamate receptor subunit 1, which has been shown to be increased after chronic antidepressant treatments (Martinez-Turrillas et al., 2002, 2005; Du et al., 2004). Because BDNF can be induced by AMPA receptor activation (Zafra et al., 1990) and contributes to antidepressant action (Duman, 2002; Manji et al., 2003; Angelucci et al., 2005), we also examined the levels of its expression in WT and CN98 animals.

In CN98 mice, we found a significant increase ($F(1,12)=5.47$, $P<0.05$) of hippocampal GluR1 levels compared with WT in the synaptosomal preparation (Fig. 3). No difference was observed for GluR1 in other regions (substantia nigra, ventral tegmental area, striatum, cortex), or for other proteins (glycogen synthase kinase 3 (GSK3), extracellular signal-regulated kinase (ERK), DARPP32) analyzed (data not shown).

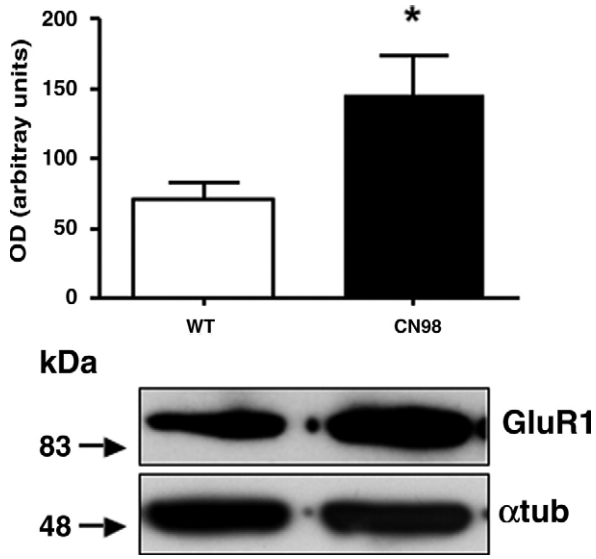

Fig. 3. Increased basal levels of GluR1 in hippocampal synaptosomes of CN98 mice. Western blot and quantification graph showing the level of GluR1 in hippocampal synaptosomal preparations from WT mice (n=8) or CN98 mice (n=7). * $P<0.05$ for WT mice versus CN96 mice (one-way ANOVA). A representative Western blot experiment is shown, expected molecular weights are of 106 kDa for GluR1 and 50 kDa for α-tubulin.

Under basal conditions the expression of BDNF mRNA was increased (45%±9) in hippocampus CA2/3 region in CN98 mice as compared with WT (Fig. 4). This increase was specific to the hippocampal sub-structure and no change in BDNF mRNA expression was observed in other forebrain structures.

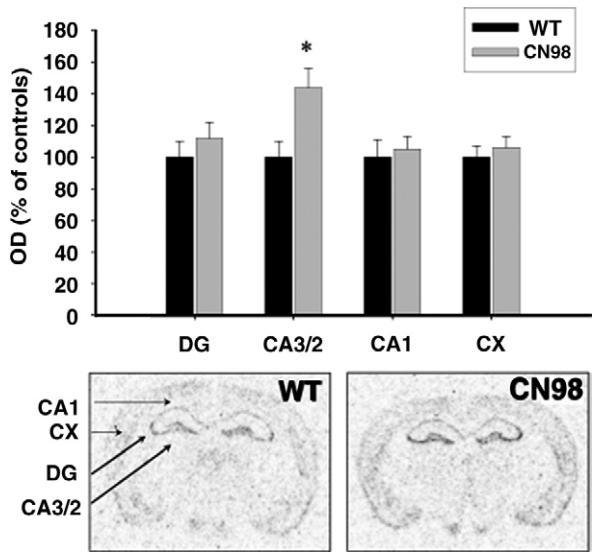

Fig. 4. Increased BDNF expression in the CA2/3 hippocampal area in CN98 mice. Representative sections and respective quantification of the autoradiographic signal for BDNF mRNA in the dentate gyrus (DG), hippocampal areas CA2/3 and CA1, and cortex (CX) of WT (black bars) and CN98 (gray bars) mice; n=5–6 animals per group. * $P<0.05$ for WT versus CN98 animals (one-way ANOVA).

DISCUSSION

Four distinct sets of evidence strongly support the contribution of calcineurin in signaling cascades underlying the action of antidepressant drugs: i) the increased calcineurin expression following chronic treatment with fluoxetine, ii) the depressive-like behaviors induced by pharmacological calcineurin inhibition, as well as iii) the enhanced behavioral sensitivity to fluoxetine and desipramine and iv) alterations of basal BDNF and GluR1 levels in mice overexpressing calcineurin. Altogether these results suggest that calcineurin would be an indirect target of antidepressants and that, in some aspects, its increased activity would mimic some molecular consequences of a chronic antidepressant treatment.

At the behavioral level, we investigated calcineurin involvement in antidepressant action using the TST. During the test, when mice are suspended by the tail, alternating immobility and agitation periods are observed and it is well established that antidepressant administration decreases the duration of immobility (Steru et al., 1985). Pharmacological inhibition of calcineurin by FK-506 administration induced depressed-like behaviors in mice, while genetic activation of calcineurin in CN98 mice increased the antidepressant-like effect of two structurally different compounds, fluoxetine and desipramine, demonstrating an increased sensitivity of CN98 mice to the behavioral actions of antidepressants in the TST. Chronic administration of the SSRI fluoxetine increased hippocampal calcineurin expression, a finding that indirectly links calcineurin with antidepressant drug action. It should be noted that in a recent study Rushlow et al. (2005) have shown that subchronic antipsychotic treatment decreases calcineurin expression in the striatum, substantia nigra and cortex, indicating that calcineurin-regulated signaling pathways are important mediators of the effects of CNS medications.

Interestingly at the present time, despite considerable efforts, the molecular mechanisms of action of antidepressants are not fully understood. Conventional antidepressants and electroconvulsive therapy impact central neurotransmission of monoamines by increasing their synaptic availability (Skolnick et al., 2001; Iversen, 2006). However recent evidence suggests that the molecular changes seen in CN98 mice (i.e. increased GluR1 and BDNF expression) are relevant to depression. Potentiators of the AMPA receptor have antidepressant like activity (Li et al., 2003; Alt et al., 2005). Furthermore, chronic administration of antidepressants, including AMPA potentiators, increases BDNF levels in the hippocampus (Nibuya et al., 1995) while acute BDNF administration into either the midbrain or the hippocampus produces an antidepressant-like effect in the learned helplessness and forced swim test paradigms (Siuciak et al., 1997; Shirayama et al., 2002). Other compounds that target calcineurin-related second messenger cascades such as PKA activators (O'Donnell and Zhang, 2004; Duman, 2004), GSK-3 and PKC inhibitors have been proposed as putative mood regulators (c.f, Payne et al., 2004), and the MAP/MEK/ERK/RSK2 pathway has also been shown to be involved in chronic antidepressant effects (Tiraboschi et al., 2004).

Accumulating evidence implicates protein kinases and phosphatases in the phosphorylated/dephosphorylated status of several molecular targets that might mediate antidepressant activity. The glutamate ionotropic AMPA receptors, in particular GluR1 subunits (Svenningsson et al., 2002), whose insertion in the synaptic membrane and internalization are regulated by phosphorylation (Ehlers, 2000), have been proposed among these downstream targets. Chronic treatment of rats with imipramine produces increases in synaptosomal GluR1 levels (Du et al., 2004) and chronic treatment with desipramine or paroxetine increases GluR1 levels in membrane-enriched fractions from rat hippocampus (Martinez-Turrillas et al., 2002, 2005). Since these findings are not accompanied by changes in the total levels of these proteins, it appears that these antidepressants enhance the trafficking of AMPA receptor from intracellular pools to synaptic compartments (Martinez-Turrillas et al., 2002, 2005). Interestingly, potentiators of the AMPA receptor not only exhibit per se antidepressant-like activity in preclinical tests but also markedly augment the activity and perhaps the onset of the therapeutic effects of biogenic amine and second messenger-based antidepressants such as desipramine and fluoxetine (Li et al., 2003). Here we showed in CN98 an increase of GluR1 levels in the synaptosomal fraction of hippocampus, when compared with WT. These changes in molecular homeostasis may underlie the increased behavioral sensitivity of CN98 mice to acute antidepressant administration.

As stated above it is believed that antidepressants exert some of their therapeutic effects by increasing BDNF levels in the hippocampus. In our present study, we show that overexpression of calcineurin upregulates the levels of BDNF mRNA selectively in the CA2/3 layer of the hippocampus. Over-expression of activated calcineurin might directly account for increased BDNF, since BDNF expression in the hippocampus is positively auto-regulated by a feedforward loop involving calcineurin and nuclear factor of activated T cells (NFAT) activation (Saarelainen et al., 2003; Groth and Mermelstein, 2003). Again, this result indicates that calcineurin overexpression in forebrain neurons mimics some aspects of behavioral and biochemical effects of chronic administration of antidepressants.

It might seem paradoxical that CN98 mice, whose GluR1 synaptosomal content and BDNF expression are increased in basal state, do not show differences in immobility in the TST under baseline conditions (saline-treated CN98 mice), especially since we show that chronic fluoxetine treatment increases calcineurin expression. Interestingly, we demonstrate that CN98 mice are more responsive to antidepressants. Our results indicate that calcineurin overexpression facilitates/potentiates antidepressant effects but does not affect depression-like behavior baseline. This suggests that calcineurin could be less related to a steady state antidepressant-related phenotype but would be necessary for the dynamic expression of the behavioral effects of antidepressants, as it has been

proposed for one of its downstream effectors, namely BDNF-mediated signaling (Saarelainen et al., 2003).

However, our results do not exclude the possibility that CN98 mice will exhibit altered antidepressant-like behaviors in a chronic depression model (e.g. chronic mild stress). Experiments addressing this issue as well as experiments investigating the effects of calcineurin inhibitors on the effects of antidepressants in chronic and acute depression models are warranted to further investigate our hypothesis.

It should be noted that a comorbidity of depression and deficits in cognition exist and it has been documented that antidepressants can alleviate cognitive dysfunction in depressed patients (c.f., Doraiswamy et al., 2003). The fact that calcineurin has been extensively implicated in learning, memory and synaptic activity processes suggests that this phosphatase might constitute a common substrate for affective and cognitive dysregulations that characterize depressive pathologies.

Altogether, the results we have obtained in this study support the hypothesis that calcineurin-regulated signaling pathways could be involved in antidepressant effect. In patients, at least three weeks of daily antidepressant treatment are needed to improve depressive symptoms. It is therefore crucial to gain a better understanding of the intracellular pathways activated by antidepressants in order to propose alternative targets for novel antidepressant therapies. This study proposes that calcineurin and its downstream proteins might be relative to targets of such efficient drugs that would eventually reduce the lag-phase of antidepressant action.

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