

Chronic Antidepressants Induce Redistribution and Differential Activation of α CaM Kinase II between Presynaptic Compartments

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Changes in synaptic plasticity are involved in pathophysiology of depression and in the mechanism of antidepressants. Ca^{2+} /calmodulin (CaM) kinase II, a protein kinase involved in synaptic plasticity, has been previously shown to be a target of antidepressants. We previously found that antidepressants activate the kinase in hippocampal neuronal cell bodies by increasing phosphorylation at Thr²⁸⁶, reduce the kinase phosphorylation in synaptic membranes, and in turn its phosphorylation-dependent interaction with syntaxin-1 and the release of glutamate from hippocampal synaptosomes. Here, we investigated the chronic effect of different antidepressants (fluoxetine, desipramine, and reboxetine) on the expression and function of the kinase in distinct subcellular compartments in order to dissect the different kinase pools affected. Acute treatments did not induce any change in the kinase. In total tissue extracts chronic drug treatments induced activation of the kinase; in hippocampus (HC), but not in prefrontal/frontal cortex, this was partially accounted for by increased Thr²⁸⁶ phosphorylation, suggesting the involvement of different mechanisms of activation. In synaptosomes, all drugs reduced the kinase phosphorylation, particularly in HC where, upon fractionation of the synaptosomal particulate into synaptic vesicles and membranes, we found that the drugs induced a redistribution and differential activation of the kinase between membranes and vesicles. Furthermore, a large decrease in the level and phosphorylation of synapsin I located at synaptic membranes was consistent with the observed decrease of CaM kinase II. Overall, antidepressants induce a complex pattern of modifications in distinct subcellular compartments; at presynaptic level, these changes are in line with a dampening of glutamate release.

Neuropsychopharmacology (2007) **32**, 2511–2519; doi:10.1038/sj.npp.1301378; published online 14 March 2007

Keywords: antidepressant; CaM kinase II; glutamate; neurotransmitter release; synaptic plasticity

INTRODUCTION

It is well known that, although most antidepressants exert their initial effects by increasing the extraneuronal levels of serotonin and/or norepinephrine, their therapeutic effects occur only after chronic administration. Several different lines of evidence in recent years have suggested that a cascade of postreceptor downstream effects is ultimately responsible for their therapeutic efficacy. These effects involve selective modulation of intracellular signaling cascades, and in turn modifications in gene expression, dendritic remodeling, synaptic plasticity, and neurogenesis (Manji *et al*, 2001; Coyle and Duman, 2003; Zarate *et al*,

2003; Wong and Licinio, 2004; Spedding *et al*, 2005; Tardito *et al*, 2006). Several signaling pathways regulating cellular resilience and neuroplasticity have been implicated in the mechanism of action of antidepressants, including cyclic adenosine mono phosphate-protein kinase A (cAMP-PKA), mitogen-activated protein kinases, Ca^{2+} /calmodulin (CaM)-dependent kinases, protein kinase C (PKC), glycogen synthase kinase-3, and phosphatidylinositol 3-kinase/Akt.

CaM kinase II is an abundant protein kinase in brain, regulating neuronal response to calcium fluxes with a pivotal role in neuroplasticity (Hudmon and Schulman, 2002; Lisman *et al*, 2002). The kinase is activated by the binding of Ca^{2+} /CaM that generates Ca^{2+} -dependent enzymatic activity. Upon activation, depending on the extent of calcium elevation and duration of stimulation, the kinase autophosphorylates at Thr²⁸⁶, thereby generating Ca^{2+} -independent activity that outlasts the calcium signal and is involved in synaptic plasticity. CaM kinase II is a multifunctional and ubiquitous enzyme and it was shown recently that targeting of the kinase to distinct subcellular

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Received 12 September 2006; revised 26 January 2007; accepted 29 January 2007

compartments is crucial to its specificity of action in terms of space and time (Griffith *et al*, 2003). Targeting of the kinase to the nucleus, cytoskeleton, sarcoplasmic reticulum, and postsynaptic density of dendritic spines is achieved by several mechanisms, including alternative splicing of sequences containing nuclear localization signal, change of α/β isoform ratio, alternatively splicing-generated anchoring protein (α CaM kinase II association protein, α KAP), activity-dependent-regulated dendritic translation, and binding to *N*-methyl-D-aspartate (NMDA) receptors (Srinivasan *et al*, 1994; Bayer *et al*, 1998; Ouyang *et al*, 1999; Shen *et al*, 1998, 2000; Fink *et al*, 2003).

A number of studies have addressed the role of CaM kinase II in antidepressant mechanisms, by exploring its expression and function in limbic and cortical areas (Popoli *et al*, 1995; Pilc *et al*, 1999; Celano *et al*, 2003; Tiraboschi *et al*, 2004a; Bonanno *et al*, 2005). Chronic, but not acute, treatment with different antidepressants upregulated the Thr²⁸⁶ autophosphorylation and the enzymatic activity in neuronal cell bodies of hippocampus (HC), without increasing the total expression of α CaM kinase II (Tiraboschi *et al*, 2004a). A consistent finding was that chronic antidepressants also upregulated the expression level, autophosphorylation and enzymatic activity of the kinase localized to synaptic vesicles in both HC and prefrontal/frontal cortex (P/FC) (Celano *et al*, 2003). Recently, by using Percoll gradient-purified synaptosomes, we found that chronic antidepressants downregulated, rather than upregulate, Thr²⁸⁶ phosphorylation of α CaM kinase II in purified synaptic terminals (synaptosomes) and synaptic membranes of HC. The decrease in the kinase phosphorylation reduced its interaction with the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) protein syntaxin-1, thereby changing protein-protein interactions at glutamatergic presynaptic terminals and reducing depolarization-evoked release of glutamate (Bonanno *et al*, 2005).

Taken together, these results suggested a complex regulation of CaM kinase II by these drugs, with different state of activation/inactivation in distinct subcellular compartments, likely related to different functions of the kinase. Therefore, in order to understand better the different cellular functions affected by antidepressants and identify nodal points in signaling that may be related to the therapeutic action of these drugs, in the present study we systematically investigated CaM kinase II in different cellular compartments. In particular, the main aim of the present work was to investigate the state of CaM kinase II at synaptic site as opposed to total tissue, and within different presynaptic compartments. By using purified synaptosomes and fractionating this pure preparation in different membrane compartments, we report here that after chronic antidepressants the kinase redistributed between the synaptic membranes and the total pool of synaptic vesicles, and that its activation (Thr²⁸⁶ phosphorylation) was complementary reduced in synaptic membranes and increased in total synaptic vesicles of drug-treated rats. Furthermore, we observed that likely as a consequence of the redistribution of α CaM kinase II, the level of synapsin I was also greatly reduced in synaptic membranes, a finding that may further explain the reduction of glutamate release induced by antidepressants in HC (Bonanno *et al*, 2005).

MATERIALS AND METHODS

Animals and Drug Treatments

Experiments complied with guidelines for use of experimental animals of European Community Council Directive 86/609/EEC. Groups of 12 male Sprague-Dawley rats (170–200 g) were anesthetized and subcutaneously implanted with osmotic minipumps Alzet 2ML2 (release 5 μ l/h, capacity 2 ml) (Charles River, Wilmington, MA), containing either vehicle (5% ethanol) or reboxetine (RBX), a selective norepinephrine reuptake inhibitor (NRI), fluoxetine (FLX), a selective serotonin reuptake inhibitor (SSRI) or desipramine (DMI), a tricyclic antidepressant mainly inhibiting norepinephrine release. Drug dosage was 10 mg/kg daily for each drug. Acute treatment was carried out by injecting rats (250–270 g) intraperitoneally. Animals were killed after 3 h for acute treatment and after 14 days for chronic treatment.

Preparation of Total Homogenate and Synaptosomes

Animals were killed and the HC was quickly dissected on ice. The whole frontal lobe was separated by a coronal section in correspondence to the optic chiasm (Glowinski and Iversen, 1966), and referred to as P/FC. The tissue was homogenized in 10 volumes of 0.28 M sucrose buffered at pH 7.4 with Tris, containing 20 mM NaF, 5 mM Na₄P₂O₇, 1 mM Na₃VO₄ (protein phosphatase inhibitors), and 2 μ l/ml of protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO), using a glass-teflon tissue grinder (clearance 0.25 mm). Purified synaptosomes were prepared essentially according to Dunkley *et al* (1986), with minor modifications. The homogenate was centrifuged (5 min, 1000 g at 4°C) to remove nuclei and debris. The supernatant was centrifuged 5 min at 12 000 g; the resultant pellet was resuspended in homogenization buffer and gently stratified on a discontinuous Percoll gradient (2, 6, 10, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33 500 g for 5 min. The layer between 10 and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation. To obtain cytosolic fraction (S3), the supernatant of 12 000 g centrifugation was ultracentrifuged at 135 000 g for 1 h. The synaptosomes were resuspended in lysis buffer: 120 mM NaCl, 20 mM HEPES pH 7.4, 0.1 mM EGTA, 0.1 mM DTT, containing 20 mM NaF, 5 mM Na₂PO₄, 1 mM Na₃VO₄, and 2 μ l/ml of protease inhibitor cocktail (Sigma-Aldrich). Further fractionation of purified synaptosomes into synaptic membrane- (LP1) and synaptic vesicle-fraction (LP2) was carried out by differential centrifugation and ultracentrifugation, essentially according to Huttner *et al* (1983), as previously reported in Popoli and Paternò (1991) and Popoli *et al* (1995). The LP1 and LP2 fractions, previously characterized with regard to morphology and protein markers by several groups, were enriched with N-cadherin (a plasma membrane marker) and synaptophysin (a synaptic vesicle marker), respectively (Figure 1a).

Assay of Ca²⁺-Dependent and -Independent Activity of CaM Kinase II

Ca²⁺-dependent activity of CaM kinase II was assayed by measuring initial rate of phosphate incorporation in the selective peptide substrate autocalmitide-3 (Biosource, Camarillo, CA), as described previously (Celano *et al*, 2003). For

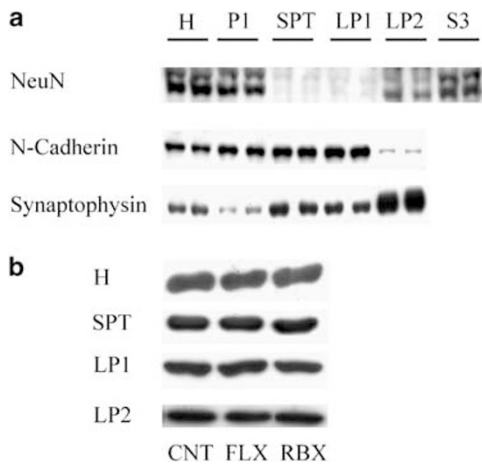


Figure 1 Characterization of subcellular fractions and β -actin levels after drug treatments. (a) Subcellular distribution of protein markers. Proteins from HC subcellular fractions were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blot with antibodies for NeuN, a soluble nuclear marker that is also found in cytosol (Mullen *et al*, 1992); N-cadherin, a marker for cell plasma membrane; synaptophysin, a marker for synaptic vesicles; H, total HC extract; P1, nuclear fraction; SPT, purified synaptosomes; LP1, synaptic membranes; LP2, synaptic vesicles; S3, high speed cytosol. (b) Immunoreactive bands for β -actin in total extract and subcellular fractions as above in rats treated with vehicle, FLX or RBX.

assay of Ca^{2+} -independent activity the samples contained, in the place of $\text{Ca}^{2+}/\text{CaM}$, 2 mM EGTA, 2 μM heat-stable cAMP-dependent protein kinase inhibitor (New England, Beverly, MA), and 5 μM PKC (fragment 19–36) inhibitor (Sigma-Aldrich). Blanks were incubated in the absence of peptide.

Western Blot

Western-blot analysis was carried out as previously described (Verona *et al*, 2000; Tiraboschi *et al*, 2004b), by incubating poly-vinylidene difluoride membranes containing electro-phoresed proteins with monoclonal or polyclonal antibodies in appropriate dilutions. Monoclonal antibodies used were: antibody for αCaM kinase II 1:3000, NeuN 1:500 (both from Chemicon International, Temecula, CA), βCaM kinase II 1:500, N-Cadherin 1:1000 (both from Zymed, South San Francisco, CA), synapsin I 1:2000, synaptophysin 1:350 (both from Synaptic Systems, Goettingen, Germany), and β -actin 1:5000 (Sigma-Aldrich). Polyclonal antibodies were: antibody for phospho-Thr²⁸⁶ αCaM kinase II 1:1000 (Promega, Milan, Italy) and phospho-Ser⁶⁰³ synapsin I 1:1000 (Chemicon). The membranes were blocked with 5% milk and incubated with primary antibody. Following incubation with peroxidase-coupled secondary antibodies, protein bands were detected by using ECL (Amersham Biosciences, Piscataway, NJ) or Super Signal Dura West (Pierce Biotechnology Inc., Rockford, IL). Standard curves were obtained by loading increasing amounts of samples on gels as described previously (Bonanno *et al*, 2005). All protein bands used were within linear range of standard curves, and normalized for actin level in the same membrane. Actin level did not change with drug treatment in any of the subcellular fractions analyzed (Figure 1b). Quantity One software (BioRad Laboratories, Hercules, CA) was used for

standardization and quantitation, as previously reported (Tiraboschi *et al*, 2004b).

RESULTS

Expression and Function of αCaM Kinase II in Total HC and P/FC after Chronic Antidepressant Treatments

We sought to investigate the outcome of long-term treatment with different representative antidepressants on expression and function of CaM kinase II in distinct cellular compartments. To this aim we first assayed the basal enzymatic activity of the kinase in total extracts prepared from HC and P/FC of rats treated with FLX, DMI, and RBX (Figure 2). Both basal Ca^{2+} -dependent and Ca^{2+} -independent activity of CaM kinase II were measured. Ca^{2+} -dependent activity is induced by the binding of $\text{Ca}^{2+}/\text{CaM}$ to pseudosubstrate inhibitory domain and consequent activation of the kinase; Ca^{2+} -independent activity is generated when the activated kinase autophosphorylates at Thr²⁸⁶ residue in the autoinhibitory domain, an event allowing the kinase to remain persistently activated when calcium concentration falls to basal levels (Lisman *et al*, 2002). Acute treatments did not induce any change in this or other mechanisms investigated here (not shown). After chronic treatment, basal Ca^{2+} -dependent activity was slightly but significantly elevated by DMI in HC (32%) and P/FC (35%) and by FLX in HC (34%), with DMI inducing the highest increase in P/FC (Figure 2a). Basal Ca^{2+} -independent activity showed greater changes after drug treatments, particularly in HC (FLX 140%, DMI 65%, and RBX 105%) (Figure 2b); in P/FC only DMI significantly increased the activity (60%). We further investigated the changes induced by drug treatments, measuring the expression level of αCaM kinase II (the major isoform in forebrain) and the basal phosphorylation level of Thr²⁸⁶ residue (Figure 2c and d). None of the drugs increased total expression level of the kinase in the two areas (except RBX in P/FC; 19%); rather, RBX (–13%) slightly decreased the total level of αCaM kinase II in HC. In both areas, Thr²⁸⁶ phosphorylation was not significantly changed by the three drugs. Overall, the results with Ca^{2+} -independent activity are compatible with and extend our previous data showing that chronic antidepressants activate CaM kinase II in neuronal cell bodies of hippocampal neurons (Tiraboschi *et al*, 2004a). The same did not apply to P/FC in the present work, where the increase of Ca^{2+} -independent activity observed was significant only for DMI and not paralleled by any increase of Thr²⁸⁶ phosphorylation by the same drug. This suggests that mechanisms different from phosphorylation are responsible for the kinase activation by antidepressants (see Discussion).

Expression and Function of αCaM Kinase II in Synaptosomes after Chronic Antidepressant Treatments

To investigate the action of antidepressants on expression and function of CaM kinase II at synaptic sites, we purified synaptic terminals (synaptosomes) from rats long-term treated with the three drugs as above, by centrifugation on Percoll gradients as reported previously (Bonanno *et al*, 2005). Basal Ca^{2+} -dependent activity of the kinase in

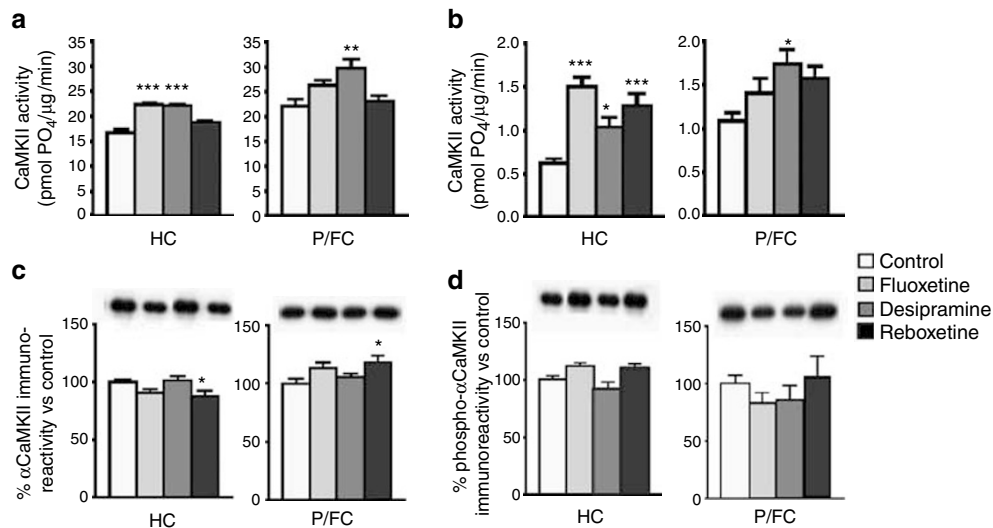


Figure 2 Enzymatic activity, expression level, and phosphorylation of CaM kinase II in total HC and P/FC. (a) Ca^{2+} -dependent enzymatic activity of CaM kinase II in total HC or P/FC extract from rats chronically treated with vehicle (Control), FLX, DMI or RBX. HC, hippocampus; prefrontal/frontal cortex, P/FC. Activity expressed as pmoles of PO_4 incorporated per minute per μg protein (mean \pm SEM). (b) Ca^{2+} -independent enzymatic activity of CaM kinase II as in (a). (c) Western-blot analysis of total αCaM kinase II in HC and P/FC. The band intensities were normalized for β -actin. Data expressed as % immunoreactivity vs control (mean \pm SEM). (d) Western-blot analysis of phospho-Thr²⁸⁶ αCaM kinase II as in (c). Insets: representative immunoreactive bands from Western blots. Statistics: one-way analysis of variance followed by Newman-Keuls *post hoc* tests. *p*-values reported refer to drug treated vs control tests (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; $n = 3$ in triplicate or quadruplicate).

synaptosomes was significantly elevated by FLX (18%) and DMI (54%) in HC, and by DMI (78%) and RBX (37%) in P/FC (Figure 3a). FLX exerted a minimal effect on Ca^{2+} -dependent activity in synaptosomes from both areas. Conversely, basal Ca^{2+} -independent activity was robustly increased in both brain areas by all drugs, with the only exception of FLX in P/FC (Figure 3b). In P/FC, only pronoradrenergic drugs increased the kinase enzymatic activity (DMI, 130%; RBX, 162%). In HC, the results showed no selectivity of effect for drug classes (FLX, 280%; DMI, 110%; RBX, 365%). The changes in enzymatic activity were not justified by corresponding changes in the levels of synaptic kinase (Figure 3c). Interestingly, the effects of the drugs on Thr²⁸⁶ phosphorylation were different in the two brain areas (Figure 3d). Whereas in HC all drugs markedly and significantly decreased phosphorylation of the activator residue (FLX, -36%; DMI, -62%; RBX, -42%), in P/FC the reduction of phosphorylation was more modest and not significant (Figure 3d).

Furthermore, the complete absence of upregulation of Thr²⁸⁶ phosphorylation in the synaptosomes from both areas suggested that the augmentation of Ca^{2+} -independent activity induced by the drugs cannot be owing to this phosphorylation change. However, the present results confirm our previous data showing that antidepressants decrease Thr²⁸⁶ phosphorylation in HC synaptosomes (Bonanno *et al*, 2005).

Differential Expression Level and Activation of αCaM Kinase II in Presynaptic Compartments after Chronic Antidepressant Treatments

To further investigate the changes induced in the regulation of CaM kinase II at presynaptic level, we fractionated purified synaptosomes by using differential centrifugation and ultracentrifugation in the two particulate compartments containing the kinase: synaptic vesicles and synaptic

membranes. The remaining part of this work was conducted on HC, because in this area the reduction of kinase phosphorylation was more marked and significant (see Figure 3d). For further characterization of subsynaptic compartments, we used rats treated with two (FLX and RBX) of the three drugs used in the first part of this work, representative of different primary mechanisms (SSRI and NRI, respectively). Preparation of synaptic vesicles and membranes must be carried out using freshly prepared synaptosomes, and requires further centrifugation and ultracentrifugation. Using additional samples from DMI-treated rats would have slowed down the procedure; for this reason we chose to limit the second set of experiments to controls, FLX- and RBX-treated rats. Overall, by this way the second part of this study compares the effects of two drugs with different and complementary mechanisms on synaptic effectors.

Surprisingly, we found that long-term treatment with both drugs induced opposite changes in local expression level and Thr²⁸⁶ phosphorylation of αCaM kinase II in the two presynaptic compartments (Figure 4). In synaptic vesicles, both expression level and phosphorylation of the kinase were markedly and significantly increased by drug treatments, whereas in synaptic membranes protein level and, particularly, Thr²⁸⁶ phosphorylation were reduced. Although part of the effect seems to be owing to actual changes in protein level, in both cases phosphorylation changes largely exceeded protein level changes. We recently showed that the down-regulation of kinase autophosphorylation in synaptic membranes induces a redistribution of the binding of the SNARE protein syntaxin-1 between αCaM kinase II and Munc-18, a molecular event with a physiological counterpart represented by a decrease of depolarization-evoked release of glutamate (Bonanno *et al*, 2005). Our present results suggest that two antidepressants with different primary mechanisms (eg, SSRI and NRI) may

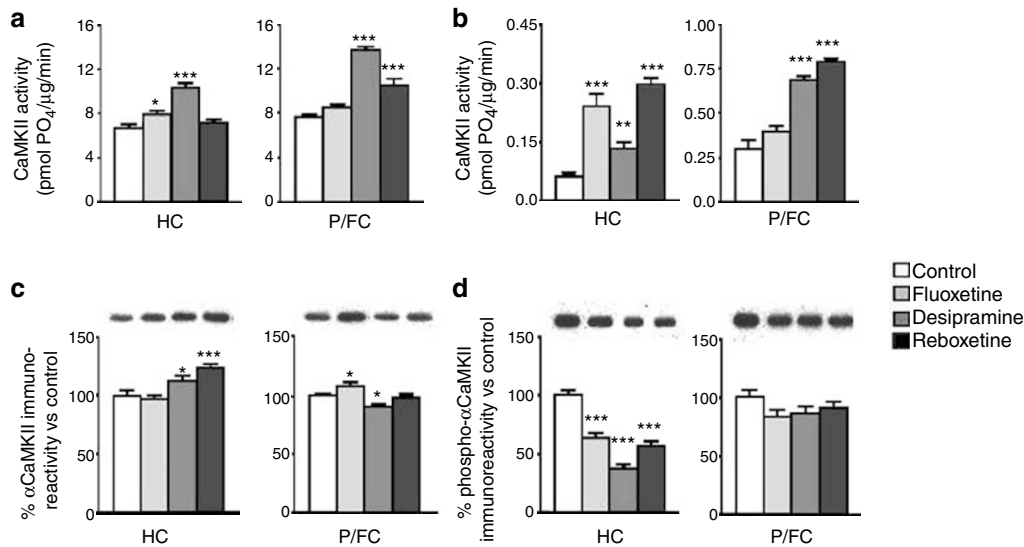


Figure 3 Enzymatic activity, expression level, and phosphorylation of CaM kinase II in synaptosomes from HC and P/FC. (a) Ca^{2+} -dependent enzymatic activity of CaM kinase II in purified synaptosomes from HC or P/FC, from rats chronically treated as in Figure 1. (b) Ca^{2+} -independent enzymatic activity of CaM kinase II in purified synaptosomes as in (a). (c) Western-blot analysis of total α CaM kinase II in purified synaptosomes. The band intensities were normalized for β -actin. Data expressed as % immunoreactivity vs control (mean \pm SEM). (d) Western-blot analysis of phospho-Thr²⁸⁶ α CaM kinase II in purified synaptosomes as in (c). Insets: representative immunoreactive bands from Western blots. Statistics as in Figure 2 (* p < 0.05, ** p < 0.01, and *** p < 0.001; n = 3 in triplicate or quadruplicate).

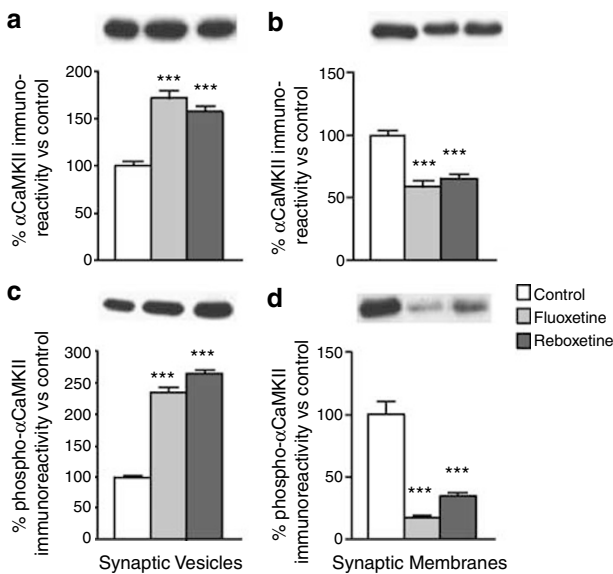


Figure 4 Expression level and phosphorylation of α CaM kinase II in synaptic vesicles and membranes from HC. Western-blot analysis of α CaM kinase II in synaptic vesicles and synaptic membranes isolated from hippocampal synaptosomes of rats chronically treated with vehicle (Control), FLX, or RBX. (a) Western-blot analysis of total α CaM kinase II in synaptic vesicles. The band intensities were normalized for β -actin. Data expressed as % immunoreactivity vs control (mean \pm SEM). (b) Western-blot analysis of total α CaM kinase II in synaptic membranes. (c) Western-blot analysis of phospho-Thr²⁸⁶ α CaM kinase II in synaptic vesicles. (d) Western-blot analysis of phospho-Thr²⁸⁶ α CaM kinase II in synaptic membranes. Insets: representative immunoreactive bands from Western blots. Statistics as in previous figures (*** p < 0.001; n = 3 in triplicate or quadruplicate).

alter the function of the presynaptic machinery by inducing redistribution of α CaM kinase II and of its basal phosphorylation level between synaptic membranes and synaptic vesicles. The lack of major changes in total expression level

(Figure 2c) and synaptosomal expression level (Figure 3c) of the kinase supports this conclusion.

Regulation of Synapsin I and β CaM Kinase II in Presynaptic Compartments after Chronic Antidepressant Treatments

We speculated that the redistribution of total and phosphorylated α CaM kinase II between synaptic membranes and synaptic vesicles induced by antidepressants could be due to a number of reasons, including: (1) translocation or different targeting of the kinase (change in the binding to other membrane-associated proteins or to the hydrophobic core of the membrane); (2) changes in the α - vs β -subunit composition of the kinase holoenzyme that was shown to be sensitive to changes in neuronal activity and NMDA/ α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors activation (Thiagarajan *et al*, 2002); (3) changes in local α CaM kinase II synthesis (possibly involving kinase associated with portions of postsynaptic densities that copurify with presynaptic terminals).

(1) While it was shown that both α - and β CaM kinase II copurifying with synaptic vesicles are tightly associated with the vesicle membrane, suggesting that this is due to either covalent modification or high-affinity association with other membrane-associated components (Benfenati *et al*, 1996), no data are available regarding the association of the kinase with the presynaptic membrane. However, because in presynaptic terminals the particulate kinase is tightly bound to the vesicles, it can be speculated that the association with presynaptic membranes is mainly due to the presence of vesicles docked to the plasma membrane (see Discussion).

It has been demonstrated that α CaM kinase II is an anchoring protein for synapsin I, a presynaptic protein

regulating the vesicle cycle and neurotransmitter release, and a major substrate for α CaM kinase II (Benfenati *et al*, 1992). We sought further evidence for changes in the membrane targeting of the kinase by measuring the level of synapsin I and its phosphorylation at Ser⁶⁰³ (the consensus site for CaM kinase II) in the rats chronically treated with FLX and RBX. Although the drugs did not reduce synapsin I in the total HC (Figure 5a,d), both the level and CaM kinase II-dependent phosphorylation of synapsin I (likely as a consequence of protein level decrease) were reduced in synaptosomes and synaptic membranes after treatment (Figure 5b,e and c,f). In particular, the level of synapsin I in synaptic membranes of FLX- and RBX-treated rats was reduced by 70–80%. First, these results suggest that the reduction of synapsin I associated with synaptic membranes is a likely consequence of the downregulation of the anchoring site for this protein (eg, CaM kinase II) in this compartment after drug treatments. Second, the drugs also reduced the localization of synapsin I to synaptic terminals *in toto*, in the absence of reduction in total expression.

- (2) Interestingly, the expression of β CaM kinase II in total HC was robustly increased by the drug treatments (Figure 6a), showing a regulation different from the α -isoform (compare with Figure 2c). Furthermore, no major changes in the level of β CaM kinase II were found in synaptosomes and synaptic membranes, with the exception of a small downregulation by FLX in the latter (Figure 6b, c). Therefore, both at total and synaptosomal levels, β CaM kinase II was differently regulated compared to α CaM kinase II.
- (3) Portions of the dendritic postsynaptic membrane, containing postsynaptic densities enriched with α CaM

kinase II, are a typical contaminant of synaptosomes. It has been shown that neuronal activation induces local dendritic synthesis of α CaM kinase II (Ouyang *et al*, 1999; Bagni *et al*, 2000); therefore, changes in local synthesis of the kinase might contribute to the changes observed here following drug treatments. In a parallel study, we subjected purified synaptosomes from RBX-treated rats as above to the analysis of mRNA expression and of mRNA translation for several different mRNAs known to be present and locally translated at synapses. Although we found significant changes for some mRNAs, the efficiency of translation for α CaM kinase II transcript was unchanged in the RBX-treated rats (Veneri *et al*, manuscript in preparation), ruling out an involvement of dendritic α CaM kinase II synthesis in the modifications observed here.

DISCUSSION

Chronic Antidepressant Treatments Activate CaM Kinase II in HC and P/FC by Canonical and Non-Canonical Mechanisms

The main aim of this work was a complete description of the changes in enzymatic activity, expression level, and Thr²⁸⁶ phosphorylation of CaM kinase II in distinct subcellular compartments after chronic antidepressant treatments. We found that drugs with different primary mechanisms (serotonin and/or norepinephrine reuptake inhibition) upregulated CaM kinase II enzymatic activity in total extracts from HC and P/FC; the increase was more remarkable for Ca²⁺-independent activity in HC (see Figure 2a and b). In this area, the kinase activation was

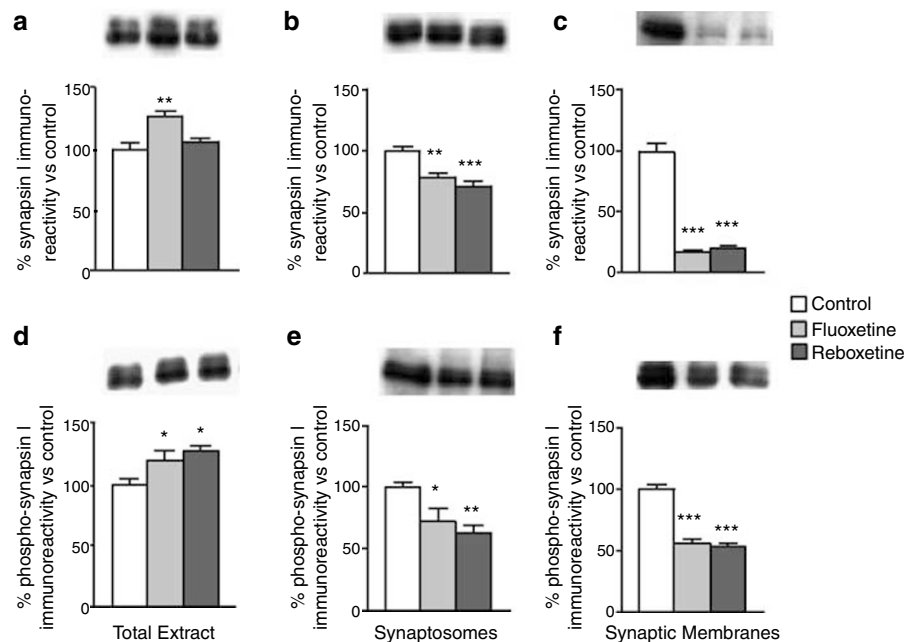


Figure 5 Expression level and phosphorylation of synapsin I in total extract, synaptosomes, and synaptic membranes from HC. Western-blot analysis of total synapsin I in: (a) total extract; (b) synaptosomes; and (c) synaptic membranes from HC of rats chronically treated with vehicle (Control), FLX, or RBX. Western-blot analysis of phospho-Ser⁶⁰³ synapsin I in: (d) total extract; (e) synaptosomes; and (f) synaptic membranes from HC of rats as above. Insets: representative immunoreactive bands from Western blots. Statistics as in previous figures (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; $n = 3$ in triplicate or quadruplicate).

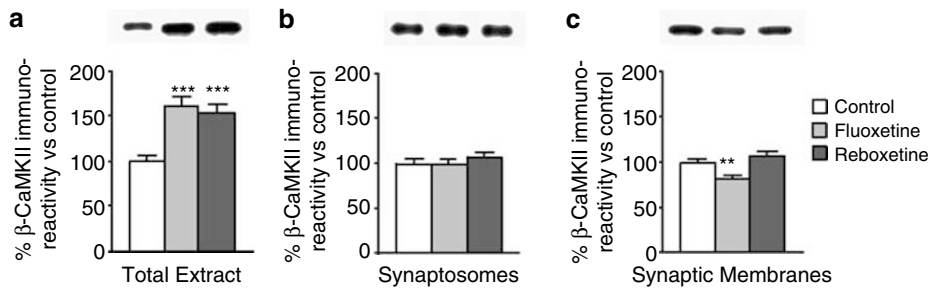


Figure 6 Expression level of β CaM kinase II in total extract, synaptosomes, and synaptic membranes from HC. Western-blot analysis of total β CaM kinase II in: (a) total extract; (b) synaptosomes; and (c) synaptic membranes from HC of rats chronically treated with vehicle (Control), FLX, or RBX. Insets: representative immunoreactive bands from Western blots. Statistics as in previous figures (** $p < 0.01$ and *** $p < 0.001$; $n = 3$ in triplicate or quadruplicate).

accompanied by a slight but not significant increase of autophosphorylation at Thr²⁸⁶ (for FLX and RBX), which represents the canonical mechanism for activity-induced activation of this kinase (Hudmon and Schulman, 2002; Lisman *et al*, 2002). We reported previously upregulation of CaM kinase II activity by pronoradrenergic antidepressants in cell bodies of pyramidal and granular neurons of HC (Tiraboschi *et al*, 2004a); our previous and present results strongly suggest that the modifications observed in total HC extract are mainly related to cell bodies and that this mechanism seems to be common to pronoradrenergic (DMI and RBX) and proserotonergic (FLX) antidepressants. However, in total HC, the increase of kinase autophosphorylation did not reach significance; and in P/FC, the kinase activation (significant only for DMI) was not accompanied by any increase of Thr²⁸⁶ phosphorylation. These findings suggest that other mechanisms of kinase activation are involved in both brain areas. CaM kinase II can be persistently activated by interacting with other proteins, including the NMDA receptor and ether-a-go-go (Eag) K⁺ channel (Strack *et al*, 2000; Bayer *et al*, 2001; Sun *et al*, 2004). These non-canonical mechanisms of activation of CaM kinase II could be a preferential target of antidepressants, because they also seem to be involved at synaptic level (see next section).

Chronic Antidepressant Treatments Induce a Redistribution of CaM Kinase II between Synaptic Membranes and Synaptic Vesicles in HC

First, antidepressants upregulated CaM kinase II enzymatic activity in synaptosomes from HC and P/FC. The increase was particularly remarkable for Ca²⁺-independent activity (in P/FC restricted to noradrenergic drugs). However, in both areas again the kinase activation was neither related to an increase of expression level nor of Thr²⁸⁶ phosphorylation. By contrast, the kinase autophosphorylation was reduced in synaptosomes from both areas, particularly in HC where up-regulation of Ca²⁺-independent activity was maximal. This suggests that the modulation of CaM kinase II activity by antidepressants at synaptic terminals is independent from autophosphorylation and may involve protein-protein interactions (Strack *et al*, 2000; Bayer *et al*, 2001; Sun *et al*, 2004). Indeed, it has been shown that interaction of CaM kinase II with subunits of the NMDA receptor or the Eag potassium channel activates the kinase independent on autophosphorylation (Bayer *et al*, 2001; Sun *et al*, 2004). A future study of CaM kinase II interaction with

NR2A/B and NR1 subunits of NMDA receptor, or with Eag potassium channel, in synaptosomes of antidepressant-treated rats will verify this hypothesis. Therefore, the present results showed that activation of HC CaM kinase II by antidepressants in synaptosomes is obtained by a mechanism independent on Thr²⁸⁶ phosphorylation.

Second, antidepressants induced a redistribution of α CaM kinase II between synaptic membranes and synaptic vesicles of HC. Based on previous studies, it can be argued that the presence of CaM kinase II in synaptic membranes is mainly due to the presence of a pool of vesicles docked to the plasma membrane (Mehta *et al*, 1996). This morphologically defined pool of vesicles has been correlated with the functionally defined readily releasable pool (RRP), containing the vesicles that fuse with the membrane and release glutamate upon stimulation (Schikorski and Stevens, 2001). Overall, our results would suggest that the redistribution is due to a translocation or a change in the targeting of the kinase during the synaptic vesicle cycle. This is supported by the following results: (a) the total level of α CaM kinase II in synaptosomes was little or not changed after drug treatments; (b) the level of synapsin I, which is anchored to CaM kinase II in synaptic vesicles (Benfenati *et al*, 1992), was dramatically reduced in synaptic membranes; (c) the level of β CaM kinase II in synaptic membranes was not increased by the drug treatments, ruling out a replacement of α - with β -isoform (but see next paragraph); and (d) local protein synthesis of α CaM kinase II was unchanged (Veneri *et al*, manuscript in preparation). Similar to our previous results showing a downregulation of the interaction α CaM kinase II/syntaxin-1 in synaptic membranes (Bonanno *et al*, 2005), the extent of kinase redistribution we observed is large enough to involve glutamatergic synaptic terminals (estimated to represent up to 60% of hippocampal terminals).

With regard to β CaM kinase II, it is interesting that, although not replacing the α CaM kinase II missing at synaptic membranes, the changes observed in total expression of β after drug treatments will alter the normal ratio between α and β isoform in HC (normally $\approx 3:1$; Benfenati *et al*, 1996). In particular, the large increase of β expression in total HC will likely affect the kinase function in neuronal cell bodies, whereas the lack of expression changes in β in synaptic membranes in the face of a large reduction of α will still considerably change the ratio between subunits. It has been shown that in HC neurons, the α/β ratio increases during periods of higher electrical activity and decreases with reduced

activity. The expression level of α -isoform was found to be regulated by the activity of NMDA receptor (Thiagarajan *et al*, 2002). The reduction induced by antidepressants in the α/β ratio at both total and synaptic levels could be related to a drug-induced reduction of glutamate neurotransmission. It is worth mentioning that these same drug treatments also induced a robust reduction in the expression level of NR1 subunit of NMDA receptor in synaptic membranes, a finding that could be functionally coupled to the reduction of α CaM kinase II in the same compartment (not shown).

Functional Changes Induced by Antidepressants in the Presynaptic Machinery of HC: Implications for Synaptic Plasticity

Preclinical and clinical studies have shown that molecular/cellular mechanisms underlying synaptic plasticity are involved in both stress-related disorders and in the action of antidepressants (McEwen, 1999; Sapolsky, 2000; Popoli *et al*, 2002; Shakesby *et al*, 2002; Zarate *et al*, 2002). With regard to presynaptic mechanisms, chronic stress has been shown to induce in HC terminals a redistribution of synaptic vesicles suggesting increased glutamate release, and microdialysis *in vivo* studies reported increased extracellular glutamate following stress paradigms (Bagley and Moghaddam, 1997; Magarinos *et al*, 1997). Conversely, we showed that different antidepressants reduce depolarization-evoked release of glutamate from hippocampal synaptosomes, by reducing syntaxin-1/CaM kinase II interaction and increasing syntaxin-1/Munc-18 interaction (Bonanno *et al*, 2005). Therefore, although independent evidence suggests that stress induces an increase of glutamate release in HC and cortical areas, antidepressants seem to exert their restorative action partly by dampening glutamate release. The combination of these presynaptic effects with the documented drug-induced postsynaptic effects on different types of glutamate receptors (Zarate *et al*, 2003; Popoli *et al*, 2002) could explain the impact of antidepressants on synaptic plasticity (Popoli *et al*, 2002; Shakesby *et al*, 2002).

Overall, our present and previous (Bonanno *et al*, 2005) data suggest that the functional presynaptic compartments involved here are the RRP of vesicles (associated with the synaptic membrane) and the reserve pool of synaptic vesicles (largely coincident with the vesicle fraction). We previously showed that the decreased kinase phosphorylation in the RRP brings about consistent changes in the presynaptic machinery, leading to a decrease of depolarization-evoked glutamate release (Bonanno *et al*, 2005). Therefore, the antidepressant-induced change in α CaM kinase II phosphorylation seems to be a crucial event in the molecular modifications leading to modulation of glutamate release. Indeed, α CaM kinase II has been proposed to regulate probability of release in central synapses during high-frequency presynaptic stimulation (Hinds *et al*, 2003); the drug-induced molecular and functional changes we found related to α CaM kinase II are in line with this role for the enzyme. The mechanism whereby antidepressants may induce the adaptive changes we observed in α CaM kinase II is at present unknown. A possibility is that these drugs affect calcium fluxes, because in preliminary experiments with Fura-2 employing synaptosomes from chronic FLX-treated

rats we found consistent upregulation of basal and depolarization-induced calcium fluxes (not shown).

However, our results also suggest an additional functional change that may be a consequence of the large decrease of synapsin I in synaptic membranes, likely due to the decrease of its anchoring protein (α CaM kinase II). It has been shown that synapsin I is required for stimulation of glutamate release by brain-derived neurotrophic factor (BDNF) (Jovanovic *et al*, 2000). BDNF expression is increased and indirect evidence showed that more BDNF is released after chronic antidepressant treatments (Saarelainen *et al*, 2003). Now, if antidepressants increase the synaptic concentration of BDNF one would expect an upregulation of glutamate release rather than a downregulation, as we observed. However, if synapsin I is nearly absent from the RRP of vesicles after drug treatment ($\approx 80\%$ of synapsin I was gone, see Figure 5), elevated BDNF will not increase glutamate release, but at the same time will be able to induce different neurotrophic effects. This mechanism may represent an additional explanation for the antidepressant-induced dampening of glutamate release.

In summary, CaM kinase II is a target of antidepressants, which induce a complex pattern of modifications in the kinase at different subcellular compartments. At presynaptic level, these changes are consistent with the dampening of glutamate release chronically induced by these drugs. It will be crucial in order to understand if these mechanisms can be targeted by novel pharmacological strategies, to identify the anchoring protein for the kinase in synaptic vesicles, and the factors regulating phosphorylation changes in the presynapse.

ACKNOWLEDGEMENTS

This study was supported by Grants from NARSAD (USA) to MP and from Ministry of University, PRIN # 2001054224 and 2003053993 to GR and MP (Italy). RG was founded by the PhD Program in Neuropharmacology, University of Catania Medical School.

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