Abnormalities in α/β-CaMKII and related mechanisms suggest synaptic dysfunction in hippocampus of LPA₁ receptor knockout mice

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Abstract

Lysosphatidic acid (LPA) is a natural lysosphopholipid that regulates neuronal maturation. In mice, the deletion of the LPA₁ receptor causes some phenotypic defects partly overlapping with those found in schizophrenia. In this study, we identified molecular abnormalities in hippocampal synaptic mechanisms involved in glutamatergic neurotransmission, which allow further characterization of synaptic aberrations in LPA₁ knockout (KO) mice. At the synaptic level, we found dysregulation of Ca²⁺/calmodulin (CaM)-dependent kinase II (CaMKII) activity and phosphorylation, with markedly higher Ca²⁺-dependent enzyme activity, probably related to increased expression levels of the β isoform of CaMKII. Conversely, although the synaptic Ca²⁺-independent activity of the enzyme was unchanged, autophosphorylation levels of both α and β isoforms were significantly increased in LPA₁ KO mice. Moreover, in LPA₁ KO mice the α/β isoform ratio of CaMKII, which plays a key role in neuronal maturation during development, was markedly decreased, as found previously in schizophrenia patients. At post-synaptic level, LPA₁ KO mice showed changes in expression, phosphorylation and interactions of NMDA and AMPA receptor subunits that are consistent with basal strengthening of glutamatergic synapses. However, we measured a reduction of nuclear cAMP responsive element-binding protein phosphorylation, suggesting that activation of the NMDA receptor does not occur at the intracellular signalling level. At the presynaptic level, in line with previous evidence from schizophrenia patients and animal models of pathiology, LPA₁ KO mice showed accumulation of SNARE protein complexes. This study shows that CaMKII and related synaptic mechanisms at glutamatergic synapses are strongly dysregulated in LPA₁ KO mice.

Introduction

The aetiology of schizophrenia is thought to involve mechanisms influencing neuronal connectivity (Andreasen, 2000; Gaspar et al. 2009; Harrison & Eastwood, 2001; Lewis et al. 2005; Lisman et al. 2008; Selemmon & Goldman-Rakic, 1999).

The LPA₁ receptor, the most widely expressed receptor for lysosphatidic acid (LPA), is a receptor coupled with G₁₅α, G₉ and G₁₂/₁₃ (Fukushima et al. 1998). LPA induces cell proliferation, adenyl cyclase inhibition, serum-response element, mitogen-activated protein kinases, phospholipase C, Akt and Rho activation and calcium mobilization (for a review, see Ishii et al. 2004). The targeted deletion of the LPA₁ gene in mice caused significant morphological, behavioural and neurological alterations. LPA₁ receptor knockout (KO) mice display craniofacial dysmorphology (Contos et al. 2000), a marked deficit in prepulse inhibition and decreased tissue levels of amino acids and monoamines in frontal cortex and hippocampus (Harrison et al. 2003). Moreover, in LPA₁ KO mice, although electrophysiological properties of hippocampal neurons...
and synaptic communication within CA1 appeared normal (Harrison et al. 2003), depolarization-evoked release of glutamate and GABA in the hippocampus and electrically evoked serotonin efflux were significantly decreased (Roberts et al. 2005). It was also demonstrated that gamma frequency oscillations in the superficial layers of entorhinal cortex and parvalbumin-immunopositive GABAergic cell bodies in layer II are reduced in LPA1 KO mice (Cunningham et al. 2006). Together, these previous results suggest that loss of LPA1 receptor leads to a range of phenotypic defects partly overlapping with those found in schizophrenia (Braff & Geyer, 1990; Falkai et al. 2000; Harrison, 1999; Harrison & Eastwood, 2001; Lewis et al. 2005; Waddington et al. 1999).

Post-mortem and neuroimaging studies in schizophrenia patients demonstrated abnormal glutamatergic transmission and hippocampal structure and function (Harrison, 1999; Heckers, 2004; Nelson et al. 1998; Shenton et al. 2001). In the present study, to better characterize synaptic dysfunction in LPA1 KO mice, we analysed molecular alterations in some postsynaptic mechanisms involved in glutamatergic neurotransmission in the hippocampus.

Ca2+/calmodulin (CaM)-dependent kinase II (CaMKII) is a Ca2+-dependent enzyme that plays a key role in the regulation of neurotransmission. This kinase is necessary for the induction of long-term potentiation (LTP) and regulates neuronal excitability, synaptic transmission and plasticity (Hudmon & Schulman, 2002; Lisman et al. 2002). The neuronal CaMKII is a holoenzyme composed by a variable α and β subunit ratio (Miller & Kennedy, 1985). Modifications in the α/β-CaMKII ratio regulate subcellular localization of the holoenzyme and also have consequences on synaptic activity and strength (Fink et al. 2003; Thiagarajan et al. 2002). Activity-dependent CaMKII activation by Ca2+/CaM allows the kinase to phosphorylate specific residues (Thr286 in α, Thr287 in β), promoting Ca2+-independent (autonomous) enzymatic activity. CaMKII autonomy is considered a form of molecular memory and has been shown to be involved in mechanisms of synaptic plasticity (Lisman et al. 2002). Once CaMKII becomes Ca2+-independent, secondary sites (Thr305–306 in α, Thr306–307 in β) can be phosphorylated, acting to prevent further stimulation and resulting in a catalytically inactive kinase (Hudmon & Schulman, 2002).

In light of the synaptic role of CaMKII in the regulation of glutamatergic transmission and of the known involvement of abnormal glutamatergic transmission and hippocampal structure and function in schizophrenia (Harrison, 1999; Heckers, 2004; Nelson et al. 1998; Shenton et al. 2001), we analysed activity and regulation of the kinase and related synaptic molecular mechanisms in the hippocampus of LPA1 KO mice.

Methods

Animal model and subcellular fractionation

LPA1 KO mice used in the present study were generated in E14.1 embryonic stem cells by homologous recombination of the majority of the coding region of the LPA1 gene with an IRESlacZ expression/neomycin resistance cassette (Harrison et al. 2003). Germline chimeras were crossed onto C57B1/6j females to generate heterozygotes, which were intercrossed giving rise to N1F1 offspring homozygous for the LPA1 targeted mutation (Harrison et al. 2003). The mice were interbred at N6 to produce the study cohort. Wild-type (WT) and KO mice were separated (n=5 per cage) and maintained under standard conditions [12-h light/dark cycle (lights on 07:00 hours); 22±1°C ambient temperature; 60% relative humidity; food and water available ad libitum]. Experiments complied with guidelines for use of experimental animals of European Community Council Directive 86/609/EEC. Groups of 25 WT and 25 KO male animals (aged 9–10 wk) were sacrificed; the hippocampus was quickly excised on ice and homogenized with a glass-Teflon Potter tissue grinder (clearance, 0.25 mm) in homogenization buffer, 0.28 m sucrose, 10 m M Hepes, 0.1 mM EGTA, 20 mM NaF, 5 mM Na3PO4, 1 mM NaVO4 and 2 μl/ml protease inhibitor cocktail (Sigma Aldrich, USA), pH 7.4. From total homogenate of each area, fraction P1, enriched in nuclei and fraction P2, enriched in synaptosomes were prepared by means of differential centrifugation as described previously (Bonanno et al. 2005). Purified synaptic terminals (synaptosomes) were prepared by centrifugation on Percoll gradients (Dunkley et al. 1986), with minor modifications (Bonanno et al. 2005; Musazzi et al. 2010a). Purity of synaptosomes and other subcellular fractions was checked by measuring subcellular distribution of protein markers, as previously shown (Barbiero et al. 2007). Synaptosomes were resuspended in lysis buffer: 120 mM NaCl, 20 mM Hepes, 0.1 mM EGTA, 0.1 mM DTT, containing 20 mM NaF, 5 mM Na3PO4, 1 mM NaVO4 and 2 μl/ml of protease inhibitor cocktail (Sigma-Aldrich), pH 7.4.

Assay of Ca2+-dependent and Ca2+-independent CaMKII enzymatic activity

Ca2+-dependent activity of CaMKII was assayed by measuring initial rate of phosphate incorporation
in the selective peptide substrate autocamtide-3 (Biosource, USA), as previously described (Musazzi et al. 2005). Five micrograms of protein/sample were incubated in standard phosphorylation buffer containing 20 μM AC-3, 0.5 mM CaCl₂, 20 μg/ml CaM (Biomol, USA) and 20 μM [γ-32P]ATP (0.6 Ci/mmole, GE Healthcare, USA). Reaction was carried out for 30 s at 30 °C and stopped by ice-cold trichloroacetic acid (final concentration 5%). After centrifugation, 10 μl of supernatant were spotted on phosphocellulose P81 paper (Whatman, UK). Filters were washed in 75 mM phosphoric acid, dried and counted for liquid scintillation. For Ca²⁺-independent activity assay samples contained 2 mM EGTA, 2 μM heat-stable cAMP-dependent protein kinase inhibitor (New England, USA), 5 μM protein kinase C (fragment 19-36) inhibitor (Sigma Aldrich), 10 μg protein/sample and no Ca²⁺/CaM. Blanks were incubated in the absence of peptide.

**Western blot analysis**

Western blot analysis was performed as described previously (Bonanno et al. 2005; Ryan et al. 2009; Tardito et al. 2009) by incubating PVDF membranes containing electrophoresed proteins with monoclonal or polyclonal antibodies. Monoclonal antibodies used were: antibodies for α-CaMKII 1:1000 (Chemicon International, USA), β-CaMKII 1:500 (Invitrogen, USA), syntaxin-1 1:5000 (Sigma Aldrich), synapsin 1 1:2000, SNAP-25 1:4000 and synaptobrevin 1:4000 (Synaptic Systems GmbH, Germany) and β-actin 1:10 000 (Sigma Aldrich). Polyclonal antibodies were: antibodies for phospho-Thr²⁸⁶ α-CaMKII 1:500 (Affinity BioReagents Inc., USA), phospho-Thr³⁶⁵–³⁶⁶ α-CaMKII 1:4000 and AMPA glutamate receptor subunit 1 (GluR1) 1:1000 (Cell Signaling Technology, USA), phospho-Ser³⁸³ synapsin 1 1:2000, NMDA receptor subunit 1 (NR1) 1:1000, NMDA receptor subunit 2A/2B (NR2A/2B) 1:1000 and AMPA glutamate receptor subunit 2/3 (GluR2/3) 1:500 (Chemicon International), phospho-Thr³⁶⁵–³⁶⁶ GluR1 1:1000 (Affinity Bioreagents, USA), CREB 1:1000 and phospho-Ser³⁸⁵ CREB 1:1000 (Cell Signalling Technology). For the detection of the SDS-resistant SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) complexes, Western blotting was performed on samples of electrophoresed presynaptic membranes (not-boiled before gel loading) (Chen et al. 1999; Matveeva et al. 2003; Musazzi et al. 2010a), incubating PVDF membranes containing blotted proteins with the antibody for syntaxin-1 1:5000 (Sigma-Aldrich).

Following incubation with peroxidase-coupled secondary antibodies, protein bands were detected with ECL (GE Healthcare) or Super Signal Dura West (Pierce Biotechnology Inc., USA). All protein bands were normalized for β-actin level detected in the same membrane and quantitation was performed with Quantity One software (Bio-Rad Laboratories, USA), as reported previously (Musazzi et al. 2010a).

**Co-immunoprecipitation of α-CaMKII with β-CaMKII, NMDA receptor subunits NR2A/2B and NR1, β-actin, AMPA receptor subunit GluR1 and syntaxin-1**

α-CaMKII was immunoprecipitated as described previously (Bonanno et al. 2005; Musazzi et al. 2010b). Synaptosomes (100 μg) were incubated in immunoprecipitation buffer: 200 mM NaCl, 10 mM EDTA, 10 mM Na₂HPO₄, 0.5% SDS, pH 7.4, in a final volume of 200 μl (Sigma Aldrich) and antibody against α-CaMKII overnight at 4 °C with slow end-over-end rotation. Protein A- sepharose (Sigma Aldrich; 10 mg/tube), rinsed in immunoprecipitation buffer, was added and incubation continued for 2 h at 4 °C. The beads were collected by centrifugation and washed with immunoprecipitation buffer. Sample buffer for SDS-PAGE was added and the mixture was boiled. The beads were pelleted by centrifugation and 25 μl of supernatant was applied to 10% polyacrylamide gels for SDS-PAGE. For the detection of co-immunoprecipitated proteins, polyacrylamide gels were stained with SYPRO Ruby (Bio-Rad Laboratories) and revealed with VersaDoc™ Imaging System (Bio-Rad Laboratories) or electro-blotted on PVDF membranes. Membranes were incubated with primary antibodies for α-CaMKII 1:3000, β-CaMKII 1:500, NR2A/2B 1:500, NR1 1:250, β-actin 1:1000, GluR1 1:500 or syntaxin-1 1:2000, incubated with suitable secondary antibodies and revealed with ECL or Super Signal Dura West. All co-immunoprecipitated protein bands were normalized for immunoprecipitated α-CaMKII level detected in the same SDS–PAGE lane and quantitation was as above (Bonanno et al. 2005; Musazzi et al. 2010b).

**Statistical analysis**

All data were analysed using Student’s t test for unpaired samples. Significance was assumed at p < 0.05.

**Results**

**Modification of enzymatic activity, expression and phosphorylation levels of CaMKII in hippocampus of LPA₁ KO mice**

In order to investigate if the deletion of LPA1 gene caused general modifications in CaMKII regulation,
Interestingly, the increased Ca\textsuperscript{2+}insets: show representative immunoreactive bands from Western blots. Data expressed as % intensity units/mm\textsuperscript{2}phosphorylation levels at Thr\textsuperscript{286}+ independent activity +dependent activity + dependent activity.

Panels (a) and (d), activity expressed as % pmol of PO\textsubscript{4} incorporated per minute per μg protein (mean \pm S.E.M.). (b), (c), (e), (f), insets: show representative immunoreactive bands from Western blots. Data expressed as % intensity units/mm\textsuperscript{2}(mean \pm S.E.M.). Statistics: Student’s t test for unpaired samples (** p<0.01, *** p<0.001, n=4 in duplicate).

we first assessed the kinase activity in total homogenate of hippocampus from WT and LPA\textsubscript{1} KO mice. Ca\textsuperscript{2+}-dependent and, especially, Ca\textsuperscript{2+}-independent activity were markedly higher in these animals (Ca\textsuperscript{2+}-dependent activity +25.31 \pm 3.99\% vs. WT; Ca\textsuperscript{2+}-independent activity +62.95 \pm 9.31\% vs. WT) (Fig. 1a). Interestingly, the increased Ca\textsuperscript{2+}-dependent activity of CaMKII holoenzyme was accompanied by a selective increase of the expression levels of β isoform (+58.56 \pm 12.54\% vs. WT), with no changes in the expression of α-CaMKII (the major isoform in forebrain) (Fig. 1b, c). The increase of the enzymatic activity was accompanied by marked higher auto-phosphorylation levels of Thr\textsuperscript{286} in α isoform (+40.11 \pm 5.09\% vs. WT) and Thr\textsuperscript{287} in β isoform (+56.03 \pm 5.79\% vs. WT) (Fig. 1b, c). No changes were observed in the phosphorylation levels at Thr\textsuperscript{305–306} of α-CaMKII (Fig. 1b).

To analyse if CaMKII was also dysregulated at the synaptic level in hippocampus of LPA\textsubscript{1} KO mice, where the enzyme modulates glutamatergic transmission, we tested the kinase in purified synaptic terminals (synaptosomes). In synaptosomes of LPA\textsubscript{1} KO mice the alterations of CaMKII regulation were different from those measured in total homogenate. We observed a markedly higher Ca\textsuperscript{2+}-dependent activity of CaMKII (+70.36 \pm 3.49\% vs. WT), but no changes in Ca\textsuperscript{2+}-independent activity (Fig. 1d). The study of subunit expression and activation showed that, as observed in total homogenate, the increase of Ca\textsuperscript{2+}-dependent activity in synaptosomes was probably due to a selective increase of β-CaMKII expression (+39.18 \pm 8.84\% vs. WT). Surprisingly, although Ca\textsuperscript{2+}-independent activity was similar to WT in LPA\textsubscript{1} KO synaptosomes, the phosphorylation of Thr\textsuperscript{286} in α isoform (+50.26 \pm 11.04\% vs. WT) and Thr\textsuperscript{286} in β isoform (+26.79 \pm 3.39\% vs. WT) was increased (Fig. 1e, f). The reason for this discrepancy is unknown, although it should be mentioned that in lysed synaptosomes, used for the enzymatic assay, different pools of CaMKII might have different access to the peptide substrate; but in electrophoresed samples for Western analysis synaptosomal proteins were completely denatured in SDS-containing buffer.
shown in Fig. 1, we found a 39.65% of co-precipitating a-CaMKII and β-CaMKII in the immunoprecipitate were analysed by Western analysis. Representative immunoreactive bands are shown. Data represent the means ± S.E.M. (percentage ratio β-CaMKII/α-CaMKII). (b) Staining with SYPRO Ruby of SDS–PAGE of immunoprecipitation of α-CaMKII in synaptosomes from hippocampus: line 1, molecular weight standards; line 2, immunoprecipitate from WT mice; line 3, immunoprecipitate from LPA1 KO mice; line 4, 5 μg of synaptosomes from hippocampus of WT mice. The three bands of higher intensities (arrows) to the right of lane 4 are described in the Results section. (c) α-CaMKII was immunoprecipitated; α-CaMKII and β-actin in the immunoprecipitate were analysed by Western analysis. Representative immunoreactive bands are shown. Data represent the means ± S.E.M. (percentage ratio β-actin/α-CaMKII). Statistics as in Fig. 1 (* p < 0.05, ** p < 0.001, n = 4 in duplicate).

However, in the case of β-CaMKII, the increase in the phosphorylated form was similar to the total protein and probably due to higher expression.

**Decreased α/β-CaMKII ratio and increased interaction of CaMKII with β-actin in the hippocampus of LPA1 KO mice**

Recent studies demonstrated that modifications in the α/β-CaMKII ratio play a role in subcellular localization of the holoenzyme and also have consequences for synaptic activity and strength. It has been shown previously that the increase of α-CaMKII level correlates positively, while increase in β-CaMKII correlates negatively, with the increase in electrical activity (Fink et al. 2003; Thiagarajan et al. 2002). Our results showed that in hippocampus of LPA1 KO mice, although α isoform expression is not changed, there is a higher expression of β isoform, suggesting an imbalance of the α/β subunit ratio. Therefore, we measured the α/β ratio in synaptosomes through immunoprecipitation of α-CaMKII and measurement of co-precipitating β-CaMKII. In line with results shown in Fig. 1, we found a 39.65 ± 14.18% (vs. WT) higher β/α ratio (Fig. 2a).

The localization of α-CaMKII at synapses is a regulated and dynamic process. α-CaMKII has been shown to interact with a number of synaptic proteins, which may affect both its localization and activity (Griffith et al. 2003). Our results so far demonstrated a dysregulation of synaptic CaMKII activity and subunit composition in LPA1 KO mice. In order to test interactions of CaMKII with structural proteins we immunoprecipitated α-CaMKII from synaptosomes and, after SDS–PAGE of immunoprecipitates, the electrophoretic gels were stained with SYPRO Ruby, a highly sensitive fluorescent protein detection reagent. As shown in Fig. 2b, the main band in the immunoprecipitate was α-CaMKII (a) (confirmed by Western Blot, not shown). We observed in the immunoprecipitate two additional bands with high intensities (b and c) that were more marked in samples from LPA1 KO mice, suggesting increased interaction of these proteins with α-CaMKII. β-actin can interact directly with CaMKII holoenzyme, through binding of F-actin with the β-CaMKII isoform (Fink et al. 2003; Shen et al. 1998; Tao-Cheng et al. 2001). Because of this direct interaction, and because we found that two proteins are enriched in the CaMKII immunoprecipitate from LPA1 KO (see above), we wondered whether one of these CaMKII-interacting proteins might be β-actin. Therefore, we immunoprecipitated α-CaMKII and found, by staining with specific antibodies, a markedly higher level (74.30 ± 7.37% vs. WT)
of co-precipitating β-actin in LPA1 KO mice (Fig. 2c). A limitation of these experiments is that they rely solely on one-directional immunoprecipitation. However, it is interesting to note that the immunoprecipitation results closely match those of the expression experiments. Indeed, LPA1 KO mice showed higher β/α ratio and β-actin/α-CaMKII ratio, in line with higher expression of β-CaMKII.

**Modification in the expression and level of interaction of α-CaMKII with ionotropic glutamate receptor subunits**

In light of the synaptic function of CaMKII and the implication of altered glutamatergic neurotransmission in schizophrenia (Andreasen, 2000), we investigated in LPA1 KO mice if the changes observed in CaMKII also have consequences on the mechanisms regulating localization of the enzyme and on glutamatergic receptor function. It has been shown previously that two distinct mechanisms play a role in CaMKII/β-actin interaction, regulating CaMKII synaptic localization. First, β-actin can interact directly with CaMKII holoenzyme, localizing the α/β heteromers to the cytoskeleton (Fink et al. 2003; Shen et al. 1998; Tao-Cheng et al. 2001). Thus, the higher β/α-CaMKII ratio, and the consequent disproportion of β vs. α isoform levels observed in LPA1 KO mice, could be involved in the increase of α-CaMKII/β-actin interaction measured in these animals. Second, β-actin is implicated in the localization of AMPA receptor (AMPA) at synapses and, binding both protein 4.1 and actin, can link CaMKII/actinin complex to protein 4.1/SAP97/GluR1 complex, forming a macromolecular scaffold that favours AMPAR anchoring to the plasma membrane (Lisman & Zhabotinsky, 2001). It has been shown that activated α-CaMKII phosphorylates GluR1 AMPAR subunit at Ser831, enhancing the conductance of AMPARs during LTP (Barria et al. 1997). Mature AMPARs are heteromers composed mainly of GluR1 and GluR2 subunits (Wenthold et al. 1996).

First, we investigated the expression levels of AMPAR subunits and the phosphorylation of GluR1 subunit at Ser831 in LPA1 KO mice (Fig. 2c). We found a significant increase of synaptic expression (+54.53% ± 8.87% vs. WT) of GluR1 subunit (GluR2/3 expression levels were unchanged). The significant increase of phosphorylation (+19.18 ± 4.65% vs. WT) appears ascribable to the increase in expression (Fig. 3a). These data suggest a different regulation of the channel conductance in LPA1 KO mice. To evaluate the formation of the AMPAR macromolecular scaffold, since post-synaptic density (PSD)-associated portions of post-synaptic membrane co-purify with synaptic terminals (Dunkley et al. 1986), we immunoprecipitated α-CaMKII and measured co-immunoprecipitated GluR1. We found a 33.49 ± 10.78% (vs. WT) higher α-CaMKII/GluR1 interaction level (Fig. 3b), suggesting that the increase of α-CaMKII/β-actin interaction was also accompanied by a modification in the interaction of AMPARs with CaMKII-containing scaffold. The increased interaction of CaMKII with GluR1 essentially appears to reflect the increased GluR1 expression level.

Moreover, upon activation, CaMKII can translocate to PSD and bind to NMDA receptors (NMDARs), improving the persistently enhanced transmission (Barria & Malinow, 2005; Bayer et al. 2001). In the forebrain NMDARs are composed of one NR1 subunit and one or more NR2A or NR2B subunits. We measured the expression levels of NMDAR subunits and, although NR1 expression was not altered, NR2A/B expression was markedly and significantly higher (+50.55 ± 7.86% vs. WT) (Fig. 3c). We assessed the interaction between α-CaMKII and NMDARs, by immunoprecipitating the kinase and measuring the co-precipitating NR2A/B (affinity of α-CaMKII for the NR2B subunit is >10-fold higher than for NR2A or NR1 (Ryan et al. 2009; Strack & Colbran, 1998)). In LPA1 KO mice, we found a markedly higher (+91.74 ± 15.82% vs. WT) α-CaMKII/NR2A/B interaction (Fig. 3d). To verify if this reflected an increase of the binding of α-CaMKII with correctly assembled NMDARs, we also measured the interaction between α-CaMKII and the core subunit NR1. Remarkably, the α-CaMKII/NR1 interaction was higher in LPA1 KO (+80.37 ± 18.35% vs. WT) and was similar to that measured for α-CaMKII/NR2A/B (Fig. 3e), suggesting higher interaction of α-CaMKII with assembled NMDARs and a persistently higher translocation of the enzyme to PSD.

Overall, in LPA1 KO mice we found marked alterations in the interaction of CaMKII with ionotropic glutamatergic receptors, suggesting increased AMPAR/NMDAR synaptic localization and increased conductance of AMPARs. Activation of synaptic NMDARs can activate gene expression mediated by cAMP responsive element-binding protein (CREB) and promote pro-survival events (Shaywitz & Greenberg, 1999). We attempted to assess if the synaptic changes observed in NMDARs also caused modifications in related intracellular signalling pathways. We assessed protein expression and activation (phosphorylation) of CREB and found reduced CREB phosphorylation at Ser133 in nuclear fraction from the hippocampus of LPA1 KO mice (–21.08 ± 6.10% vs. WT) (Fig. 4), which
suggests that activation of NMDARs is not conveyed at the level of intracellular signalling.

**LPA<sub>1</sub> KO mice show accumulation of SNARE complexes in hippocampal synaptosomes**

In addition to post-synaptic roles of CaMKII, the enzyme modulates synaptic vesicle trafficking and neurotransmitter release in the presynapse. Synapsin I is an abundant presynaptic protein that regulates the availability of vesicles for exocytosis (Hosaka et al. 1999). When the protein is phosphorylated by different kinases, including CaMKII, it allows vesicles to enter the readily releasable pool, which is released upon arrival of appropriate stimulus. Moreover, presynaptic phospho-Thr<sup>286</sup>α-CaMKII binds specifically to syntaxin-1, a protein that is a component of the exocytotic SNARE complex, which mediates fusion of synaptic vesicles (Jahn & Scheller, 2006; Rizo & Rosenmund, 2008; Sudhof, 2004). This binding has been suggested to represent a step in the regulation of exocytosis because α-CaMKII/syntaxin-1 interaction promotes the binding of syntaxin-1 to other SNARE proteins (Agid et al. 2007; Bonanno et al. 2005; Nomura et al. 2003; Ohyama et al. 2002).

We measured, in synaptosomes from hippocampus of WT and LPA<sub>1</sub> KO mice, the expression and
phosphorylation of synapsin I at Ser\(^{603}\) (the specific consensus site for \(\alpha\)-CaMKII), the expression of syntaxin-1 and, by co-immunoprecipitation, the interaction between \(\alpha\)-CaMKII and syntaxin-1. We did not find any modifications in these presynaptic proteins (Fig. 5a–c), suggesting that the alterations in CaMKII observed in synaptosomes have no measurable consequences on the presynaptic mechanisms analysed.

Furthermore, we measured the formation of the SNARE complex. Three presynaptic proteins compose the SNARE complex: the two plasma membrane SNAREs, syntaxin-1 and SNAP-25, and the vesicle-associated membrane protein 2 (VAMP-2 or synaptobrevin). Although no modulation of syntaxin-1 expression was observed (Fig. 5b), the synaptic expression of both SNAP-25 and synaptobrevin was higher in LPA\(_1\) KO mice (SNAP-25 +19.14 ± 5.93% vs. WT; synaptobrevin +33.81 ± 17.34% vs. WT) (Fig. 5d, e). We also quantitated the level of the assembled SNARE complex in synaptosomes. The SNARE complex is SDS-resistant and can be visualized by loading in SDS–PAGE samples treated at 25 °C instead of 100 °C and performing Western blot with syntaxin-1 antibody. Quantification of (g) 80 kDa SNARE complex and (h) 100 kDa SNARE complex, in synaptosomes from hippocampus of LPA\(_1\) KO mice vs. WT. Insets: representative immunoreactive bands from Western blots. Data expressed as % intensity units/mm\(^2\) (mean ± S.E.M.). Statistics as in Fig. 1 (* \(p < 0.05\), \(n = 4\) in duplicate).

**Fig. 5.** In hippocampal synaptosomes of LPA\(_1\) KO mice the accumulation of presynaptic SNARE complex is increased. (a) Expression and phosphorylation levels at Ser\(^{603}\) of synapsin I (SYN). (b) Expression levels of syntaxin-1 (SYX). (c) \(\alpha\)-CaMKII was immunoprecipitated; \(\alpha\)-CaMKII and syntaxin-1 were analysed by Western analysis. Data represent the means ± S.E.M. (percentage ratio SYX/\(\alpha\)-CaMKII). Expression levels of (d) SNAP-25 and (e) synaptobrevin (SYB). (f) Western blot analysis of SNARE complexes in WT (\(n = 1\)) and LPA\(_1\) KO mice (\(n = 2\)) stained with syntaxin-1 antibody. Quantification of (g) 80 kDa SNARE complex and (h) 100 kDa SNARE complex, in synaptosomes from hippocampus of LPA\(_1\) KO mice vs. WT. Insets: representative immunoreactive bands from Western blots. Data expressed as % intensity units/mm\(^2\) (mean ± S.E.M.). Statistics as in Fig. 1 (* \(p < 0.05\), \(n = 4\) in duplicate).
of LPA1 KO mice (+44.08 ± 15.84% vs. WT) (Fig. 5g), while 100 kDa SNARE complex was not significantly different from WT (Fig. 5h).

**Discussion**

The present study provides evidence that deletion of the LPA1 receptor in the mouse hippocampus induces marked synaptic alterations in CaMKII-related molecular mechanisms regulating glutamatergic neurotransmission.

**CaMKII is dramatically dysregulated and α/β ratio markedly decreased in synaptosomes from hippocampus of LPA1 KO mice**

In hippocampal synaptosomes from LPA1 KO mice we found a marked dysregulation of CaMKII. A main result was the finding of an imbalance of α/β subunit ratio with a relative increase of β-CaMKII that leads to a marked augmentation of Ca2+-dependent activity. In contrast, although both α and β subunits were more autophosphorylated at Thr domains, Ca2+-independent activity was not different from WT. It is of interest that previous work on rats sensitized with amphetamine, a drug which enhances striatal dopamine release and may induce psychotic behaviour, demonstrated an increase of Ca2+-dependent (but not Ca2+-independent) CaMKII activity in striatal synaptosomes, with no changes in α-CaMKII expression levels (Iwata et al. 1997).

CaMKII plays a key role in the modulation of synaptic activity, neurogenesis, neuronal maturation and neuroplasticity (Lisman et al. 2002). In particular, the control of α/β subunit ratio in CaMKII holoenzyme is essential because distinct subunits have different functions: while α isofrom regulates synaptic strength, β isofrom is more involved in the control of dendritic morphology and the formation and number of synapses (Fink et al. 2003). A role of the β subunit is to bind the CaMKII holoenzyme to the actin cytoskeleton and, upon stimulation, translocate the whole functional unit to the dendritic spines, thereby regulating the subcellular localization of the enzyme (Okamoto et al. 2007; Shen et al. 1998; Shen & Meyer, 1999). In addition, the α/β ratio is inversely regulated in response to neuronal activity: the α subunit levels rise during higher firing levels, while β subunit levels increase during lower activity periods (Thiagarajan et al. 2002). The concentration of α- and β-CaMKII is critical in the regulation of neuronal maturation and pruning of neurons during neurodevelopment. Indeed, α/β ratio changes with a specific pattern during the postnatal period; in rats, β expression starts earlier compared to α and, while at postnatal day 10 the α/β ratio is 1:1, in adult animals the ratio is approximately 3:1 (Kelly et al. 1987, Miller & Kennedy, 1985). Recent studies suggested that alterations of α/β-CaMKII levels and regulation, affecting neuronal morphology, connectivity and activity, may be relevant to the development of schizophrenia (Greenstein et al. 2007; Novak et al. 2000, 2006). Indeed, the timing of onset of schizophrenia coincides with the change in CaMKII holoenzyme subunit composition and the absence of this maturation leads to neuropathology and behavioural defects in adulthood (Keshavan et al. 1994). This hypothesis was supported by recent findings demonstrating that β-CaMKII is increased both in a different animal model of schizophrenia, amphetamine-sensitized rats (Greenstein et al. 2007), and in schizophrenia patients (Novak et al. 2000, 2006). Moreover, the deletion of LPA1 receptor could affect dendritic morphology and pruning of neurons. Indeed, one prominent cellular response evoked by LPA is rearrangement of the actin cytoskeleton, influencing cell morphology in various cell types, including neurons (for a review, see Fukushima 2004). It was demonstrated that LPA induces process/membrane retraction through Rho/actomyosin pathways and inhibits migration of young, post-mitotic neurons (Fukushima et al. 2002). These cellular phenomena may reflect collapse and retraction of the basal processes of immature neurons that are involved in the proper neuronal maturation, a phenomenon that may be implicated in brain pathology. In future studies, it will be interesting to analyse the synapse morphology of LPA1 KO mice in fixed brain samples.

**LPA1 KO mice show molecular changes consistent with basal strengthening of glutamate synapse**

CaMKII is the most abundant protein of the PSD, where it is activated by calcium influx and regulates glutamatergic synapses and activity-dependent strengthening of neurotransmission (Lisman et al. 2002). Biochemical studies have demonstrated a high-affinity binding between the catalytic domain of CaMKII and the NR2B subunit of NMDARs (Bayer et al. 2001; Strack & Colbran, 1998). Interaction of CaMKII and NR2B renders the enzyme constitutively active (Bayer et al. 2001) and could be a key component of the persistently enhanced transmission (Lisman et al. 2002). CaMKII is also involved in AMPAR trafficking (Maletic-Savatic et al. 1998), anchoring (Lisman & Zhabotinsky, 2001) and conductance (Barria et al. 1997). Indeed, CaMKII stimulates the exocytosis of AMPAR-containing vesicles and phosphorylated CaMKII can
enhance transmission through a structurally mediated assembly process. This process leads to the incorporation of additional GluR1 binding proteins into the synapse, which then strengthens the synapse by anchoring additional AMPARs by CaMKII and found that in hippocampal synaptosomes from LPA1 KO mice the CaMKII/NMDAR interaction, the CaMKII/GluR1 interaction, and the phosphorylation of GluR1 at Ser831 are significantly increased. Taken together, these observations, along with the measured increase of Ca2+-dependent activity and autophosphorylation of CaMKII, suggest a post-synaptic strengthening of glutamatergic synapses in the hippocampus of these animals. However, although the opening of post-synaptic NMDARs activates signalling that induces phosphorylation of the transcription factor CREB (Hardingham & Bading, 2003; Malenka & Nicoll, 1999), in LPA1 KO mice, we measured a reduction of CREB phosphorylation. These combined results would suggest that, while biochemical characteristics of the post-synaptic membrane are altered in ways that imply greater synaptic reactivity, actual synaptic activity (and therefore recruitment of these enhanced post-synaptic mechanisms) is decreased. Similarly, it was shown previously that, while basal glutamate release is increased in the hippocampus of LPA1 KO mice, the depolarization-evoked release is decreased (Roberts et al., 2005), suggesting a reduction of the responsiveness to stimuli carried through the glutamatergic system, leading to hypofunction. Another possibility is that L-type Ca2+ channels, which significantly contribute to an increase of intracellular calcium and induction of CREB regulating signalling, are not sufficiently activated in LPA1 KO (Shaywitz & Greenberg, 1999).

Current hypotheses on the pathophysiology of schizophrenia suggest that, in the presence of dysfunction of NMDARs, glutamatergic pyramidal neurons lose the tonic regulation by fast-spiking GABAergic interneurons, thereby producing an augmentation of sensory input, and a decrease in the signal-to-noise ratio (Gaspar et al. 2009; Lisman et al. 2008). In line with this suggestion a deficit in gamma rhythm generation and associated synchrony has been shown in the neocortex of schizophrenia patients during visual and auditory processing tasks (Spencer et al. 2003, 2004). Intriguingly, recent studies have found a marked decrease in the power of gamma oscillations in superficial entorhinal cortex layers in brain slices from WT mice acutely treated with a NMDA antagonist and in brain slices from LPA1 KO mice (Cunningham et al. 2006). All these observations, including the present results, suggest that specific functional deficits in GABAergic interneurons, due to altered glutamatergic function, may underlie some of the behavioural correlates of cortical dysfunctions found in schizophrenia.

Deletion of LPA1 receptor induces accumulation of presynaptic SNARE complexes in hippocampus

At the presynaptic level, we found an increase of the expression of two of the three SNARE proteins and a parallel accumulation of SNARE complexes in synaptosomes of the hippocampus of LPA1 KO mice. Previous evidence showed that exocytotic neurotransmitter release is dependent on the cyclic docking and un-docking of vesicles to the presynaptic membrane and SNARE complexes formed between proteins located on vesicles and membrane are essential for docking and fusion (for a review see Rizo & Rosennmund, 2008; Sudhof, 2004). The basal accumulation of presynaptic SNARE complexes found in LPA1 KO mice is in line with a previous study reporting that, in hippocampal synaptosomes in superfusion, basal glutamate release is higher compared to WT (Roberts et al. 2005). Higher basal levels of SNARE complexes found in hippocampus of LPA1 KO mice could represent a molecular correlate of the increase of basal glutamate release. Interestingly, on the contrary, Roberts et al. (2005) showed that depolarization-evoked glutamate release is decreased in LPA1 KO compared to WT mice. This would suggest that at the presynaptic level sensitivity to depolarizing stimuli could be decreased as for the post-synaptic level where we hypothesize a reduction of responsiveness of glutamatergic synapses due to basal hyperactivation.

Moreover, an increasing body of evidence from neuropathological, human genetics and animal studies suggested that the SNARE complex may play a role in the pathophysiology of schizophrenia (for a review, see Johnson et al. 2008). Post-mortem studies on schizophrenia patients revealed modifications of SNARE proteins and SNARE complex levels and regulation. Interestingly, the changes observed do not seem to be due to altered synaptic density but rather to selective alterations of the presynaptic machinery regulating neurotransmitter release. In particular, Honer et al. (2002) found an increase of SNARE complexes in frontal cortex of schizophrenia and depressed individuals with suicide as cause of death.
More recently, the same group found that SNARE complex accumulation is also increased in striatal areas from schizophrenia patients (Barakauskas et al. 2010).

A recent hypothesis on the pathophysiology of schizophrenia, the so-called ‘synaptic hypothesis’, was derived from the dopaminergic and glutamatergic hypotheses and from neurogenetic studies, which implicated genes influencing synaptic plasticity (Owen et al. 2005). Schizophrenia is a neurodevelopmental disorder involving genetic predisposition and environmental insults, which lead to aberrant synaptogenesis and continued dysfunction of mature synapses. LPA1 KO mice, linking a developmentally expressed receptor with some endophenotypes partly resembling those associated with schizophrenia, were proposed as a preclinical animal model of the pathology (Contos et al. 2000; Cunningham et al. 2006; Harrison et al. 2003; Roberts et al. 2005). Our findings demonstrated that the deletion of the LPA1 receptor generates persistent molecular abnormalities of the glutamatergic system in adult mice that are partly similar to those previously found in other animal models of schizophrenia.

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Statement of Interest

None.

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