FEBS Letters

FEBS PRESS science publishing by scientists

Elucidating the role of the pLG72 R30K substitution in schizophrenia susceptibility

Silvia Sacchi[1,2], Pamela Cappelletti[1,2], Luciano Pirone[3], Giovanni Smaldone[4], Emilia Pedone[3] and Loredano Pollegioni[1,2]

1 Department of Biotechnology and Life Sciences, Università degli Studi dell'Insubria, Varese, Italy
2 The Protein Factory, Politecnico di Milano and Università degli Studi dell'Insubria, Italy
3 Institute of Biostructures and Bioimaging, Italian Research National Council, Naples, Italy
4 IRCCS SDN, Naples, Italy

Correspondence
L. Pollegioni, Department of Biotechnology and Life Sciences, Università degli Studi dell'Insubria, via J.H. Dunant 3, 21100 Varese, Italy
Fax: +39 0332 421500
Tel: +39 0332 421506
E-mail: loredano.pollegioni@uninsubria.it

(Received 17 December 2016, revised 24 January 2017, accepted 28 January 2017, available online 18 February 2017)

doi:10.1002/1873-3468.12585

Edited by Ned Mantei

In the human brain, pLG72 interacts with the flavoenzyme D-amino acid oxidase (hDAAO), which is involved in catabolism of D-serine, a co-agonist of *N*-methyl-D-aspartate receptors (NMDAR). Here, we investigated the wild-type pLG72, the R30K variant associated with schizophrenia susceptibility, and the K62E variant. The protein conformation, oligomeric state, ligand-, and hDAAO-binding properties are only slightly modified by the substitutions. All pLG72 variants inhibit hDAAO and lead to an increase in cellular (D/D+L)-serine. However, the R30K pLG72 is significantly more prone to degradation than the R30 and the K62E variants in a cell system, thus possessing a lower ability to interact/inhibit hDAAO. This links R30K pLG72 with the hyperactivity of hDAAO, the decreased D-serine level, and NMDAR hypofunction observed in schizophrenia-affected patients.

Keywords: D-amino acid oxidase; D-serine; protein–protein interaction; regulation; schizophrenia

The *G72* gene is located in a 5-Mb region on the long arm of chromosome 13, giving strong evidence for linkage with several neuropsychiatric disorders, including bipolar affective disorder and schizophrenia [1]. The *G72* gene, encoding a small protein (153 amino acids) named pLG72 or D-amino acid oxidase activator (DAOA), is of utmost interest as it is present only in primates; no other homologs have been identified in the databases, and putative functions were not inferred from sequence analysis of the putative ORFs [1,2]. Previous functional data have indicated that pLG72 specifically interacts in the human brain with the flavoenzyme D-amino acid oxidase (hDAAO, EC 1.4.3.3)—encoded by what has been termed a 'master gene' for schizophrenia susceptibility [3]—modulating both activity and half-life of this oxidase [4–7]. The main role of hDAAO in the brain is modulation of D-serine levels by catalyzing its oxidative deamination [8,9]. D-serine is an endogenous modulator of the glutamate receptors of the *N*-methyl-D-aspartate subtype (NMDAR) [10]: D-serine signaling plays a key role in developing brain wiring and regulating higher brain functions [11]. Alterations in D-serine-dependent NMDAR activation have been reported in various neurological diseases: excessive production/release of D-serine is implicated in acute and chronic degenerative disorders, while low D-serine levels have been detected in patients affected by schizophrenia or bipolar disorders [12–16]. Based on biochemical evidence, we proposed that a decrease in the endogenous levels of pLG72 might increase hDAAO activity, producing an excessive depletion of D-serine and thus NMDAR

Abbreviations
ANS, 8-anilinonaphtalene-1-sulfonate; CD, circular dichroism; CPZ, chlorpromazine; FAD, flavin adenine dinucleotide; hDAAO, human D-amino acid oxidase; NLS, *N*-lauroylsarcosine; NMDAR, *N*-methyl-D-aspartate receptor; SPR, surface plasmon resonance.

hypofunction, which predisposes these individuals to schizophrenia [1,5,9]. It is remarkable that pLG72 was proposed to be a mitochondrial protein [17] located on the cytosolic surface of the external membrane [6], where it might interact with newly synthesized hDAAO (before its final targeting to peroxisomes) and modulate the morphology of the mitochondrial network, indirectly affecting function of this organelle [17].

pLG72 is a small protein largely constituted by α-helices and encompassing disordered regions. It is present in solution as a 37-kDa homodimer and interacts with large, hydrophobic compounds and hDAAO. The latter interaction forms a ≈ 200-kDa complex with a K_d in the micromolar range, and yields a time-dependent loss of hDAAO activity [5,18–20]. By using low-resolution techniques, we recently proposed a three-dimensional model of pLG72 as well as the mode of interaction with hDAAO [20]. Notably, in cross-linking experiments, we identified a covalent bond between Thr182 in hDAAO and Lys62 in pLG72.

In order to delve into the structure–function relationships of this human protein and to shed light on its contribution to pathological conditions, we investigated the effect of two point mutations reported in the dbSNP NCBI database. The pLG72 protein containing an arginine at position 30 corresponds to the wild-type sequence. Despite this, when the literature refers to pLG72, the SNP rs2391191 (M15), which is related to schizophrenia susceptibility as reported earlier [4], is usually meant, i.e., the substitution of arginine 30 with a lysine (R30K), which has been related to decreased thickness of brain cortex in patients with schizophrenia [21] and with poor episodic memory function [22]. Here, the wild-type protein (pLG72 R30) was studied to clarify the properties altered by the pathological R30K substitution. The K62E pLG72 variant was also investigated, as this substitution is encoded by the rs9558562 SNP (not directly associated with a specific pathology) and involves a position corresponding to the residue cross-linked to hDAAO [20].

Materials and methods

Preparation of pLG72 variants

The gene encoding the R30 pLG72 variant was produced by mutagenic PCR (QuikChange II XL Site-Directed Mutagenesis kit; Agilent Technologies, Santa Clara, CA, USA) using as template the pET11a vector (Novagen, Madison, WI, USA) containing the *G72* R30K gene [18]. The ensuing plasmid was the template to generate the gene encoding the K62E pLG72 variant. The sequence of the amplification products was confirmed by DNA sequencing. For protein expression, the different constructs were used to transform BL21-Codon plus®(DE3)-RIL (Stratagene, San Diego, CA, USA) *Escherichia coli* cells. Cells growth and protein expression were as stated in Ref. [18].

pLG72 variant proteins were isolated from insoluble inclusion bodies obtained following cell lysis by sonication and centrifugation, as reported in Ref. [18]. Proteins were prepared in 20 mM Tris/HCl, pH 8.5, 100 mM NaCl, 5% (v/v) glycerol, and 5 mM 2-mercaptoethanol.

Structural and spectral analyses

The oligomeric state of pLG72 variants was studied by size-exclusion chromatography on a Superdex 200 10/30 column (GE Healthcare, Uppsala, Sweden) equilibrated in 20 mM Tris/HCl, pH 8.5, 100 mM NaCl, 0.1% (w/v) *N*-lauroylsarcosine (NLS), and 5 mM dithiothreitol. The interaction of pLG72 variants with hDAAO was investigated by the same procedure using 25 nmol of hDAAO, different amounts of pLG72 variants (12.5, 25, 50, and 75 nmol) and the following elution buffer: 20 mM Tris/HCl, pH 8.5, 150 mM NaCl, 5% (v/v) glycerol, 40 μM flavin adenine dinucleotide (FAD), 5 mM 2-mercaptoethanol, and 0.06% (w/v) NLS [5]. The presence of the two proteins in the eluted peaks was evaluated by western blot analysis by using anti-hDAAO and anti-pLG72 antibodies [5]. Recombinant hDAAO was produced as stated in Ref. [23]; hDAAO activity was assayed by the oxygen consumption assay [5,24].

Real-time binding assays were performed on a Biacore 3000 Surface Plasmon Resonance (SPR) instrument (GE Healthcare) as stated in Ref. [20]. Recombinant hDAAO (20 $\mu g \cdot mL^{-1}$) was immobilized on a CM5 Biacore sensor chip by using EDC/NHS chemistry. Binding assays were carried out by injecting 60 μL of different concentrations of pLG72 variants at 20 $\mu L \cdot min^{-1}$, using 10 mM Hepes, pH 7.5, 0.1 M NaCl, 3 mM EDTA, and 0.005% (v/v) P20 detergent as running buffer. The values for the SPR response at equilibrium (RU_{max} value) vs. the ligand concentration were fit based on the one-site binding equation.

UV-Visible absorbance spectra were recorded with a Jasco V-560 spectrophotometer (Jasco Europe, Cremello, Italy). Protein fluorescence and ligand-binding experiments were carried out using a Jasco FP-750 spectrofluorimeter at 0.25 or 0.5 $mg \cdot mL^{-1}$ protein concentration in 20 mM Tris/HCl, pH 8.5, 100 mM NaCl, 5% (v/v) glycerol, 0.06% (w/v) NLS, and 5 mM 2-mercaptoethanol as reported in Ref. [25]. During titration experiments, spectra were corrected for contribution of the ligand. In this regard, CPZ and FAD show a strong absorbance at ~ 260 nm and thus could result in quenching of fluorescence intensity: similar dissociation constant values for these compounds were previously obtained both by fluorescence changes and SPR analyses [5]. 8-anilinonaphtalene-1-sulfonate (ANS) fluorescence emission spectra were recorded in the 400- to 600-nm range (excitation at 370 nm) [26].

Circular dichroism (CD) spectra were recorded on a J-810 Jasco spectropolarimeter. Protein samples were used at 0.1 mg·mL^{-1} in 20 mM Tris/HCl, pH 8.5, 100 mM NaCl, 5% (v/v) glycerol or at 0.4 mg·mL^{-1} in 20 mM Tris/HCl, pH 8.5, 0.1 M NaCl, and 5% (v/v) glycerol for far-UV and near-UV CD measurements, respectively. All measurements were performed at 15 °C [18,25].

Temperature ramp experiments, in the 20–100 °C range, were carried out following the change on CD signals at 210 and 265 nm using a Peltier thermostatic system (temperature ramp of 0.5 °C·min^{-1}).

GTP/ATPase assay

pLG72 (10, 25, or 50 μg) was incubated in 500 μL of 10 mM HEPES, 0.5 mM $MgCl_2$, 20 mM KCl, 0.5 mM dithiothreitol, and 1 mM GTP or ATP at 37 °C for 1 h; 10% (w/v) SDS was then added to stop the reaction. An equal volume of a solution containing 1% (w/v) ammonium molybdate, 6% (w/v) ascorbic acid, 2% (w/v) sodium citrate, and 2% (v/v) acetic acid was added to the assay mixture, followed by incubation at 45 °C for 25 min. The release of inorganic phosphate was quantified by measuring absorbance at 660 nm [27–29]. ATPase/GTPase activity is reported as amount of released phosphate.

Docking analysis

Molecular docking studies were performed by employing AUTODOCK VINA, a package based on an iterated local search global algorithm [30]. The structure of the ligand GTP and the structure of a pLG72 model [20] produced by I-Tasser were used for docking simulations. Docking results were analyzed using PYMOL software (www.pymol.org).

Cell studies

U87 human glioblastoma cells (ATCC, LCG Standards, Teddington, UK) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin, and amphotericin B (Euroclone, Milano, Italy) at 37 °C in a 5% CO_2 incubator as reported in Refs. [5,6]. Cells were transfected using the FuGENE HD transfection reagent (Promega, Milan, Italy) and 2 μg of pCDNA3-pLG72 constructs encoding the different protein variants. The transfected cells were used to select stable clones by adding 0.4 mg·mL^{-1} G418. Protein expression levels were monitored by western blot analysis using anti-pLG72 and anti-actin antibodies (Sigma-Aldrich, St. Louis, MO, USA). The immunoreactivity signals were detected by enhanced chemiluminescence (ECL Plus; GE Healthcare) and the Odyssey Quantitative Imaging system (LI-COR, Lincoln, NE, USA). The intensity of anti-pLG72 signals were normalized to the values obtained with anti-actin antibody.

The stability of pLG72 variants at the cellular level was investigated using the different U87 pCDNA3 pLG72 clones treated with 100 μg·mL^{-1} cycloheximide (CHX; Sigma-Aldrich). At different time points, cells were harvested for western blot analysis [6,7].

Cellular D- and L-serine levels were determined employing 2.5×10^5 U87 cells expressing the different pLG72 variants and HPLC chromatography using a 5-μm Waters C8 reversed-phase column (Waters Corp., Milford, CT, USA) [5,7,31]. The analyses were replicated at least three times for each sample. Variation between groups was evaluated by one-way ANOVA, and *post hoc* significance tests were performed using a Student's *t*-test (significance was assessed at $P < 0.05$).

Results

R30K and K62E substitutions do not alter the structural/conformational properties of pLG72

The R30 (wild-type) and K62E pLG72 proteins were expressed in *E. coli* cells and purified using the condition previously established for the R30K variant [18]: an overall production of 250 mg of purified protein·L^{-1} fermentation broth was obtained for both variants (vs. a figure of 150 mg·L^{-1} for the R30K pLG72).

The size-exclusion chromatography elution profile at 0.1% (w/v) NLS of the R30K and K62E pLG72 variants resembled that of the wild-type (R30) protein, i.e., all pLG72 variants eluted in a main peak at ≈ 14.5 mL. Light-scattering analysis of the eluted peak demonstrated that all pLG72 variants were dimeric: estimated molecular mass ≈ 35 kDa (data not shown).

Analysis of the spectral properties revealed that both point substitutions altered to a limited extent the pLG72 protein conformation as compared to the wild-type counterpart. Tryptophan fluorescence showed an emission maximum at 339 nm for all pLG72 variants: the emission intensity was slightly lower for the R30K pLG72 than for the wild-type (R30) and K62E variants (the latter showing the comparatively higher intensity, Fig. 1A). Analogously, both the near- and the far-UV CD spectra showed overlapping signals for all pLG72 variants (Fig. 1C).

For all protein variants, binding of the fluorescent compound ANS increased the fluorescence of this hydrophobic probe remarkably, accompanied by a red shift in its emission fluorescence maximum (from 467 to 475 nm). The ANS fluorescence intensity at (the same) saturating probe concentration was slightly higher in K62E pLG72 than in R30 and R30K proteins (Fig. 1B). The K_d value determined for the binding of ANS was slightly higher in the R30K and K62E

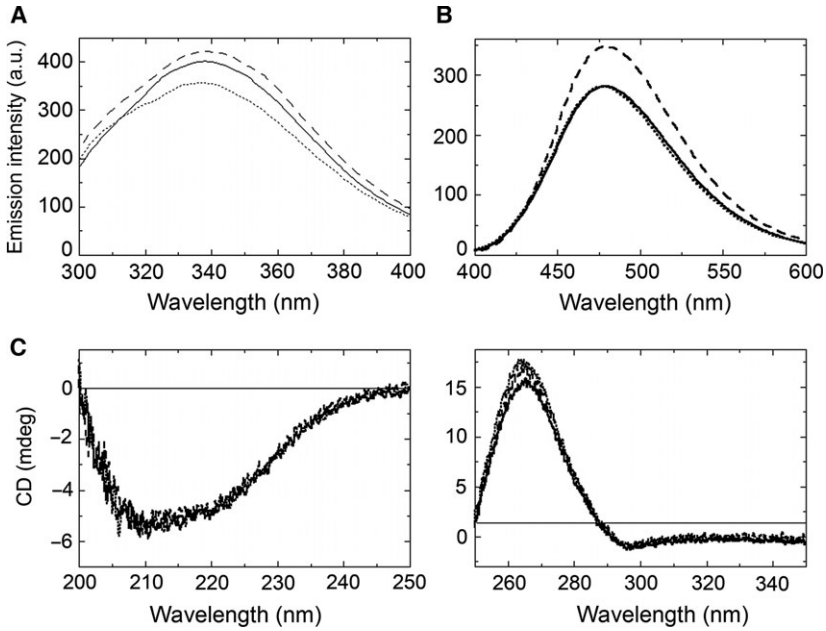

Fig. 1. Analysis of spectral properties related to the protein conformation of different pLG72 variants. (A) Protein fluorescence spectra (excitation at 280 nm), recorded at 0.75 mg·mL^{-1} protein concentration. (B) ANS fluorescence following the addition of 0.3–0.6 mM (saturating concentration) ANS to 0.25 mg·mL^{-1} pLG72 variants. (C) Far-UV CD spectra recorded at 0.1 mg·mL^{-1} protein concentration (left) and near-UV CD spectra recorded at 0.4 mg·mL^{-1} protein concentration (right). All the spectra were recorded at 15 °C. (——): R30 pLG72; (···): R30K pLG72; (– –): K62E pLG72.

variants than in the R30 pLG72; likewise, the calculated $\Delta E_{max}/K_d$ ratio was also decreased (Table 1). These results indicate that the two pLG72 variants expose similar amounts of hydrophobic surfaces with a comparatively larger accessibility for the wild-type protein.

Temperature-induced unfolding of pLG72 variants

The temperature sensitivity of pLG72 structural determinants was evaluated in temperature ramp experiments by following the near-UV CD signal and the protein fluorescence intensity at 340 nm. Monitoring the CD signal at 265 nm, the tertiary structure of all the protein variants was substantially modified upon shifting from the native to the unfolded state. The highest transition temperature was determined for R30K pLG72 (~ 3 °C higher, Table 1). Analogously, following the protein fluorescence signal at 340 nm, the melting temperature for the R30K variant was ~ 8 °C higher than for R30 pLG72. Intermediate T_m values were apparent for the K62E variant. Notably, the melting temperature values indicate that the change in protein fluorescence comes before that of the near-UV signal.

R30K and K62E substitutions do not alter ligand binding to pLG72

R30K pLG72 was previously reported to bind large, hydrophobic molecules such as the drug

Table 1. Comparison of binding of the hydrophobic probe ANS to three pLG72 variants and of the melting temperatures related to loss of tertiary structure. Values are reported as mean ± SD (n = 3). a.u., arbitrary units (relative intensity).

pLG72 variants									
	R30 (wild-type)			R30K			K62E		
ANS titration	ΔE_{max} (a.u.)	K_d (μM)	$\Delta E_{max}/K_d$	ΔE_{max} (a.u.)	K_d (μM)	$\Delta E_{max}/K_d$	ΔE_{max} (a.u.)	K_d (μM)	$\Delta E_{max}/K_d$
	273	51.3 ± 2.1	5.32	274	73.2 ± 15.3	3.74	335	93.5 ± 8.3	3.58
	Melting temperature (°C)								
Thermal unfolding									
Near-UV CD (265 nm)	59.5 ± 2.7			61.4 ± 1.7			60.5 ± 1.6		
Protein fluorescence (340 nm)	48.3 ± 4.8			56.5 ± 8.6			53.2 ± 3.7		

chlorpromazine (CPZ) and the cofactors FAD and FMN [5]. CPZ binding to the R30 and K62E variants of pLG72 was investigated by following the changes in protein fluorescence at increasing concentrations of this compound (results for R30 shown in Fig. 2A). A similar CPZ binding affinity was observed for all the pLG72 variants (Table 2). By the same experimental procedure, binding of FAD and FMN to the different pLG72 variants was also investigated, again showing quite similar K_d values (Table 2). Notably, the overall protein conformation of all pLG72 variants (as evaluated by recording far- or near-UV spectra) was not altered by the presence of a hydrophobic ligand such as FAD (40 μM) or by 0.5 mM Mg^{2+} (data not shown).

To shed light on the ability of pLG72 to bind nucleotides, the binding of GTPγS, a nonhydrolyzable form of nucleotide triphosphate, to the different pLG72 variants was studied (Fig. 2B). The K_d value estimated for the R30K pLG72 is 1.5-fold lower than for the R30 and K62E variants (Table 2). Indeed, pLG72 variants also bind GDP but with a 4- to 10-fold lower affinity compared to GTPγS.

Interestingly, docking analysis performed by means of AUTODOCK VINA software on a three-dimensional model of the pLG72 structure [20] shows a close proximity of the R30 side chain with the triphosphate moiety of the nucleotide (Fig. 2C). The ability of pLG72 to hydrolyze GTP was assessed by assaying the amount of phosphate released from 1 mM GTP after 60 min of incubation at 37 °C [27–29]. For all variants, the absorbance intensity at 660 nm could not be distinguished from the control performed in the absence of pLG72. Similarly, no ATPase activity was detected under identical conditions.

R30K and K62E substitutions alter to a limited extent the binding of pLG72 to hDAAO

The size-exclusion chromatography profile of all pLG72 variants at 0.06% (w/v) NLS (a detergent concentration that preserves full solubility of pLG72) [5] showed a main peak at ~ 14.7 mL and a second one in the dead volume (whose intensity corresponds to 25–30% of the principal peak). As previously observed for the R30K protein [18], the titration of a fixed amount of hDAAO (elution volume of 14.2 mL) with increasing amounts of wild-type (R30) or K62E pLG72 variants resulted in the formation of a ~ 200 kDa complex at 12.8 mL that is assumed to contain two hDAAO dimers and one pLG72 dimer (2 × 80 kDa + 2 × 18 kDa) (Fig. 3A–C). A saturation of the 200-kDa peak area was observed at 1 : 2 hDAAO : pLG72 molar ratio for R30K and K62E variants and at a larger hDAAO molar ratio for the R30 pLG72. The interaction between hDAAO and pLG72 was also investigated by SPR technology. From the response at increasing pLG72 concentration a stronger interaction was determined for the R30K variant (K_d = 2.2 ± 0.5 μM): a slightly weaker interaction was apparent for both the R30 and K62E pLG72 proteins, but the decrease was not statistically significant (Table 2).

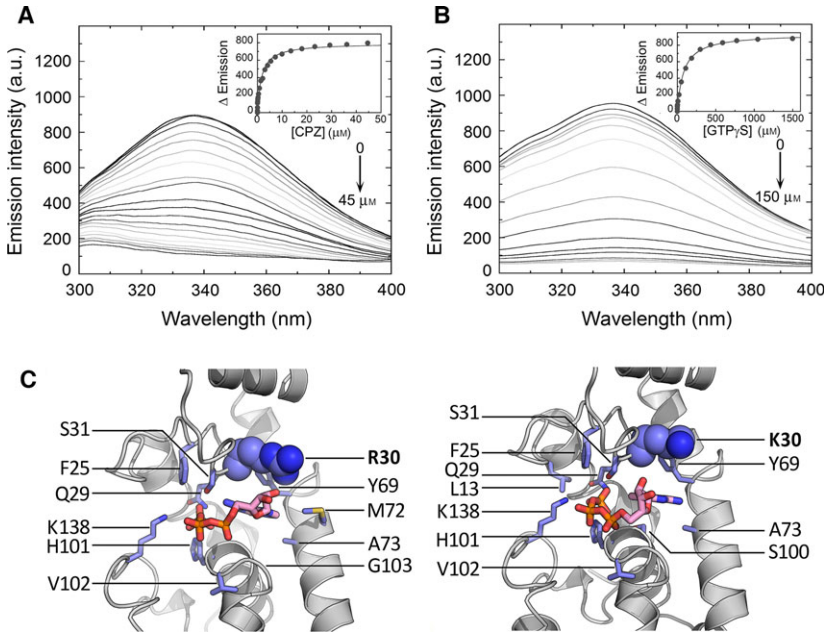

Fig. 2. Binding of ligands to pLG72 variants. (A) Increasing amounts of CPZ (0–45 μM) were added to a fixed amount of R30 pLG72 (0.8 μM) and the changes in protein fluorescence were recorded. (B) Titration of R30 pLG72 with increasing amounts of GTPγS (0–1.5 mM). Insets show the change in fluorescence intensity at 340 nm vs. ligand concentration. All measurements were carried out in 20 mM Tris/HCl, pH 8.5, 100 mM NaCl, 5% (v/v) glycerol, 0.06% (w/v) NLS, and 5 mM 2-mercaptoethanol, at 15 °C. (C) Proposed complex between GTP and pLG72 model [20] corresponding to the R30 (left) and R30K variant (right). Residues interacting with GTP are shown as sticks; the residue at position 30 is highlighted.

Table 2. Binding of different ligands to three pLG72 variants.

pLG72 variants			
	R30 (wild-type)	R30K	K62E
$K_{d,CPZ}$ (μM)	1.69 ± 0.09	1.56 ± 0.20	1.28 ± 0.03
$K_{d,FAD}$ (μM)	54.2 ± 9.3	50.7 ± 3.5	84.4 ± 3.2
$K_{d,FMN}$ (μM)	68.9 ± 1.9	36.4 ± 3.2	50.5 ± 4.5
$K_{d,GTP\gamma S}$ (μM)	72.5 ± 14.9	40.0 ± 8.2	64.6 ± 14.8
$K_{d,GDP}$ (μM)	287 ± 22	546 ± 82	690 ± 55
$K_{d,hDAAO}$ (μM)[a]	3.30 ± 1.00	2.15 ± 0.45	3.40 ± 1.07

Binding was determined following changes in protein fluorescence. The K_d values are the average of three independent experiments (mean ± SD).
[a]Determined from SPR analysis.

A main effect of R30K pLG72 binding on hDAAO function is a time-dependent inactivation of the flavoenzyme [5]. As shown in Fig. 4A, the R30 pLG72 was the less-effective variant in inhibiting hDAAO after 30 min of incubation. In particular, at 1 : 2 hDAAO : pLG72 molar ratio, residual activity was 15% and < 10% with the R30K and K62E variants vs. 50% for the R30 pLG72. Indeed, the time course of hDAAO inactivation slightly differed for the various pLG72 variants at different molar ratios (Fig. 4B–D): the R30K variant showed the fastest and most elevated inactivation at 1 : 0.5 molar ratio, while at higher ratios, the K62E pLG72 induced the fastest inactivation. Altogether, at hDAAO : pLG72 ratios > 1 : 4 and after 120 min of incubation, all pLG72 variants fully inactivated the flavoenzyme.

Cellular studies: D-serine concentration and pLG72 half-life

U87 glioblastoma cells stably transfected for R30K pLG72 and/or hDAAO fused to ECFP and EYFP, respectively, were previously used to investigate the effect of their expression levels on the cellular D-serine concentration [5,6,31]. Now, U87 cell clones stably transfected for R30, R30K, or K62E untagged pLG72 variants were selected based on the level of expression (i.e., the chosen clones possessed comparable levels by western blot analysis, not shown). In all cases, the relative level of cellular D-serine (expressed as D-/(D+L)-serine ratio) moderately increases as compared to cells transfected with the empty pcDNA3 plasmid (Table 3), indicating inhibition of endogenous hDAAO by overexpressed pLG72 variants. The modest increase in D-serine level as compared to control is due to the very low level of DAAO expression in this cell system [6,32]. Notably, the observed increase

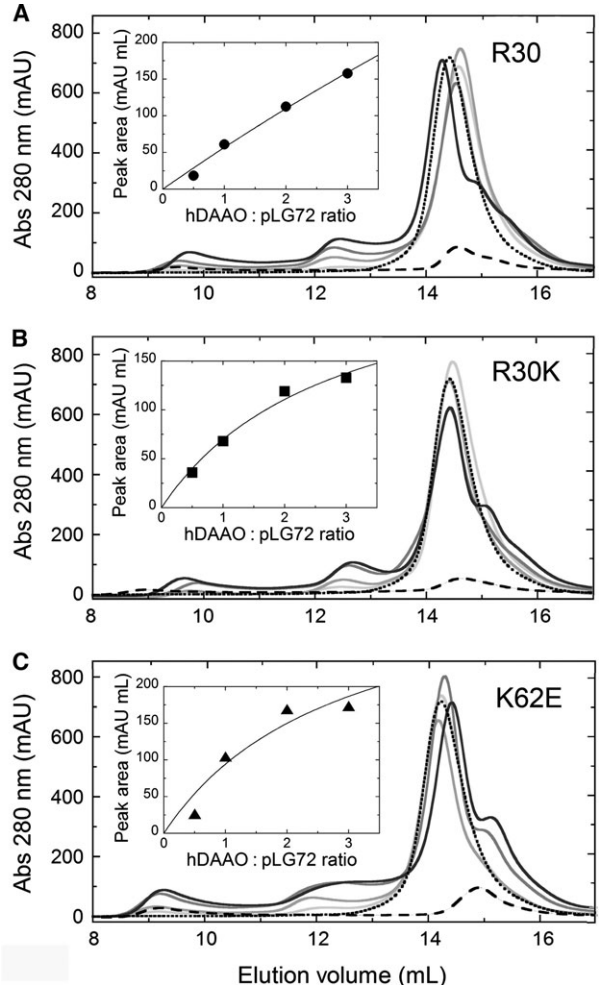

Fig. 3. Analysis by size-exclusion chromatography of pLG72 variants binding to hDAAO. (A–C) Elution profiles of mixtures containing 25 nmol of hDAAO (peak at 14.2 mL) and different amounts of pLG72 variants (from 12.5 to 75 nmol, gray curves; peak at ~ 14.7 mL): panel (A) R30 pLG72; panel (B) R30K pLG72; panel (C) K62E pLG72. The 200-kDa hDAAO : pLG72 complex shows an elution volume of 12.8 mL. Analyses were performed in 20 mM Tris/HCl, pH 8.5, 150 mM NaCl, 5% (v/v) glycerol, 40 μM FAD, 5 mM 2-mercaptoethanol, and 0.06% (w/v) NLS. Protein mixtures were incubated for 10 min at 4 °C and centrifuged before injection. Dotted curves: 25 nmoles hDAAO; dashed curves: 25 nmoles pLG72 variants. Insets: effect of hDAAO : pLG72 ratio on the area of the peak corresponding to the protein complex.

was stronger for the clones expressing the R30K and K62E pLG72 variants than the wild-type (R30) protein; in all cases, the change was not statistically significant ($P > 0.05$).

We investigated the half-lives of pLG72 variants in U87 glioblastoma cells stably expressing the human protein following cycloheximide treatment. Previous studies revealed that pLG72-ECFP is rapidly degraded

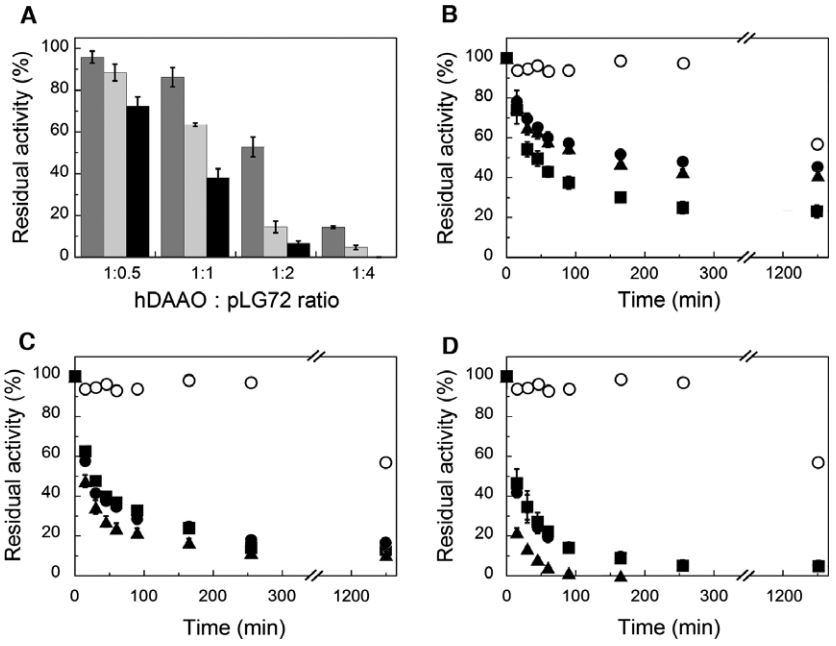

Fig. 4. Effect of pLG72 binding on hDAAO activity. (A) Residual activity of hDAAO (1.3 nmol) 30 min after adding increasing amounts of pLG72 variants (0.65, 1.3, 2.6, and 5.2 nmol) at 25 °C. Results for R30, R30K, and K62E pLG72 are represented by gray, light gray, and black bars, respectively. (B–D) Time course of hDAAO inactivation (4.55 nmol) when increasing amounts of pLG72 variant are added (B: 2.275; C: 4.55; D: 9.1 nmol) at 25 °C. Results are shown for hDAAO alone (○), and with addition of the variants R30 (●), R30K (■), or K62E pLG72 (▲). The enzymatic activity was assayed by the oxygen consumption method with 100 mM D-serine, and withdrawing aliquots of the incubation mixtures at different times. The activity of hDAAO alone at the beginning of the incubation was fixed as 100%. Experimental values are reported as mean ± SD (n = 3).

Table 3. Effect of stable overexpression of three different pLG72 variants on cellular D-serine level in human glioblastoma U87 cells.

	Control	pLG72 variants		
	Empty vector	R30 (wild-type)	R30K	K62E
D-/(D+L)-serine × 100 (%)	2.81 ± 0.63	3.09 ± 1.06	3.52 ± 1.07	3.68 ± 0.97

The values are the average of three independent experiments (mean ± SD). In all cases, the change was not statistically significant ($P > 0.05$).

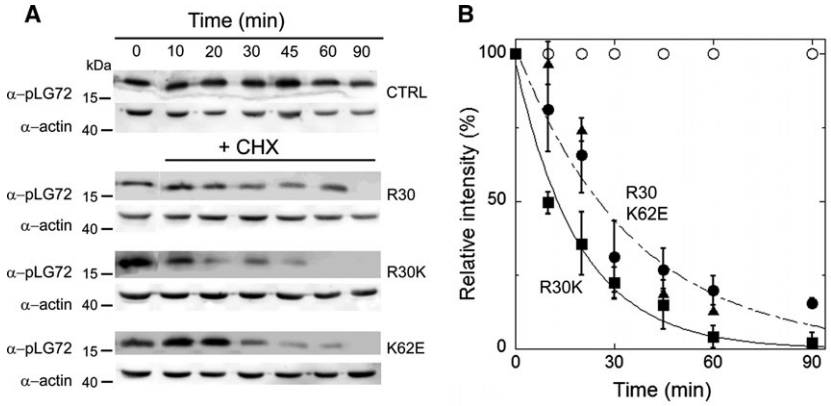

Fig. 5. Analysis of pLG72 degradation rate. U87 cells stably expressing pCDNA3-pLG72 R30, -pLG72 R30K, or -pLG72 K62E were treated with 100 μg·mL^{-1} CHX (or 0.1% DMSO as control). Cells were harvested at the times shown and the proteins were separated by SDS/PAGE and immunoblotted. (A) Western blot analysis using an anti-pLG72 antibody reveals that the pLG72 signal strongly and quickly decreases following CHX treatment. (B) The values from the densitometric analysis were fitted using a single exponential decay: control (no CHX addition, ○); pLG72 R30 (●); R30K (■); K62E (▲). Values are the mean ± SD (n = 3), normalized to actin and expressed relative to the untreated control.

in CHX-treated U87 cells (within 1 h) [6] and a half-life of ~ 40 min was estimated [7]. As shown in Fig. 5, the untagged R30K pLG72 shows a significantly faster turnover than the R30 and the K62E variants (13.6 ± 3.1, 23.9 ± 3.4, and 21.0 ± 0.9 min, respectively).

Discussion

In the last few years, a number of candidate genes have been proposed that may be responsible for increased susceptibility to schizophrenia. Now it seems that schizophrenia is influenced by genetic variation of hundreds of different loci [33]. With the discovery of the correlation between *G72* and *DAAO* genes and schizophrenia, a biochemical system was identified that might be responsible for symptoms of a psychiatric disorder by affecting NMDAR function [1,4,5]. In order to demonstrate a link between the pLG72-hDAAO system and schizophrenia, we compared the features of three pLG72 variants: the R30 pLG72 corresponding to the wild-type protein, the R30K pLG72 variant that was associated to schizophrenia susceptibility (SNP rs2391191, M15) [4], and the K62E point variant corresponding to SNP rs9558562.

All pLG72 proteins were dimeric and their conformation was only slightly modified by the substitution at position 30 or 62 (Table 1). A moderately more rigid conformation for the pathological R30K variant was apparent as assessed by thermal denaturation, as its melting temperature was 2–8 °C higher than that of wild-type pLG72 (Table 2). The small alterations in protein conformation did not affect the binding of hydrophobic ligands such as CPZ, FAD, FMN, or GDP/GTPγS (Table 3). Notably, no GTPase or ATPase activity was seen for any of the pLG72 variants. Concerning the binding of the physiological protein partner hDAAO, all pLG72 variants formed the 200-kDa complex (as shown by size-exclusion chromatography, Fig. 3) and interacted with similar affinity with hDAAO, as was apparent by SPR analyses.

A significant difference among pLG72 variants was apparent following the time course of hDAAO inactivation at increasing pLG72 molar ratios: the R30 pLG72 was less effective at inhibiting the flavoenzyme after 30 min of incubation, while the K62E variant gave the highest degree of inhibition (Fig. 3A). However, at hDAAO : pLG72 ratios $> 1 : 4$ and after 120 min of incubation, the flavoenzyme was fully inhibited by all the three pLG72 variants. Notably, the stronger *in vitro* hDAAO inhibition by the two single-point variants as compared to the R30 pLG72 agrees with the observed increase in cellular D-/(D+L)-serine levels in human glioblastoma U87 cells stably overexpressing the different pLG72 variants (Table 3).

Overall, the small alteration in protein conformation due to the conservative substitution of R30 with a lysine—a residue proposed to line the binding pocket for hydrophobic ligands [20]—results in a more rigid conformation, yielding a stronger hDAAO inhibition at the lowest molar ratios. On the other hand, the nonconservative substitution of K62—a residue proposed to be located on the protein surface [20]—with a glutamate yields a stronger inhibition at the highest hDAAO : pLG72 ratios. This can be due to the additional charge-dipole interaction between E62 in the pLG72 variant and the side chain of T182 in hDAAO, the interaction residues identified by cross-linking experiments [20].

All pLG72 variants show the ability to inhibit hDAAO, yielding an increase in D-serine concentration when overexpressed in a cellular system. This generates a condition opposite than that reported in schizophrenia, i.e., a decrease in D-serine concentration [34,35]. This apparent discrepancy is solved taking into consideration the half-life of pLG72 variants. The R30K variant is significantly more prone to degradation compared to its R30 counterpart (half-life of 13.6 vs. 23.9 min). This seems to be a specific effect of the R30K substitution, as it was not observed for the K62E pLG72. The faster turnover of R30K pLG72 decreases the cellular concentration of this negative modulator of the flavoenzyme, leading to a less effective inactivation of hDAAO and in turn generating a decrease in D-serine cellular concentration, a condition that affects neurotransmission. This observation matches with NMDAR hypofunction, which is a central component in the glutamatergic hypothesis of schizophrenia onset [36].

hDAAO activity has been reported to be increased in schizophrenia [37,38] and D-serine levels reduced in specific brain areas and in serum [34,35,37]. pLG72 affects the neuromodulator availability at synapses by influencing on hDAAO activity and stability. Accordingly, we previously proposed that pLG72 hypoexpression promotes NMDAR hypofunctionality and schizophrenia onset [1,5]. On the other hand, the properties of wild-type (R30) and R30K variants do not correlate with the proposal that pLG72 is an inactive inhibitor of hDAAO [21] or an activator of hDAAO function [4,39]. Concerning the hypothesis that pLG72 binds to the FMN-containing oxidoreductase of complex I in mitochondria and thus modulates its activity, as proposed earlier [40], we can exclude that the pathological R30K substitution favors this direct interaction with the cofactor as K_d values for FMN/FAD were similar for wild-type and variants of pLG72.

Elucidating the role of pLG72 is of utmost relevance as the pharmacological modulation of hDAAO-pLG72 interaction is expected to open up possibilities to target complex diseases by acting on D-serine levels [9,24,41]. Modulation of hDAAO activity exerted by pLG72 binding was recently investigated by developing

novel classes of hDAAO inhibitors. 'Class C' compounds are less active when pLG72 is present, while 'class A' compounds are more potent hDAAO inhibitors when pLG72 is present [24].

Acknowledgements

We thank Dr Gianluca Molla for docking analyses. This work was supported by grants from Fondo di Ateneo per la Ricerca to SS and LP. We thank the support from Consorzio Interuniversitario per le Biotecnologie.

Author contributions

SS and LoP planned the experiments and wrote the paper. SS and PC prepared pLG72 variants and performed biochemical characterization; and PC performed cell studies. LuP, GS, and EP performed spectral, size-exclusion chromatography, and SPR analyses.

References

1 Sacchi S, Binelli G and Pollegioni L (2016) G72 primate-specific gene: a still enigmatic element in psychiatric disorders. *Cell Mol Life Sci* **73**, 2029–2039.
2 Owen MJ, Williams NM and O'Donoval MC (2004) The molecular genetics of schizophrenia: new findings promise new insights. *Mol Psychiatry* **9**, 14–27.
3 Yang H, Liu C, Liu Y, Chen C, Chang CC, Fann CSY, Chiou J-J, Yang U-C, Chen C-H, Faraone SV *et al.* (2013) The *DAO* gene is associated with schizophrenia and interacts with other genes in the Taiwan Han Chinese population. *PLoS One* **8**, e60099.
4 Chumakov I, Blumenfeld M, Guerassimenko O, Cavarec L, Palicio M, Abderrahim H, Bougueleret L, Barry C, Tanaka H, La Rosa P *et al.* (2002) Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proc Natl Acad Sci USA* **99**, 13675–13680.
5 Sacchi S, Bernasconi M, Martineau M, Mothet JP, Ruzzene M, Pilone MS, Pollegioni L and Molla G (2008) pLG72 modulates intracellular D-serine levels through its interaction with D-amino acid oxidase: effect on schizophrenia susceptibility. *J Biol Chem* **283**, 22244–22256.
6 Sacchi S, Cappelletti P, Giovannardi S and Pollegioni L (2011) Evidence for the interaction of D-amino acid oxidase with pLG72 in a glial cell line. *Mol Cell Neurosci* **48**, 20–28.
7 Cappelletti P, Campomenosi P, Pollegioni L and Sacchi S (2014) The degradation (by distinct pathways) of human D-amino acid oxidase and its interacting partner pLG72–two key proteins in D-serine catabolism in the brain. *FEBS J* **281**, 708–723.
8 Pollegioni L and Sacchi S (2010) Metabolism of the neuromodulator D-serine. *Cell Mol Life Sci* **67**, 2387–2404.
9 Sacchi S, Caldinelli L, Cappelletti P, Pollegioni L and Molla G (2012) Structure-function relationships in human D-amino acid oxidase. *Amino Acids* **43**, 1833–1850.
10 Snyder SH and Kim MP (2000) D-amino acids as putative neurotransmitters: focus on D-serine. *Neurochem Res* **25**, 553–560.
11 Machado-Vieira R, Manij HK and Zarate CA (2009) The role of the tripartite glutamatergic synapse in the pathophysiology and therapeutics of mood disorders. *Neuroscientist* **15**, 525–539.
12 Mundo E, Tharmalingham S, Neves-Pereira M, Dalton EJ, Macciardi F, Parikh SV, Bolonna A, Kerwin RW, Arranz MJ, Makoff AJ *et al.* (2003) Evidence that the N-methyl-D-aspartate subunit 1 receptor gene (GRIN1) confers susceptibility to bipolar disorder. *Mol Psychiatry* **8**, 241–245.
13 Sasabe J, Chiba T, Yamada M, Okamoto K, Nishimoto I, Matsuoka M and Aiso S (2007) D-serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis. *EMBO J* **26**, 4149–4159.
14 Bagetta V, Ghiglieri V, Sgobio C, Calabresi P and Picconi B (2010) Synaptic dysfunction in Parkinson's disease. *Biochem Soc Trans* **38**, 493–497.
15 Demuro A, Parker I and Stutzmann GE (2010) Calcium signaling and amyloid toxicity in Alzheimer's disease. *J Biol Chem* **285**, 12463–12468.
16 Labrie V and Roder JC (2010) The involvement of the NMDA receptor D-serine/glycine site in the pathophysiology and treatment of schizophrenia. *Neurosci Biobehav Rev* **34**, 351–372.
17 Kvajo M, Dhilla A, Swor DE, Karayiorgou M and Gogos JA (2008) Evidence implicating the candidate schizophrenia/bipolar disorder susceptibility gene *G72* in mitochondrial function. *Mol Psychiatry* **13**, 685–696.
18 Molla G, Bernasconi M, Sacchi S, Pilone MS and Pollegioni L (2006) Expression in *Escherichia coli* and in vitro refolding of the human protein pLG72. *Protein Expr Purif* **46**, 150–155.
19 Caldinelli L, Molla G, Bracci L, Lelli B, Pileri S, Cappelletti P, Sacchi S and Pollegioni L (2010) Effect of ligand binding on human D-amino acid oxidase: implications for the development of new drugs for schizophrenia treatment. *Protein Sci* **19**, 1500–1512.
20 Birolo L, Sacchi S, Smaldone G, Molla G, Leo G, Caldinelli L, Pirone L, Eliometri P, Di Gaetano S, Orefice I *et al.* (2016) Regulating levels of the neuromodulator D-serine in human brain: structural insight into pLG72 and D-amino acid oxidase interaction. *FEBS J* **283**, 3353–3370.

21 Schultz CC, Nenadic I, Koch K, Wagner G, Roebel M, Schachtzabel C, Mühleisen TW, Nöthen MM, Cichon S, Deufel T *et al.* (2011) Reduced cortical thickness is associated with the glutamatergic regulatory gene risk variant DAOA Arg30Lys in schizophrenia. *Neuropsychopharmacology* **36**, 1747–1753.
22 Donohoe G, Morris DW, Robertson IH, McGhee KA, Murphy K, Kenny N, Clarke S, Gill M and Corvin AP (2007) DAOA ARG30LYS and verbal memory function in schizophrenia. *Mol Psychiatry* **12**, 795–796.
23 Molla G, Sacchi S, Bernasconi M, Pilone MS, Fukui K and Pollegioni L (2006) Characterization of human D-amino acid oxidase. *FEBS Lett* **580**, 2358–2364.
24 Terry-Lorenzo RT, Masuda K, Sugao K, Fang QK, Orsini MA, Sacchi S and Pollegioni L (2015) High-throughput screening strategy identifies allosteric, covalent human D-amino acid oxidase inhibitor. *J Biomol Screen* **20**, 1218–1231.
25 Caldinelli L, Iametti S, Barbiroli A, Bonomi F, Fessas D, Molla G, Pilone MS and Pollegioni L (2005) Dissecting the structural determinants of the stability of cholesterol oxidase containing covalently bound flavin. *J Biol Chem* **280**, 22572–22581.
26 Caldinelli L, Iametti S, Barbiroli A, Bonomi F, Ferranti P, Pilone MS and Pollegioni L (2004) Unfolding of the peroxisomal flavoprotein D-amino acid oxidase. *J Biol Chem* **279**, 28426–28434.
27 Matambo TS, Odunuga OO, Boshoff A and Blatch GL (2004) Overproduction, purification, and characterization of the *Plasmodium falciparum* heat shock protein 70. *Protein Expr Purif* **33**, 214–222.
28 Dang W, Zhang M and Sun L (2011) *Edwardsiella tarda* DnaJ is a virulence-associated molecular chaperone with immunoprotective potential. *Fish Shellfish Immunol* **31**, 182–188.
29 Cappelletti P, Binda E, Tunesi M, Albani D, Giordano C, Molla G and Pollegioni L (2016) Recombinant human Tat-Hsp70-2: a tool for neuroprotection. *Protein Expr Purif*, doi: 10.1016/j.pep.2016.07.005.
30 Trott O and Olson AJ (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J Comput Chem* **31**, 455–461.
31 Caldinelli L, Sacchi S, Molla G, Nardini M and Pollegioni L (2013) Characterization of human DAAO variants potentially related to an increased risk of schizophrenia. *Biochim Biophys Acta* **1832**, 400–410.
32 Shoji K, Mariotto S, Ciampa AR and Suzuki H (2006) Mutual regulation between serine and nitric oxide metabolism in human glioblastoma cells. *Neurosci Lett* **394**, 163–167.
33 Sullivan PF, Daly MJ and O'Donovan M (2012) Genetic architectures of psychiatric disorders: the emerging picture and its implications. *Nat Rev Genet* **13**, 537–551.
34 Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N, Nakazato M, Kumakiri C, Okada S, Hasegawa H *et al.* (2003) Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. *Arch Gen Psychiatry* **60**, 572–576.
35 Bendikov I, Nadri C, Amar S, Panizzutti R, De Miranda J, Wolosker H and Agam G (2007) A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia. *Schizophr Res* **90**, 41–51.
36 Lisman JE, Coyle JT, Green RW, Javitt DC, Benes FM, Heckers S and Grace AA (2008) Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia. *Trends Neurosci* **31**, 234–242.
37 Madeira C, Freitas ME, Vargas-Lopes C, Wolosker H and Panizzutti R (2008) Increased brain D-amino acid oxidase (DAAO) activity in schizophrenia. *Schizophr Res* **101**, 76–83.
38 Habl G, Zink M, Petroianu G, Bauer M, Schneider-Axmann T, von Wilmsdorff M, Falkai P, Henn FA and Schmitt A (2009) Increased D-amino acid oxidase expression in the bilateral hippocampal CA4 of schizophrenic patients: a post-mortem study. *J Neural Transm* **116**, 1657–1665.
39 Chang SLY, Hsieh CH, Chen YJ, Wang CM, Shih CS, Huang PW, Mir A, Lane HY, Tsai GE and Chang HT (2014) The C-terminal region of G72 increases D-amino acid oxidase activity. *Int J Mol Sci* **15**, 29–43.
40 Otte DM, Sommersberg B, Kudin A, Guerrero C, Albayram O, Filiou MD, Frisch P, Yilmaz O, Drews E, Turck CW *et al.* (2011) N-acetyl cysteine treatment rescues cognitive deficits induced by mitochondrial dysfunction in G72/G30 transgenic mice. *Neuropsychopharmacology* **36**, 2233–2243.
41 Sacchi S, Rosini E, Pollegioni L and Molla G (2013) D-amino acid oxidase inhibitors as a novel class of drugs for schizophrenia therapy. *Curr Pharm Des* **19**, 2499–2511.