Cellular levels of D-amino acid oxidase are modulated through pLG72 interaction

Cappelletti P., Pollegioni L. and Sacchi S.

Dipartimento di Biotecnologie e Scienze della Vita, Università degli studi dell'Insubria, via J.H. Dunant 3, 21100 Varese, and Centro Interuniversitario di Ricerca in Biotecnologie Proteiche "The Protein Factory", Politecnico di Milano, ICRM-CNR Milano and Università degli studi dell'Insubria, Italy

Address correspondence to: Sacchi Silvia, Tel.: 0332 421504; Fax: 0332 421500; E-Mail: silvia.sacchi@uninsubria.it

Abbreviations

CHX, cicloheximide; DAAO, D-amino acid oxidase; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; FAD, flavin adenine dinucleotide; hDAAO, human D-amino acid oxidase; NMDAr, N-methyl-D-aspartate receptor; SR, serine racemase; UB, ubiquitin; UPS, ubiquitin proteasome system.

Enzymes

D-Amino acid oxidase, EC 1.4.3.3

Keywords

D-serine; neuromodulator; schizophrenia; degradation pathway; regulation.

Abstract

D-amino acid oxidase (DAAO) is a FAD-dependent peroxisomal flavoenzyme significantly enriched in the mammalian brain. DAAO has been proposed to play (together with serine racemase) an essential role in the metabolism of D-serine, an "atypical", key signalling molecule acting in the nervous system as an allosteric activator of the N-methyl-D-aspartate-type glutamate receptor. Human DAAO (hDAAO), itself and through the interacting partner pLG72, has been related to schizophrenia, an highly disabling psychiatric disorder in which a dysfunction of NMDA mediated

neurotransmission is widely assumed to occur. We previously demonstrated that a major effect of the hDAAO-pLG72 complex formation is the progressive destabilization and inactivation of the flavoenzyme. Furthermore, we provided convergent lines of evidence that D-serine cellular concentration depends on hDAAO and pLG72 expression levels, and that newly synthesized hDAAO interacts with its (negative) modulator in the cytosol. Here we investigated the degradation pathways of hDAAO or pLG72 in U87 human glioblastoma cells stably expressing EYFP-hDAAO, hDAAO-EYFP or pLG72-ECFP fusion proteins. In our cellular system, hDAAO is a stable “long-lived protein”: the largest part of the flavoprotein is degraded by the lysosomal/endosomal pathway while a minor amount is polyubiquitinated and targeted to the proteasome. On the contrary, pLG72 is characterized by rapid turnover ($t_{1/2} \sim 25$ minutes) and is essentially degradated through the UPS. Overexpression of pLG72 increases the turnover of hDAAO thus suggesting that pLG72 binding to hDAAO yields an instable complex prone to degradation, in turn playing a protective role against excessive D-serine depletion. Our results uncover basic molecular aspects of D-serine metabolism and open novel perspectives for therapeutic strategies to treat schizophrenia by targeting the catabolic side of D-serine metabolism.

Introduction

Over time, an increasing body of evidence strongly supports a mutual communication between neurons and glia (1, 2). Astroglial cells control synapse development (3), regulate synaptic function (4), and overall synchrony in neuronal network (5, 6). In particular, astrocytes are in close structural and functional relation with neurons: their processes unsheath the synapses (7); they sense the level of synaptic activity through a plethora of ion channels, high affinity transporters and receptors expressed at their surface; they respond to changes in neuronal activity through intracellular calcium elevation, which in turn triggers other signalling mechanisms (8, 9). It has become clear that a significant mechanism of this astrocytes-to-neuron “intimate dialogue” is mediated by the release of signalling molecules that act as neuromodulators, since they can regulate synaptic function and neuronal excitability (2). Among them worthy of note is D-serine. This molecule, previously considered an “unnatural amino acid”, is a key brain regulator by acting as a physiological ligand for the

strychnine-insensitive glycine modulatory site of N-methyl-D-aspartate receptors (NMDAr), a pivotal receptor for excitatory transmission and cognitive function (10-12). Together with glutamate, endogenous D-serine mediates several NMDAr-dependent processes (13, 14) including excitotoxicity (15) and synaptic plasticity (16). Its removal decreases NMDAr responses (13), and it is required for the long term potentiation of the synaptic transmission in the hippocampus, which is thought to be involved in memory formation (17). This neuromodulator is produced by the isomerization reaction from L-serine catalyzed by the pyridoxal-5' phosphate dependent enzyme serine racemase (SR) (18, 19); its degradation is due to SR (through an α,β-elimination reaction) (20, 21) and/or by the FAD-containing flavoprotein D-amino acid oxidase (DAAO); for extensive reviews see (22, 23).

D-Serine signalling dysregulation might be involved in the NMDAr dysfunction that occurs in several pathologies, including neurodegenerative and neuro-psychiatric diseases, such as schizophrenia (24, 25). A number of recent studies indicated that the level of endogenous D-serine may be decreased in schizophrenic patients (26-28), and that the administration of D-serine greatly ameliorated the typical positive, negative and cognitive symptoms of schizophrenia when associated with conventional neuroleptics (29-31). Indeed, among the identified possible schizophrenia susceptibility genes, polymorphisms (SNPs) in the genes encoding for DAAO and SR have been reported, as reviewed in (23). Moreover, an association between schizophrenia and nucleotide variations in the new human gene G72 was also reported: this gene encodes for pLG72, a protein present in primates only, expressed mainly in brain, and that specifically interacts with human DAAO (hDAAO) (32).

Despite the important roles proposed for the endogenous D-serine, up to now little is known about the regulation of its cellular levels by DAAO-mediated degradation process. Several evidences strongly support the intimate relationship between glutamatergic neurotransmission and DAAO activity: D-serine levels are inversely related to the regional expression of DAAO during development (33) and application of DAAO to brain slices or cell cultures has a considerable effect on NMDAr-dependent synaptic transmission and neuronal plasticity (13, 16, 17, 34) or protects neurons from the NMDAr-related excitotoxicity (35-37). We recently showed that hDAAO and pLG72 are both expressed in human frontal cortex and they are localized in the same cell type (astrocytes). We also demonstrated that *in vitro* pLG72 acts as “inactivator” of hDAAO (38) and, by using U87

glioblastoma cells trasfected with plasmids encoding for hDAAO and/or pLG72, established that the cellular concentration of D-serine depends on the expression of the active form of this flavooxidase. Furthermore, the newly synthesized hDAAO is transiently cytosolic (and active), where it can interact with pLG72 (likely located on the external membrane of mitochondria) before being targeted to peroxisomes (39). Both *in vitro* (40) and cellular studies (39), indicated that pLG72 binding alters hDAAO stability and significantly affects its cellular half life. We proposed a model to explain the role of hDAAO and pLG72 on the D-serine cellular level (and thus on schizophrenia onset): a decrease in pLG72 expression yields to an anomalous high level of hDAAO activity and therefore to an excessive decrease in the local concentration of D-serine (38). All together these findings highlight an outstanding role of DAAO in the modulation of NMDAr activity: since the co-agonist binding site of the receptor is not saturated under normal conditions (41), it is likely that even small changes in D-serine level might exert remarkable effects on the functionality of the ionic channel.

In order to clarify the mechanisms that modulate the NMDAr-mediated neurotransmission (and the related pathological states) by D-serine, the clarification of factors affecting the amount and activity level of SR and DAAO enzymes is a crucial topic. SR is degraded by the ubiquitin proteasome system (UPS) (42): moreover, Golga3 (a protein associated to the cytosolic surface of the Golgi apparatus) modulates SR ubiquitination significantly increasing its half life. Notably, nothing is known about the mechanism(s) by which hDAAO and pLG72 expression/degradation is regulated. Thus, we here report on the investigation of the degradation pathway of this key human flavoenzyme (both the peroxisomal EYFP-hDAAO enzyme form and its cytosolic hDAAO-EYFP counterpart) and of its interacting protein pLG72.

Results

pLG72 is a short-lived protein whereas hDAAO is a stable protein (within U87 cells)

To deep insight on the mechanisms modulating hDAAO and pLG72 cellular levels, we investigated their half lives in U87 glioblastoma cells stably expressing the fluorescent-tagged chimeric proteins EYFP-hDAAO,

hDAAO-EYFP or pLG72-ECFP (U87 EYFP-hDAAO, U87 hDAAO-EYFP and U87 pLG72-ECFP cells, respectively) treated with cicloheximide (CHX). A previous investigation revealed that pLG72-ECFP is rapidly degraded in treated cells (within 1 hour) (39). Accordingly, U87 pLG72-ECFP expressing cells were analyzed up to 90 minutes upon the addition of the ribosomal inhibitor: at the end of the treatment the amount of pLG72-ECFP detected by Western blot analysis was ≈10% with respect to control cells (Fig. 1A, left panel). Densitometric analysis (shown in Fig. 1A right panel) highlighted a fast degradation of pLG72-ECFP, suggesting that pLG72 is a short-lived protein, with an estimated half life of ≈25 minutes. This result is not related to the degradation of the fluorescent ECFP tag itself since the time course of control experiments performed on U87 ECFP-expressing cells did not show any change in its relative cellular concentration (not shown): in our cellular system the fluorescent protein is highly stable.
On the other hand, the same experiments performed on U87 EYFP-hDAAO and hDAAO-EYFP (prolonged up to 12 hours from CHX addition) suggest that hDAAO is a long-lived protein: at the end of the incubation, no significant change in the level of the expressed protein was detected (Fig. 1B, C, top panels). Densitometric analyses of the corresponding immunorecognition signals estimated an half life of ≈ 60 hours for both EYFP-hDAAO and hDAAO-EYFP (Fig. 1B, C, bottom panels).
Taken together these results suggest that when hDAAO and pLG72 are individually overexpressed in U87 cells they show a different degradation kinetics, pointing to different degradation processes.

hDAAO degradation takes place largely in lysosomes

Since hDAAO is known as a peroxisomal protein and such a sub-cellular localization has been confirmed for EYFP-hDAAO fusion protein in the U87 EYFP-hDAAO cells (39), it is reasonable to assume that it might be mainly degraded through macroautophagy processes involved in the disposal of proteins and complexes, as well as whole organelles, through the lysosomal/endosomal system. To investigate whether EYFP-hDAAO is effectively targeted to this degradation pathway, we incubated U87 EYFP-hDAAO cells overnight in starvation conditions -DMEM + 1% FBS- in order to maximize the effect of the subsequent treatment. Then we added chloroquine (75 μM, a strong inhibitor of the lysosome degradation pathway): cells were collected at different intervals up to 6 hours after

treatment. In treated cells the amount of EYFP-hDAAO detected by Western blot analysis was significantly higher than in control ones: an up to 3-fold increase at 3 hours from the addition of the inhibitor was observed (Fig. 2A). Analogous treatments with a different inhibitor (NH_4Cl, 10 mM) confirmed the accumulation of EYFP-hDAAO (~ 4-fold, data not shown) following the blockade of the lysosomal degradation pathway.

The experiments with chloroquine and NH_4Cl were repeated on U87 hDAAO-EYFP. In these cells the expressed EYFP-tagged protein is cytosolic since the fluorescent protein mask the PTS1 targeting sequence (-SHL) required to address hDAAO to peroxisomes. Noteworthy, in this case the time course of hDAAO-EYFP cellular levels did not show variation following the treatment although a significant lower amount of the protein was immunodetected in treated cells with respect to control ones (~50%, Fig. 2B). This observation suggests that the localization of the protein is a key factor in establishing the pathway of hDAAO degradation (and regulating its cellular concentration).

In order to address the signal that drives hDAAO to degradation, we investigated the ubiquitination state of the chimeric protein. Samples of U87 EYFP-hDAAO cells treated for 3 hours with chloroquine were used for immunoprecipitation experiments using rabbit anti-hDAAO polyclonal antibodies. The amount of the immunoprecipitated EYFP-DAAO was significantly higher (~ 3-fold, Fig. 2C) in treated cells than in control (untreated) ones, confirming a substantial accumulation of the EYFP-tagged hDAAO upon the addition of the lysosome inhibitor. Interestingly, the same increase (~ 3-fold) in the total ubiquitination signal (detected by the anti-P4D1 antibody that recognizes both mono- and polyubiquitin chains) was evident in the immunoprecipitated samples from treated cells with respect to control ones (Fig. 2C). On the other hand, no change was observed for the anti-FK1 immunorecognition signal which specifically detects the presence of polyubiquitin chains. These results indicate that the blockage of the lysosomal/endosomal degradation pathway affects extent the ubiquitination level of EYFP-hDAAO to a significant: i.e. hDAAO is likely labelled through the binding of a single ubiquitin molecule.

Overexpressed hDAAO is (partially) degraded by the proteasome machinery

In order to verify if hDAAO cellular levels are regulated by degradation mechanisms alternative to the processes mediated by the lysosomes system, U87 EYFP-hDAAO cells were treated with MG132 and ALLN, two proteasomal inhibitors which bind to the “chymotrypsin–like” component blocking one or more of the peptidase activities within the 20S proteasome core (43) but which do not affect the activity of protein-ubiquitinating and -deubiquitinating enzymes. Intriguingly, MG132 treatment (25 μM in the starvation culture medium) resulted in a gradual increase of the observed EYFP-hDAAO cellular level. As shown by Western blot analysis, at 6 hours from the addition of the inhibitor the amount of the expressed chimeric fluorescent protein was 1.7-fold higher in treated than in untreated cells (Fig. 3A), suggesting that in U87 EYFP-hDAAO cells a fraction of the expressed chimeric protein is eventually targeted to the UPS and proteolyzed. The same MG132-induced effect on EYFP-tagged protein cellular levels is observed in U87 hDAAO-EYFP cells (Fig. 3B): an up to 2-fold increase in the immunorecognition signal corresponding to cytosolic hDAAO has been detected 6 hours after the addition of the inhibitor.
Since proteins designed to be degraded through the proteasome machinery are labelled by polyubiquitin chains (44) we investigated the ubiquitination state of EYFP-hDAAO in U87 EYFP-hDAAO MG132-treated cells by immunoprecipitation experiments. At 6 hours from addition of the proteasome inhibitor, Western blot analysis shows that the treatment yielded a remarkable increase (up to 4-fold) in the ubiquitination signal detected by both anti-FK1 and anti-P4D1 antibodies (Fig. 3C, α-FK1 and α-P4D1 panels). We confirmed the observed results by treatment with a different proteasome inhibitor (ALLN, 60 μM). The amount of immunoprecipitated EYFP-hDAAO protein, as well as the corresponding immunodetected polyubiquitination signal increased significantly in treated cells ($\sim$ 4-fold and $\sim$ 1.7-fold, respectively, relatively to control samples, data not shown). The higher amount of immunoprecipitated EYFP-hDAAO following ALLN treatment, as compared to that detected in MG132 treated samples, is probably due to ALLN ability to (partially) inhibit lysosomal calpaine and cathepsin (45), that prevents EYFP-hDAAO degradation.

The inhibition of the lysosome degradation system yields to the accumulation of active hDAAO

Interestingly, in U87 EYFP-hDAAO cells treated with chloroquine a significant increase in hDAAO activity (assayed by means of the Amplex UltraRed fluorescent assay) was apparent with respect to control ones (Fig. 4): the inhibition of the lysosomal system yielded to the accumulation of the EYFP-hDAAO active form within the cells. On the contrary, no change in hDAAO activity was detected in MG132-treated cells extracts (Fig. 4), this suggesting that the polyubiquitinated EYFP-hDAAO protein accumulating upon MG132 treatment might be inactive.

The UPS is responsible for pLG72 degradation

The results of the experiments performed using the inhibitors of the proteasome machinery (MG132 and ALLN) and U87 EYFP-hDAAO cells raise an interesting issue: is hDAAO directly ubiquitinated or is it addressed to the UPS through the interaction with other proteins, i.e. substrates of ubiquitinating enzymes? In this perspective, the hDAAO interactor pLG72 seems to be a plausible candidate since it has been demonstrated to be a short-lived protein which speeds up the degradation of EYFP-hDAAO (39). Moreover, the UPS is known to mediate the selective degradation of many short-lived proteins (46).
To substantiate this hypothesis, we repeated the MG132 treatment on U87 pLG72-ECFP expressing cells. A large increase in the cellular level of pLG72-ECFP was detected by Western blot analysis: 6 hours after MG132 addition the amount of pLG72-ECFP is $\sim$ 8-fold higher than that detected in untreated cells (Fig. 5A, α-GFP panel). Noteworthy, no change in the fluorescent protein level of control cells expressing ECFP alone (U87 ECFP stable clone) has been observed upon the addition of the inhibitor and during the time course of the experiment (data not shown). These results strongly indicate that pLG72 is largely degraded through the proteasome pathway. Experiments performed using ALLN confirmed this observation, albeit the increase in the pLG72-ECFP levels is less pronounced ($\sim$ 2.5-fold with respect to the corresponding control cells, data not shown). To further straighten our conclusion Western blot analysis on U87 pLG72-ECFP cells treated with CHX alone or with CHX and MG132 (both the inhibitors were added to the complete culture medium) and their relative controls was performed. The addition of MG132 together with ribosomal

inhibitor restored the steady state levels of the chimeric protein, further confirming the role of the UPS in pLG72-ECFP degradation (Fig. 5B).
On the contrary, no increase in the chimeric protein cellular levels is instead observed treating U87 pLG72-ECFP cells with the inhibitors of the lysosomal degradation pathway (i.e. chloroquine and NH_4Cl). Similarly to what observed in the experiments performed on U87 hDAAO-EYFP cells, the amount of pLG72-ECFP is halved in treated cells with respect to the control ones (Fig. 5C, α-GFP panel). As discussed above, the rationale of this result is not clear. We suggest that although starvation conditions do not cause any variation in protein cellular levels (data not shown), the addition of a further stress such as the blockade of the lysosomal acidic hydrolases may raise pLG72-ECFP degradation processes involving the proteasome machinery and thus yield to a substantial decrease of the cellular levels of ECFP-tagged protein.

Discussion

Over- and down-stimulation of NMDARs are crucial initial (pathological) events in a number of neurodegenerative and psychiatric disorders. Worthy of note, diminished glutamatergic neurotransmission mediated by NMDAR has been implicated in the biological mechanisms underlying schizophrenia: genetic studies have identified several risk genes for schizophrenia influencing NMDAR activity (47, 48). These include genes encoding for enzymes involved in the metabolism of D-serine, a selective endogenous NMDAR glycine site agonist (32, 49). D-Serine is a key contributor to NMDAR activation: a reduction in D-serine concentration has been shown to down-regulate the receptor mediated signaling (13, 16, 17). These findings suggest that diminished NMDAR function in schizophrenia might be related to lower D-serine availability. In brain cells, endogenous D-serine concentration is determined by the enzymatic reactions catalyzed by SR and DAAO (which synthesized and degrade the neuromodulator), and by various glial and neuronal transporters (11, 20). Different SR regulation mechanisms have been recently unravel (50, 23). In particular, SR is ubiquitinated and degraded by the UPS; its ubiquitination state might be modulated through the interaction with the Golgin subfamily A member 3 (Golga3) protein, which significantly increases SR half-life and steady state levels (42). On the other hand, the modulation of DAAO enzymatic activity and stability has not been clarified yet.

For this reason, here we reported about the degradation pathway of hDAAO and pLG72. In U87 cells stably expressing EYFP-tagged hDAAO and pLG72-ECFP we observed that hDAAO is a long-lived proteins, while pLG72 is a short-lived one (Fig. 1). These results suggest that the two protein are degraded by different cellular processes. Subsequent investigations confirmed this hypothesis: for the peroxisomal form of hDAAO, the cellular steady state levels are largely controlled by the lysosome/endosome system (Fig. 2A), as demonstrated by the strong accumulation of the active form of the protein in U87 cells expressing EYFP-hDAAO treated with inhibitors of this degradation pathway (Fig. 4). It is tempting to speculate that this could happens through pexophagy i.e. those macroautophagy processes involved in peroxisomes “turn-over” and renewal: further investigations are needed to confirm this assumption. Moreover, immunoprecipitation analyses revealed that EYFP-hDAAO is ubiquitinated (mono- or multiple mono-ubiquitinated, but not polyubiquitinated) before being addressed to the lysosomal degradative pathway (Fig. 2C). This finding agrees with the knowledge that ubiquitin tagging also participates to selective autophagic degradation - monoubiquitination or/and multiubiquitination are required in some cases for cargo proteins to entry into vesicles of the lysosomal/endosomal degradative pathway (51).

On the other hand, the blockade of the lysosomal system determined a decrease in the level of the cytosolic form of the flavoprotein (hDAAO-EYFP, Fig. 2B). Beside sharing some of the ubiquitin recognizing molecules or shuttle factors, an increasing body of evidences suggest a cross-talk between the UPS and the lysosome system. Cells respond to blockage of the UPS by up-regulating macroautophagy, whereas persistent blockage of macroautophagy has been shown to affect UPS activity (52). Our results suggest that the hDAAO subcellular localization might be a determinant factor for the addressing of the flavoprotein to a specific degradation pathway.

Unexpectedly, by treating U87 cells stably expressing EYFP-hDAAO or hDAAO-EYFP chimeric proteins with inhibitors of the UPS, a significant increase in their cellular level was observed, thus indicating that the enzyme degradation is partially prevented under these conditions: the hDAAO cellular levels might be regulated by this degradative pathway too (Fig. 3A,B). Immunoprecipation analyses performed on U87 EYFP-hDAAO cells treated with MG132 revealed that the accumulated protein is labeled by polyubiquitin chains (Fig. 3C) and thus is likely addressed to the

proteasome machinery. These data suggest that cytosolic hDAAO is mainly targeted to the UPS for its degradation, while the peroxisomal form of the flavoprotein is largely degraded by the lysosomal/endosomal pathway but, to a lower extent, also by the ubiquitin dependent system.
This latter assumption raises an important question: is hDAAO itself directly ubiquitinated or is it addressed to UPS through its interaction with pLG72? The second possibility is plausible since we have demonstrated that pLG72 is a short-lived protein and thus it should speed up the degradation of EYFP-hDAAO. Moreover, we demonstrated that pLG72 is a mitochondrial protein (supposed to be exposed on the cytosolic side of the external membrane) (39) and, in agreement with studies indicating that mitochondrial outer membrane proteins are mostly ubiquitinated and degraded through the UPS (51), it is presumable that pLG72 degradation is mediated by the proteasome. This latter hypothesis is strongly supported by results obtained treating U87 pLG72-ECFP cells with inhibitors of the proteasome machinery: in this condition a strong increase (up to 8-fold) in the cellular amount of the protein was apparent (Fig. 5A). Furthermore, the treatment of cells with CHX and MG132 simultaneously restored pLG72-ECFP level (rapidly and substantially decreased in CHX treated cells) to a value comparable with that observed in untreated cells (Fig. 5B). Further experiments aimed to elucidate the effective mechanism of pLG72-driven degradation of hDAAO are currently in progress.
In conclusion, these studies clarify how the steady state cellular levels of hDAAO and pLG72 are regulated, a prerequisite to shed light on their role in D-serine metabolisms. Since alteration of the cellular concentration of this neuromodulator have been related with the schizophrenia, the definition of the mechanism of regulation of the proteins involved in its metabolism will promote therapeutic approaches targeting the molecular pathogenesis rather than the symptoms.

Experimental Methods

Expression vectors

hDAAO was expressed in human glioblastoma cells by using pEYFP-hDAAO-C3 or –N1 expression vectors. The constructs were obtained as reported previously (39). Briefly, the pEGFP-hDAAO vector (38) was used as template for subcloning hDAAO cDNA into the pEYFP-C3 (EcoRI and

HindIII restriction sites) mammalian expression vector (Clontech Laboratories). The pEYFP-hDAAO-N1 construct was instead obtained by the amplification of the region encoding for hDAAO was amplified using 5'-CTCAAGCTT*GCCACC*ATGCGTGTGGTGGTGATTGG-3' (introducing a HindIII restriction site and the Kozak consensus sequence upstream to the hDAAO ORF) and 5'-CGGTACCGTGAGGTGGGATGGTGGCATTCTG-3' (which suppresses a stop signal and introduce a KpnI restriction site downstream the coding region) as primers. These vectors express of hDAAO fused with EYFP downstream (EYFP-hDAAO) or upstream (hDAAO-EYFP) to the fluorescent protein.

Cell culture and transfection

All experiments were performed using the U87 human glioblastoma cell line (ATCC), and in particular stable clones as reported in (39), constitutively expressing EYFP-hDAAO or hDAAO-EYFP or pLG72-ECFP. These cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin/streptomycin and amphotericin B (Euroclone) at 37 °C in a 5% CO_2 incubator (38). Indeed, U87 and U87 pLG72-ECFP cells were transiently transfected using the FuGENE HD transfection reagent (6 µL, Roche) and 2 µg of pEYFP-hDAAO (-C3 and -N1). The levels of expression of the fusion proteins were monitored by detecting the corresponding fluorescence emission using a FITC filter on a fluorescence microscope (Olympus IX51). For immunoprecipitation experiments or Western blot analyses the cells were detached, counted, collected, and stored at -80 °C. To investigate hDAAO and pLG72 degradation pathways, U87-EYFP-hDAAO, U87 DAAO-EYFP or U87-pLG72-ECFP cells were treated with specific inhitors of the ubiquitin-proteasomal system or the endosomal/lysosomal pathway. The cells were grown one night in starvation condition (a culture maintained in DMEM supplemented with 1% fetal bovine serum) and then added with the proteasomal inhibitors MG132 (Z-Ley-Leu-Leu-A, 25 µM) or ALLN (N-Acetil-L-Leucil-L-Norleucinal, 60 µM), or the lysosomal hydrolases inhibitors chloroquine (75 µM) or NH_4Cl (10 µM). All inhibitors were purchased by Sigma. In order to study the stability of hDAAO and pLG72 within the cell and assess their rate of degradation, the same cell line were treated with the potent inhibitor of protein synthesis cycloheximide (100 µg/mL, Sigma). Control experiments were performed on U87 cells stably trasfected with

pECFP-C3 plasmid and thus constitutively expressing ECFP fluorescent protein (U87 ECFP cells).

Immunoblot and immunoprecipitation

For Western blot analysis both stably transfected clones and transiently transfected cells were resuspended in SDS-PAGE sample buffer (25 mM Tris-HCl, pH 6.7, 6% SDS, 10% glycerol and 25 mM DTT) to have 2500 cells/µL and 20 µL of each sample were subjected to SDS-PAGE electrophoresis and Western blot analysis.
For immunoprecipitation experiments, the cells were resuspended in lysis buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 0.06% NLS, 1 mM EDTA) supplemented with protease inhibitors (2 µM leupeptin, 1 µM pepstatin, 500 µM phenylmethylsulfonyl fluoride) and subjected to sonication. The cell lysates were centrifugated at 16000 g for 30 min at 4 °C (Pre-IP). The total protein concentration in the supernatants was quantified by the Bradford assay (Sigma) based on the classical method of Lowry. Equivalent amount of total proteins were subjected to immunoprecipitation. The samples were precleared with 50 µL of Dynabeads Protein G (Invitrogen) for 1 hour at 4 °C with rotation. At the meantime, 10 µg of rabbit anti-DAAO andibody (Davids Biotecnologie) were crosslinked to 50 µL of Dynabeads Protein G (Invitrogen) using dimethyl pimelimidate (20 mM DMP dissolved in 0.2M triethanolamine pH 8.2, 30 min incubation at room temperature with rotation). The cross linker was subsequently removed and the Dynabeads were incubated with 50 mM Tris-HCl pH 7.5 for 15 min at room temperature with rotation and then extensively washed with PBS + 0.1% Tween. The precleared samples were added to the Dynabeads-Ab complex and incubated over night at 4 °C with rotation. The supernatant (Post-IP) was collected through separation on the magnet. The beads were extensively washed with lysis buffer and resuspended in 50 µL of non-reducing SDS-PAGE sample buffer (IP) and heat denaturated. 20 µg total protein of Pre-IP and Post-IP samples and 35 µL of the IP samples were subjected to SDS-PAGE electrophoresis (on a 8-15% polyacrylamide gel) and used for Western blot analysis. The polyvinylidene difluoride membranes (Immobilon-P, Millipore) were incubated 2 hours at room temperature or overnight at 4 °C in a blocking solution containing 4% dried milk in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 8.0, 0.5 M NaCl) to which 0.1% Tween-20 (TTBS) was added and then incubated for 1.5 hours at room temperature with the primary antibodies. Membranes were then

washed extensively in TTBS containing 2% dried milk and incubated 1 hour at room temperature with specific peroxidase-conjugated immunoglobulins. The immunoreactivity signals were detected by enhanced chemiluminescence (ECL plus, GE Healthcare). Both the primary and the secondary antibodies were diluted in TBS added with 0.05% Tween-20 and 2% dried milk. Primary antibodies were at the following diluition: rabbit polyclonal anti-DAAO (1:5000, Davids Biotecnologie), mouse monoclonal anti-GFP (1:200, Santa Cruz Biotechnology), mouse monoclonal anti-P4D1 (1:400, Santa Cruz Biotechnology) and rabbit anti-actin (1:200, Santa Cruz Biotechnology), mouse IgM anti-FK1 (1:1000, Enzo Life Science).

Densitometric analysis

For the quantification of the different immunorecognition signals the Quantity One software (BioRad) which provides a densitometric estimation of bands intensity, was used. In cellular stability experiments and studies of degradation pathways following Western blot analysis of treated and control cells, the intensity values immunodetected by anti-GFP, anti-hDAAO or anti-P4D1 antibodies was normalized for the values obtained with the anti-actin antibody. After normalization, the ratio between the signals corresponding to treated and control cells at the same time of incubation was calculated. In immunoprecipitation experiments the results of Western blot analysis (using anti-FK1, anti-GFP or anti-P4D1 antibodies) and densitometric evaluation, are expressed as the ratio between treated and control samples.

Activity assay

DAAO activity measurements on U87 EYFP-hDAAO cells treated or not with inhibitors of degradation pathways were performed by the Amplex UltraRed kit (Invitrogen) based on the detection of H_2O_2 by the peroxidase mediated oxidation of the fluorogenic Amplex UltraRed Dye. 30,000 cells were suspended in 750 µL of 50 mM sodium phosphate buffer pH 7.4, containing 1 µM pepstatin, 2 µM leupeptin and 10 µM FAD, sonicated and cleared by centrifugation at 16000 g for 30 min (4 °C). Each solution was then diluted 1:2 in the activity assay solution containing 50 µM Amplex UltraRed, 0.2 units/mL horseradish peroxidase, 10 mM NaN_3, 10 µM FAD, 50 mM D-serine and incubated for 1 hour in the dark at room temperature

in agitation. The reaction was blocked adding 20 µL of Amplex UltraRed stop reagent, and fluorescence emission at 590 nm was measured. For each sample a control without the substrate D-serine was prepared; DAAO activity was expressed as the difference in fluorescence emission between sample and control assay mixture. Furthermore, 1 mM sodium benzoate (a well known DAAO inhibitor) (22, 46) was added to verify whether the observed fluorescence changes were effectively due to DAAO activity.

Calculation and statistics

Kinetic data for the estimation of pLG72 half-life were fit to a single exponential decay equation by using KaleidaGraph™ (Sinergy software). Each experiment was replicated 3-4 times and statistical analyses were performed using KaleidaGraph™ (Sinergy software). Variation between groups was evaluated by one-way ANOVA, and post-hoc significance tests were performed using a Student's t test.

Acknowledgments

This work was supported by grants from MIUR prot. 2008N2FM4A001 to L. Pollegioni, and Fondo di Ateneo per la Ricerca to L. Pollegioni and S.Sacchi. P. Cappelletti is a student of the PhD program in Biotechnology, School in Biological and Medical Sciences, University of Insubria.

References:

1. Fields RD, Stevens-Graham B. New insights into neuron-glia communication. 2002. 298:556-562.
2. Volterra A, Meldolesi J. Astrocytes, from brain glue to communication elements: the revolution continues. 2005. Nat Rev Neurosci. 6:626-640.
3. Ullian EM, Christopherson KS, Barres BA. Role for glia in synaptogenesis. 2004. Glia. 47:209-216.
4. Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG. Astrocytic purinergic signaling coordinates synaptic networks. 2005. Science. 310:113-116.

5. Angulo MC, Kozlov AS, Charpak S, Audinat E. Glutamate released from glial cells synchronizes neuronal activity in the hippocampus. 2004. J Neurosci. 24:6920-6927.
6. Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G. Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. 2004. Neuron. 43:729-743.
7. Ventura R, Harris KM. Three-dimensional relationships between hippocampal synapses and astrocytes. 1999. J Neurosci. 19:6897-6906.
8. Verkhratsky A, Steinhäuser C. Ion channels in glial cells. 2000. Brain Res Brain Res Rev. 32:380-412.
9. Perea G, Araque A. Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes. 2005. J Neurosci. 25:2192-2203.
10. Mustafa AK, Kim PM, Snyder SH. D-Serine as a putative glial neurotransmitter. 2004. Neuron Glia Biol. 1:275-281.
11. Martineau M, Baux G, Mothet JP. D-serine signalling in the brain: friend and foe. 2006. Trends Neurosci. 29:481-491.
12. Wolosker H. D-serine regulation of NMDA receptor activity. 2006. Sci STKE. 2006:pe41.
13. Mothet, J. P., Parent, A. T., Wolosker, H., Brady, R. O., Jr., Linden, D. J., Ferris, C. D., Rogawski, M. A., and Snyder, S. H. (2000) d-serine is an endogenous ligand for the glycine site of the N-methyl-Daspartate receptor. Proc. Natl. Acad. Sci. U. S. A. 97, 4926–4931
14. Gustafson, E. C., Stevens, E. R., Wolosker, H., and Miller, R. F. (2007) Endogenous d-serine contributes to NMDA-receptor- mediated light-evoked responses in the vertebrate retina. J. Neurophysiol. 98, 122–130
15. Kartvelishvily, E., Shleper, M., Balan, L., Dumin, E., and Wolosker, H. (2006) Neuron-derived D-serine release provides a novel means to activate N-methyl-d-aspartate receptors. J. Biol. Chem. 281, 14151–14162
16. Panatier, A., Theodosis, D. T., Mothet, J. P., Touquet, B., Pollegioni, L., Poulain, D. A., and Oliet, S. H. (2006) Gliaderived d-serine controls NMDA receptor activity and synaptic memory. Cell 125, 775–784
17. Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C, Poo M, Duan S. Contribution of astrocytes to hippocampal long-term potentiation

through release of D-serine. 2003. Proc Natl Acad Sci U S A. 100:15194-15199.
18. Wolosker H, Sheth KN, Takahashi M, Mothet JP, Brady RO Jr, Ferris CD, Snyder SH. Purification of serine racemase: biosynthesis of the neuromodulator D-serine. Proc Natl Acad Sci U S A. 1999a. 96:721-725.
19. Wolosker H, Blackshaw S, Snyder SH. Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. 1999. Proc Natl Acad Sci U S A. 96:13409-13414.
20. Foltyn VN, Bendikov I, De Miranda J, Panizzutti R, Dumin E, Shleper M, Li P, Toney MD,
21. Kartvelishvily E, Wolosker H.Serine racemase modulates intracellular D-serine levels through an alpha,beta-elimination activity. 2005. J Biol Chem. 280:1754-6173.
22. Pollegioni L, Piubelli L, Sacchi S, Pilone MS, Molla G. Physiological functions of D-amino acid oxidases: from yeast to humans. 2007. Cell Mol Life Sci. 64:1373-1394.
23. Pollegioni L, Sacchi S. Metabolism of the neuromodulator D-serine. 2010. Cell Mol Life Sci. 67:2387-2404.
24. Kantrowitz JT, Javitt DC. N-methyl-d-aspartate (NMDA) receptor dysfunction or dysregulation: the final common pathway on the road to schizophrenia? 2010. Brain Res Bull. 83:108-121.
25. Labrie V, Wong AH, Roder JC. Contributions of the d-serine pathway to schizophrenia. 2011. Neuropharmacology. Feb 2 In press
26. Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N, Nakazato M, Kumakiri C, Okada S, Hasegawa H, Imai K, Iyo M. Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. 2003. Arch Gen Psychiatry. 60:572-576.
27. Hashimoto K, Engberg G, Shimizu E, Nordin C, Lindström LH, Iyo M. Reduced D-serine to total serine ratio in the cerebrospinal fluid of drug naive schizophrenic patients. 2005. Prog Neuropsychopharmacol Biol Psychiatry. 29(5):767-769.
28. Bendikov I, Nadri C, Amar S, Panizzutti R, De Miranda J, Wolosker H, Agam G. A CSF and postmortem brain study of D-serine metabolic parameters in schizophrenia. 2007. Schizophr Res. 90:41-51.

29. Heresco-Levy U, Javitt DC, Ebstein R, Vass A, Lichtenberg P, Bar G, Catinari S, Ermilov M. D-serine efficacy as add-on pharmacotherapy to risperidone and olanzapine for treatment-refractory schizophrenia. 2005. Biol Psychiatry. 57:577-585.
30. Javitt DC. Management of negative symptoms of schizophrenia. 2001. Curr Psychiatry Rep. 3:413-417.
31. Tsai G, Yang P, Chung LC, Lange N, Coyle JT. D-serine added to antipsychotics for the treatment of schizophrenia. 1998. Biol Psychiatry. 44:1081-1089.
32. Chumakov I, Blumenfeld M, Guerassimenko O, Cavarec L, Palicio M, Abderrahim H, et al. Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. 2002. Proc Natl Acad Sci U S A. 99:13675-13680.
33. Schell MJ, Molliver ME, Snyder SH. D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release. 1995. Proc Natl Acad Sci U S A. 92:3948-3952.
34. Stevens ER, Esguerra M, Kim PM, Newman EA, Snyder SH, Zahs KR, Miller RF.D-serine and serine racemase are present in the vertebrate retina and contribute to the physiological activation of NMDA receptors. 2003. Proc Natl Acad Sci U S A. 100:6789-6794.
35. Wu S, Barger SW. Induction of serine racemase by inflammatory stimuli is dependent on AP-1. 2004. Ann N Y Acad Sci. 1035:133-1346.
36. Katsuki H, Nonaka M, Shirakawa H, Kume T, Akaike A. Endogenous D-serine is involved in induction of neuronal death by N-methyl-D-aspartate and simulated ischemia in rat cerebrocortical slices. 2004. J Pharmacol Exp Ther. 31:836-84.
37. Shleper M, Kartvelishvily E, Wolosker H. D-serine is the dominant endogenous coagonist for NMDA receptor neurotoxicity in organotypic hippocampal slices. 2005. J Neurosci. 25:9413-9417.
38. Sacchi S, Bernasconi M, Martineau M, Mothet JP, Ruzzene M, Pilone MS, Pollegioni L, Molla G. pLG72 modulates intracellular D-serine levels through its interaction with D-amino acid oxidase: effect on schizophrenia susceptibility. 2008. J Biol Chem. 283:22244-22256.
39. Sacchi S, Cappelletti P, Giovannardi S, Pollegioni L. Evidence for the interaction of d-amino acid oxidase with pLG72 in a glial cell line. 2011. Mol Cell Neurosci. 48:20-28.
40. Caldinelli L, Molla G, Bracci L, Lelli B, Pileri S, Cappelletti P, Sacchi S, Pollegioni L. Effect of ligand binding on human D-amino acid oxidase:

implications for the development of new drugs for schizophrenia treatment. 2010. Protein Sci. 19:1500-1512.
41. Danysz W, Parsons CG. Glycine and N-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. 1998. Pharmacol Rev. 50:597-5664.
42. Dumin E, Bendikov I, Foltyn VN, Misumi Y, Ikehara Y, Kartvelishvily E, Wolosker H.Modulation of D-serine levels via ubiquitin-dependent proteasomal degradation of serine racemase. 2006. J Biol Chem.;281:20291-302.
43. Groll M, Huber R. Inhibitors of the eukaryotic 20S proteasome core particle: a structural approach. 2004. Biochim Biophys Acta. 1695:33-44.
44. Thrower JS, Hoffman L, Rechsteiner M, Pickart CM. Recognition of the polyubiquitin proteolytic signal. 2000. EMBO J. 19:94-102.
45. Koh YH, von Arnim CA, Hyman BT, Tanzi RE, Tesco G. BACE is degraded via the lysosomal pathway. 2005, 280:32499-32504
46. Hershko A, Ciechanover A. The ubiquitin system. 1998. Annu Rev Biochem. 67:425-479.
47. Molla G, Sacchi S, Bernasconi M, Pilone MS, Fukui K, Pollegioni L. Characterization of human D-amino acid oxidase. 2006 FEBS Lett. 580:2358-2364
48. Coyle JT. Glutamate and schizophrenia: beyond the dopamine hypothesis. 2006. Cell Mol Neurobiol. 26:365-84.
49. Morita Y, Ujike H, Tanaka Y, Otani K, Kishimoto M, Morio A, Kotaka T, Okahisa Y, Matsushita M, Morikawa A, Hamase K, Zaitsu K, Kuroda S. A genetic variant of the serine racemase gene is associated with schizophrenia. 2007. Biol Psychiatry. 61:1200-3.
50. Baumgart F, Rodríguez-Crespo I. D-amino acids in the brain: the biochemistry of brain serine racemase. 2008. FEBS J. 275:3538-45.
51. Hicke L. A new ticket for entry into budding vesicles-ubiquitin. 2001. Cell. 106:527-30.
52. Wong E, Cuervo AM. Integration of clearance mechanisms: the proteasome and autophagy. 2010. Cold Spring Harb Perspect Biol. Epub 2010 Nov 10.

Figure legends:

Fig. 1: Analysis of pLG72 and hDAAO degradation rate. U87 cells stably expressing pLG72-ECFP, EYFP-hDAAO (peroxisomal) or hDAAO-EYFP (cytosolic) proteins were treated with 100 μg/mL cycloheximide (CHX). A) The pLG72-ECFP signal detected using the anti-GFP antibody strongly decreases following CHX treatment (left panel). The quantified values were fit using a single exponential decay: an half life of ≈ 25 minutes was estimated (right panel). B-C) No significant variation in the signal detected by the anti-DAAO antibody in U87 EYFP-hDAAO (B) or hDAAO-EYFP (C) CHX-treated cells was apparent up to 12 hours upon the addition of the inhibitor with respect to control ones. Quantification analysis confirmed that the levels of the expressed chimeric proteins are substantially unchanged during the time course of the experiment (bottom panels). For both EYFP-hDAAO and hDAAO-EYFP an half life of ≈ 60 hours was estimated. Values are the average ± standard error (n=3), normalized to actin and expressed relatively to controls value (lane C in Western blot panels).

Fig. 2: Effect of chloroquine treatment on U87 cells expressing EYFP-hDAAO or hDAAO-EYFP. U87 EYFP-hDAAO and U87 hDAAO-EYFP cells were incubated in starvation conditions and treated with 75 μM chloroquine or 0.1% PBS as a control and incubated for up to 6 hours. A) The chimeric EYFP-hDAAO (peroxisomal) protein is largely degraded through the lysosome pathway. Western blot analysis using anti-DAAO antibody (left, top panel) and quantitative analysis (right panel) demonstrate that the fusion protein significantly accumulates at 3 h upon the inhibitor addition (~ 3-fold with respect to control cells). B) Western blot and quantification analyses indicate that the amount of the hDAAO-EYFP (cytosolic) expressed protein is decreased in treated cells with respect to control ones (up to ~ 2-fold) but its level does not modify up to 6 hours from treatment with the lysosomial inhibitor. C) Western blot analysis shows an increase (up to ~ 3-fold) in immunoprecipitated protein (by using anti-GFP antibodies, α-GFP panel) and a similar increase in the ubiquitination signal immunodetected using the anti-P4D1 antibody (α-P4D1 panel). On the contrary, no increase was apparent in the polyubiquitination signal (using the anti-FK1 antibodies, α-FK1 panel). Values are the average ± standard error (n=3), normalized to actin and expressed relatively to controls value (lane C in Western blot panels).

Fig. 3: Effect of MG132 treatment on U87 cells expressing EYFP-hDAAO and hDAAO-EYFP. U87 EYFP-hDAAO or U87 hDAAO-EYFP cells were incubated in starvation conditions and treated with 25 μM MG132 or 0.1% DMSO as a control, and incubated for up to 6 hours. A (minor) fraction of EYFP-hDAAO is addressed to the proteasome degradation pathway. A) After 6 h from MG132 addition a significant accumulation of EYFP-hDAAO (~ 2-fold) is observed in U87 EYFP-hDAAO cells as detected by Western blot analysis (α-DAAO panel) and the quantification of the corresponding signal. B) The treatment with MG132 induces a similar effect on U87 hDAAO-EYFP cells (expressing the cytosolic EYFP-tagged hDAAO): Western blot and quantification analyses reveal the same accumulation observed in U87 EYFP-hDAAO cells. C) Immunoprecipitation experiments further support that EYFP-hDAAO is partially addressed to the proteasome machinery. The immunoprecipitation was performed on cell lysates from treated and untreated cells as a control (100 μg total protein), using rabbit anti-hDAAO polyclonal antibody. An increase in the amount of the immunoprecipitated protein is apparent in treated cells with respect to control ones (~ 1.5-fold, α-GFP panel). This change is associated with an increase in the signal corresponding to polyubiquitinated (α-FK1 panel) and to generally ubiquitinated proteins (α-P4D1 panel).

Fig. 4: Effect of inhibitors treatment on the activity level of EYFP-hDAAO. The accumulation of EYFP-hDAAO in chloroquine treated cells as active enzyme was confirmed by the activity values on the different cell lysates using the Amplex UltraRed reagent. No change in DAAO activity was instead measured in MG132-treated cells compared to control ones. The activity values are reported as relative activity of treated cells with respect to mean activity value of the corresponding control, and as mean ± st. dev. (n=5, *p<0.015).

Fig. 5: Effect of inhibitors treatment on pLG72 cellular levels. U87 pLG72-ECFP cells were incubated in starvation conditions and treated with different inhibitors, as detailed previously. The quantification of pLG72-ECFP immunorecognition signals indicates that the ECFP-tagged protein is mainly degraded through the UPS. A) The amount of pLG72-ECFP detected by anti-GFP antibody strongly increases following MG132 treatment (up to 9-fold at 6 hours from the addition of the inhibitor). B)

Treatment of U87 pLG72-ECFP cells with CHX and MG132 simultaneously substantiates the key role of the UPS in the degradation of the chimeric protein. The addition of the proteasome inhibitor to CHX-treated cells maintained the cellular level of the expressed pLG72-ECFP at the steady state level whereas the addition of CHX alone yields to a fast degradation of the chimeric protein. C) Chloroquine affects the cellular level of pLG72-ECFP. The signal detected by the anti-GFP antibody at 1 hour from the addition of the inhibitor is halved with respect to that immunodetected in control ones. The cellular level of the protein then remains stable until the end of the treatment. Values are the average ± standard error (n=3), normalized to actin and expressed relative to controls value (lane C in Western blot panels).

Fig. 1

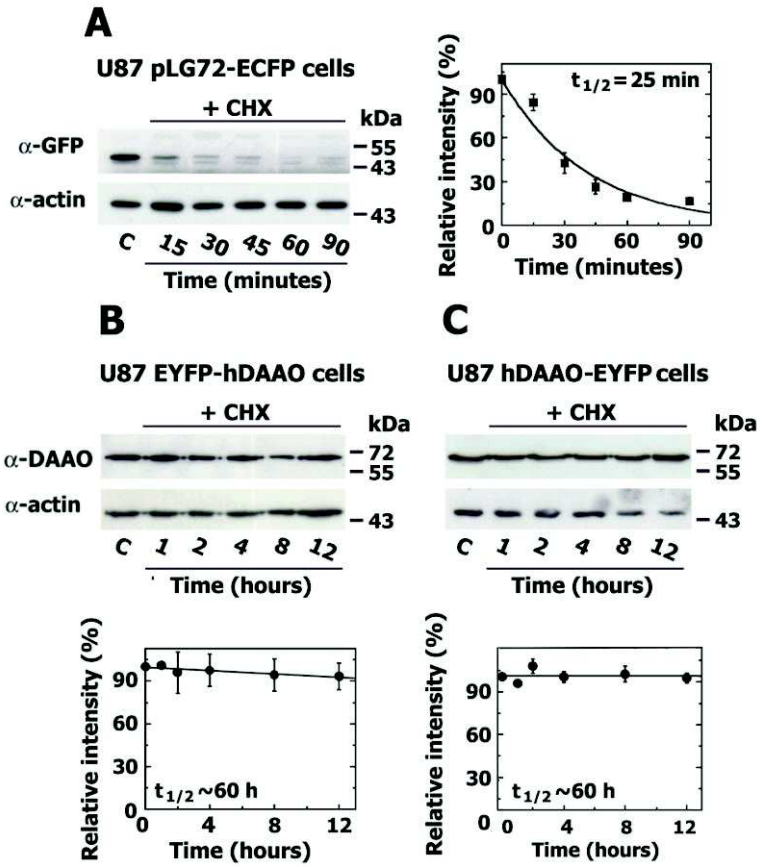

A
U87 pLG72-ECFP cells
+ CHX
kDa
α-GFP
α-actin
55
43
43
C 15 30 45 60 90
Time (minutes)
Relative intensity (%)
t1/2 = 25 min
90
60
30
0
0 30 60 90
Time (minutes)
B
U87 EYFP-hDAAO cells
+ CHX
kDa
α-DAAO
α-actin
72
55
43
C 1 2 4 8 12
Time (hours)
Relative intensity (%)
t1/2 ~60 h
90
60
30
0
0 4 8 12
Time (hours)
C
U87 hDAAO-EYFP cells
+ CHX
kDa
72
55
43
C 1 2 4 8 12
Time (hours)
Relative intensity (%)
t1/2 ~60 h
90
60
30
0
0 4 8 12
Time (hours)

Fig. 2

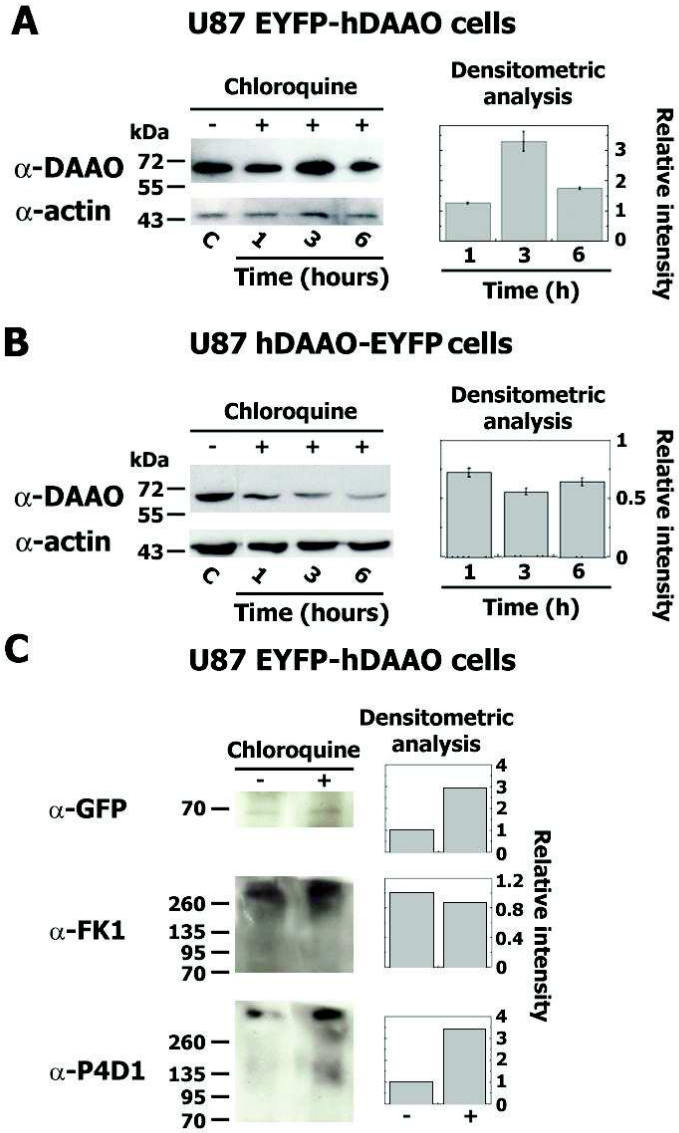

A
U87 EYFP-hDAAO cells
Chloroquine
- + + +
kDa
α-DAAO
72
55
α-actin
43
C 1 3 6
Time (hours)
Densitometric analysis
Relative intensity
3
2
1
0
1 3 6
Time (h)
B
U87 hDAAO-EYFP cells
Chloroquine
- + + +
kDa
α-DAAO
72
55
α-actin
43
C 1 3 6
Time (hours)
Densitometric analysis
1
0.5
0
Relative intensity
1 3 6
Time (h)
C
U87 EYFP-hDAAO cells
Chloroquine
- +
Densitometric analysis
α-GFP
70
4
3
2
1
0
α-FK1
260
135
95
70
1.2
0.8
0.4
0
Relative intensity
α-P4D1
260
135
95
70
4
3
2
1
0
- +

Fig. 3

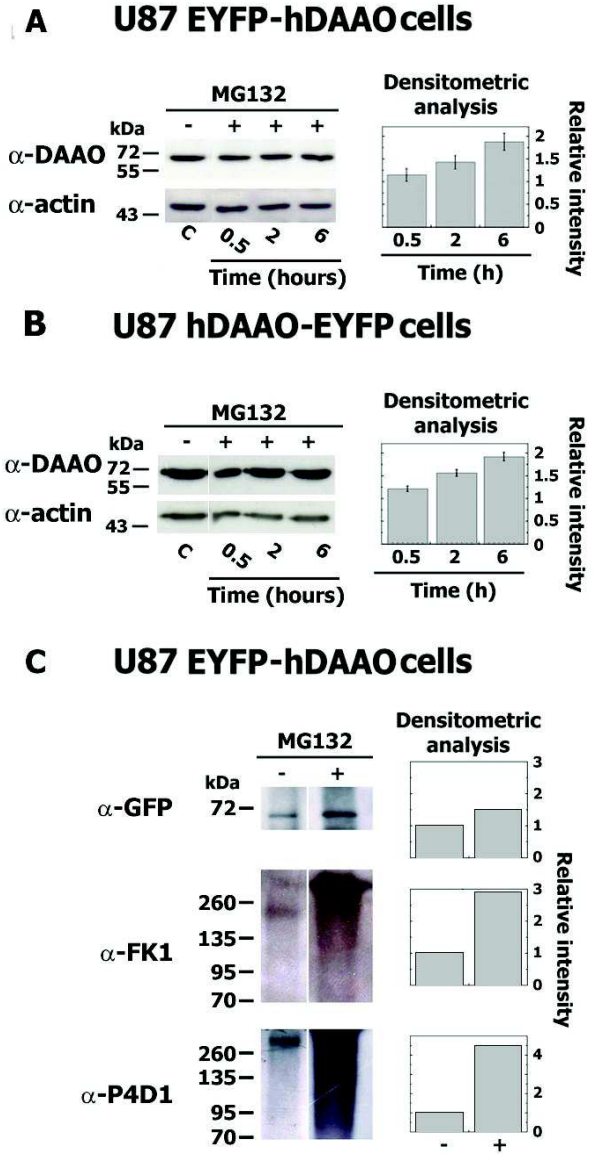

A
U87 EYFP-hDAAO cells
MG132
kDa
- + + +
α-DAAO
72—
55—
α-actin
43—
C 0.5 2 6
Time (hours)
Densitometric analysis
Relative intensity
0.5 2 6
Time (h)
B
U87 hDAAO-EYFP cells
MG132
kDa
- + + +
α-DAAO
72—
55—
α-actin
43—
C 0.5 2 6
Time (hours)
Densitometric analysis
Relative intensity
0.5 2 6
Time (h)
C
U87 EYFP-hDAAO cells
MG132
kDa
- +
α-GFP
72—
α-FK1
260—
135—
95—
70—
α-P4D1
260—
135—
95—
70—
Densitometric analysis
Relative intensity
- +

Fig. 4

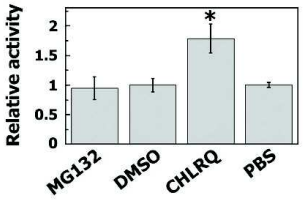
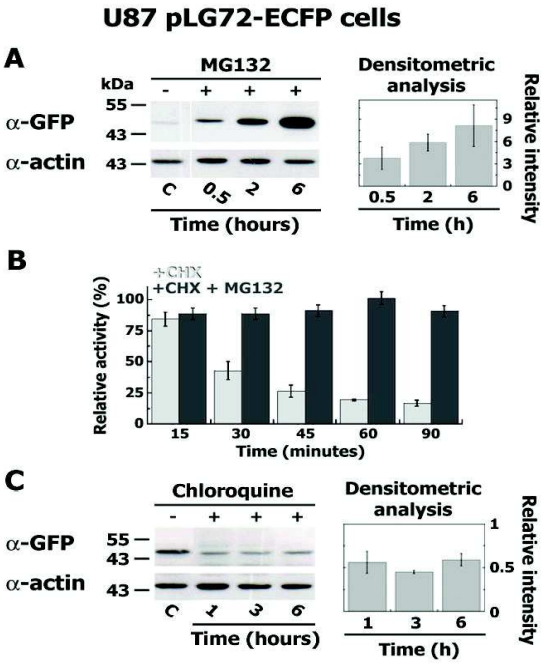

Relative activity
2
1.5
1
0.5
0
*
MG132
DMSO
CHLRQ
PBS

Fig. 5


U87 pLG72-ECFP cells
A
MG132
kDa
- + + +
55
43
α-GFP
43
α-actin
C 0.5 2 6
Time (hours)
Densitometric analysis
9
6
3
0
Relative intensity
0.5 2 6
Time (h)
B
+CHX
+CHX + MG132
Relative activity (%)
100
75
50
25
0
15 30 45 60 90
Time (minutes)
C
Chloroquine
- + + +
55
43
α-GFP
43
α-actin
C 1 3 6
Time (hours)
Densitometric analysis
1
0.5
0
Relative intensity
1 3 6
Time (h)

Synapses are the sites of neurons-glia intense cross-talk: glial cells sense and integrate the levels of synaptic activity responding to their excitation state by the release of several signaling molecules. Among them, worthy of note is D-serine, a D-amino acid present at high concentration in mammalian brain where it acts as an endogenous coagonist of NMDA receptors (42). A relationship between D-serine signaling deregulation, NMDAR dysfunction and neuropsychiatric diseases is widely assumed (34, 35). In particular, hypoactivation of NMDAR is related to the onset of schizophrenia (43), a severe debilitating disorder that affects ~ 1% of the population worldwide (138).

In neurons and glial cells D-serine concentration is regulated by the PLP-dependent enzyme serine racemase (SR) and the FAD-containing flavoenzyme D-amino acid oxidase (DAAO). SR is responsible for both the synthesis of the D-amino acid (through the isomerization from the L-isomer) and its elimination (through an α-elimination reaction) (60), while DAAO catalyzes the oxidative deamination of the neuromodulator (56, 77).

Genetic studies have identified several risk genes for schizophrenia influencing NMDAR activity (139, 146), including those encoding the enzymes involved in D-serine metabolism. This yields to the hypothesis that an imbalance between D-serine production and degradation determined by alterations in the expression level and/or activity of SR and DAAO could be responsible for an abnormal decrease of its availability in the proximity of synapses, thus entailing NMDAR dysfunction and consequently to the onset of the disease.

Notably, while there are still weak evidences that SR might be a risk gene and that alteration in its expression levels occurs in affected individuals, consistent data from genetic and biochemical studies indicate an involvement of DAAO in schizophrenia susceptibility. In 2002, Chumakov and collaborators reported that pLG72, a small protein encoded by the newly identified human gene G72 linked to schizophrenia through a single nucleotide polymorphism (SNPs) analysis, interacts with DAAO and modulates its activity (117). Afterwards, DAAO itself was associated with schizophrenia in a number of studies, see (43) for a review.

Our laboratory recently, confirmed *in vitro* the specific interaction of human DAAO (hDAAO) and pLG72 recombinant proteins. We demonstrated that pLG72 affects the flavoenzyme activity: the main effect of hDAAO-pLG72 complex formation is a time-dependent inactivation of the flavoenzyme resulting from the modification of its tertiary structure (107). This observation prompted our group to propose a model to explain the

relationship between hDAAO-pLG72-D-serine and schizophrenia susceptibility: an anomalous hypoexpression of pLG72 yields to an increase of hDAAO activity, then to a decrease of D-serine released at the synapse and finally to the hypoactivation of NMDAr (107).

This PhD project was focused on the analysis of the interaction between hDAAO and pLG72 in a model cell line system represented by U87 human glioblastoma cells and on the study of the mechanisms that control the cellular steady state level of the two proteins, in order to shed light on the regulation of D-serine levels (and NMDAR activity) in the brain.
In a critical review focused on the neurobiology of hDAAO, Verral and collaborators emphasized that "*the subcellular distribution of hDAAO is relevant to the question of how it 'sees' its substrates. For hDAAO located outside of the peroxisomes, accessibility of hDAAO to its substrates would likely not be an issue"* (91). Moreover, D-serine regulation mechanism relying on hDAAO-pLG72 interaction does not match with the subcellular localization proposed for hDAAO (peroxisomes) and pLG72 (mitochondria). Here we provide convergent line of evidences solving this apparent discrepancy: we demonstrated that the newly synthesized hDAAO is transitorily present in the cytosol before being delivered to the peroxisomes, and that it colocalizes and interacts with pLG72, which we propose to be expressed on the cytosolic side of the external membrane of mitochondria. We suggest that the hDAAO-pLG72 complex formation is transitory and dependent on specific spatiotemporal stimuli are to be elucidated.
We also demonstrated, for the first time to our best knowledge, that cytosolic hDAAO is active and that its specific activity resembles that of the peroxisomal form of the enzyme. The activity of hDAAO in the cytosol has relevant implications since, in this way, hDAAO could directly impact on D-serine cellular concentration; the concomitant formation of H_2O_2 in the reaction catalyzed by hDAAO on D-serine could be deleterious and responsible for the induction of cytotoxicity and oxidative stress within the cell. Our results also indicated that pLG72 binding to cytosolic hDAAO negatively affects its half-life by boosting the enzyme degradation. We thus support our previous hypothesis that pLG72 binding plays a protective role against hDAAO-induced depletion of D-serine and in case of cellular oxidative stess.
Another interesting issue is the comprehension of how the proteins involved in D-serine metabolism are regulated at cellular level. Recently, Dumin and collaborators reported that SR is degraded by the ubiquitin proteasome

system (UPS) (63); however nothing in known about the mechanisms responsible for the regulation of the cellular level of DAAO.
We demonstrated that pLG72 is a short-lived protein degraded by the UPS whereas hDAAO has a long half-life and its degradation takes place largely in the lysosomes. However, the hDAAO cellular levels are partially regulated also by the proteasome through the binding of polyubiquitin chains to the flavoprotein. This latter observation raises the important question whether hDAAO is directly ubiquitinated or if it is addressed to the UPS through its interaction with pLG72. The second possibility is plausible since we demonstrated that pLG72 is a short-live protein degraded by the UPS and that it is able to speed up the degradation of hDAAO. One possible explanation is that pLG72 could interact with a still unidentified ubiquitin ligase (a class of proteins that promote the transfer of activated ubiquitin to proteins) (149). Further investigations are needed in order to investigate if pLG72 plays a role in targeting hDAAO to proteasome; in this way the pLG72-hDAAO interaction would be crucial for both the modulation of the flavoenzyme activity and regulation of its steady state cellular levels. Therefore, pLG72 could play an additional role in the regulation of hDAAO and thereby in D-serine cellular concentration. In order to answer to this important question, immunoprecipitations experiments on U87 EYFP-hDAAO cells, transiently transfected with pLG72-ECFP and treated with proteasomal inhibitors will be performed in order to verify if the presence of pLG72 leads to an alteration in the steady state and/or the polyubiquitinaton levels of hDAAO.
It has been suggested that the ubiquitin system is implicated in the protein turnover at synaptic site, but the mechanisms regulating synaptic proteins by degradation only started to be unveiled recently (150). Popiolek and collaborators have suggested a novel localization of hDAAO in the presynaptic active zone, where the authors had identified 24 putative hDAAO-interacting proteins (from rat cerebellum). The most robust interaction occurred with Basson (BSN, a known component of the prsynaptic active zone) leading to a significant inhibition of hDAAO enzymatic activity (124).

Given the fundamental and unquestioned role of hDAAO in D-serine metabolism, its enzyme activity is likely to be finely tuned. The analysis of hDAAO interactome, as well as the investigation of the mechanisms regulating the enzyme functionality might provide key information for understanding the molecular mechanisms responsible for the regulation of

the expression and the acquisition of the enzyme's catalytic activity. On this regard, preliminary data obtained by our team through mass spectrometric analysis carried out on immunoprecipitated samples from U87 cMyc-hDAAO expressing cells, identified 48 new putative hDAAO interactors. Moreover, the identification of post-translational forms of hDAAO and the definition of how these modifications might affect the enzyme functionality are also important findings that have to be analyzed in order to understand how they could eventually impact on D-serine cellular concentration. Interestingly, Shoji and collaborators proposed that in U87 cells nitrosylation regulates the activity of both SR and hDAAO: they proposed that NO inhibits SR but enhances DAAO activity (80), thus tightly controlling the D-serine cellular levels in glia. However, the molecular effect of nitrosylation on the structural and functional properties of the flavoenzyme is still unknown. Finally, it will be of interest to understand if there are differences in the hDAAO-nitrosylation mechanisms as well as the effect of this post-translational modification on the flavoenzyme's activity in various brain areas.

In conclusion, the definition of the mechanisms that regulate the levels of D-serine within the cell by acting on the catabolic enzyme hDAAO (and on its interacting partner) might allow the identification of new molecular targets for the treatment of pathologies in which the NMDAR mediated neurotransmission is likely to be affected by D-serine level, such in schizophrenia. In fact, current treatments for this psychiatric disease is only symptomatic and novel approaches are urgently needed. Inhibitors of hDAAO have recently begun to emerge as potential drugs: they were proposed to be a safer alternative to D-serine addition to antipsycothic treatments, as hDAAO catabolism of high doses of D-serine has been shown to produce metabolites with a cytotoxic effect (151).

1. Araque A., Carmignoto G., Haydon P.G. “Dynamic signaling between astrocytes and neurones”. 2001, Annu. Rev. Physiol. 63:795-813

2. Fields R.D., Stevens-Grahm B. “New insight into neuron-glia communication”. 2002, Science 298:556-562

3. Haydon P.G. “Listening and talking with the synapse”. 2001, Nat. Rev. Neurosci. 2:185-193

4. Volterra A., Mendolesi J. “Astrocytes, from brain glue to communication elements: the revolution continues”. 2005, Nat. Rev. Neurosci. 6(8):626-640

5. Haydon P.G., Carmignoto G. “Astrocytes control of synaptic transmission and neurovascular coupling”. 2006, Physiol. Rev. 86:1009-1031

6. Perea G., Araque A. “Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes”. 2005, J.Neurosci. 25:2192-2203

7. Wang X., Luo N., Xu Q., Tian G.F., Peng W.G., Han X., Kang J., Takano T., Nedergaard M. “Astocytic Ca2+ signaling evoked by sensory stimulation in vivo” 2006, Nat. Neurosci. 9:816-823

8. Fellin T.,Pascual O., Haydon P.G. “Astrocytes coordinate synaptic networks: balance of excitation and inhibition”. 2006, Physiology 21:208-215

9. Genoud C., Quairiaux C., Steiner P., Hirlig H., Welker E., Knott G.W. “Plasticity of astrocytic coverage and glutamate transporter expression in adult mouse cortex” 2006, PloS. Biol. 4:343

10. Winship I.R., Plaa N., Murphy T.H. “Rapid astrocyte calcium signals correlate with neuronal activity and onset of the hemodynamic response in vivo. 2007, J. Neurosci. 27:6268-6272

11. Shummers J., Yu H., Sur M. “Tuned responses of astrocytes and their influence on hemodynamic signals in the visual cortex”. 2008, Science 320:1638-1643

12. Halassa M.M., Fellin T., Haydon P.G. “Tripartite synapse: role for astrocytic purines in the control of synaptic physiology and behavior”. 2009, Neuropharmacology 57:343-346

13. Charles A.C., Merril J.E., Dirksen E.R, Sanderson M.J. “Intracellular signaling in glial cell: calcium waves and oscillation in response to mechanical stimulation and glutamate”. 1991, Neuron 6:983-992

14. Cornell-Bell A.H., Finkbeiner S.M, Cooper M.S, Smith S.J. “Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling”. 1990, Science 247:470-473

15. Bushong E.A., Martone M.E., Jonez Y.Z., Elissman M.H. “Protoplasmatic astrocytes in Ca1 stratum radiatum occupy separate anatomical domain”. 2002, J. Neurosci. 22:183-191

16. Halassa M.M., Fellin T., Takano H., Dong J.H., Haydon P.G. “Synaptic island defined by territory of a single astrocyte” 2007, J.Neurosci. 27:6473-6477

17. Ullian E.M., Christopherson K.S., Barres B.A. “Role for glia in synaptogenesys”. 2004, Glia 47:209-216

18. Allen N.J., Barres B.A. “Signaling between glia and neurones: focus on synaptic plasticity”. 2005, Curr. Opin. Neurobiol. 15:542-548

19. Rouach N., Koulakoff A., Abudara V., Willecke K., Giaume C. “Astroglial metabolic network sustain hippocampal synaptic transmission”. 2008, Science 322:1551-1555

20. Robertson J.M. “The astrocentric hypothesis: proposed role of astrocytes in consciousness and memory formation”. 2002, J. Physiol. 96:251-255

21. Machado-Vieira R., Manij H.K., Zarate C.A. “The role of the tripartite glutamatergic synapse in the pathophysiology and therapeutics of mood disorders”. 2009, Neuroscientist. 15:525-539

22. Verkhratsky A., Steinhauser C. “Ion channel in glial cells”. 2000, Brain Res. Rev. 32:380-412

23. Araque A., Martin E.D., Perea G., Arellano J.I. Bruno W. “Synaptically released acetylcholine evokes Ca^{2+} elevations in astrocytes in hippocampal slices”. 2002, J. Neurosci. 22:2443-2450.

24. Johnson J.W., Ascher P. “Glycine potentiates the NMDA response in cultured mouse brain neurones”. 1987, Nature 325:529-531

25. Hille B. “Ionic channels of excitable membrane”. 1992, (2^{nd} edn) Sinauer Associates Inc.

26. Pereira A., Furlan F.A. “Astrocytes and human cognition: modeling information integration and modulation of neuronal activity”. 2010, Prog. Neurobiol. 92:405-420

27. Perea G., Navarrete M., Araque A. “Tripartite synapse: astrocytes process and control synaptic information”. 2009, Trens Neurosci. 32:421-31

28. Witcher M.R., Kirov S.A., Harris K.M. “Plasticity of perisynaptic astroglia during synaptogenesis in the mature rat hippocampus” 2007, Glia 55:13-23

29. Eroglu C., Barres B.A. “Regulation of synaptic connectivity by glia”. 2010, Nature 468:23231

30. Huntley G.W., Vickers J.C., Morrison J.H. “Cellular and synaptic localization of NMDA and non-NMDA receptor subunits in neocortex: organizational features related to cortical circuitry, function and disease”. 1994, Trends Neurosci. 17:536-543

31. Seebury P.H. “The tips/tins lecture: the molecular biology of mammalian glutamate receptor channels”. 1993, Trends Pharmacol. Sci. 14:35-40

32. Cotman C.W., Monaghan D.T., Ganong A.H. “Excitatory amino acids and neurotransmission: NMDA receptor and Hebb-type synaptic plasticity”. 1988, Annu. Rev. Neurosci. 11:61-80

33. Reid I.C., Morris R.G.M. “NMDA receptor and learning: a frame-work for classifying some recent studies”.1991, Excitatory amino acids 521-53

34. Coyle J.T., Tsai G., Goff D.C. “Ionotropic glutamate receptors as therapeutic targets in schizophrenia”. 2002, Curr. Drug Target CNS Neurol. Disord. 1:183-189

35. Coyle J.T., Tsai G., Goff D. " Converging evidences of NMDA receptor hypofunction in the pathophysiology of schizophrenia". 2003, Ann. N.Y. Acad. Sci. 1003:318-327

36. Wenthold R.J., Prybylowski K., Standley S., Sans N., Petralia R.S. "Trafficking of NMDA receptors". 2003, Annu. Rev Pharmacol. Tpxicol. 43:335-358

37. Prybylowski K., Wenthold R.J. "N-methyl-D-aspartate receptors: subunit assembly and trafficking to the synapse". 2004, J. Biol. Chem. 279:9673-9676

38. Kemp J.A., Mckeran R.M. "NMDA receptor pathways as drug targets". 2002, Nat. Neurosci. 5:1038-1042

39. Inanobe A., Furukawa H., Gouaux E. "Mechanisms of partial agonist action at the NR1 subunit of NMDA receptors". 2005, Neuron 47:71-84

40. Fucs S.A., Berger R., de koning T.J. "D-serine: the right or wrong isoform?". 2011, Brain Research 1401:104-117

41. McBain C.J., Kleckner N.W., Wyrick S., Dingledine R. "Structural requirements for activation of the glycine coagoniste site of N-methyl-D-aspartate receptors expressed in Xenopus oocytes". 1989, Mol. Pharmacol. 36:556-565

42. Martineau M., Baux G., Mothet J.P. " D-serine signaling in the brain: friend and foe". 2006, Trends Neurosci. 29:481-491

43. Pollegioni L., Sacchi S. "Metabolism of the neuromodulator D-serine". 2010, Cell. Moll. Life Sci. 67:2387-2404

44. Hashimoto A., Oka T., Nishikawa T. "Extracellular concentration of endogenous free D-serine in the rat brain as revealed by in vivo microdialisys". 1995, Neuroscience 66:635-643

45. Hashimoto A., Oka T., Nishikawa T. "Endogenous D-serine in rat brain: N-Methyl-D-aspartate receptor-related distribution and aging".1993, J. Neurochem. 60:783-786

46. Shell M.J., Brady J.R.O., Molliver M.E., Snyder S.H. "D-serine as a neuromodulator: regional and developmental localization in rat brain glia resemble NMDA receptors". 1997, J. Neurosci. 17:1604:1615

47. Wolosker H., Panizzuti R., de Miranda J. "Neurobiology through the looking-glass: D-serine as a new glial-derived transmitter". 2002, Neurochemistry Internat. 41:327-332

48. Furukawa H., Gouaux E. "Mechanisms of activation, inhibition and specificity: crystal structures of the NMDA receptor NR1 ligand-binding core". 2003, EMBO 22:2873-2885

49. Panatier A., Theodosis D.T., Mothet J.P., Touquet B., Pollegioni L., Poulain D.A., Oliet S.H. "Glia-derived D-serine controls NMDA receptor activity and synaptic memory". 2006, Cell 125:775-784

50. Wolosker H., Dumin E., Balan L., Foltyn V.N. "D-amino acids in the brain: D-serine in neurotransmission and neurodegeneration". 2008, FEBS J. 3514-4526

51. Hashimoto A., Oka T., Nishikawa T. “Anatomical distribution and postnatal changes in endogenous free D-aspartate and D-serine in rat brain and periphery” 1995, Eur. J. Neurosci. 7:1657-1663

52. Halassa M.M., Haydon P.G. “Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior”.2010, Annu. Rev. Physiol. 72:335-355

53. Wolosker H. “Serine racemase and the serine shuttle between neurones and astrocytes”. 2011, Biochim. Byophis. Acta in press

54. Puyal J., Martineau M., Mothet J.P., Nicolas M.T., Paymond J. “Changes in D-serine levels and localization during postnatal development of the rat vestibular nuclei”. 2006, J. Comp. Neurol. 497:610-621

55. Oliet S.H.R., Mothet J.P. “Molecular determinats of D-serine-mediate gliotransmission: from release to function”. 2006, Glia 54:726-737

56. Wolosker H., Blackshaw S., Snyder S.H. “Serine racemase, a glial enzyme synthesizing D-serine to regulate glutamate-N-Methyl-D-aspartate neurotransmission”. 1999, Proc. Natl. Acad. Sci. USA 96:13409-13414

57. Kartvelishvily E., Shleper M., Balan L., Dumin E., Wolosker H. “Neuron-derived D-serine release provides a novel means to activate N-methyl-D-aspartate receptots”. 2006 J. Biol. Chem. 281:14151-14162

58. Foltyn V.N., Bendikov I., de Miranda J., Panizzuti R., Dumin E., Shleper M., Toney M.D., Kartvelishvily E., Wolosker H. “Serine racemase modulates intracellular D-serine levels through an α,β-elimintion activity”. 2005, J. Biol. Chem 280:1754:1763

59. Strisovsky K., Jiraskova J., Mikulova A., Rulisek L., Konvalinka J. “Dual substrate and reaction specificity in mouse serine racemase: identification of high-affinity dicarboxilate substrate and inhibitors and analyses of the β-eliminase activity”. 2005, Biochemistry 44:13091-13100

60. Oliet S.H.R., Mothet J.P. “ Regulation of N-methyl-D-aspartate receptors by astrocytic D-serine”. 2009, Neuroscience 158:275-283

61. de Miranda J., Santoro A., Englender S., Wolosker H. “Human serine racemase:molecular cloning, genomic organization, and functional analyses”. 2000, Gene 256:183-188

62. Martineau M., Baux G., Mothet J.P. “Gliotransmission at central glutamatergic synapse: D-serine on stage”. 2006, J. Physiol. 99:103-110

63. Dumin E. Bendikov I., Foltyn V.N., Misumi Y., Ikehara Y., Kartvelishvily E., Wolosker H. “Modulation of D-serine levels via ubiquitin-dependent proteasomal degradation of serine racemase”. 2006, J. Biol. Chem 281:20291-20302

64. Cook S.P., Galve-Rophern I., Martinez del Pozo A., Rodriguez-Crespo I. “Direct calcium binding results in activation of brain serine racemase”. 2002, J. Biol. Chem. 277:27782:27792

65. Dunlop D.S., Neidle A. “Regulation of serine racemase activity by amino acids”. 2005, Brain Res. Mol. Brain Res. 133:208-214

66. Mustafa A.K., Kumar M., Selvakumar B., Ho G.P., ehmsen J.T., Barrow R.K., Amzel L.M., Snyder S.H. “Nitric oxide S-nitrosylates serine racemase, mediating

feedback inhibition of D-serine formation". 2007, Proc. Natl. Acad. Sci. USA 104:1413-1416

67. Kim P.M., Aizawa H., Kim P.S., Huang A.S., Wickramasinghe S.R., Kashani A.H., Barrow R.K., Huganir R.L., Ghosh A., Snyder S.H. "Serine racemase, activating by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration". 2005, Proc. Natl. Acad. Sci. USA 102:2105-2110

68. Fujii K., Maeda K., Hikida T., Mustafa A.K., Balkissoon R., Xia J., Yamada T., Ozeki Y., Kawahara R., Okawa M., Huganir R.L., Ujike H., Snyder S.H., Sawa A. "serine racemase binds to PICK1:potential relevance to schizophrenia." 2006, Mol. Psychiatry 11:150-157
69. Balan L., Foltyn V.N., Zehl M., Dumin E., Dikopostolev E., Knoh D., Ohno Y., Kihara A., Jensen O.N., Radzishevsky I.S., Wolosker H. "Feedback inactivation of D-serine synthesis by NMDA receptor-elicted translocation of serine racemase to the membrane". 2009, Proc. Natl. Acad. Sci. USA 106:7589-7594

70. Shell M.J., Molliver M.E., Snyder S.H. "D-serine, an endogenous synaptic modulator: localization to astrocytes and glutamate-stimulated release". 1995, Proc. Natl. Acad. Sci. USA. 92:3948-3952

71. Martineau M., Galli T., Baux G., Mothet J.P. "Confocal imaging and tracking of the exocytotic routes for D-serine-mediated gliotransmission". 2008, Glia 56:1271-1284

72. Mothet J.P., Pollegioni L., Ouanounou G., Martineau M., Fossier P., Baux G. "Glutamate receptor activation triggers a calcium-dependent and SNARE protein-dependent release of the gliotransmitter D-serine". 2005 Proc. Natl. Acad. Sci. USA 102:5606-5611

73. Ribeiro C.S., Reis M., Panizzuti R., de Miranda J., Wolosker H. "Glial transport of neuromodulator D-serine". 2002, Brai Res. 929:202-209.

74. Hayashi F., Takahashi K., Nhishikawa T. "Uptake of D- and L-serine in C6 glioma cells". 1997, Neurosci. Lett. 239:85-88

75. Javitt D.C., Balla A., Sershen H. "A novel alanine-insensitive D-serine transporter in rat brain synaptosomal membranes". 2002, Brain Res. 941:146-149

76. Yamamoto N., Tomita U., Umino A., Nishikawa T. "Uptake of D-serine by synaptosomal P2 fraction isolated from rat brain". 2001, Synapse 42:84-86

77. Hamase K., Konno R., Morikawa A., Zaitsu K. "Sensitive determination of D-amino acids in mammals and the effect of the D-amino acid oxidase on their amount". 2005, Biol. Pharm Bull. 28:1578-1584

78. Morikawa. A., Hamase K., Inoue T., Konno R., Niwa A., Zaitsu K. "Determination of free D-aspartic acid, D-serine and D-alanine in the brain of mutant mice lacking D-amino acid oxidase activity". 2001, J. Chromatogr. B. Biomed., Sci. Appl. 757:119-125

79. Verral L., Walker M., Rawlings N., Benzel I., Kew J.N., Harrison P.J., Burnet P.W. "d-Amino acid oxidase and serine racemase in human brain: normal distribution and altered expression in schizophrenia". 2007, Eur. J. Neurosci. 26:1657-1669

80. Shoji K., Mariotto S., Ciampa A.R., Suzuki H. "Regulation of serine racemase activity by D-serine and nitric oxide in human glioblastoma cells". 2006, Neurosci. Lett. 392:75-78

81. Shoji K., Mariotto S., Ciampa A.R., Suzuki H. “Mutual regulation between serine and nitric oxide metabolism in human glioblastomal cell”. 2006, Neurosci. Lett. 394: 163-167

82. Dunlop D.S., Neidle A. “The origin and turnover of D-serine in the brain”. 1997, Biochem. Byophis. Res. Commun. 235:26-30

83. Labrie V., Fukumura R., Rastogi A., Fick L.J., Wang W., Boutros P.C., Kennedy J.L., Semeralul M.O., Lee F.H., Baker G.B., Belsham D.D., Barger S.W., Gondo Y., Wong A.H., Roder J.C. “Serine racemase is associated with schizophrenia susceptibility in humans an in a mouse model”. 2009, Hum. Mol. Genet. 18:3227-3243

84. Hirabayashi Y., Furuya S. “Role of L-serine and sphingolipids synthesis in brain development and neuronal survival”. 2008, Prog. Lipid Res. 47:188-203

85. Yamasaki M., Yamada K., Furuya S., Mitoma J., Hirabayashi Y., Watanbe M. “3-Phosphoglycerate dehydrogenase, a key enzyme for l-serine biosynthesis, is preferentially expressed in the radial glia/astrocyte lineage and olfactory ensheathing glia in the mouse brain.” 2001, J. Neurisci. 21: 7691-7704

86. Yoshida K., Furuya S., Osuka S., Mitoma J., Shinoda Y., Watanabe M., Azuma N., Tanaka H., Hashikawa T., Itohara S., Hirabayashi Y. “Targeted disruption of the mouse 3-phosphoglycerate dehydrogenase gene causes severe neurodevelopmental defects and results in embryonic lethality.” 2004, J. Biol. Chem 279:3573-2577.

87. Yang J.H., Wada A., Yoshida K., Miyoshi Y., Sayano T., Esaki K., Kinoshita M.O., Tomonaga S., Azuma N., Watanabe M., Hamase K., Zaitsu K., Machida T., Messig A., Itohara S., Hirabayashi Y., Furuya S. “Brain-specific Phgdh deletion reveals a pivotal role for L-serine biosynthesis in controlling the level of D-serine, an N-methyl-D-aspartate receptor coagonist, in adult brain”. 2010, J. of Biol. Chem. 53:41380-41390

88. Krebs H.A. “Metabolism of amino acids: deamination of amino acids”. 1935, Biochem. J. 29:1620-1644

89. Pilone M.S “D-amino acid oxidase: new findings”. 2000 Cell. Moll. Life Sci. 57:1732-1747

90. Pollegioni L., Piubelli L., Sacchi S., Pilone M.S., Molla G. “Physiological functions of D-amino acid oxidase: from yeast to humans”. 2007, Cell. Moll. Life. Sci. 64:1373-1394

91. Verral L., Burnet P.W.J., Betts J.F., Harrison P.J. “The neurobiology of D-amino acid oxidase (DAO) and its involvement in schizophrenia”. 2010, Mol. Psychiatry 15:122-137

92. Ohide H., Miyoshi Y., Maruyama R., Hamase K., Konno R. “D-amino acid metabolism in mammals: Biosynthesis, degradation and analytical aspects of the metabolic study”. 2011, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. article in press

93. Khoronenkova S.V., Tishkov V.I. “D-amino acid oxidase: physiological role and applications”. 2008, Biochemistry 13:1511-1518

94. Gould S.J., Keller G.A., Subramani S. “Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins”. 1988, J. Cell Biol. 107:897-905

95. Caldinelli L., Iametti S., Barbiroli A., Bonomi F., Piubelli L., Ferranti P., Picariello G., Pilone M.S., Pollegioni L. “Unfolding intermediate in the peroxisomal flavoprotein D-amino acid oxidase”. 2004, J. Biol. Chem. 279:28426-28434

96. Fukui K., Miyake Y. “Molecular cloning and chromosomal localization of a human gene encoding D-amino acid oxidase”. 1992, J. Biol. Chem. 267:18631-18638

97. Momoi K., Fukui K., Watanabe F., Miyake Y. “Molecular cloning and sequence analysis of cDNA encoding human kidney D-amino acid oxidase”. 1988, FEBS Lett. 238:180-184

98. Cline M. J., Lehrer R.I. “D-amino acid oxidase in leukocytes: a possible D-amino-acid-linked antimicrobial system”. 1969, Proc. Natl. Acad. Sci. USA 62:756-763

99. Robinson J. M., Briggs R.T., Karnowsky M.J. “Localization of D-amino acid oxidase on the cell surface of human polymorphonuclear leukocytes”. 1978, Proc. Natl. Acad. Sci. USA 77:59-71

100. Gossrau R., Frederiks W.M., van Noorden C.J., Klebe S., Ruhnke M. “Light microscopical detection of H2O2-generating oxidases using cerium ions and aqueous incubation media”. 1991, Acta Histochem. 90:27-37

101. Horiike K., Tojo H., Arai R., Nozaki M., Maeda T. “D-amino acid oxidase is confined to the lower brain stem and cerebellum in rat brain: regional differentiation of astrocytes”. 1994, Brain res. 652:297:303

102. Horike K., Tojo H., Arai R., Yamano T., Nozaki M., Maeda T. “Localization of D-amino acid oxidase in Bergmann glial cells and astrocytes of rat cerebellum”. 1997, Brain Res. Bull. 19:587-596

103. Urai Y., Jinnouchi O., Kwak K.T., SuzueA., Nagahiro S., Fukui K. “Gene expression of D-amino acid oxidase in cultured rat astrocytes: regional and cell type specific expression”. 2002, Neurosci Lett. 324:101-104

104. Moreno S., Nardacci R., Cimini A., Cerù M.P. “Immunocytochemical localization of D-amino acid oxidase in rat brain”. 1999, J. Neurocytol. 28:169-185

105. Kappor R., Kappor V. “Distribution of D-amino acid oxidase (DAO) activity in medulla and thoracic spinal cord of the rat: implications for a role for D-serine in autonomic functions”. 1997, Brain Res. 771:351-335

106. Ono K., Shishido Y., Park H.K., Kawazoe T., Iwana S., Chung S.P., Abou El-Magd R.M., Yorita K., Okano M., Watanabe T., Sano N., Bando Y., Arima K., Sakai T., Fukui K. “Potential pathophysiological role of D-amino acid oxidase in schizophrenia: immunohistochemical and in situ hybridization study of the expression in human and in rat brain”. 2009, J. Neural. Transm 116:1335-1347

107. Sacchi S., Bernasconi M.G., Martineau M., Mothet J.P., Ruzzene M., Pilone M.S., Pollegioni L., Molla G. “pLG72 modulates intracellular D-serine levels through its interaction with D-amino acid oxidase”. 2008; J. Biol. Chem. 32:22244-22256

108. Molla G., Sacchi S., Bernsconi M., Pilone M.S., Fukui K., Pollegioni L. “Characterization of human D-amino acid oxidase”. 2006, FEBS Lett. 580:2358-2364

109. Kawazoe T., Tsuge H., Pilone M.S., Fukui K. “Crystal structure of human D-amino acid oxidase:context-dependent vriability of the backbone conformation of the VAAGL hydrophobic stretch located at the si-face of the flavin ring”. 2006, Protein Sci. 15:2708-2717

110. Subramani S. “Protein import to peroxisomes and biogenesis of the organelle”. 1993, Annu. Rev. Cell. Biol. 9:445-478

111. Mattevi A., Vanoni M.A., Todone F., Rizzi M., Teplyakov A., Coda A., Bolognesi M., Curti B. “Crystal structure of D-amino acid oxidase: a case of active site mirror-image convergent evolution with flavocytochrome b2”. 1996, Proc. Natl. Acad. Sci. USA 93:7496-7501

112. Caldinelli L., Molla G., Sacchi S., Pilone M.S., Pollegioni L. “Relevance of weak flavin binding in human D-amino acid oxidase”. 2009, Protein Sci. 18:801-810

113. Caldinelli L., Molla G., Bracci L., Lelli B., Pileri S., Cappelletti P., Sacchi S., Pollegioni L. “Effect of ligand binding on human D-amino acid oxidase: Implications for the development of new drugs for schizophrenia treatment”. 2010, Protein Sci. 19:1500-1512

114. Konno R., Yasumara Y. “Mouse mutant deficient on D-amino acid oxidase activity”. 1983, Genetics 103:277-285

115. Sakata K., Fukushima T., Minje L., Ogurusu T., Taira H., Mishina M., Shingai R. “Modulation by L- and D-isoforms of amino acid of the L-glutamate response of N-methyl-D-aspartate receptors”. 1999, Biochemistry 38:10099-10106

116. Konno R., Okamura T., Kasai N., Summer K.H, Niwa A. “Mutant rat strain lacking D-amino acid oxidase”. 2009, Amino Acids 37:367

117. Chumakov I., Blumenfeld M., Guerassimenko O., Cavarec L., Palicio M., Abderrahim H., Bougueleret L., Barry C., Tanaka H., La Rosa P., Puech A., Tahri H., Cohen-Akenine A., Delabrosse S., Lissarrague S., Picard F.P., Maurice K., Essioux L., Millasseau P., Grel P., Debailleul V., Simon A.M., Caterina D., Dufaure L., Malekzadeh K., Belova M., Luan J.J., Bouillot M., Sambucy J.L., Primas G., Saumier M., Boubkiri N., Martin-Saumier S., Nasroune M., Peixoto H., Delaye A., Pinchot V., Bastucci M., Guillou S., Chevillon M., Sainz-Fuertes R., Meguenni S., Aurich-Costa J., Cherif D., Gilmac A., van Duijn C., Gauvreau D., Oullette G., Fortier I., Raelson J., Sherbatich T., Riazanskaia N., Rogaev E., Raeymaekers P., Aerssens J., Konings F., Luyten W., Macciardi F., Sham P.C., Straub R., Weinberger D. R., Coehn N., Coehn D. “Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia”. 2002, PNAS 21:13675-13680

118. Ohi K., Hashimoto R., Yasuda Y., Yoshida T., Takahashi H., Iike N., Fukumoto M., Takamura H., Iwase M., Kamino K., Ishii R., Kazui H., Sekiyama R., Kitamura Y., Azechi M., Ikezawa K., Kurimoto R., Kamagata E., Tanimukai H., Tagami S., Morihara T., Ogasawara M., Okochi M., Tokunaga H., Numata S., Ikeda M., Ohnuma T., Ueno S., Fokunaga T., Tanaka T., Kudo T., Arai H., Ohomori T., Iwata N., Takeda M. “Association study of the G72 gene with schizophrenia in Japanese population: a multicenter study”. 2009, Schizophr. Res. 109:80-85

119. Chen F.C., Li W.H. “Genomic divergences between humans and other hominoids and the effective population size on the common ancestor of humans and chimpanzee”. 2001, Am. J. Hum. Genet. 68:444-456

120. Molla G, Bernasconi M., Sacchi S., Pilone M.S., Pollegioni L. “Expression in Escherichia coli and in vitro refolding of the human protein pLG72”. 2006, Protein expr. Purif. 46:150-155

121. Korostishevsky M., Kaganovich M., Cholostoy S., Ashkenazy M., Ratner Y., Dahary D., Bernstein J., Bening-Abu-Shach U., Ben-Asher E., Lancet D., Ritsner M., Navon R. “Is the G72/G30 locus associated with schizophrenia? Single nucleotide polymorphism, haplotypes, and gene expression analysis”. 2004, Biol. Psychiatry. 56:169-176

122. Benzel I., Kew J.N., Viknaraja R., Kelly F., de Belleroche J., Hirsch S., Sanderson T.H., Maycox P.R.”Investigation of G72 (DAOA) expression in the human brain”. 2008, 11:8-94

123. Otte D.M., Bilkei-Gorzo A., Filiou M.D., Turck C.W., Ylmaz O., Holst M.I., Schilling K., Abou-Jamra R., Schumacher J., Benzel I., Kunz W.S., Beck H., Zimmer A. “Behavioral changes in G72/G30 transgenic mice”. 2009, Eur. Neuropharmacol. 19:339-348

124. Popiolek M., Ross J.F., charych E., Chanda P., Gundelfinger E.D., Moss S.J., Brandon N.J., Paush M.H. “D-amino acid oxidase activity is inhibited by an interaction with Basson protein at the presynaptic active zone”. 2011, J. Biol. Chem 286:28867-28875

125. Mossner R., Shumaker A., Wagner M., Quednow B.B., Frommann I., Kuhn K.U. “DAOA/G72 predicts the progression of prodromal syndromes to the first episode psychosis”. 2010, Eur. Arch., Psychiatry Clin. Neurosci. 260:209-215

126. Burnet P.W., Eastwood S.L., Bristow G.C., Godlewska B.R., Sikka P., Walker M., Harrison P.J. “D-amino acid oxidase activity and expression are increased in schizophrenia”. 2008, Mol. Psychiatry 13:658-660

127. Kapoor R., Lim K.S., Cheng A., Garrick T., Kapoor V. “Preliminary evidence for a link between schizophrenia and NMDA-glycine site receptor ligand metabolic enzymes, d-amino acid acid oxidase (DAAO) and kynurenine aminotransferase-1(KAT-1)”. 2006, Brain Res. 1106:205-210

128. Habl G., Zink F., Petroianu G., Bauer M., Shneider-Axmann T., von W.M., Falkai P., Henn F.A., Shmitt A. “Increased D-amino acid oxidase expressionin the bilateral hippocampal CA4 of schizophrenic patients: a post- mortem study”. 2009, J. Neural. Transm. 116:76-83

129. Harrison P.J., Weinberg D.R. “Schizophrenia genes, gene expression, and neuropathology: on the matter on their convergences”. 2005, Mol. Psychiatry 10:40-68

130. Cloninger C.R. “The discovery of susceptibility genes for mental disorders”. 2002, Proc. Natl. Acad. Sci. USA 99:13365-13367

131. Bendikov I., Nadri C., Amar S., Panizzuti R., de Miranda J., Wolosker H. “A CSF and post mortem brain study of D-serine metabolic parameters in schizophrenia” 2007, Schizophr. Res. 90:365-368

132. Hashimoto K., Engbrg G., Shimizu E., Nordin C., Lindstrom L.H., Iyo M. “Reduced D-serine to total serine ratio in the cerebrospinal fluid of drug naïve schizophrenic patients”. 2005, Biol. Psychiatry 29:767-769

133. Hashimoto K., Fukushima T., Shimizu E., Komatsu N., Wutanabe H., Shinoda N., Nakazato m., Kumakiri C., Okada S., Hasegawa H., Imai K., Iyo M. “Decreased serum levels of D-serine in patients with schizophrenia: evidence in support the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia”. 2003, Arch. Gen Psychiatry 60:572-576

134. Heresco-Levy U., Javitt D.C., Ebstein R., Vass A., Lichtenberg P., Bar G., Catinari S., Ermilov M. “D-serine efficacy as add-on pharmacotherapy to risperidone and olanzapine for treatment-refractory schizophrenia”. 2005, Biol Psychiatry 57:577-585

135. Goltsov A.Y., Loseva J.G., Andreeva T.V., Grigorenko A.P., Abramova L.I., Kaleda V.G., Orlova V.A., Loliaka Y.K., Rogaev E.I. “Polymorphism in the 5'-promoter region of serine racemase gene in schizophrenia”. 2006, Mol. Psychiatry 11:325-326

136. Morita Y., Ujieke H., Tanaka Y., Otni K., Kishimoto M., Moiro A., Kotaka T., Okahisa Y., Mutsushita M., Morikawa A., Hamase K., Zaitsu K., Kuroda S. “A genetic variant of th serine racemase gene is associated with schizophrenia”. 2007, Biol. Psychiatry 61:1200-1203

137. Otte D.M., Sommersberg B., Kudin A., Guerrero C., Albyram O., Filiou M.D., Frish P., Ylmaz O., Drews E., Turk C.T., Bilkei-Gorzo A., Kunz W.S., Beck H., Zimmer A. “N-acetyl Cysteine treatment rescues cognitive deficits induced by mitochondrial dysfunction in G72/G30 transgenic mice”. 2011, Neuropsychopharmacol. 36:2233-2243

138. Tandon R., Keshavan M.S., Nasrallah H.A. “Schizophrenia, “Just the fact” what we know in 2008. 2. Epidmiology and etiology”. 2008, Scizophr. Res. 102:1-18

139. Ross C.A., Margolis R.L., Readings S.A.J., Pletnikov M., Coyle J.T. “Neurobiology of schizophrenia”. 2006, Neuron 52:139-153

140. Lang U.E., Puls I., Muller D.J., Strutz-Seebohm N., Gallinat J. “Molecular mechanisms of schizophrenia”. 2007, Cell Physiol. Biochem. 20:687-702

141. Niemi L.T., Suvisaari J.M., Tuulio-heriksson A., Lonnqvist J.K. “Childhood developmental abnormalities in schizophrenia: evidence from high-risk studies” 2003, Schizophr. Res. 60:239-258

142. Niznikiewicz M.A., Kubicke M., Shenton M.E. “Recent structural and functional imaging finding in schizophrenia”. 2003, Curr. Opin. Psych. 16:123-147

143. Jackob H., Beckmann H. “Gross and hystological criteria for developmental disorders in brains of schizophrenics”. 1989, J. R., Soc. Med. 82:466-469

144. Roberts R.C., Roche J.K., Conley R.R. “Synaptic differences in the patch matrix compartments of subjects with schizophrenia: a postmortem ultrastructural study of the striatum”. 2005, Neurobiol. Dis. 20:69-80

145. Hirvonen M., Laasko A., Nagren K., Rinne J.O., Pohjalainen T., Hietala J. “C957T polymorphism of the dopamine D2 receptor (DRD2) gene affects striatal DRD2 availability in vivo”. 2004, Mol. Psychiatry 9:1060-1061

146. Coyle J.T. “Glutamate and schizophrenia: beyond the dopamine hypothesis”. 2006, Cell Mol. Pharmacol. 26:365-384

147. Kantrowitz J.T., Javitt D.C. “N-methyl-D-aspartate (NMDA) receptor dysfunction or dysregulation: the final common pathway on the road of schizophrenia?”. 2010, Brain Res. Bull. 83:108-121

148. Akbarian S., Kim L., Poktin S., Hagman J., Tafazzoli A., Bunney B.J., Jones E.G. “Gene expression for glutamic acid decarboxilase is reduced without loss of neurons in prefrontal cortex of schizophrenics”. 1995, 52:258-266

149. Hershko A., Chiechanover A. “The ubiquitin system”. 1998, Annu. Rev. Biochem. 67:425-479

150. Yi J.J., Ehlers M.D. “Ubiquitin and protein turnover in synapse function”. 2005, Neuron 47:629-632

151. Chung S.P., Soquabe K., Park H.K., Song Y., Ono K., Abou El-Magd R.M., Shishido Y., Yorita K., Sakai T., Fukui K. “Potential cytotoxic effect of hydroxipiruvate produced from D-serine by astroglial D-amino acid oxidase”. 2010, J. Biochem. 48:743-753