UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE

XXIV CICLO

Novel mechanisms in the modulation of D-serine cellular concentration: hDAAO-pLG72 interaction

Docenti guida: Prof. **Loredano Pollegioni**

Dott.ssa **Silvia Sacchi**

Tesi di dottorato di:

Pamela Cappelletti

The Protein Factory

DBSV-Università degli Studi dell'Insubria

Anno accademico 2010-2011

Ala: alanine

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolapropionic acid receptor

ASC1: alanine-serine-cysteine transporter 1

ASCT: amino acid transporter type transporters

ATP: adenosine triphosphate

BAC: bacterial artificial chromosome

BD: bipolar disorder

Bp: base pairs

BS: bassoon

Cb: cerebellum

CD: Circular dichroism

CNS: central nervous system

CF_3-D-Ala: trifluoro-D-alanine

COS-7: african green monkey fibroblast or fibroblast-like cells

CPZ: chlorpromazine

C-terminal: carboxyl terminal

CTRL: control

Ctx: cerebral cortex

DAAO: d-amino acid oxidase

DAOA: d-amino acid oxidase activator

DSM-IV: diagnostic and statistical manual of mental disorder 4^{th} edition

EAAT:excitatory amino acid transporter

E.Coli: Escherichia coli

ECFP: enhanced cyan fluorescent protein

EGFP: enhanced green fluorescent protein

ERC1: ELKS/RAB6-interacting/CAST family member 1

EYFP: enhanced yellow fluorescent protein

FAD: flavin adenine nucleotide

fMRI: Functional magnetic resonance imaging

GABA: gamma-aminobutyric acid

GAT: GABA transporter

GFAP: glial fibrillary acid protein

Gln: glutamine

Glu: glutamate

GRIP: glutamate receptor interacting protein

Golga 3: golgin subfamily A member 3

G-protein: guanine nucleotide-binding proteins

hDAAO: human D-amino acid oxidase

Hp: hippocampus

Hyp: hypothalamus

K_d: dissociation costant

KDa: kilodalton

K_m: Michaelis constant

Lys: lysine

LTP: long term potentiation

mGluR: Metabotropic glutamate receptor

mRNA: messanger ribonucleotide acid

NMDAR: N-methyl-D-aspartate receptor

NO: nitric oxide

N-terminal: amino terminal

Ob: olfactory bulb

ORF: open reading frame

PCP: phencyclidine

PDZ domain: post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)

Phgdh: phosphoglycerate dehydrogenase

PICK1: protein interacting with C-kinase

PIP2: phosphatidylinositol(4,5)biphosphate

pkDAAO: porcine kidney's D-amino acid oxidase

PLP: pyridoxal 5'-phosphate

PTS1: peroxisomal targeting signal 1

$P2X_7$: purinergic receptor P2X, ligand-gated ion channel 7

RAPGEF4: Rap guanine nucleotide exchange factor (GEF) 4

RgDAAO: Rhodotorula gracilis D-amino acid oxidase

RT-PCR: Reverse transcription polymerase chain reaction

SEM: standard error of the mean

SHL: Serine-Histidine-Lysine sequence

SLMV: synaptic-like microvescicles

SNARE: SNAP (Soluble NSF Attachment Protein) Receptor

SNIP: SNAP-25-interacting protein

SNP: single nucleotide polymorphysm

SR: serine racemase

St: striatum

T: thalamus

TCA: tricarboxilic acid

TNFα: tumor necrosis factor α

UPS: ubiquitin-proteasome system

UTR: untranslated region

UV: ultraviolet

U87: human glioblastoma cell line

U251: human glioblastoma cell line

VAMP: Vesicle associated membrane proteins

V_{max}: maximum reaction rate

1. Not only neurotransmission: the trypartite synapse model

The chemical synapse is the structure where transmission of information between neurons occurs: in fact it is a place of contact of two neurons in series (defining the pre- and post- synaptic compartments), and neurotransmitters released upon depolarization of the presynapse, and of the binding to specific receptors on the post synaptic target cell (Oliet, Glia 2006).

This physiological concept is now profoundly changed by recent findings describing the role of glial cells (and particularly astrocytes) as the third element of the chemical synapse (1-5).

Astrocytes are a star-shaped subtype of glial cells present in the central nervous system (CNS), envrapping the synapses. Although they compose at least one half of human brain tissue volume, until two decades ago they were supposed to be devoted only to passive functions such as giving structural, functional and metabolic support to neurons (6-12). The "astrocytic revolution" in current neuroscience began early in the 1990s. Pioneering studies employed the fluorescence techniques to monitor intracellular Ca^{2+} levels in living astrocytes revealed that cultured astrocytes display a form of "excitability" based on variations of the intracellular Ca^{2+} concentration (13, 14). Moreover, three-dimensional reconstruction of dye-filled astrocytes showed that these glial cells extend thousands of intricate processes that are organized into large, non-overlapping anatomical domains. It has been estimated that a single astrocyte can associate with multiple neurons and over 1,000,000 synapses (15, 16). Now it has been role out that glia actively communicate with pre- and postsynaptic neurons thus influencing functions that have long been thought to be under neuronal control. Bidirectional signaling between neurons and astrocytes influence synaptic functionality and plasticity in mature brain. This cross-talk plays also a pivotal role in the developing brain during the dynamic period (when neuronal circuits were formed and refined), influencing the formation and elimination of synapses, as well as synaptic morphology and structural plasticity (17, 18). All these findings are collected in the concept of the "**tripartite synapse**" (Fig 1) which state the importance of astroglia as an integral part of central and peripheral synapses. Astrocytes participating in trypartite synapse are coupled by gap-junction forming a network that can support brain large-scale integrative functions, from dynamic glucose delivery (19) to cognitive information processing (6, 20).

The term “tripartite” encompasses presynaptic neurones, postsynaptic neurones, and glia and includes several targets involved in the regulation of synaptic and extrasynaptic glutamate levels, such as excitatory amino acid transporters (EAATs), postsynaptic density proteins, AMPA-, NMDA- and kainite-receptors (21).

Moreover, it has been demonstrated that astrocytes express membrane receptors to neurotransmitters and several evidences strongly suggested that they release their own chemical messenger named **“gliotransmitters”**. According to this model glial cells first sense synaptic activity through the receptors expressed at their surface (22). Synaptic activation of astrocytes then triggers intracellular second messenger pathway including Ca^{2+} increases. In turn, activation of these second messenger pathway induce the release of gliotransmitters (23). Whereas neurons base their cellular excitability on electrical signal generated across the plasma membrane, astrocytes responsiveness depend on variations of Ca^{2+} concentration in the cytoplasm. This Ca^{2+} waves in turn, yield to the release of signaling molecules: compelling evidences indicated that gliotransmitters are released in a Ca^{2+}-dependent manner through vesicles and lysosomes exocytosis. Ultrastructural studies shown that astrocytic processes contain small synaptic-like vesicles, which are located in close proximity to synapses. The mechanism of neuromodulator release is however still debated: alternative release mechanisms, including reversal of glutamate transporters, connexin/pannexin hemichannels, pore-forming P2X7 receptor and swelling-induced activation of volume-regulated anion channels, have also been proposed (24, 25).

By means of the release of a set of signaling molecules (glutamate, D-serine, ATP, adenosine, GABA, tumor necrosis factor α -TNFα-, prostaglandins, proteins and peptides), astrocityc modulation of neuronal activity contributes to substain, reinforce or depress pre- and post-synaptic (26) and thus influence neuronal and synaptic physiology. Glutamate was one of the first gliotransmitters released from astrocytes to be identified and has been reported to exert many effects on neuronal excitability (27).

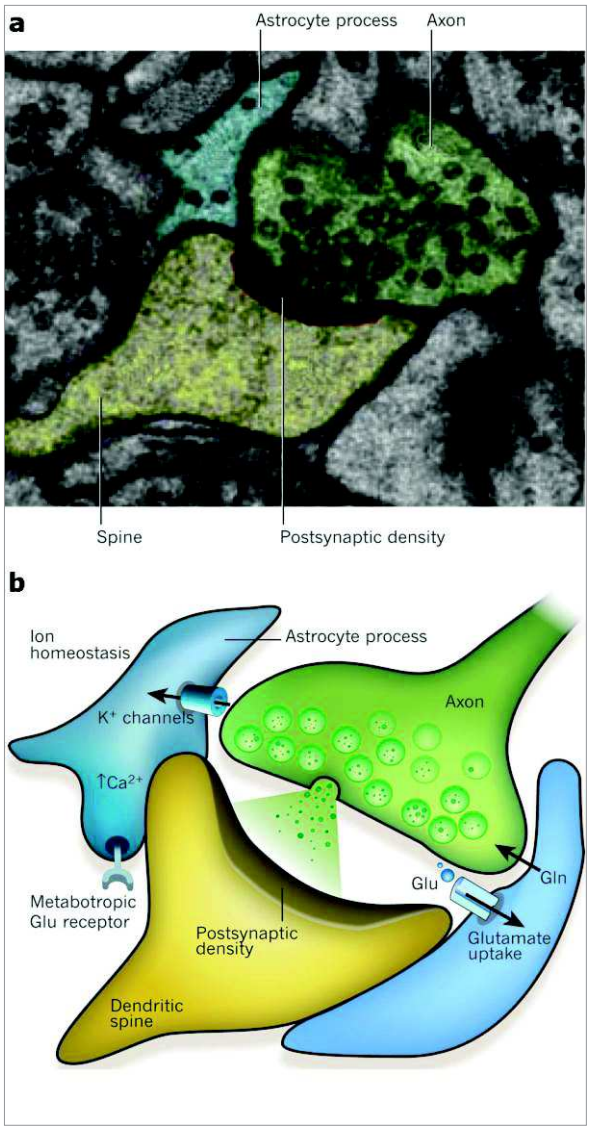

Fig 1. The trypartite synapse. The processes of astrocytes are intimately associated with synapses. This association is both structural and functional. **a)** Electron micrograph showing a tripartite synapse in the hippocampus. The astrocyte processes (blue) ensheaths the perisynaptic area. The axon of the neuron is shown in green, with the dendritic spine in yellow and the postsynaptic density in red and black (28). **b)** Schematic representation of a tripartite synapse. Perisynaptic astrocyte processes contain transporters that take up glutamate (Glu, green circles) that has been released into the synapse and return it to neurons in the form of glutamine (Gln). Glutamate receptors on astrocytes (such as metabotropic glutamate receptors) sense synaptic glutamate release, which in turn induces a rise in Ca^{2+} concentration in the astrocytes. One of the main function of glia at the synapse is to maintain ion homeostasis, for example regulating extracellular K^+ concentration and pH (29).

Appendix I: neurotransmission and N-methyl-D-aspartate receptors (NMDAR)

In the brain, most of the excitatory neuronal transmission is mediated by endogenous excitatory amino acids: glutamate, aspartate and homocysteine. Their actions is connected at distinct families of metabotropic receptors (that are G-protein coupled, tyrosine kinase and guanylin cyclase receptors) and three subtypes of ionotropic receptors: amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors (30, 31). **Glutamatergic receptors** play an important role in several key brain functions, including learning and memory, synaptic and developmental plasticity, sensory information and coordinated movement patterns. Their function is also essential to neurite outgrowth, synaptogenesis, and maturation of synapse in the developing brain (32, 33).

In particular, **NMDA receptors** are among the most important synaptic receptors in the CNS: broadly distributed throughout the brain, they play a major role in glutamatergic synaptic transmission and participate in synaptic events associated with the outstanding physiological progresses detailed above (34, 35). NMDA receptors has been found at both synaptic and extrasynaptic sites, but are present at much higher density at the synapse (36).

They are organized as heteromeric complexes of different subunits (NR1, NR2a, NR2b, NR3), composed of spliced variant of NR1 subunit, combined with at least one of four NR2 subunits. A third subunits, NR3, which is less common, can co-assemble with NR1/NR2 complex. The C-terminal domain of the receptor is the site of numerous protein-protein interactions determining the trafficking and synaptic organization of the receptor. The channel pore is formed by three transmembrane domains and a hairpin bend within the membrane. The N-terminus and the loop between the two transmembrane domains contain S1-S2 region, which forms the binding pocket for the agonist. The NMDA receptors are unique in their requirement for more than one agonist to operate: the receptor channel is blocked by Mg^{2+}, depolarization removes this block and enables ion flux when the receptor binds both glutamate (on NR2 subunit) and a co-agonist (on NR1 subunit) (37-39). This regulation of NMDAR activity by a second agonist may be regarded as a safety mechanism to protect against the deleterious effects of overstimulation of the NMDAR (excitotoxicity) (24, 36, 40, 41). Co-agonist binding increases the receptor's affinity for glutamate, decrease its desensitization and promotes NMDAR turnover by internalization. In the past, the NMDAR co-agonist site was thought to be occupied by glycine, hence, the co-agonist site is also generally referred as the “glycine site” (Fig 2). Now **D-serine** is widely recognized as the main physiological ligand of the NMDAR co-agonist site, mediating several NMDA-dependent processes.

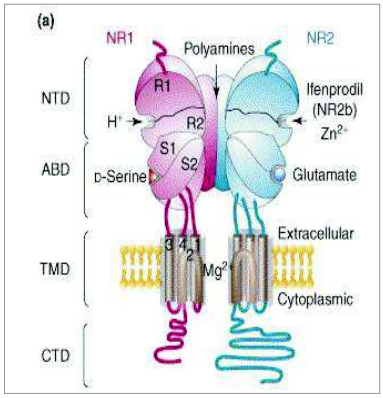

Fig 2. A model of NR1 and NR2 NMDA receptor subunits, depicting functional domains and binding site (42).

Notably, over- or down- stimulation of NMDAR is implicated in psychiatric disorders, such as schizophrenia and in many acute and chronic degenerative disorders, including stroke, epilepsy, peripheral neurophaties, and Parkinson's and Alzheimer's disease (43).

Appendix II: D-serine vs. glycine role in activating NMDA receptors

Both D-serine and glycine are present in the human CNS in similar concentration (44) and both were found to colocalize with NMDAR. However, D-serine mimics the anatomical localization of NMDAR more precisely. Immunohistochemical studies demonstrated that the regional distribution of D-serine in rat is well defined in cranial area of the brain and overlaps almost perfectly with that of NMDAR; while the intensity of D-serine corresponding signal is much lower in the caudal part of the brain (45, 46). In contrast, glycine immunoreactivity is higher in that brain's area where the density of NMDAR is lower. The inverse localization of D-serine and glycine suggested that endogenous D-serine is physically closer to NMDAR than glycine.

Moreover, it has been observed that D-serine is up to three-fold more potent than glycine as a co-agonist of NMDAR (47), this indicating D-serine prominent role in modulating the functionality of the receptor.

A structural explanation for the higher potency of D-serine in modulating NMDAR activity comes from the analysis of the crystal structure of the binding core of the NR1 subunit. D-Serine binds more tightly to the receptor in comparison with glycine, since it makes three additional hydrogen bonds and displaces a water molecule from the binding pocket (48).

A direct demonstration that D-serine is the principal physiological NMDAR co-agonist arose from experiments in which D-serine metabolic enzyme (D-amino acid oxidase, DAAO) was employed to remove in a selective manner, endogenous D-serine from brain slices and cells cultures, leaving the levels of glycine unchanged. The selective depletion of D-serine from immature rat cerebellar slices, rat hippocampus slices and primary hippocampal cell culture yield to a decrease (up to 70%) in the spontaneous activity attributed to post-synaptic NMDAR. Additionally, electrophysiological experiments performed in the supra-optic nucleus of the hypothalamus demonstrated that endogenous D-serine degradation blocked

NMDAR responses in hypothalamic slices, as opposed to endogenous glycine enzymatic depletion (49). Taken together, these studies rule out the importance of D-serine in mammalian brain physiology, as a modulator of NMDAR mediated neurotransmission.
The difference between the relative activities of exogenous D-serine and glycine to active NMDAR has been attributed to the presence at synapse of a glycine transporter that set concentration of glycine within the cleft well below that required to saturate the co-agoniste site of the receptor. On the other hand, specific transporters for D-serine have not been yet discovered and the affinity of neutral amino acid transporters is low to moderate (50). The absence of a specific transporter for D-serine could explain the elevated levels of the D-amino acid, despite glycine, at the synaptic cleft.

2. D-Serine: a transmitter-like molecule

Only a few decades ago, D-amino acids were considered to be restricted to some bacteria and insects. In the last few years, evidence is accumulating that D-amino acids occur in mammals. Recently, most of research has focused on D-serine, which was found to occur in a significant high concentration in mammalian brain. D-serine is synthesized endogenously by the enzymes serine racemase (SR) and metabolized by both SR and D-amino acid oxidase (DAAO). In the CNS, D-serine function as a neurotransmitter: it fulfills all criteria to be the major ligand for the strychnine-intensive glycine modulatory binding site of NMDA receptors (40).

2.1. Regional distribution in tissues

D-Serine is present in significant amount in the mammalian brain, but it is detected also at lower levels in peripheral organs outside the nervous systems such as heart, pancreas, kidney and blood (51).
In the brain the distribution of the neuromodulator is heterogenous, with the highest concentrations detected in the telencephalon (as showed in Fig 3a) and in the developing cerebellum. Its distribution in the rat brain significantly changes with post-natal development: it is almost homogenous immediately after birth, but approaches the pattern of mature stage during the successive three weeks period (51). At adult stage, the highest concentration of the D-amino acid has been observed in the cerebrum cortex (particularly in gray matter), followed by the thalamus, the striatum, the hippocampus, the hypothalamus and the amygdala. Moreover, moderate to low concentrations of D-serine are detected in diencephalon

and midbrain and trace levels in the pons-medulla and cerebellum. It has been observed that, in the brain, the distribution of the D-amino acid closely resemble that of NR2A/B subtype of NMDA receptors.

2.2. Cellular distribution

D-Serine is mostly associated with astrocytes (Fig 3b) that ensheath synapses; it colocalizes with glial fibrillary acid protein (GFAP) and with glutamine synthetase, both well documented glial cell marker. D-serine is more abundant in neuropil regions where protoplasmatic astrocytes are prominent, however a significant amount of the neuromodulator has also been observed in astrocytes sourrounding blood vessels (46) and, trace amounts were detected in quiescent and activated microglia cells and oligodendrocytes (45). Interestingly, the cellular distribution of D-serine in the CNS is developmentally regulated: in the developing cerebellum D-serine is localized in Bergmann glia while in adults declines to negligible levels (46).

Although glial D-serine is prominent, the presence of the D-amino acid is reported also in neurons (52), particularly in those of the cerebral cortex, vestibular nuclei and retina (53). At this point it is not clear if D-serine localized and released by astrocytes plays a different physiological role with respect to neuronal one. An interesting model formulated recently by Wolosker, “the serine shuttle” suggests that neurons perfom the role of D-serine “producer” while astrocytes play that of storage site for the neuromodulator (see appendix III). Moreover, it is intriguing to speculate that glial and neuronal D-serine may be differentially regulated during the development: it has been reported that in the rat vestibular nuclei, the localization of D-serine shift from glia to neurons in older rat (54).

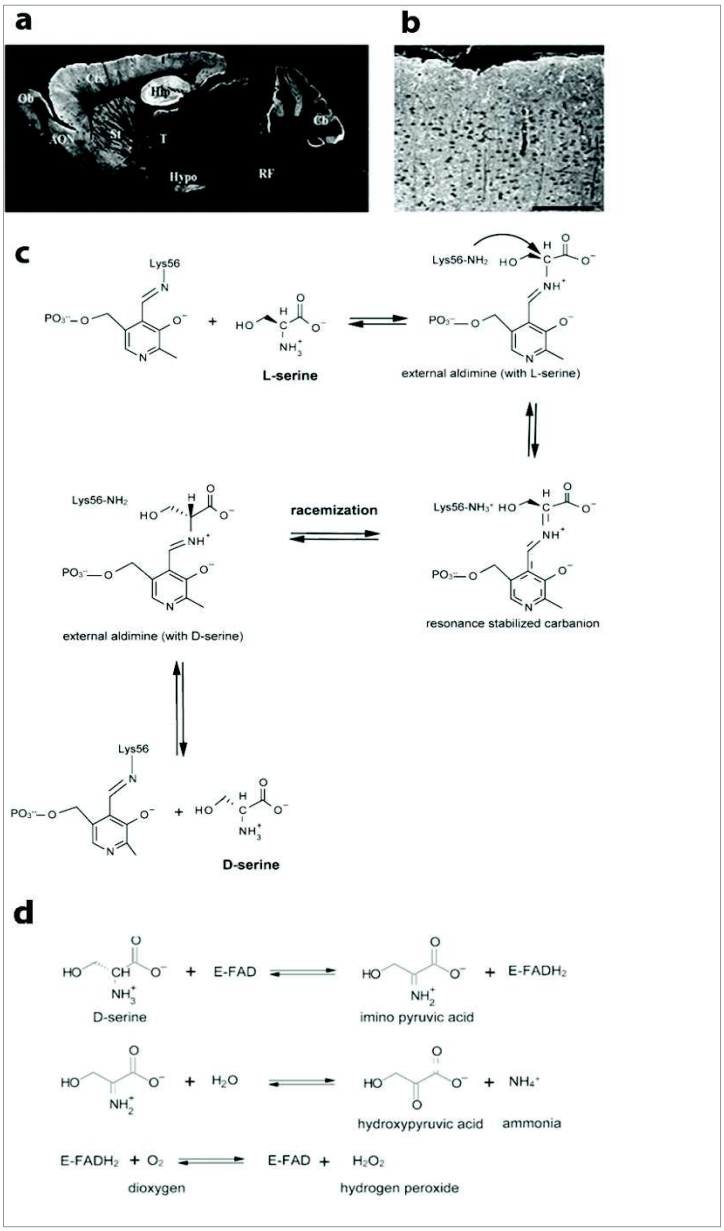

Fig 3. D-Serine distribution and metabolism in the brain. a) D-Serine immunoreactivity is mostly retrieved in structures of the telencephalon. Highest levels are found in the cerebral cortex (Ctx), olfactory bulb (Ob), hippocampius (Hp), striatum (St), thalamus (T), hypothalamus (Hyp), and in the molecular layer of the cerebellum (Cb), (55). **b)** Strong immunoreactivity is evident in astrocytes cells, (55). **c)** Reaction mechanism for D-serine synthesis by SR. PLP bound to SR through an internal aldimine with Lys56 reacts with L-serine yielding an external aldimine; α-proton abstraction from this intermediate gives a resonance-stabilized carbanion. The reprotonation of the intermediate on the opposite face of the planar carbanion generates the external aldimine which release D-serine via

transimination with Lys56 (43). **d)** Degradation of D-serine by hDAAO. The hydride-transfer of the α-proton of D-serine to the oxidized N(5) FAD position yields imino pyruvic acid and reduced anionic flavin. The imino pyruvic acid is then non-enzymatically hydrolized to pyruvate and ammonia; the reduced flavin is re-oxidized by dioxygen yiealding hydrogen peroxide (43).

Appendix III: the "serine shuttle" model

The type of the cells that express SR and synthesize D-serine is a contentious issue. Initial studies localized SR to astrocytes (49, 56). However, immunolocalization using new antibody against SR and *in situ hybridization* studies recently demonstrated that SR is predominantly expressed in neurons. Only weak SR immunoreactivity was observed in astrocytes. (50, 53, 57). Thus, it would have been expected that neurons would posses more D-serine than astrocytes. However, as discussed before (see 2.3 "Cellular distribution"), the opposite localization was observed: astrocytic D-serine is more prominent than neuronal D-serine in most cerebral regions (46, 54, 57). Moreover, interesting, further analysis indicated that D-serine is released from neurons upon membrane depolarization (50, 53, 57).

In order to explain the opposite localizations of SR and D-serine, the double activity of SR on D-serine needs to be taken into account: the enzyme catalyzes both racemization of D-serine (from L-serine) and α,β-elimination (of water) from D-serine (as extensively discussed in 2.3. "D-serine-synthesis") (58, 59). The efficiency of the α,β-elimination reaction is surprisingly high and is consistent with the notion that it limits the achievable concentration of D-serine in the cells. Since the brain L-serine concentration is much below the K_m of SR, physiological levels of L-serine do not significantly inhibit the degradation of D-serine by α,β-elimination. This reaction provides a rationale for the lower levels of D-serine in neurons when compared with astrocytes.

On other hand, it has been demonstrated that D-serine is stable when physically separated from SR (and its elimination activity). In cells expressing SR, the release of D–serine to the extracellular medium prevents its destruction by SR (58). So, the release of neuronal D-serine, followed by its uptake in astocytes, provides a highly efficient barrier to shield D-serine from the α,β-elimination activity of SR. Thus, it is convincible that the buildup of D-serine in astrocytes comes from uptake from the extracellular medium associated to a relative low metabolism in these cells (53).

Altogether, this findings point to the existence of an astrocytic-neuron metabolic cross-talk model that Wolosker and collaborators defined **"The serine shuttle"** (Fig 4). In this model D-serine synthesis take place in neurons and require the uptake of L-serine, synthesized and exported by astrocytes, because neurons cannot synthesize L-serine (part of L-serine provided to neurons through the serine shuttle may be then stored in phospholipids and later used for D-serine synthesis). Neuronal SR produces D-serine which is then released by neurons and subsequently taken up by astrocytes (that can accumulate higher levels of the neuromodulator because of the poor expression of SR); finally astrocytes release D-serine in an activity dependent manner (53).

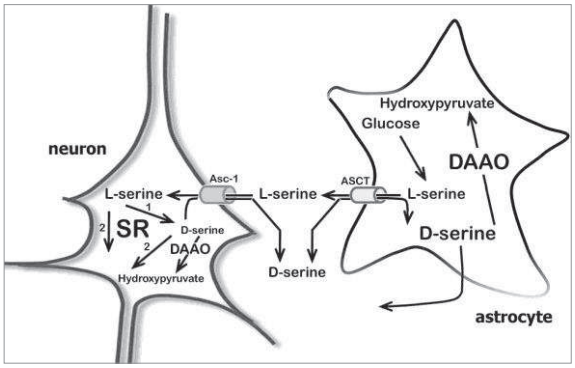

Fig 4. The serine shuttle pathway between neurons and astrocytes. L-Serine is mostly synthesized from glucose in astrocytes and exported to neurons via a neutral amino acid transporter (ASCT or similar). Synthesis of D-serine by neurons requires the uptake of L-serine from the extracellular medium via neutral amino acid transporter (Asc-1 or similar). Neuronal SR produces D-serine, pyruvate and ammonia by α,β-elimination. This reaction also limits the achievable D-serine concentration in neurons and provides a rationale for the limited accumulation of D-serine in neurons. D-Serine produced and released by neurons is subsequently taken up by astrocytes, where its cellular concentration might be regulated by DAAO, modified from (53).

2.3. D-Serine synthesis

Although previously brain D-serine was proposed to derive from diet, gastrointestinal bacteria, or from cleavage of metabolically stable proteins, more recently it was demonstrated that this signaling molecule in the mammalian brain is generated by the activity of a pyridoxal 5'-phosphate (PLP)-dependent enzyme, serine racemase (SR) (60) (Fig 3c).

SR belongs to the fold type II of PLP-dependent enzymes and structurally resembles bacterial serine/threonine dehydratases, rather then classical racemase (53). It synthesizes D-serine directly converting L-serine (generated primarily via the phosphorilated pathway) to the D-enantiomer (Fig 3c); it also converts D- to L-serine, albeit with lower affinity. L-serine is the only source for endogenous D-serine in the brain and the enzyme is highly selective, failing to racemize other amino acids (56).

As a general rule, PLP-dependent enzymes have the ability to catalyze more than one reaction type with a single substrate, with one reaction being more effective than others. An intriguing feature of SR in fact, is the ability of catalyze not only the racemization of D-serine from L-serine, but also the production of pyruvate (and ammonia), through the α,β-elimination of water from both L- or D-serine (58, 61). Three molecules of pyruvate are synthesized for each molecule of D-serine produced by SR (62). SR α,β-elimination reaction provides an unusual mechanism to control intracellular

D-serine levels (especially in the forebrain where SR expression is high) (53). While the elimination reaction starting from L-serine shows a higher kinetic efficiency than the racemization reaction, the V_{max}/K_m ratio for racemization of D-serine into L-serine is threefold higher than for elimination to pyruvate (43).

In the CNS the distribution of SR closely resembles that reported for endogenous D-serine, with the strongest protein expression in the forebrain and negligible levels in the brainstem. The highest amounts of protein were found in the hippocampus and corpus callosum, intermediate levels were reported in substantia nigra and caudate, and trace amounts were observed in the amygdala, thalamus and subthalamic nuclei (60).

Worthy of note, the half life of the overexpressed serine racemase is relatively short (4 hours), much shorter than that of NMDAR (20 hours) or D-serine itself (16 hours) (63).

Appendix IV : serine racemase regulation

SR activity is modulated by different molecular mechanism such as post-translational modifications (phosphorylation and nitrosylation) and the binding of a variety of interacting proteins and small effector molecules. It has been demonstrated that Mg^{2+}, Ca^{2+} and adenosine triphosphate (ATP) increase the rate of D-serine synthesis (61, 64). In contrast, SR activity might be negatively regulated by glycine and a series of metabolites related to L-aspartic acid that competitively inhibit the enzyme. Considering that glycine is present in astrocytes at relatively high concentration (59, 65) it could play a significant role on D-serine synthesis.

Activation of NMDAR might also affect the production of D-serine: NMDAR-mediated calcium influx at postsynaptic neurons yields to the activation of nitric oxide syntase and consequent production of nitric oxide (NO) which inhibits the activity of the PLP-dependent enzyme (66). Furthermore, in primary neuronal culture from rat, NMDAR activation promotes the traslocation of cytosolic SR to the plasma membrane and dendrites (a process requiring atypical palmytolation of SR at serine/threonine residues), that results in a substantial inactivation of the enzyme (66).

Protein-protein interaction is another important mechanism of SR regulation. Yeast two-hybrid system studies, identified glutamate receptor-interacting protein (GRIP), as well as protein interacting with C-kinase (PICK1) and Golgin subfamily A member 3 (Golga 3) as binding partners. GRIP proteins play a major role in the trafficking of the glutamate receptors of the AMPA/kainate type. SR binds specifically to the PDZ6 domain of GRIP by means of its C-terminal PDZ-binding motif, and it is activated by this interaction. Moreover, GRIP-SR interaction modulates neuronal migration along Bergmann glia in the developing cerebellum, a process blocked by degradation of D-serine or SR inhibition (67). On the other hand, PICK1 is a scaffolding protein that has been proposed to regulate subcellular

localization and membrane expression of various binding partner. The binding of PICK1 to SR requires the PDZ domain of PICK1 and the C-terminus of SR. SR-PICK1 Interaction reduces the activity of the PLP-dependent enzyme by the phosphorylation at specific serine residues (68). Furthermore, it has been reported that SR is targeted to the ubiquitin-proteasome system (UPS) for degradation process regulated through Golga3 interaction. This member of the Golgin subfamily binds to the N-terminal position of SR leading to a decrease in its ubiquitination state and degradation rate, probably interfering with the binding of a still unidentified E3-ubiquitin ligase. SR-Golga3 interaction promotes D-serine synthesis increasing SR cellular steady-state levels (63).
The binding of SR to phosphatidylinositol(4,5)biphosphate (PIP2) represents another mechanism for sequestering an inactive enzyme to the membrane in glial cells, a process that inhibits the enzyme according to a competitive mechanism with ATP (69). For a complete scheme of SR regulation mechanism see Fig 6.

2.4. Regulation mechanisms of D-serine concentration at synapse

The mechanisms by which D-serine is released from glial cells and neurons, and those involved in the clearance of the transmitter molecule from the synaptic cleft is a debated “hot” topic and has not been elucidated yet (62).

2.4.1. D-Serine release

Pioneering experiments performed during last decade, revealed that the efflux of radiolabeled D-serine from pre-loaded astrocytes can be induced by the activation of metabotropic and non-NMDA glutamate receptors. Activation of AMPAR triggers the binding of GRIP to SR, which causes a major activation of SR and efflux of D-serine from astrocytes (72). These observations strengthen the existence of a regulated release for D-serine from glial cell, with AMPAR being the principal stimulatory pathway. In addition to AMPAR also the activation of kainate and metabotropic receptors trigger a Ca^{2+} and SNAREs-dependent release of D-serine from astrocytes (66). Recently, by confocal analysis, it has been demonstrated that within glial cells a large pool of D-serine is present in the vesicles of the regulated secretory pathway: the D-amino acid colocalizes with two vSNAREs proteins (synaptobrevin/VAMP2 and cellubrevin/VAMP3) located at the membrane of the same population of secretory vesicles. This pool of D-serine can be mobilized upon increase in intracellular calcium: pharmacological stimulation yields to an elevation of calcium concentration, which in turn trigger a recruitment of synaptobrevin/VAMP2 to the plasma

membrane with a concomitant disappearance of D-serine from the regulated secretory pathway strongly, thus supporting the involvement of a Ca^{2+}-dependent exocytotic pathway in the release of the gliotransmitter (71). Moreover, by immunogold cytochemistry, it has been shown that D-serine (with glutamate) accumulates in synaptic-like microvesicles (SLMVs) in the perisynaptic processes of astrocytes. Endoplasmatic reticulum is regularly found in close vicinity of SLMVs, suggesting that astrocytes contain functional nanodomains, where a local Ca^{2+} increase can trigger release of glutamate and/or D-serine (71).

However, other mechanisms of D-serine release has not been excluded. Although, a specific transporter for D-serine has not yet been identified, the release of cytosolic D-serine can operate through neutral amino acids transporters present on the membrane of astrocytes (72). In primary cultures, D-serine fluxes are coupled to counter-movements of L-serine and, to lesser extent, of other small neutral amino acids, suggesting an antiporter mechanism for D-serine transport (73). Astrocytes may also use the chemical gradient to drive under physiological conditions the release of cytosolic D-serine through activated $P2X_7$ receptors, connexin-formed hemichannel, or volume-anion channel as proved for glutamate (4) (Fig 5).

On the other hand, it has been shown that in neuron D-serine release is mainly induced by depolarization: varatridine (a depolarizing agent) and KCl induce D-serine release from cultured neurons, but not from astrocytes. Even AMPAR evoke D-serine release, but their effect is much smaller. Though in this case the molecular mechanism of release requires further investigation, the Asc1 transporter (which promotes unidirectional amino acid efflux) is the natural candidate to mediate D-serine release from neurons (62).

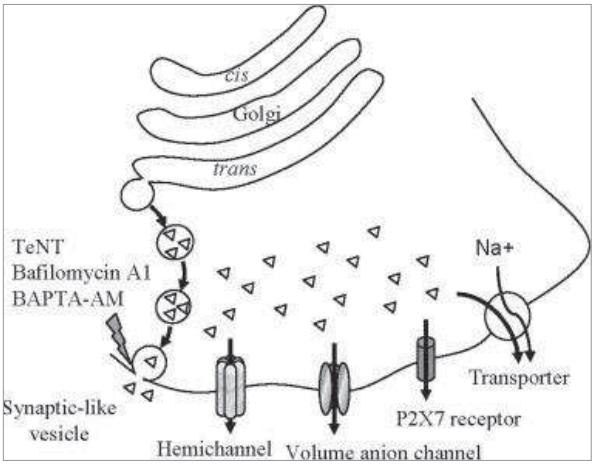

Fig 5. Possible pathway for glial D-serine release. Astrocytes can release neurotransmitters through reversal Na^+-dependent transport or via cell swelling-induced anion channel activation. Moreover, astrocytes can also utilize the $P2X_7$ receptors, a particular type of ATP receptors or the hemichannel to drive gliotransmitters outside the cell. Another pathway is represented by vesicle-mediated exocytotic release which is induced by intracellular Ca^{2+} elevation and fusion of filled vesicles. D-Serine release can occur from a cytosolic and a vesicular pool by reversal of transporters and by a calcium- and SNAREs-dependent mechanism (62).

2.4.2. D-Serine uptake

Similarly to others neurotransmitters, D-serine signaling should be terminated by its clearance from the synaptic cleft by transporter proteins expressed by neurons and/or glia. In primary astrocyte culture, D-serine uptake is dependent on a transport with Na^+ ions, that exhibits both low affinity and low specificity for D-serine. The kinetic of D-serine transport resembles that of B amino acid transporter type transporters (ASCT). D-Serine uptake is associated with an efflux of L-serine and, to a less extent, of other small amino acids suggesting an antiporter mechanism for glial D-serine transport (73). In neurons, the sodium independent alanine-serine-cysteine transporter 1 (Asc-1), that is confined to presynaptic terminals, dendrites and somata of neurons, presents a high affinity for D-serine and its cellular localization suggests that it could contribute to the synaptic clearance of the D-amino acid (74). Finally, a Na^+/Cl^- sensitive transporter has been described in rat synaptosomes. In contrast to ASCT system, which has broad substrate selectivity, this serine transporter has limited affinity for other neutral amino acids (75, 76).

2.5. D-Serine degradation

The termination of signaling by a neurotransmitter normally requires, beyond its re-uptake, its degradation. The metabolic pathway of D-serine degradation however, remains more elusive then the synthetic one.

D-Serine can be metabolized, and converted into pyruvate, by the flavoprotein D-amino acid oxidase (DAAO) expressed in brain (77) (Fig 3d). Physiological degradation of D-serine by DAAO was suggested by the marked regional and developmental variation in D-serine concentration which appears to be related to DAAO presence: the levels of the D-amino acid are inversely related to the regional expression of DAAO during development and in adult rat brain (46). Moreover, adult DAAO-deficient mice display increased D-serine concentration especially in areas where the flavoenzyme's levels are normally low (77). On the contrary, the amount of D-serine was relatively unchanged in the forebrains of $DAAO^{-/-}$ mice, implying that in these areas D-serine concentration is tuned by other mechanisms (78). Both in mice and humans, DAAO expression in the cortex is robust but the activity practically negligible. Two possible reasons have been previously proposed: DAAO is inactive because it is only a residue from an earlier developmental function or in the cortex the flavoenzyme might be active on a substrate other than D-serine and catalyze a reaction not detected by the commonly used functional assays (79). However, our opinion is that in the cortex some kind of negative effector is present along with DAAO: a small ligand or an interacting protein that is able to down-regulate its activity. This fit nicely with the difficulties encountered in detecting the DAAO activity in tissue slices and it would also explain why the levels of D-serine in the forebrain, and particulary in cortex, are relatively unchanged in adult DAAO-deficent mice (see Appendix V and 43).

The real problem in understanding whether D-serine degradation is exerted by DAAO, is due to the subcellular localization of the flavoprotein. In fact, it is known that the major amount of D-serine is present as a cytosolic pool or it is loaded in secretory vesicles, whereas DAAO is a known peroxisomal enzyme. Notwithstanding, we recently observed that a significant amount of this flavoenzyme is localized outside these organelles (38). Furthermore, it was argued that in brain regions lacking DAAO activity, D-serine concentration is tuned by other mechanisms. In these areas, SR would offer an alternative pathway to regulate the intracellular content of the neuromodulator (43), since the enzyme catalyze the α,β-elimination of water from both L-serine and D-serine to form pyruvate and ammonia, see

paragraph 2.3 and (58). It has also been demonstrated that the elimination reaction competes with the isomerization reaction for regulating intracellular D-serine levels, especially in forebrains areas which lack DAAO activity (43) (Fig 6).

Interestingly, the two enzymes involved in the metabolism of D-serine, when present in the same cells or at least in the same synapse, do not work independently: their activities are regulated in opposite ways by the gaseous transmitter nitric oxide (NO). In fact, NO inhibits SR but enhances DAAO activities, thus tightly controlling the levels of D-serine in glia (80, 81). In turn, D-serine, through the activation of NMDAR, stimulates the production of NO in neurons. Neuronal NO could thus represent an inhibitory feedback mechanism tightly regulating D-serine metabolism in astrocytes, thereby preventing its overproduction and excessive stimulation of NMDAR (60).

This fine regulation of DAAO and SR is not surprising considering the importance of the role of the neuromodulator D-serine in physiological and pathological processes (Fig 6).

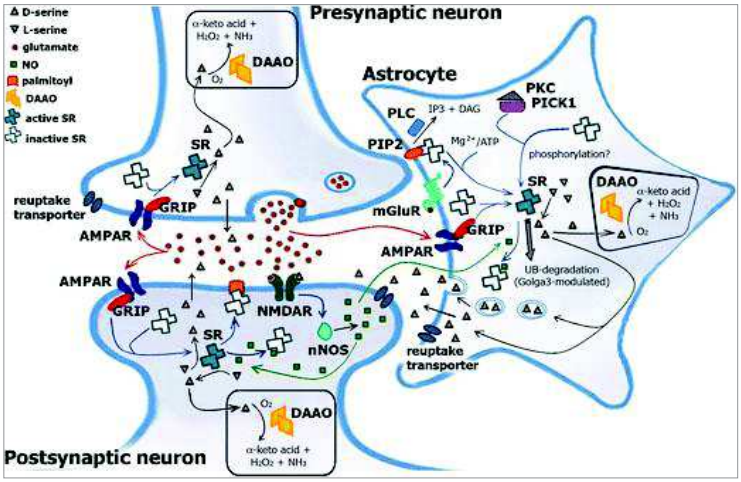

Fig 6. D-serine metabolism in the brain. Upon depolarization of the nerve terminal, glutamate is released and activates the non-NMDAR leading an influx of calcium ions through AMPA and kainate receptors or to intracellular calcium release (mGluR). This increase in intracellular calcium concentration triggers the exocytosis of D-serine and/or its synthesis. Activation of glial SR presumably is due to an association with GRIP proteins and/or phosphorylation. Since GRIP-1 is expressed in neurons where the large majority of AMPAR are found, this mechanism may also activate neuronal SR. In the synaptic cleft, D-serine binds to the glycine-binding site on the NMDAR and in conjunction with glutamate results in the opening of the receptor channel. Clearance of D-serine from the synaptic cleft is assured by Na^+-dependent and -independent transporters and its degradation by both DAAO and SR (43).

3. The role of L-serine as the precursor of D-serine in the brain

Although the importance of D-serine level in the NMDA mediated neurotransmission, the source of the precursor L-serine and its role in D-serine metabolism in adult brain have not be determined up to recently. In rats and mice, synthesis of D-serine from L-serine is restricted to the brain of adult individuals; other sources of D-serine via blood circulation seem not to play a major role in establishing steady-state levels of this amino acid (82). Genetic deletion of SR in mice results in a marked decrease in brain D-serine content, providing *in vivo* evidence that D-serine is synthesized from L-serine by SR in adult brain (83).
L-Serine can derive from the diet, glycine, protein degradation, and/or *de novo* biosynthesis from the glycolitic intermediate 3-phosphoglycerate (84). In the first step of L-serine biosynthesis, D-3-phosphoglycerate dehydrogenase (Phgdh) catalyzes the formation of 3-phosphohydroxypyruvate from 3-phosphoglycerate. In the rodent nervous system Phgdh is expressed specifically in the glial cell lineage throughout ontogeny. Neurons, in contrast, seems to have a diminished capacity for L-serine synthesis. In mature brain, L-serine appears to be supplied to neurons from external sources, such as astrocytes and/or circulation (85).
Systemic deletion of the Phgdh gene results in embryonic mortality (86), however recently it has been developed a conditional Phgdh mouse strain. Brain specific Phgdh deletion resulted in a marked simultaneous decrease in the L- and D-serine content at cerebral cortex and hippocampus, demonstrating unequivocally that in the adult brain L-serine synthesized endogenously via the phosphorylated pathway is the principal precursor of D-serine. Single dose supplementation of excess of L-serine did not fully restored the content of D- and L-serine: this observation agrees with the finding that L-serine uptake is extremely inefficient in adult brain (87).

4. D-Amino acid oxidase

The FAD-dependent flavoenzyme D-amino acid oxidase (DAAO) was discovered and first purified from porcine kidney in 1935 from Krebs by fresh pig kidney after he observed that amino acid belonging to the "*d-series were deaminated much more rapidly than the natural isomers"* (88). This is due to the DAAO's strict stereoselectivity towards D-isomers of amino acids which are deaminated to give α-ketoacids and ammonia.

DAAO has been the focus of an overwhelming body of research and has become a model for the oxidase class of flavoenzymes: the chemical aspects of enzyme reactivity have been unraveled by different groups who studied the recombinant pig kidney protein (pkDAAO), and the enzyme from different microbial sources, in particular from the yeast *Rhodotorula gracilis* (RgDAAO) (89, 90).

DAAO exhibits maximal activity toward neutral amino acids whereas only low activity is detected using basic amino acids as substrates; acidic D-amino acids are selectively oxidized by another flavoenzyme: D-aspartate oxidase (89, 90). DAAO oxidises D-amino acids with small, neutral side chain, namely: D-serine, D-alanine, D-proline, and D-leucine (91). During the initial step of catalysis, the D-amino acid is oxidized to an imino acid and FAD is concomitantly reduced. The reduced FAD is subsequently oxidized by oxygen to release hydrogen peroxide, and the imino acid is non-enzymatically hydrolyzed to produce the corresponding 2-oxo acid and ammonia (92) (Fig 7).

The “dark side” of this very well studied enzyme was its physiological role. This topic was long debated until the 90's, when an high concentration of D-amino acids in the brain and other peripheral tissues (such as pituitary gland, liver, kidney and blood) was detected (45).

In the various organisms in which it is expressed, DAAO fulfills distinct physiological functions (90). In yeast cells a catabolic role has been suggested: the enzymatic reaction catalyzed by the flavoprotein allows them to grow on D-amino acids as carbon and nitrogen sources. In eukaryotic cells and higher organisms, DAAO maintains the physiological level of D-amino acids, which play important roles in the regulation of many processes such as aging, neural signaling, hormone secretion etc. (93). In particular, the flavoenzyme has a regulatory role in the brain where it modulates the levels of the atypical neuromodulator D-serine (see paragraph 2.5 and and references therein).

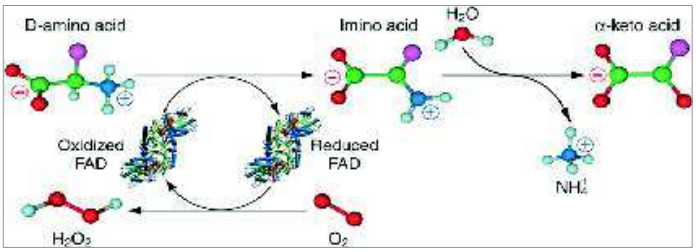

Fig 7. Scheme of the reaction catalyzed by D-amino acid oxidase (90).

4.1. DAAO expression in eukariotic organisms

DAAO is almost ubiquitous in eukaryotic organisms (from the simplest one such as fungi, to fishes and mammals). At the subcellular level DAAO is compartmentalized to peroxisomes: the peroxisomal targeting signal PST1 (-SHL), at the C-terminus of the protein sequence (94). Unfolding studies performed on RgDAAO by our group suggested that the flavoprotein might be targeted to these organelles as a partially folded inactive intermediate (95). The virtual inactive non-compartimentalized form of DAAO is presumably beneficial since the production of hydrogen peroxide by its catalyzed reaction in other cellular compartments, would be deleterious (96).

The overall amino acid sequence is well conserved among mammalian DAAOs (~ 80% sequence identity) (90). The corresponding gene is present in a single copy in the mammalian genome and encodes a 39 kDa protein (92). The human DAAO (hDAAO) gene is located on chromosome 12q24 and comprises 11 exons and 10 introns: the full length transcript comprises 1595 bp (97). Although only a single DAAO mRNA has been identified up to date, different isoforms of DAAO might be present. The potential for additional DAAO transcripts is suggested by the presence of transcription initiation sequences in the first intron, which may be relevant to the production of a brain hDAAO mRNA variant with a 5' untranslated region (UTR) deletion (96).

4.2. Regional and cellular distribution of DAAO

In mammals, the flavoprotein has been detected mainly in kidney, liver, brain, and to a lesser extent in leukocytes, small intestine, epididymis, and preputial and adrenal glands (98-100).

In the CNS the DAAO localization in tissue and cells has been investigated in rat by different methods. In particular, the first papers published reported a regional heterogeneity of DAAO distribution by staining the enzyme for its activity: it appears more abundant in the cerebellum and brainsteam with respect to the forebrain. Moreover, a significant signal corresponding to DAAO activity was detected diffusely in the gray matter of medulla and thoracic spinal cord with the exception for discrete areas (101, 102). DAAO has been described as being a glial enzyme since different groups provided evidences that its activity is localized in astrocytes, particularly in Bergmann glia within the cerebellum. Glial DAAO gene expression was also investigated by RT-PCR in cultured rat astrocytes: high levels were detected in type-2 cells (103).

Subsequently, because of the development of more sensible antibodies, the presence of the flavoenzyme has been detected in other brain regions beyond the cerebellum, and a complete map of the flavoenzyme distribution in the rat brain has been obtained: DAAO immunoreactivity was observed in cortical regions, hippocampus and midbrain (104). Interestingly, the same study reported the presence of the protein in both neuronal and glial cells albeit at different concentrations. Regional differences have also been detected: astroglial cells of the caudal brainstem and of the cerebellum are generally more immunoreactive than those located in forebrain and the neuronal immunoreactivity is generally stronger in the hindbrain than in the forebrain (104).

When the same analysis were performed on human tissues, the results substantially confirmed what previously observed in rat: hDAAO is most highly expressed in the cerebellum, but immunohistochemistry and *in situ* hybridization have confirmed the presence of relative high amounts of messenger and protein in specific cells throughout the brain, including cortex (although in dorsolateral prefrontal cortex the detection was extremely variable), thalamus, choroid plexus and hippocampus (79, 104, 105, 106). Interestingly, outside from the cerebellum, hDAAO appears to be significantly expressed in neurons. Particularly, the presence of hDAAO has been observed in putative dopaminergic neurons (of the substantia nigra pars compacta) suggesting that the enzyme plays a role also in dopaminergic functions (104). Moreover, recently our group confirmed the presence of hDAAO in human brain cortex by Western blot analysis, immunoprecipitation experiments, and immunolfluorescence localization both in tissue slices and secondary culture astrocytes (107).

Interestingly, although hDAAO expression in human cortex is robust, the enzyme activity is virtually undetectable. This could be related to the weak FAD binding of the human enzyme (see next paragraph), but also raises the possibility that forebrain DAAO might have different and as yet unidentified functions with respect to hindbrain hDAAO. Our hypothesis is that in cortical brain region specific mechanisms (i.e. the interaction with regulatory proteins) contribute to maintain the level of hDAAO activity close to the detection limit, allowing a fine modulation of D-serine degradation (43, 107).

4.3. Human DAAO: biochemical characterization of the recombinant enzyme

In the past few years, hDAAO was overexpressed in *E.coli*, purified as a stable and active holoenzyme and thoroughly in depth characterized in our laboratory (108). The purified native protein showed the classical properties of the dehydrogenase-oxidase class of flavoproteins: it reacts quickly with oxygen in the reduced state and it stabilizes the anionic red semiquinone. hDAAO contains one molecule of non-covalently bound FAD per protein monomer and tightly binds the classical DAAO ligands, such as benzoate, anthranilate and sulfite.

The apparent kinetic parameters indicate that hDAAO posseses a low catalytic efficiency and substrate affinity on the putative physiological substrate D-serine (a higher activity was determined on D-alanine and D-proline) (Table 1). Analogously to pkDAAO the human oxidase uses a sequential kinetics mechanism in which the rate-limiting step is represented by the product release from the reoxidized enzyme. In contrast to the porcine and yeast enzyme, the hDAAO homodimer is stable even in the apoprotein form (108).

The hDAAO structures contains 11 α-helices and 14 β-strands that fold into two domains, the FAD-binding domain and the interface domain (109), which forms the contact area with a second monomer in the crystallographic dimer (Fig 8a). The FAD cofactor is buried inside the protein, it adopts an elongated conformation and is involved in a number of stabilized interactions (110).

In contrast to yeast DAAO, the active site of pkDAAO and hDAAO are covered by a loop acting as an “active site lid” (Fig 8b): a change in conformation of this loop regulates the active site accessibility and thus substrate binding and product release (108).

Among all known DAAOs, the human enzyme possesses some peculiar properties:

- the binding of the FAD cofactor in the absence of an active site ligand is the weakest among other DAAOs;
- it is the only DAAO apoprotein present in solution as a dimer;
- hDAAO shows a significantly slower rate of flavin reduction than the pig enzyme (90).

As for pkDAAO, hDAAO dimeric structure shows a head-to-head mode of monomer-monomer interaction. Concerning hDAAO, the frequency of substitution at monomer-monomer interface is higher than the overall substitution frequency (33% vs. 15%, respectively). This yields to a

significant change in the eletrostatic surface potential in this region: at the dimeric interface the human enzyme is negatively charged while pkDAAO is positively charged. It has been proposed that this alteration accounts for the different aggregation state (monomeric) of the apoprotein form of hDAAO (109, 111).

In all DAAOs the FAD binding domain contains the conserved βαβ motif (Rossmann fold) and the isoalloxazine ring is located at the interface of the two DAAO domains, with the re-face facing the inner part of the substrate binding cavity (109). As expected from the 85% sequence identity between the human and the porcine enzyme, the active sites were fully conserved at the re-face of the flavin ring. On the other hand, at the si-face of the flavin ring the conformation of the hydrophobic stretch VAAGL (residues 47-51) differs in hDAAO: this short string of residues is shifted away from FAD resulting in the loss of the H-bond between the flavin N(5) and the backbone N atom of Ala49. Since no further remarkable differences were observed within 6 Å from FAD, it was argued that the VAAGL stretch plays an important role in determining the (low) affinity for the cofactor (109).

Because of the weak interaction with the FAD cofactor (K_d = 8 μM) (Table 1), the recombinant hDAAO exists in solution as an equilibrium of holo- and apoprotein forms. Moreover, this feature raised the focal question of how much active hDAAO is possibly present *in vivo*, particularly in brain tissue (108). Worthy of note, in the presence of a saturating concentration of the inhibitor benzoate the K_d for FAD binding decreases to 0.3 μM and, 90% of the flavoenzyme in solution is in the holoenzymatic form (at 1mg/ml protein concentration, Table 1). Considering the overall physiological concentration of D-serine and FAD in human brain tissue, *in vivo* hDAAO should be largely present in the inactive apoprotein form. It is tempting to speculate that the low affinity of hDAAO for the cofactor has been selected to control D-serine concentration in the brain, avoiding an excessive degradation of the neuromodulator (43, 112). From a physiological point of view, this FAD-hDAAO weak interaction may represent a means of evolving an enzyme that is largely present in the holoenzyme form only in the presence of a ligand (i.e. the substrate D-serine).

Furthermore, the stability of hDAAO protein has been analyzed in our laboratory through limited proteolysis and spectroscopic studies. During limited proteolysis experiments the hDAAO apoprotein form is fully degraded in a fast, monophasic process (complete degradation is observed in ~ 10 minutes). On the other hand, proteolysis of the holoenzyme is a biphasic and slower process: first there is a rapid phase that accounts for

the degradation of ~ 68% of the protein which is followed by a slow phase. Adding the FAD cofactor largely protects hDAAO from trypsin cleavage: more than 75% of the intact enzyme is still present after 30 minutes of incubation. Indeed, the apoprotein too, is largely protected from proteolysis by previously incubation with the cofactor: the sensitivity to trypsin of the reconstituted holoprotein resembles that of the native hDAAO holoenzyme (113). The temperature sensitivity of specific structural features was compared in the holo- and apoprotein forms of hDAAO using temperature ramp experiments. The temperature sensitivity of tryptophan and FAD fluorescence and of CD signals at 220 nm show midpoint transition temperatures that are ~ 6-9°C higher for the holo than for the apoprotein form of hDAAO, suggesting that the holo-hDAAO is less sensitive to heat denaturation then the apoprotein (112) (Table 1). Subsequently, we observed that the presence of ligands profoundly affects hDAAO conformation as indicated by protein fluorescence, near-UV CD spectroscopy and limited proteolysis studies. Benzoate (a classical substrate-competitive inhibitor) and CF_3-D-Ala (the pseudo-substrate trifluoro-D-alanine) binding protect the holoenzyme from thermal denaturation and trypsinolysis. Both ligands exert a stabilizing effect by increasing the amount of hDAAO holoenzyme present in solution as the result of a 20-fold decrease in the K_d for FAD binding. On the other hand, susceptibility of hDAAO to trypsinolysis is enhanced by CPZ (an aliphatic phenotiazine that acts as a FAD competitive inhibitor) binding, in fact, the binding of this compound appears to favor a protein conformation resembling that of the apoprotein form (113).

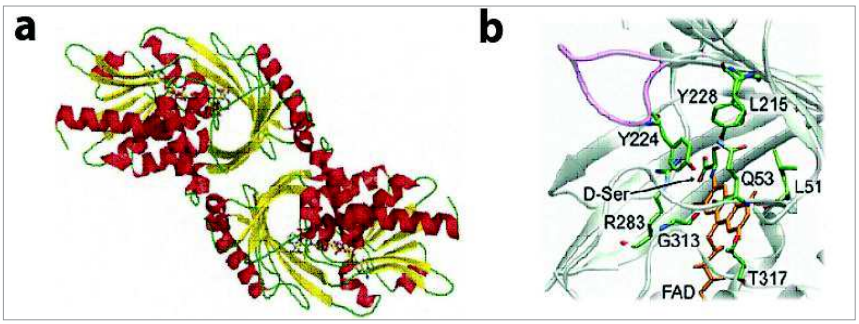

Fig. 8. Human DAAO structure. a) Cartoon depicting the hDAAO homodimer, colored by secondary structure compositon (helix in red, sheet in yellow, loop in green) (109). b) Detail of the active site of hDAAO in complex with D-serine. The principal residues in the active site of the enzyme are labelled (90).

Spectral and binding properties	Values	References
$\varepsilon_{454\ nm}$ (mM^{-1} cm^{-1})	12.2	107, 108
pK_a N(3)-H	10.3 ± 0.15	
K_d FAD (μM):		
Free form	8 ± 2	
Benzoate complex	0.3 ± 0.1	
K_d benzoate (μM)	7 ± 2	
K_d anthranilate (μM)	40 ± 10	
K_d sulfite (μM)	64 ± 9	

Substrate specificity	$V_{max,\ app}$ (s^{-1})	$K_{m,\ app}$ (mM)	$V_{max,\ Km,app}$ (mM)	References
D-Serine	3.0	7.5	0.4	107-109
D-Alanine	5.2	1.3	4	
D-Proline	10.2	8.5	1.3	
D-Aspartate	6.7	2000	0.003	
Glycine	0.9	180		
D-Phenylalanine	15.5	1.1	14.1	
D-Tyrosine	14.8	1.5	9.9	
D-DOPA	21.7	1.5	14.5	

Temperature-induced unfolding (T_m, °C)	Holoenzyme	Apoprotein	Reference
Far-UV CD (220 nm)	57.0 ± 1.0	51.0 ± 1.0	108
Trp fluorescence (335 nm)	57.7 ± 0.3	48.6 ± 1.0	

Table 1. Human DAAO properties. The $V_{max,app}$ and $K_{m,app}$ values are the kinetic parameters determined for the listed substrates at air oxygen saturation (0.25 mM). The temperature-induced unfolding of holo- and apoprotein forms of hDAAO were determined by temperature ramp experiments following the signal associated to secondary (decrease of CD signal at 220 nm) and tertiary (increase in Trp fluorescence at 335 nm) structure (43).

Appendix V: physiological significance of DAAO activity: the ddY-DAAO-mice model

Data from DAAO functional knock-out mice, "ddY-DAAO-mouse" provide further information on the role of DAAO in CNS, particularly in schizophrenia pathophysiology.

The mutant mice strain lacks DAAO activity because of a mutation within the DAAO encoding gene sequence: it produces a functionally inactive form of the enzyme because of the substitution of glycine 181 with arginine (G181R) (77, 114). Although these animals did not show any obvious abnormality, large quantities of D-amino acids were present in their organs and body fluids, this observation confirms the DAAO physiological role in the metabolism of D-amino acids. In particular, D-amino acids analysis performed on the mutant mouse brain revealed that D-serine concentration was increased (~ 10-fold) in those regions where DAAO is normally strongly expressed (namely cerebellum and brainstem). Moreover, levels of D-alanine, another NMDAR co-agonist and substrate for DAAO, were augmented ~4-fold in all the analyzed brain regions. Notably elevated

D-serine and D-alanine levels were accompanied by an increased occupancy of the NMDAR glycine modulatory site, thus supporting the regulative role proposed for DAAO on NMDA mediated neurotransmission. In fact, the DAAO G181R mutant mice present a phenotype consistent with alterated NMDAR signaling (115): they display an augmented excitatory postsynaptic currents mediated by NMDAR, this indicating an enhanced of NMDAR activity; they performed better in the Morris water maze memory test and had enhanced learning in association with fear-based tasks. Furthermore, enhanced long term potentiation (LTP) was observed in hippocampal slices of mutant mice (116).

5. hDAAO binding partners: pLG72

The product of the human gene G72 (pLG72) was first described by Chumakov in 2002, who published an analysis of single nucleotide polymorphisms (SNP) and haplotypes unraveling an association between schizophrenia and the newly identified gene. By means of yeast two hybrid system the author identified DAAO as a putative pLG72 interacting partner and in subsequent biochemical characterization experiments suggested that it acts as a modulator of the flavoenzyme activity. This hypothesis was based on functional measurements performed on pkDAAO: in the presence of the large molar excess of pLG72, pkDAAO basal levels or activity increased up to three fold (117). More recently, we failed to reproduce the effect of pLG72 on pkDAAO as reported by Chumakov. In our experiments, the activity of pkDAAO decreased on presence of pLG72 suggesting that pLG72 inactivates pkDAAO (107).

In order to investigate the effect of pLG72 on the functional properties of hDAAO, both human proteins were produced and purified in our laboratory, as recombinant proteins from *E.coli.* We confirmed the interaction between hDAAO and pLG72 by means of different experimental methods (i.e. gel permeation cromatography, quantitative pull down experiments, surface plasmon resonance analysis and coimmunoprecipitation). It has been shown that the interaction between the two proteins is specific, yielding *in vitro* to a complex of ~ 200 kDa composed of two hDAAO homodimers and two pLG72 molecules. pLG72 interacts specifically with both holo- and apoprotein forms of hDAAO and the K_d for complex formation is ~ 8 X 10^{-6} M (107).

Moreover we observed that pLG72 binding did not affect the kinetics parameters of the reaction catalyzed by hDAAO on D-serine, nor its affinity for the coenzyme, or the rate constant of FAD binding to the apoprotein.

The principal effect of interaction was a faster time course of hDAAO inactivation (i.e. a decrease in hDAAO stability) when an excess of pLG72 was present, due to a decrease in the amount of the active form of holoenzyme in solution that might be reduced by the substrate (Fig 9a) (107). This inactivation of hDAAO is not related to the depletion of FAD as the same result was obtained when an excess of free FAD was present in the assay mixture (Fig 9a). Moreover, through visible absorbance and near-UV CD spectroscopy, it has been shown that the pLG72 binding altered the tertiary structure of hDAAO, suggesting that the modification destabilizes the holoenzymatic form (107).

More recently, we analyzed the stability of the flavoenzyme when it is complexed with pLG72 through proteolysis experiments. We observed that in presence of stoichiometric amount of pLG72 the rate constant for the second phase of holoenzyme degradation increases; furthermore, the destabilization of hDAAO was most evident when a twofold excess of pLG72 was added. On the other hand, the time course of proteolysis of the apoprotein form was not affected by the addition of pLG72, indicating that the pLG72 binding mostly destabilizes the holoenzymatic form of hDAAO. Moreover, we investigated the effect of small ligands on hDAAO-pLG72 interaction: none of the ligands tested, i.e. CPZ, FAD, benzoate, D-serine, modified the formation of the complex between the two interacting proteins as judged by Surface Plasmon Resonance and gel permeation analyses, while CPZ binding increased the susceptibility to proteolysis (113).

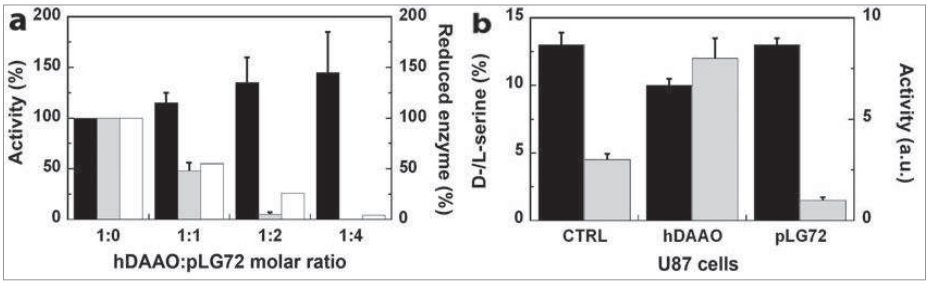

Fig. 9. Effect of pLG72 on hDAAO activity both *in vitro* (a) and in a cellular system (b). **a)** Effect of pLG72 binding on hDAAO activity: *black bars* activity measured without pre-incubation or *gray bars* after 30 min of pre-incubation of a fixed amount of hDAAO (0.1 nmol/ml) with increasing amounts of pLG72; (*white bars)* effect of pLG72 on the reactivity of the hDAAO-bound FAD. In this latter case, the oxidized hDAAO was incubated with a stoichiometric amount of free FAD and increasing amount of pLG72 and subsequentally was anaerobically reduced by adding 1 mM D-serine: the bars report the percentage of reduced flavin. **b)** (*Black bars*) Summary histogram of the D-/L- serine ratio (percentage) in U87 control cells (CTRL) and in the same cells transfected with hDAAO or pLG72: the change was significant for hDAAO (p=0.004) and not significant for pLG72. (*Gray bars*) Summary

histogram of the hDAAO activity (arbitrary units) in the same U87 cells: the change in activity with respect to the control was statistically significant for hDAAO (p=0.012) and not significant for pLG72. The data are reported as mean ± SEM (43).

At cellular level, the transient transfection of EGFP-hDAAO on U87 cell (a human glioblastoma cell line) determines a decrease of D-serine concentration (Fig. 9b), according to the function of this flavoenzyme in the catabolism of the neuromodulator. However, when EGFP-pLG72 is also cotransfected in the same cells no modification of D-serine cellular concentration was observed with respect to control/not transfected cell (Fig. 9b), further supporting the function of pLG72 as an inactivator of hDAAO (107).

Moreover, our lab reported that the two interacting proteins are present on the same astrocytes in human cortex, and that they possibly interact *in vivo,* as demonstrated by immunofluorescence and immunoprecipitation studies, respectively (107). A significant DAAO and pLG72 colocalization signal was observed also in cultured human astrocytes, although in this case the colocalization between the two proteins is very low, suggesting that pLG72 interaction with hDAAO is probably driven by specific spatiotemporal stimuli (107).

These results prompted us to modify the initial model proposed by Chumakov and collaborators (117) (Fig 9): pLG72-hDAAO complex formation modifies the tertiary structure of the flavoprotein resulting in a time-dependent inactivation of the enzyme. This “slow” modification of hDAAO activity by pLG72 binding correlates with the low, inferred *in vivo* efficiency of this flavoenzyme (due to the weak cofactor binding and low turnover number) and with the long half-life of D-serine in brain. We then proposed that an hypoexpression of pLG72 could significantly decrease D-serine concentration locally in the brain due to abnormally high activity of hDAAO (107).

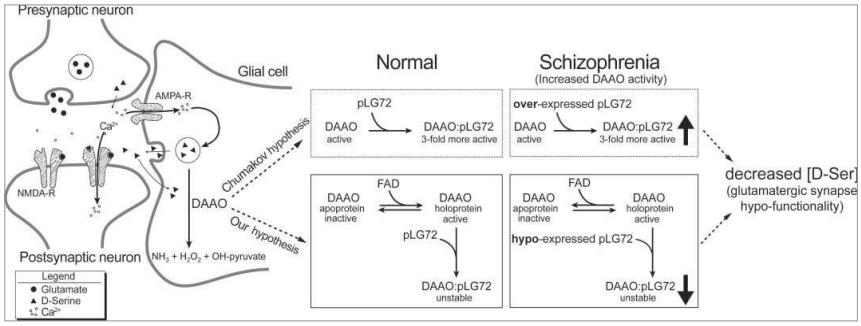

Fig 10. Hypothesis of the role of hDAAO and pLG72 on D-serine bioavailability at glutamatergic synapses under normal and pathological conditions. Upper loop: hypothesis of involvement of hDAAO and pLG72 in schizophrenia according to Chumakov and collaborators in which pLG72 is an activator of hDAAO; under pathological condition an overexpression of pLG72 leads to an increase of hDAAO activity that decreases the local concentration of D-serine. Lower loop: our hypothesis, in which pLG72 modulates the amount of active hDAAO acting on the stability of the holoenzyme. An abnormal, low expression of pLG72 under pathological conditions results in an hyperactivation of hDAAO and decrease D-serine concentration. In both hypotheses the decrease in D-serine concentration results in a lower amount of activated NMDAR and thus in a hypofunction of the glutamatergic neurotransmisson (107)

APPENDIX VI: pLG72 identification

G72 gene was first identified by Chumakov in a study using extensive genotyping and advanced statistical analysis of individual single nucleotide polymorphism (SNP) markers in two separate populations, French Canadians and Russians (117). Further genetic analysis indicated the G72/G30 locus as one of the several *loci* that account for a genetic linkage with schizophrenia susceptibility that spans a broad region on chromosome 13q (118). G72 and G30 are overlapping genes transcribed from opposite DNA strands, but only G72 appears to be actively translated (117). The G72 gene is located within a genomic region enriched in repeat elements, contains eight exons and is present only in primates. It seems to represent an exceptional case of a primate-specific gene with rapidly changing encoded protein structure, related with a rapid evolution of underlying brain functions (117, 119). The gene shows a complex alternative splicing pattern and several splicing isoforms have been described. The longest possible G72 ORF encodes for pLG72 a small proteins (152 amino acids, 18 kDa) with no recongnizable motifs and a high alpha-helix content which suggests a membrane localization (120). Potential pLG72 orthologues can be deduced for chimpanzee, gorilla and gibbons genomes. Although the N-terminus of the proteins is well conserved among anthropoid primates, variations in the C-terminus result in proteins of different size. Humans have the largest protein, followed by the gibbons (with 121 amino acids) and chimpanzees and gorillas (which have proteins consisting of only 66 amino acids) (117).

Since G72/G30 transcript levels are relatively low, only limited information about the tissue-specific expression are available (123). G72 gene have been identified in spinal cord, testis and in the adult brain; here the expression of the protein has been indicated by different groups to be maximal in the amygdala, caudate nucleus and the dorsolateral prefrontal cortex (117, 121), but the distribution pattern is still controversy (122). In the early study, the observed subcellular localization of pLG72 in transiently transfected cells was the endoplasmatic reticulum/Golgi apparatus (117). However most recently, a mitochondrial localization for this protein has been proposed. In fact, transient transfection of G72 on Cos-7 and U251 cells, as well as primary rat neurons and human astrocytes shows a typical punctuate and tubular pattern that resembles mitochondrial compartment (123).
The exact function of pLG72 still remain enigmatic. It has been shown to promote mitochondria fragmentation in several cell lines as well as in primary neurons. Furthermore, it was observed that increase in pLG72 level in immature neurons induce a robust increase in dendritic arborization which in turn could be related to mitochondria fragmentation, a process that may further enhance the organelles density in those fine processes during period of active dendritogenesis (123). Most studies proposed that pLG72 is a specific modulator of the activity of the flavoenzyme DAAO.

6. New putative hDAAO interactors

Recently, trough a co-immunoprecipition and mass spectrometry approach, 24 putative DAAO-interacting proteins from rat cerebellum have been identified (124). Many of these proteins, including Basson, Piccolo, SNIP, ERC1 and RAPGEF4, are enriched in the presynaptic active zone. Among them, the most robust interaction occurred with Basson (BSN) suggesting that a pool of hDAAO may localize outside of the peroxisomes at the synaptic junction, where it may be accessible to interact with BSN. The interaction of hDAAO with BSN has been confirmed by co-immunoprecipitation assay. Moreover, hDAAO and BNS colocalizes in cultured cerebellar granule cells and in synaptic junction membrane protein fraction derived from rat cerebellum.
The hDAAO-BSN interaction lead to an inhibition of the enzymatic activity of the flavoprotein. This interaction may be especially relevant in the forebrain, where hDAAO has been reported to be predominantly expressed in neurons. Moreover the inhibitory effect of that interaction could explain the undetectable enzymatic activity of hDAAO in that brain region. Discussing all these results, Popliolek and collaboratores suggest that synaptic D-serine concentration may be under tight regulation by BSN-

hDAAO complex, and thus propose that the observed interaction play a role in the modulation of glutamatergic signaling through NMDAR (124).
The possibility that a fraction of the expressed hDAAO is not compartmentalized within the peroxisome was also suggested by our previous work (107), since we observed by immunofluorescence localization that a significant amount of the flavoprotein is present in the cytosol, outside these organelles. If confirmed, this alternative subcellular localization of hDAAO would support the key role of the enzyme in the modulation of D-serine cellular concentration: hDAAO activity might regulate the strorage of the neuromodulator, indirectly affecting its release. This postulating that hDAAO is present in the cytosol in a stable, active form, a statement which has not been verified yet.

7. Role of DAAO, pLG72 and their interaction in schizophrenia onset: a physiopathological model

The pioneering analysis of Chumakov indicating the linkage between pLG72 and schizophrenia was followed by significant replication in different population (Germans, Palestinian Arabs, South Africans, Ashkenazy Jews, Chinese, Taiwanese, Scots, Koreans, and Irish) (118 and references therein). The majority of the reported studies have indicated significant association of alleles, genotypes or haplotypes with schizophrenia, although few of them reported no significant association. G72 was also identified as a specific genetic factor for the progression of prodromal syndromes to schizophrenia (125). Based on G72/G30 genetic analysis, this locus is considered one of the few true-positive and most robust association findings in schizophrenia so far. Supporting this finding, it has been observed that the expression of this gene increases in brain samples from schizophrenic patients compared to control subjects (121).
Subsequently, hDAAO itself was associated with this pathology and combination of G72/hDAAO genotypes showed a synergistic effect on disease risk (117). As for pLG72, functional data support genetic studies: an increase of hDAAO expression and activity was observed in cerebellum, parietal cortex and hippocampus of patients with schizophrenia (with an amount of hippocampal hDAAO that was 77% higher with respect to the control). In addition, duration of illness correlated to hDAAO expression and activity in the hippocampus and cerebellum (126). We could speculate that this increase causes a decrease in D-serine concentration in specific brain

areas thereby contributing to a NMDAR hypofunction in pathological conditions (126- 128). These findings suggest that hDAAO, through its role in the metabolism of the neuromodulator D-serine might be a potential therapeutic target for schizophrenia treatment (79). It should be noted that this was the first time that researchers succeeded in demonstrating that the interaction between two genes provides a pathogenic molecular mechanism that can potentially account for increased risk in developing schizophrenia (130).

Several lines of evidences correlate NMDAR hypofunction and schizophrenia etiology (see paragraph 8), and in particular a deficiency of D-serine signaling might contribute to the hypoactivation of the receptor (79). Reduced D-serine concentrations were determined in cerebrospinal fluid and serum of patients affected by schizophrenia (131-133). Moreover, the co-administration of D-serine and anti-psychotic drugs succeeded in ameliorating some symptoms of the disorder. In fact, therapeutics effects have been observed in some clinical trials with D-serine, the partial agonist D-cycloserine, and D-alanine, when added to traditional anti-psychotic medications, and a meta-analyses concluded that D-serine is beneficial for negative symptoms, with a trend effect on cognitive ones (134).

Notably an association with the disorder was established also for SR SNPs (83). In schizophrenic patients SR protein levels and SR/DAAO ratio protein levels were significantly decreased in frontal cortex and hippocampus. In human post-mortem brain tissue, SR was significantly increased in hyppocampus of affected individuals whereas no changes was observed in any of the cortical areas, suggesting that alteration in SR levels is region specific (83, 135, 136).

All together these observations strongly suggested that genes encoding for proteins involved in D-serine metabolism, such as serine racemase, D-amino acid oxidase and its inactivator pLG72, might potentially contribute to the pathogenesis of schizophrenia.

Appendix VII: G72/G30 transgenic mouse

In order to shed light on the controversial physiological function of pLG72 “humanized” BAC transgenic mouse lines (G72Tg1 and G72Tg2, expressing alternatively spliced G72 and G30 transcripts and pLG72 protein) have been generated (123). Since G72/G30 locus is supposed to lack in mouse, the generated transgenic lines allow to address the behavioral consequences of pLG72 expression. Intriguingly, these mice display several symptoms related to psychiatric diseases: they are characterized by increased compulsive behaviors, impaired locomotor coordination, increased sensitivity to phencyclidine and

impaired odorant discrimination (123). Animals of both lines appeared healthy, were fertile, cared for their offspring, and transmitted the transgene with the expected Mendelian frequency.
Highest G72 transcript level was observed in the brain, particularly in the cerebellum, hippocampus and cortex. G72 was expressed prominently in the cerebellar granular cell layer and a strong expression was also observed in the granular layer of the dentate gyrus and olfactory bulb, while, a weaker signal was detected throughout the forebrain with no specific regional preference (123).
The hippocampal neuron of the transgenic mouse show synaptic dysfunction characterized by an impaired short-term plasticity and inability to substain higher frequency transmission.
It was proposed that pLG72 can reduce the activity of complex I of the mitochondrial respiratory chain, thereby increasing oxidative stress in affected neurons. Considering the importance of synaptic mitochondria in maintaining transmitter release during high-frequency stimulation, neuron dysfunctions are probably due to an inability to meet the high synaptic energy demand (137).

8. Schizophrenia: a disorder of brain development and plasticity

Schizophrenia is a severaly debilitating psychiatric disorder that affect ~ 1% of the population worldwide. The annual incidence of schizophrenia averaged 15 per 100,000 and the risk of developing the pathology over one's lifetime averages 0.7% (138). It is the seventh most costly medical illness for our society. The disease is characterized by the psychotic features of delusions, hallucinations and disorganized thought as well as by profound cognitive deficits, thus affecting the most basic human processes of perception, emotion and judgment (139).
The pathology is unidentifiable with any known diagnostic laboratory test. According to DSM-IV criteria, the diagnosis is based on the concomitant appearance of at least two of the typical symptoms (see above), each presenting for a significant portion of time during a 6-month period (139, 140).
Schizophrenic symptoms are classically classified into:

negative: including a diminution of emotional expression and reaction, participation in interpersonal relationships and production of speech, apathy, with loss of energy and interest, affective flattening, alogia, inappropriate social skill, inability to make friends, social isolation;

positive: including delusions of reference, paranoid and delusions, hallucinations and catatonic behavior;

cognitive including impairments of attention, working memory, learning, verbal fluency, motor speed, and executive functions.

While negative and positive symptoms can fluctuate, cognitive deficits remain relatively stable (139, 140).

The onset of schizophrenia most commonly occurs in the second or third decade of life, though the onset age may vary from childhood to old age (141).

Genetic factors and gene-environment interactions together contribute over 80% of the liability for developing schizophrenia. Moreover, several chromosomal regions and genes have been linked to the risk to developing the disease. Thus schizophrenia can be defined as a complex disorder. In fact, the pathology is not simply related to several major genes but rather evolves from addition or potentiation of a specific cluster of gene, which subsequently determines the genetic vulnerability of an individual. Furthermore, environmental factors are linked to a higher susceptibility of developing schizophrenia, these include cannabis use, prenatal infection or malnutrition and perinatal complications. How genetic and environmental determinants interact to the onset of the disease and via which precise neurobiological mechanisms they mediate their effects is not understood yet (138).

Studies of executive functions and memory using fMRI have reported abnormalities of the dorso-lateral prefrontal cortex, medial temporal lobe, hippocampus, parahippocampal gyrus, anterior cingulate, medial frontal and posterior parietal cortex, striatum, thalamus and cerebellum (142).

Signs for disturbed neuronal connectivity and migration deficits are aberrantly located and neuron clustered in schizophrenic patients in the entorhinal cortex and neocortex. A loss of the non-neuronal elements, neuropils, acts as a correlate of brain atrophy (143, 144). These kind of abnormality are strongly indicative of an early neurodevelopmental anomaly affecting neuronal migration, survival and connectivity (142, 143).

Notably, several genes related to the disorder predispose, in various ways, but in a convergent fashion, to the central pathophysiological process: an alteration in synaptic plasticity, especially affecting NMDAR mediated glutamatergic transmission, that disrupts neural microcircuits involved in higher-order cortical function, particularly executive processing (129).

In the last decade two main hypothesis have been reported with the attempt to explain the pathophysiological processes responsible for the schizophrenia onset.

The “dopamine hypothesis” postulates that symptoms of schizophrenia might result from excess of dopaminergic neurotransmission in mesolimbic and striatal brain regions which induce positive symptoms, and dopaminergic deficits in prefrontal brain regions, which are responsible for negative symptoms (145).

Several lines of evidences however, point to the hypotheses that dopaminergic dysfunctions in schizophrenia is secondary to an underlying glutamatergic dysfunction thus, a “glutamatergic hypothesis” (Fig 11) has been formulated: an hypofunction of glutamate receptors in cortico-striatal projections leads to an opening effect in thalamo-cortical loop resulting in an exaggerated sensory flooding, which in turn induces changes in dopamine concentration and the appearance of psychotic symptoms (140). Evidences supporting a role of glutamate and glutamate receptors in schizophrenia also derived from pharmacological studies. The administration of NMDAR antagonists, such ketamine and phencyclidine (PCP), might produce psychotic and cognitive abnormalities similar to those observed in schizophrenia (146). The effect of such treatments which block NMDAR suggested that the symptoms manifested by schizophrenic patients might reflect a dysfunction or dysregulation of NMDAR mediated neurotransmission. Worthy of note, and in contrast to dopaminergic model, the wide array of schizophrenia symptoms (including both positive and negative symptoms, as well as cognitive symptoms and neurophysiological dysfunctions) are more easily explained by a glutamate/NMDA perspective. Furthermore, many of the findings coming from etiological investigations such as genetic association studies, synergize to a much greater degree with glutamatergic than dopaminergic models of the disorders (147).

Finally, based on neuropathologic studies a potential role of GABA has been unlighted. It was observed that in tissue of schizophrenic patients a particular subtype of GABA interneurones, chandelier neurons, display decreased immunostaining for the GABA transporter (GAT), which is possibly related to NMDAR hypofunction (139). Moreover, the enzymes involved in GABA byosynthesis are expressed at alterated levels in postmortem brain of subjects diagnosed with the psychiatric disorder (148).

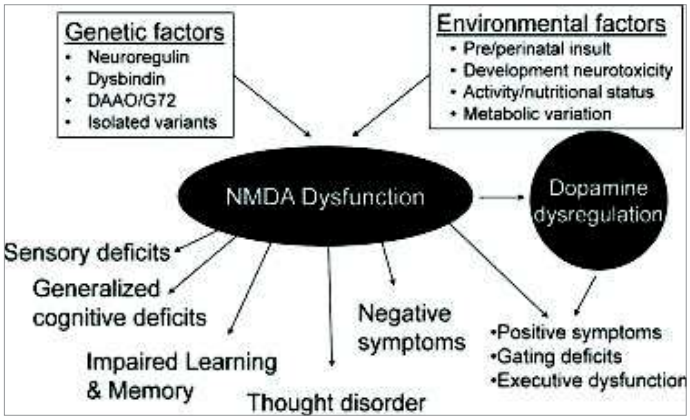

Fig 11. Summary of the proposed role of NMDAR in pathophysiology of schizophrenia. NMDAR dysfunction or dysregulation reflects final common pathway leading to the complex pattern of symptoms and cognitive deficits that characterized the pathology, with multiple potential causes of NMDAR dysfunction, including both genetic and environmental contributions (147).

Current treatment of schizophrenia disease only focuses on eliminating the symptoms of the disease. These medicaments were first introduced 50 years ago and all have as their primary mechanism of action blockade of dopamine D2 receptors. However, this first generation of antipsychotics, including chlorpromazine, haloperidol and perphenazine, had a propensity to cause extrapiramidal side effects such as rigidity, persistent muscle spasm, tremors, and restlessness. More recently, a new generation of antipsychotics, such as clozapine and olanzapine, have been developed: these drugs effectively alleviate the positive symptoms and improves the lives of many patients, but they do not cure schizophrenia (139).
It is so clear, that treatment for schizophrenia is far to be optimal. Clinical trials with agents that modulate NMDAR, including glycine, D-serine, D-cycloserine or D-alanine have indicated improvement in negative and cognitive symptoms when these agents are added to antipsychotics treatment (139). Moreover, a number of recent studies have focused on the development of hDAAO inhibitors as a therapeutic strategy for schizophrenia by modulation of D-serine metabolism in the brain (113).

D-Serine, an endogenous allosteric modulator of NMDA receptors, is synthetized by the PLP-dependent enzyme serine racemase (SR) and is degraded by SR itself and by the FAD-dependent flavoenzyme D-amino acid oxidase (DAAO). A relationship between D-serine signaling deregulation, NMDAr dysfunction and neuropsychiatric diseases is widely assumed. pLG72, a protein present in primates only, has been proposed to interact with human DAAO. Interestingly, SNPs in the human genes encoding for the two interacting proteins were associated to schizophrenia susceptibility. In our laboratory we demonstrated that *in vitro* pLG72 acts as an inactivator of hDAAO, and that D-serine cellular concentration depends on the amount of the active form of the enzyme. These observations prompted us to propose a model depicting the relationship between hDAAO-pLG72-D-serine and schizophrenia susceptibility: an hypoexpression of pLG72 triggers an abnormal increase of hDAAO activity, yielding an abnormal decrease in D-serine cellular level with the consequent decrease of the amount of the neuromodulator released at the synapse, thus resulting in the hypoactivation of NMDAr (107).

This PhD project was aimed to the investigation of the pLG72-hDAAO interaction using as a model cellular system the U87 human glioblastoma cells stably or transiently expressing the two interacting proteins (EYFP-hDAAO, hDAAO-EYFP or pLG72-ECFP). The subcellular localization of hDAAO and pLG72 represented a major concern: hDAAO is known to be a peroxisomal enzyme (94) while pLG72, albeit its subcellular localization is still controversial, has been proposed to be a mitochondrial protein. On the other hand, we recently demonstrated that human culture astrocytes display overlapping signals for pLG72 and hDAAO, although the calculated level of colocalization is low (107). Since hDAAO and pLG72 were reported to be localized in the same cortical astrocytes in human brain cortex, it was suggested that pLG72-hDAAO interaction is driven by still elusive spatiotemporal stimuli. In order to solve the apparent discrepancy between the different subcelular localization of the two putative interacting proteins, and to clarify the effect of this interaction on D-serine metabolism, the hDAAO-pLG72 complex formation was investigated by immunolocalization studies and FRET analysis in U87 pLG72-ECFP cells transfected with EYFP-hDAAO. Furthermore, the effect on hDAAO functionality and D-serine cellular concentration by the pLG72-hDAAO interaction was investigated by activity assay and HPLC analysis, respectively. Since we recently demonstrated that *in vitro* pLG72-binding to hDAAO destabilizes

the flavoprotein (113), I clarify whether pLG72 modifies the stability of hDAAO at cellular level in our model system.

Finally, with the aim to shed light on the regulation mechanisms of D-serine concentration, the degradation pathway of both hDAAO and pLG72 have been investigated. Notably, although the cellular processes involved in the degradation of SR, as well as the factors engaged in their modulation have been recently elucidated (63), nothing is known about the mechanisms that regulate the cellular steady state levels of the D-serine catabolic enzyme DAAO and of its interactor pLG72.

In order to clarify the role of hDAAO under physiological and pathological conditions our studies aimed to unravel the mechanisms of regulation of hDAAO activity in glial cells and its effects on D-serine cellular concentration. The understanding of the physiological processes involved in the modulation of D-serine metabolism might provide key information for the development of new therapeutic approaches for the treatment of neurodegenerative and psychiatric diseases, such as schizophrenia and bipolar disorder.