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The *N*-methyl D-aspartate receptor glycine site and D-serine metabolism: an evolutionary perspective

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The *N*-methyl D-aspartate (NMDA) type of glutamate receptor requires two distinct agonists to operate. Glycine is assumed to be the endogenous ligand for the NMDA receptor glycine site, but this notion has been challenged by the discovery of high levels of endogenous D-serine in the mammalian forebrain. I have outlined an evolutionary framework for the appearance of a glycine site in animals and the metabolic events leading to high levels of D-serine in brain. Sequence alignments of the glycine-binding regions, along with the scant experimental data available, suggest that the properties of invertebrate NMDA receptor glycine sites are probably different from those in vertebrates. The synthesis of D-serine in brain is due to a pyridoxal-5′-phosphate (B_6)-requiring serine racemase in glia. Although it remains unknown when serine racemase first evolved, data concerning the evolution of B_6 enzymes, along with the known occurrences of serine racemases in animals, point to D-serine synthesis arising around the divergence time of arthropods. D-Serine catabolism occurs via the ancient peroxisomal enzyme D-amino acid oxidase (DAO), whose ontogenetic expression in the hindbrain of mammals is delayed until the postnatal period and absent from the forebrain. The phylogeny of D-serine metabolism has relevance to our understanding of brain ontogeny, schizophrenia and neurotransmitter dynamics.

Keywords: glutamate; glycine; D-serine; racemase; D-amino acid; schizophrenia

1. INTRODUCTION

In their early studies of excitatory and depressant amino acids in brain, Curtis *et al.* (1961) noted that the D-isomer of serine (but not the natural L-isomer) excited preparations of toad spinal cord, while glycine was a depressant. These researchers later showed that the depressant effect of glycine was due to its action at inhibitory receptors that could be blocked by the poison strychnine (Curtis & Johnston 1970), but they did not pursue the excitatory effects of D-serine further. With the advent of receptor binding methods, it was confirmed that glycine and strychnine bound to the same receptor complex, an inhibitory chloride channel, located mainly in the brainstem and spinal cord (Young & Snyder 1973). Soon thereafter, however, it was noticed that glycine and strychnine binding sites were not always the same (DeFeudis *et al.* 1978). A number of researchers described a 'strychnine-insensitive' binding site for glycine in brain that was displaced by D-serine and was concentrated in the forebrain (Kishimoto *et al.* 1981; Bristow *et al.* 1986). These early reports remained orphan until Johnson & Ascher (1987) demonstrated that the *N*-methyl D-aspartate (NMDA) subtype of glutamate receptors required the presence of a small, heat-stable factor from their conditioned culture media in order to operate. They concluded that this factor was probably glycine.

Subsequent work has demonstrated that the NMDA receptor channel is unusual among ligand-gated ion channels in that the channel opens to allow calcium and sodium influx only when two different ligands bind the receptor simultaneously (Kleckner & Dingledine 1988). The channel complex itself is almost certainly a tetramer (Laube *et al.* 1998; Banke & Traynelis 2003), consisting of two NMDA receptor 1 (NR1) subunits and two NR2 subunits (figure 1*a*). Two molecules of the neurotransmitter glutamate bind to the NR2 subunits and two molecules of a different agonist bind to the NR1 subunit at the so-called 'glycine site' (also known as the glycineB site and reviewed comprehensively in Danysz & Parsons (1998)). The glycine site can be activated not only by glycine but also by the D-isomer of serine, and this explains the early observations of Curtis *et al.* (1961). At some glycine sites, D-serine is in fact more efficacious than glycine itself (Matsui *et al.* 1995; Priestley *et al.* 1995). D-Serine is often used in experiments as a glycine mimic because it does not bind to strychnine sites nor is it rapidly taken up into cells and metabolized.

In binding experiments using rodent cerebral cortex, the dissociation constants at the glycine site are 126 nM for glycine (Kishimoto *et al.* 1981) and 355 nM for D-serine (Danysz *et al.* 1990). Despite glycine's higher affinity, D-serine is often more effective at gating expressed channels (Matsui *et al.* 1995). Presumably this is due to the relative lack of uptake and metabolism systems for D-serine compared with those for glycine (Danysz & Parsons 1998). The concentrations of glycine in the cerebrospinal fluid are in the low micromolar range (Ferraro & Hare 1985; Westergren *et al.* 1994); likewise, microdialysis experiments of the extracellular fluid from rat frontal cortex report the concentrations of both glycine and D-serine to be *ca*. 7 μM (Matsui *et al.* 1995). Based on the reasoning that micromolar concentrations of ligand would saturate the glycine sites *in vivo*, it was suggested that the glycine site might be constantly occupied and therefore physiologically silent (Johnson & Ascher 1987), and at least one

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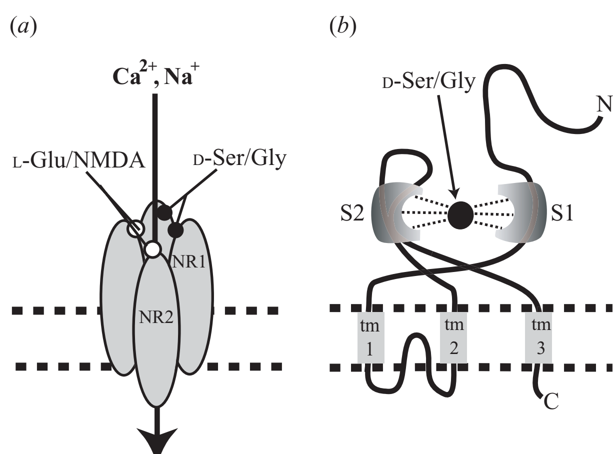

Figure 1. Schematic depictions of the NMDA receptor glycine site. (*a*) NMDA receptor channel. These channels are composed of two NR1 subunits and two NR2 subunits. Gating of the channel requires the simultaneous occupation of two types of ligand binding sites: a glutamate site on NR2 for binding L-glutamate (and also capable of binding NMDA), plus a glycine site on NR1 (also capable of binding D-serine). (*b*) Structure of a glycine binding site. The S1 and S2 regions of the NR1 subunit surround and bind the ligand, causing channel gating followed by desensitization.

study has reported that exogenous glycine has no enhancing effect on NMDA receptor activity *in vivo* (Obrenovitch *et al.* 1997). However, this view is untenable in light of the more than 40 studies in the literature demonstrating that this site is not saturated *in vivo* (Czepita *et al.* 1996; Wilcox *et al.* 1996; Nilsson *et al.* 1997; Berger *et al.* 1998; Bergeron *et al.* 1998; De Souza *et al.* 1998; Murray *et al.* 2000, Ahmadi *et al.* 2003; Chen *et al.* 2003). (See Wood (1995), Hashimoto & Oka (1997) and Danysz & Parsons (1998, table 7) for references to the many excellent earlier studies and a more detailed discussion.)

The importance of the glycine site is also emphasized by the phenotypes of transgenic mice carrying point mutations that reduce the receptor's affinity for glycine/D-serine (Kew *et al.* 2000; Ballard *et al.* 2002). A mutation that reduces the affinity 86-fold (K483Q) causes the animals to die within a few days after birth (due to failure to feed), whereas mice carrying a receptor with a less severe, fivefold reduction in affinity (D481N) survive but have behavioural deficits. The selective advantage of having a glutamate receptor that also must recognize a second, different ligand is unclear. Perhaps it functions in coincidence detection during brain development or learning. Alternatively, it might be a sort of 'fail safe' system for a receptor well known to promote excitotoxic cell death when over-stimulated (Rothman & Olney 1995).

Recently, it was reported that ligand binding at the glycine site leads to a priming of the endocytic machinery that internalizes the NMDA receptor following activation (Nong *et al.* 2003); this points to an exciting new physiological role for the glycine site. The trafficking of non-NMDA glutamate receptors regulates some forms of synaptic plasticity (Malinow 2003), and similar mechanisms might also regulate surface expression or turnover of NMDA receptors. Because the glutamate and glycine sites reside on different subunits, one can envision situations where different ligands could influence the trafficking of their cognate receptors. Whatever the reason for the existence of the glycine site, the NMDA subtype of the glutamate receptor is a key component of many developmental, learning and memory processes in mammals—and endogenous agonists acting at its glycine site participate in the modulation of these processes. However, the physiological role of the glycine site agonists is less studied compared with glutamate because many physiologists routinely supplement their recording buffers with micromolar concentrations of glycine. Under these conditions the glycine site can be conveniently ignored.

The surprising discovery that the 'wrong isomer' of serine occurs naturally in brain grew out of advances in techniques to assay small quantities of D-amino acid isomers against the high background of L-isomers present in biological samples (Hashimoto *et al.* 1992; Nagata 1992; Chouinard *et al.* 1993; reviewed in Hashimoto & Oka 1997). The high correlation between D-serine levels and NMDA receptor binding in brain suggested that D-serine might be an endogenous glycine site agonist (Hashimoto *et al.* 1993*a*). Substantial evidence for this proposal has accumulated since 1993. Immunohistochemistry revealed that endogenous D-serine is localized in astrocytes of the forebrain grey matter, near or ensheathing NMDA receptor synapses (Schell *et al.* 1995, 1997*b*). Agonists of the non-NMDA subtypes of the glutamate receptor promote the release of preloaded D-serine from cultured astrocytes (Schell *et al.* 1995). Treatment of brain slices with D-amino acid oxidase (DAO), which selectively destroys endogenous D-serine but not glycine, reduces NMDA receptor responses, demonstrating that some glycine sites are occupied by D-serine *in vivo* (Mothet *et al.* 2000). A strong case for D-serine's regulation of NMDA receptors has also been made for the retina, where D-serine is localized in the Muller glia, and DAO treatment reduces the NMDA response (Stevens *et al.* 2003). Perhaps the most compelling evidence for a physiological role for D-serine was the discovery and characterization of the brain enzyme serine racemase, which converts L-serine into D-serine (Wolosker *et al.* 1999*a*,*b*). Because D-serine has no known function in normal metabolism, has it evolved in brain specifically for the purpose of modulating NMDA receptors? To address this question it is useful to compare the phylogeny of the NMDA receptor glycine-binding site with the phylogeny of D-serine metabolism.

2. PHYLOGENY OF THE GLYCINE SITE

The ligand-binding domains of probably all glutamate receptors are structurally related to the family of bacterial periplasmic binding proteins (Madden 2002). This idea, first proposed in 1990 (Nakanishi *et al.* 1990), became supported by multiple lines of experimentation during the subsequent decade and now applies to most subtypes of glutamate receptors subunits: metabotropic receptors (O'Hara *et al.* 1993), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptors (Stern-Bach *et al.* 1994; Lampinen *et al.* 1998), NR2 subunits (Laube *et al.* 1997) and the glycine binding site of the NR1 subunit (Kuryatov *et al.* 1994; Paas *et al.* 1996). All glutamate receptors bind their ligands through the concerted action of two amino acid domains called S1 and S2. In ionotropic glutamate receptor subunits, S1 is

located roughly 400 amino acids away from the amino terminus, while S2 is located in an extracellular loop between transmembrane domains 2 and 3 (figure 1*b*). S1 and S2 grasp their amino acid ligands in a cleft and close around it upon binding in a way that has been likened variously to a Venus fly-trap (Felder *et al.* 1999), 'Pac-man', or 'a hinged, clamshell-like gorge' (Dingledine *et al.* 1999). Ligand binding initiates conformational changes in the channel that result in gating and desensitization. The related bacterial periplasmic binding proteins are soluble, and the three-dimensional structures of a number of members of this protein family have been solved (Tam & Saier 1993). Glutamate receptors, of course, are polytopic transmembrane proteins, and two transmembrane domains separate S1 and S2; only when they come together do they create a ligand-binding site. The formidable difficulty of solving the structure of a glutamate receptor-binding site was overcome by the engineering of small linkers between S1 and S2, to create a soluble protein, which can be more easily expressed (Kuusinen *et al.* 1995; Ivanovic *et al.* 1998). Remarkably, these proteins retain their pharmacological profiles (Chen & Gouaux 1997; Miyazaki *et al.* 1999). This line of investigation produced the first crystal structures of a glutamate receptor bound to its ligand (Armstrong *et al.* 1998; Armstrong & Gouaux 2000) and has now culminated in the cocrystal structure of the NR1 glycine site with its ligand (Furukawa & Gouaux 2003). As predicted from earlier work using mutagenesis and molecular modelling (Paas *et al.* 2000), the structure resembles its bacterial counterpart remarkably. Thus the glycine site can now be described structurally with high precision.

The ionotropic glutamate receptors comprise a related gene superfamily, which consists not only of NMDA, AMPA and kainate receptors but also of a more primitive plant variety of glutamate receptor (Lam *et al.* 1998; Turano *et al.* 2001; Chiu *et al.* 2002). This fact alone implies that eukaryotes used glutamate or some other amino acid as a signalling molecule before they evolved nervous systems. The plant glutamate receptor signals to mechanisms controlling carbon and nitrogen metabolism (Kang & Turano 2003). An even more primordial glutamate-gated potassium channel related to eukaryotic glutamate receptors occurs in bacteria (Chen *et al.* 1999). The various branches of the glutamate receptor phylogenetic tree nicely recapitulate the various receptor subtypes and subunits defined by pharmacology and physiology; the NR1 subunits, containing the glycine site, comprise a discrete branch of this tree (Chiu *et al.* 1999). Thus clues to when a glycine site evolved should be contained in the sequence genealogy of NR1. More explicitly, the phylogenetic information about glycine site evolution should be found in the amino acid sequences of the S1 and S2 domains that comprise the ligand-binding site.

Figure 2 is an alignment of NR1 S1 and S2 domains from various species, along with two NR3 subunits, which have recently been shown to recognize glycine (Chatterton *et al.* (2002) see below). The arrows in figure 2 point to 15 amino acids that have been shown by mutagenesis to be important for recognizing glycine (Kuryatov *et al.* 1994; Wafford *et al.* 1995; Hirai *et al.* 1996; Williams *et al.* 1996; Ivanovic *et al.* 1998; Miyazaki *et al.* 1999; Wood *et al.* 1999; Foucaud *et al.* 2003). Every key amino acid present in mammalian NR1 is conserved in the duck, fish and frog versions of NR1. Thus one can be almost certain that these vertebrates have NMDA receptors with glycine sites that resemble those in mammals. This idea is supported by the physiological data. Birds have NMDA receptors that can be enhanced by D-cycloserine, a D-serine analogue (Steele *et al.* 1996). Likewise, in the knife fish the physiological properties of the NMDA receptor resemble mammals (Harvey-Girard & Dunn 2003). Frog and turtle central neurons express NMDA receptors with properties almost identical to those of mammals (Blanton *et al.* 1990; Cline & Tsien 1991; Skatchkov *et al.* 1994), but the specific requirement for glycine has not been reported because glycine was present in all recording buffers. And of course, the initial study showing the excitatory effects of D-serine was carried out on the toad spinal cord (Curtis *et al.* 1961).

However, as one moves towards earlier-diverging eukaryotes, the presence of a glycine site becomes equivocal. Certain aromatic residues that are important for all glutamate receptors to recognize amino acids are conserved in invertebrate NR1s (asterisks in figure 2) (Paas *et al.* 2000), suggesting that these proteins probably do bind amino acid ligands but might exhibit a different pharmacology compared with mammals. Note that position F484 in *Caenorhabditis elegans* is a Y, a conservative change, and a F484Y mutation has been shown not to affect glycine binding (Laube *et al.* 1997). Overall, *Drosophila* and *C. elegans* NR1 subunits have residues identical to those in mammals at only 10 and 8 of the 15 crucial amino acid sites, respectively. At the position that corresponds to a glutamine in rat NR1A (Q405), flies have a glutamate (E) and nematodes have an aspartate (D). The effects of mutation to an acidic amino acid at this site have not been reported, but changing it to a basic lysine residue reduces the efficacy of glycine 14 000-fold (Kuryatov *et al.* 1994), while mutation to cysteine reduces efficacy 547-fold (Foucaud *et al.* 2003). The aspartate at position D481 of vertebrates is an asparagine in the nematode; this mutation (D481N) has been reported to reduce the efficacy of glycine about sevenfold (Wafford *et al.* 1995). The lysine at position K483 in vertebrates is a glutamine (Q) in the fly and a serine (S) in the nematode (the alignment of the nematode sequence in this region is somewhat ambiguous). The fly-like mutation (K483Q) reduces the efficacy of glycine 125-fold (Wafford *et al.* 1995). In the S2 loop region, position E712 in the rat is not conserved in invertebrate NR1s, and mutation to an alanine (E712A, as in flies) produces a modest 2.6-fold reduction in glycine's efficacy (Wood *et al.* 1997). Position V735 is an arginine (R) in flies and nematodes, and mutation of this site to a cysteine (a less drastic mutation) reduces the efficacy of glycine 4.4-fold (Foucaud *et al.* 2003). Finally, a phenylalanine at position F736 is a glycine in both the nematode and the fly. The effect of this mutation (F736G) has not been reported, but mutation of this site to alanine or serine reduces the efficacy of glycine about 20-fold (Hirai *et al.* 1996).

These important differences between vertebrate and invertebrate receptors raise the possibility that if invertebrates do have glycine sites, they might not have the same properties as their vertebrate counterparts. Indeed, the sequence alignment in figure 2 also reveals that some key

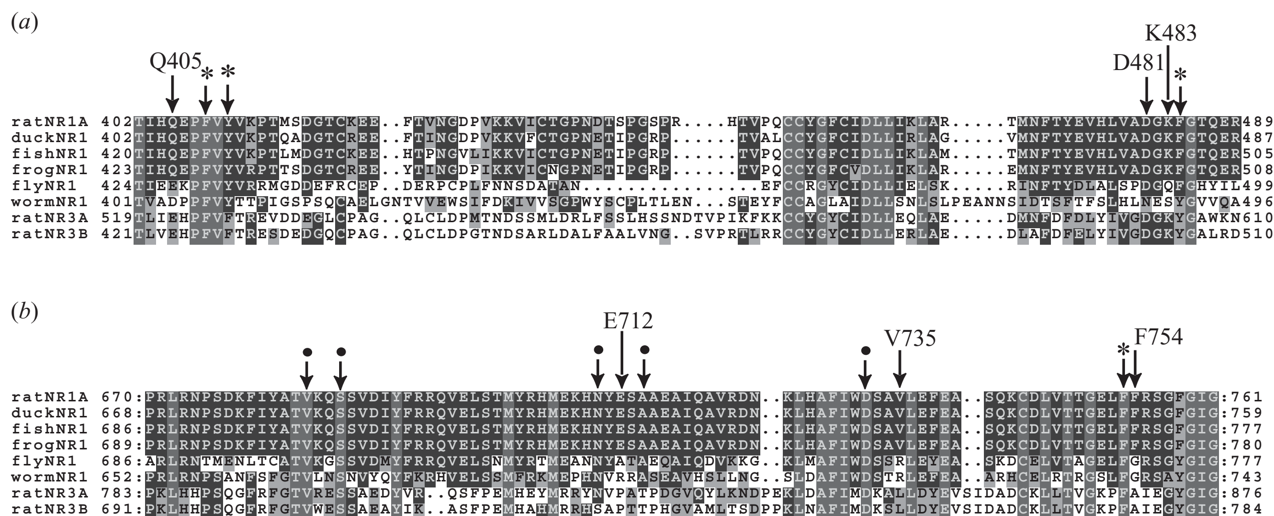

Figure 2. Alignment of the glycine site ligand binding regions of NR1 and NR3 subunits of various species: (*a*) S1 region, and (*b*) S2 region. Arrows point to amino acid residues shown by mutagenesis to be important for ligand binding. Numbered arrows point to residues not conserved between vertebrates and invertebrates, but in which mutagenesis of the vertebrate residue produces a significant reduction in the efficacy of glycine. Dots over arrows indicate residues conserved in all NR1 subunits. Asterisks over arrows denote conserved aromatic residues thought to be important for the recognition of amino acid ligands in all members of the glutamate receptor family (note that position F484 in rat is a Y in the worm; this is a conservative change that has been shown not to effect ligand binding). Amino acid numbering is based on the rat NR1 subunit (GenBank accession number NP_058706).

glycine site residues are different in the rat NR3 subunits, but that rat NR3 is more similar to rat NR1 than are nematode and fly NR1. NR3 subunits form channels with NR1 that are gated by glycine alone (with slightly lower affinity than typical NR1 glycine sites) and produce currents that desensitize very rapidly. At these channels D-serine seems to act as a partial antagonist rather than a full agonist (Chatterton *et al.* 2002). Thus even if glycine sites do exist in invertebrates, one cannot be certain that they are also D-serine sites.

What do the binding, physiological and behavioural data say about the existence of a vertebrate-like NMDA receptor with a glycine site in invertebrates? Some neuromuscular synapses in invertebrates use glutamate as a neurotransmitter, but the receptors involved do not have NMDA receptor-like properties (Heckmann & Dudel 1995; Feinstein *et al.* 1998; Fox & Lloyd 1999). NMDA-like receptors have been reported to occur at certain synapses in the central nervous systems of nematodes, arthropods and molluscs. Table 1 compiles their reported properties, focusing on those glutamate receptors that have at least one NMDA receptor-selective trait, such as activation by NMDA, blockage by MK801 or related drugs, a voltage-dependent magnesium block or a glycine requirement. Fly larvae and nematodes have a type of NMDA receptor-like channel that can be gated by NMDA but is not blocked by the glutamate site antagonist 2-amino-5-phosphonopropionic acid (AP5) (Brockie *et al.* 2001; Cattaert & Birman 2001). Perhaps surprisingly, no study, to my knowledge, has yet reported either the presence or the absence of an NMDA-associated glycine/D-serine site in these commonly studied organisms. The most complete study of NMDA receptors in *C. elegans* included 20 μM glycine in the patch pipette for all channel recordings, thereby avoiding the glycine site question (Brockie *et al.* 2001).

Some marine invertebrates possess receptors that can be activated by NMDA but not blocked by AP5. Some of these receptors have certain characteristics of NMDA receptors—such as a usage-dependent MK-801 block or a voltage-dependent magnesium block—but lack other properties of NMDA receptors in vertebrates. In the parietal ganglion of the snail, for example, co-application of glycine enhances the inward currents elicited by NMDA, but other NMDA receptor properties are not observed (Moroz *et al.* 1993). The first invertebrate NMDA-like receptor ever reported, located in the interneurons of the crayfish visual system, remains the one with properties most like its vertebrate counterparts (Pfeiffer-Linn & Glantz 1991). These receptors are activated by NMDA, blocked by AP5, blocked by Mg^{2+}, enhanced by glycine and blocked by a glycine site antagonist. The Mg^{2+} block is not relieved by depolarization and the channels appear to gate outward currents preferentially.

The final verdict on this topic awaits a more thorough testing of the pharmacology and physiology of NMDA receptor-like channels in invertebrates and in very early diverging vertebrates. Such studies will most probably come from work in *Drosophila* or *C. elegans*, because the data from sequenced genomes clearly indicates the occurrence of NR1 and NR2-like channel subunits in these creatures. In these studies, it would be interesting to test the effects of not only glycine but also D-serine. In summary, the scant experimental data available are consistent with what the sequence alignments tell us: they do not support the notion of an NMDA receptor-associated coagonist binding site similar to the glycine/D-serine site found in vertebrates; however, this possibility has not been rigorously refuted either. We are left with the tentative conclusion that, on the evolutionary time-scale, the NMDA receptor glycine site is probably a recent elaboration on an ancient amino acid binding domain and evolved some time after the divergence of arthropods but before amphibians or fishes.

Table 1. Reported occurrences of NMDA-like receptors in invertebrates.
(MK-801, NMDA-selective channel blocker).

species	preparation/assay	NMDA	MK-801	Mg^{2+} block	AP5	glycine	D-serine	reference
Caenorhabditis elegans	AVA interneuron	yes	yes	no	no	?	?	Brockie *et al.* (2001)
Drosophila larvae	central locomotor rhythm	?	yes	?	no	?	?	Cattaert & Birman (2001)
cockroach	corpus allatrum interneurons	yes	yes	yes	?	no	?	Pszczolkowski *et al.* (1999); Chiang *et al.* (2002)
crayfish	optic lobe interneurons	yes	?	yes	yes	yes	?	Pfeiffer-Linn & Glantz (1991)
snail	parietal ganglion	yes	?	no	no	yes	?	Moroz *et al.* (1993)
Aplysia	sensory neurons	blocks	?	yes	no	no	?	Dale & Kandel (1993)
lobster	olfactory organ	yes	?	?	no	?	no	Burgess & Derby (1997)
hydra	binding, feeding	?	?	?	?	yes	yes	Pierobon *et al.* (2001)

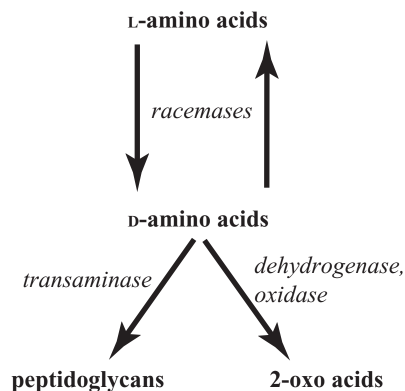

Figure 3. D-Amino acid metabolism in prokaryotes. Various types of amino acid racemases occur in bacteria and produce D-amino acids, chiefly D-alanine, D-proline and D-glutamate. These are incorporated into peptidoglycans of the cell wall via D-amino acid dehydrogenases. Bacterial D-amino acids can also be degraded to achiral 2-oxo acids via D-amino acid dehydrogenases or DAO. Not shown is bacterial D-amino acid dehydratase, which also produces achiral acid metabolites.

3. PHYLOGENY OF D-SERINE SYNTHESIS

Prokaryotic genomes contain a variety of amino acid racemases which produce D-amino acid substrates for incorporation into the cell wall peptidoglycan via the action of D-amino acid transaminase, an enzyme found only in bacteria (figure 3). Bacterial racemases can be subdivided into those that require pyridoxal-5′-phosphate (B_6) as a cofactor (alanine racemase) and those that do not (glutamate, aspartate and proline racemases). The mammalian serine racemase, which converts L-serine to D-serine (but not the inverse), requires B_6 (Wolosker *et al.* 1999*b*). Thus at first glance it might seem that the serine racemase of mammals has evolved from the alanine racemase of prokaryotes, because the two enzymes use the same cofactor to catalyse closely related reactions.

However, the bacterial proteins with the greatest amino acid similarity to mammalian serine racemase are not racemases at all but rather serine/threonine dehydratases, which also use B_6 (Wolosker *et al.* 1999*a*). A careful analysis of the evolution of the B_6 enzyme family indicates that the distinction between bacterial B_6-requiring racemases and dehydratases is not trivial (Mehta & Christen 2000). Using a combination of sequence and structural information, Christen and colleagues have classified the various B_6 enzymes into four distinct groups. The alpha group is by far the largest and consists mostly of aminotransferases and decarboxylases, but not racemases or dehydratases. Mammalian serine racemase falls into the beta group (alternatively called the fold type 2 group), which also includes serine and threonine dehydratases, threonine ammonia-lyases and diaminoproprionate ammonia lyases; see http://pfam.wustl.edu/cgi-bin/get desc?name=PALP. A third group consists of D-alanine aminotransferases, and a fourth group consists of alanine racemases. Importantly for this discussion, despite sharing a common cofactor, these four groups of prokaryotic B_6 enzymes evolved as independent lineages and had already diverged into their characteristic enzymatic groups long before the appearance of eukaryotes (Sandmeier *et al.* 1994). In other words, the four groups of B_6-using enzymes are *mutually unrelated*. In this context, the cytosolic serine racemase of mammals (being a member of the beta group rather than the alanine racemase group) is likely to be an example of convergent evolution: it catalyses a reaction similar to the alanine racemase in bacteria, but this activity has evolved independently via a separate protein lineage, and probably emerged long after the divergence of prokaryotes and eukaryotes.

Because mammalian serine racemase most closely resembles prokaryotic serine and threonine dehydratases, perhaps it is not surprising that it can also act as an eliminase and catalyse a dehydratase reaction to produce pyruvate from L-serine (Panizzutti *et al.* 2001; Neidle & Dunlop 2002; Strisovsky *et al.* 2003). The major function of this lineage of enzyme in most cells throughout most evolution was probably to produce pyruvate from L-serine or 2-oxobutanoate from L-threonine, while the production of D-serine evolved as a side reaction typical of enzymes of this class (Alexander *et al.* 1994; O'Brien & Herschlag 1999). Proteins with substantial homology to serine racemase are found in other eukaryotes, notably in yeast,

plants, and nematodes (Wolosker *et al.* 1999*a*), but it is unknown what enzymatic activity (or activities) they possess. Thus one cannot put a very precise date on when D-serine synthesis arose in eukaryotes until these putative racemases are expressed and assayed.

In lieu of this information, one can get some idea of the genealogy of racemase activity by asking which species contain substantial levels of free D-serine. Plants in particular have a serine racemase-related gene in their genomes, but only a trace of D-serine occurs in plants (Robinson 1976; Bruckner & Westhauser 2003). Likewise, serine racemase-related genes occur in yeast, *C. elegans* and *Drosophila*, but there are as yet no reports of these species producing substantial quantities of D-serine. By contrast, strong evidence exists for D-serine production in annelids and insect larvae. Earthworms (Allen & Rosenberg 1968) and silkworms (Srinivasan 1965; Uo *et al.* 1998) possess a B_6-dependent serine racemase activity; also, racemase activity appears to increase drastically in lepidopterans at the time of pupation (Corrigan & Srinivasan 1966), reviewed in Corrigan (1969). Earthworms incorporate D-serine into a compound called lombricine, which is a phosphodiester of guanidoethanol and D-serine and whose function is unknown. Thus at least some annelids and insects have the capacity to synthesize D-serine during certain times in development; whether this occurs in their nervous systems is unknown.

It has recently been suggested that under physiological conditions (in the presence of normal levels of magnesium, ATP and slightly basic pH), for every four molecules of L-serine turned over by mammalian serine racemase, three end up as pyruvate and one as D-serine (De Miranda *et al.* 2002). Because the K_m values for the dehydratase and racemase reactions are identical, perhaps it is inaccurate to categorize the racemase activity in mammals as a mere ‘side reaction’; serine racemase/dehydratase is better described as a bifunctional enzyme. No data yet exist concerning whether the enzyme can be regulated to preferentially produce one or the other product, and it is assumed that, in the tissues that express it (liver and brain), serine racemase produces a steady trickle of both pyruvate and D-serine. The cells that produce D-serine are those with a very high glycolytic activity and high L-serine levels, namely hepatocytes and astrocytes. In liver, steady-state levels of D-serine are very low despite high levels of serine racemase, and this is partly due to the presence of the enzyme DAO in the peroxisomes of hepatocytes of most species. Metabolism of D-serine by DAO produces the achiral metabolite hydroxypyruvate, which re-enters glycolytic or gluconeogenic pathways for use in normal metabolism.

4. RELEASE AND UPTAKE OF D-SERINE

D-Serine is also found in the serum, and the likely source for this D-serine is hepatic, because liver is the only other tissue besides brain that expresses appreciable levels of serine racemase (Wolosker *et al.* 1999*a*). Certain species, notably mice, have little or no DAO activity in liver (Konno *et al.* 1997), but levels of D-serine remain low in this tissue, despite the presence of serine racemase. The probable explanation is that the D-serine that is synthesized but not oxidized is released from hepatocytes into the blood. From there, the clearance of D-serine is accomplished by the kidney, where D-serine is taken up by the pars recta (the straight part) of the proximal tubule; this contrasts to kidney L-serine uptake, which occurs mainly via stereospecific transporters located in the convoluted part of the tubule (Silbernagl *et al.* 1999). The kidney pars recta transport system has a low affinity for D-serine ($K_m = 20$ mM) and low stereospecificity (Kragh-Hansen & Sheikh 1984; Silbernagl *et al.* 1999), and uses a sodium gradient to drive D-serine transport. The cells in kidney that take up D-serine possess very high levels of DAO (Usuda *et al.* 1986), which destroys the D-serine and produces H_2O_2 in the process; excessive D-serine uptake in fact leads H_2O_2-dependent oxidative damage to these cells (Silbernagl *et al.* 1997).

Despite uptake and catabolism in the kidneys, considerable quantities of D-serine pass through the kidneys and reach the urine. Natural mutant mice exist that lack DAO; D-serine levels in the serum of these mice are about five times higher than in normal mice (Hashimoto *et al.* 1993*b*), while total D-amino acid levels in these mice are about twofold higher in the liver and about 13-fold higher in the urine (Nagata *et al.* 1989). About 40% of the serine in normal urine is in the D-conformation, and the concentration of D-serine in the urine of normal humans varies between 30 and 380 μM with an average of 188 μM (Bruckner *et al.* 1994; Huang *et al.* 1998). If one assumes a typical human urine production of *ca*. $2.5\ \mathrm{l\ d^{-1}}$, then we excrete *ca*. 50 mg of D-serine daily as a normal by-product of our metabolism!

The mechanisms underlying D-serine uptake and release from tissues are only partly defined and require further study. Besides the low-affinity, sodium-dependent uptake system in the kidney, a number of other transporters and exchangers have been described in peripheral tissues which may contribute to the movements of endogenous D-serine into and out of cells; surprisingly, the system operating in hepatocytes remains undetermined (Utsunomiya-Tate *et al.* 1996; Segawa *et al.* 1999; Fukasawa *et al.* 2000; Nakauchi *et al.* 2000; Hatanaka *et al.* 2002). These systems can be broadly subdivided into those that are electrogenic (i.e. they use the energy stored in the sodium concentration gradient across the plasma membrane to drive D-serine transport), and exchangers, which move a molecule of D-serine in one direction while moving a different molecule in the opposite direction while not affecting the net charge difference (Christensen 1990). Transporters of this second class are intriguing because often the transport of one stereoisomer of serine drives the opposite movements of the other stereoisomer, creating complex situations whereby the movement of serine enatiomers through sodium-dependent transporters can influence the activity of sodium-independent ones (Verrey 2003).

Electrogenic, stereoblind transport of serine (K_m = 150 μM) occurs in colonocytes of the intestinal lumen (Hatanaka *et al.* 2002). This system is suggested to remove the small amounts of D-serine produced by intestinal bacteria. Radiolabelled L- and D-serine administered intravenously to rats distribute similarly among the tissues, with the highest levels in the pancreas (Imai *et al.* 1998). Neutral amino acid transporters of the system L amino acid transporter type 2 (LAT2) family such as Asc-1 (K_m for D-serine = 52 μM (Segawa *et al.* 1999)) function as

exchangers that require an association with the CD98 surface antigen to operate (Verrey 2003). Why some peripheral tissues produce, release and degrade/excrete D-serine is unclear. Most peripheral tissues express the NR1 subunit of NMDA receptors (Nedergaard *et al.* 2002), as do megakaryocytes (Genever *et al.* 1999) and osteoclasts (Espinosa *et al.* 1999; Itzstein *et al.* 2001); conceivably, peripheral D-serine interacts with peripheral NR1 subunits.

What about the metabolism of D-serine made in the brain? Here, the D-serine is produced mainly or exclusively in the cytosolic matrix of astroglia, which occur in close vicinity to NMDA receptors in the synapse-rich grey matter (Schell *et al.* 1997*b*). These D-serine-producing cells are also concentrated close to blood vessels, and they probably help to mediate the metabolism and transfer of fuel to serve the high-energy requirements of neurotransmission (figure 4). This phenomenon of 'metabolic coupling' is a well-established principle in the central nervous system (Schousboe *et al.* 1993; Tsacopoulos & Magistretti 1996). Glucose, the main energy source for brain, is transported from the blood and into astrocytes, where it undergoes glycolytic (anaerobic) metabolism in the cytosol. In glia, the products of this metabolism (pyruvate, lactate, glutamine, alanine and serine) are released through transporters or exchangers into the extracellular space, where they are taken up by nearby neurons to feed into aerobic mitochondrial metabolism, producing a supply of ATP for neurotransmission and carbon backbones for the synthesis of neurotransmitters. Glutamate and γ-aminobutyric acid (GABA) are transported back to glia via high-affinity uptake systems, which also serve to clear neurotransmitter from the extracellular space. The importance of metabolic coupling in brain is also emphasized by recent studies that demonstrate a neurotrophic role for glial-derived L-serine (Savoca *et al.* 1995; Mitoma *et al.* 1998; Furuya *et al.* 2000; reviewed in De Koning *et al.* 2003). In the course of such metabolic coupling, the astrocytes produce and release D-serine.

Similar to the situation in peripheral tissues, the mechanisms regulating D-serine release and uptake in brain remain incompletely defined. D-Serine injected to the lateral ventricles of rats accumulates exclusively in glia, as judged by immunohistochemistry (Wako *et al.* 1995). D-Serine is not thought to be stored in vesicles, based on the lack of ultrastructural evidence for D-serine-containing vesicles (Schell *et al.* 1997*b*). In cultured astrocytes, the uptake of radiolabelled D-serine occurs less efficiently than L-serine uptake, and depolarization with potassium chloride (KCl) does not promote the rate of basal efflux (Schell *et al.* 1995). Likewise, in microdialysis experiments in rabbit cerebral cortex, KCl does not promote D-serine efflux but D-serine levels do rise following ischaemia–reperfusion (Lo *et al.* 1998). When D-serine is preloaded into type 2 astrocytes, which express non-NMDA receptors (Gallo *et al.* 1994), treatment with agonists of these receptors (kainate and AMPA) enhances the rate of D-serine efflux (Schell *et al.* 1995). This result is probably explained by the agonists causing sodium influx through non-NMDA receptors into the glia, which then causes sodium-dependent amino acid transporters to act in reverse; this is a general mechanism for amino acid release in cultured glia (Levi & Patrizio 1992; Levi *et al.* 1992).

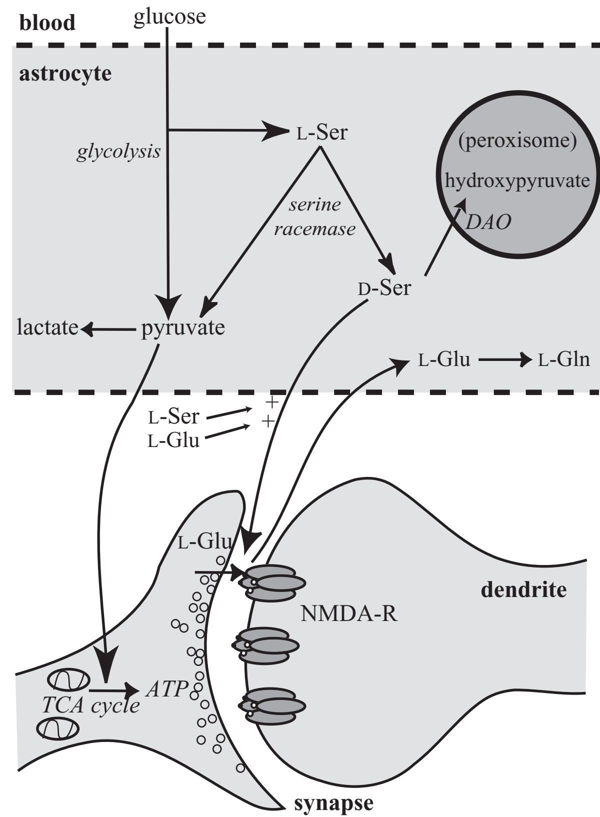

Figure 4. D-Serine metabolism in brain. Glucose from the blood is metabolized via glycolysis in glia to produce lactate, pyruvate and L-serine; these are released from glia through transporters and exchangers and are taken up into neurons to fuel aerobic metabolism via the mitochondrial tricarboxylic acid (TCA) cycle (ovals with squiggles). Glial serine racemase, when it catalyses the dehydratase reaction with L-serine, contributes to pyruvate production. When catalysing the racemase reaction with the same substrate, serine racemase produces D-serine, which escapes glia through transporters or exchangers and enhances the activation of NMDA receptors located on neurons. The precise mechanisms of D-serine release remain unclear, but in some cases D-serine release is promoted by the action of neuronal glutamate on glial glutamate receptors via reversal of sodium-dependent transporters following sodium influx (plus symbol); extracellular L-serine can also promote D-serine release, via transporter exchange (plus symbol). In some brain regions in adult brain, the D-serine produced in glia is oxidized in peroxisomes by DAO, and the product hydroxypyruvate is returned to metabolic pathways. Glutamate, following its release from neurons, is rapidly removed from the synapse by high-affinity uptake into glia, which metabolize it to glutamine through the action of glutamine synthetase.

However, in microdialysis experiments in rat striatum, kainate and also the sodium channel activator veratridine actually *reduce* the efflux of D-serine by *ca.* 50% (Hashimoto *et al.* 2000). A number of different D-serine uptake and/or release systems have been described in brain cells, and it remains unclear which of these systems predominate in physiological situations (Hayashi *et al.* 1997; Nakauchi *et al.* 2000; Yamamoto *et al.* 2001; Javitt *et al.* 2002; Ribeiro *et al.* 2002). Some require sodium and/or potassium, while others operate as exchangers. The affinities for D-serine of these various systems are generally low,

ranging between 22.8 μM (Nakauchi *et al.* 2000) and 2.5 mM (Hayashi *et al.* 1997), and they show little or no selectivity for D-serine over L-serine. Two uptake systems characterized in rat brain synaptosome fractions report K_m values in the hundreds of micromolar (Yamamoto *et al.* 2001) or low millimolar (Javitt *et al.* 2002) range. Notably, in astrocyte cultures, L-serine was reported to be more efficacious than kainate at promoting D-serine release (Ribeiro *et al.* 2002). Overall, there is currently no strong evidence to support the existence of a D-serine uptake system with high affinity and selectivity in brain. The release and uptake of D-serine in brain neuropil is likely to be both inefficient and complex, involving counterion fluxes and the movements of serine stereoisomers through multiple amino acid transport systems present on multiple cell types.

In neuropil, once D-serine is released into the extracellular spaces, it cannot be swept away by the blood (as is the case with hepatocytes), and immunohistochemical studies have reported either no (Wako *et al.* 1995; Schell *et al.* 1997*b*) or low (Schell *et al.* 1995; Yasuda *et al.* 2001) staining for D-serine in neurons, suggesting that uptake of D-serine into neurons does not represent an important mechanism for removal of D-serine from the extracellular spaces *in vivo*. Instead, astrocytic D-serine acts at the surface of neurons to activate nearby NMDA receptor glycine sites and thereby modulate their activity (figure 4). Although NMDA receptors are mainly neuronal, glial NMDA receptors also occur (Gallo & Russell 1995), especially following brain injury (Krebs *et al.* 2003). Therefore one cannot exclude a role for D-serine in some forms of autocrine or glia–glia communication. As discussed above, D-serine transport systems are low affinity. Thus compared with typical neurotransmitters such as glutamate, GABA and glycine, D-serine may remain in the extracellular spaces of brain for a very long time, its concentration at the synapse influenced mainly by synthesis and diffusion.

The relationship between the pools of D-serine in brain and peripheral tissues remains unclear. In adult rats, D-serine injected intraperitoneally raises the concentration in brain, but only when given at very high concentrations (Takahashi *et al.* 1997; Hashimoto 2002). It has long been known that D-serine crosses the blood–brain barrier poorly (Oldendorf 1973). Notably, intraperitoneal injection of L-serine, which more easily enters brain, increases the levels of D-serine, especially in juvenile animals (Takahashi *et al.* 1997; Hashimoto 2002). Presumably this is because the glial racemase (K_m of *ca.* 10 mM) is not normally saturated with substrate. Because the systemic administration of D-serine has long been known to cause nephrotoxicity (Fishman & Artom 1942; Ganote *et al.* 1974; Carone & Ganote 1975; Silbernagl *et al.* 1999), giving L-serine systemically may be the safest and best way to increase D-serine levels in brain. The therapeutic (or nootropic or neurotoxic) effects of such an approach merit further investigation.

Another interesting and unanswered question is whether endogenous D-serine moves in the other direction, from brain to blood and ultimately to the kidneys for destruction or excretion. The presence of neurotransmitter metabolites in the urine, such as those of monoamines, is well known (Goldstein *et al.* 1996). A substantial D-serine concentration gradient exists between the forebrain, where levels of D-serine approach 300 nmol g^{-1} wet weight, and peripheral tissues, where D-serine levels are below 10 nmol g^{-1} and are often undetectable (see Hashimoto & Oka (1997) and references therein). The striking difference between D-serine levels in the brain and periphery is also due to the different expression patterns of DAO, the only known enzyme capable of destroying D-serine. The phylogenetic history of DAO provides important clues about the relationship between the NMDA receptor glycine site and D-serine during evolution.

5. THE IMPORTANCE OF D-AMINO ACID OXIDASE

When Sir Hans Krebs accidentally discovered an enzyme from kidney that recognized 'non-natural' D-amino acids (but not their L-counterparts), it was not clear why nature would have created such an enzyme (Krebs 1935, 1948). Perhaps bacteria in the gut or in food produced D-amino acids and it was the job of DAO to eliminate them. Or perhaps enzymes occasionally made stereochemical mistakes and DAO destroyed the evidence. DAO is the prototypic member of the flavin adenine dinucleotide (FAD)-dependent oxidoreductase family, which also includes glycine oxidases, sarcosine oxidases and glycerol-3-phosphate dehydrogenases (http://pfam.wustl.edu/cgi-bin/getdesc?name=DAO; accession number PF01266). Most prokaryotic genomes contain at least one gene coding for an FAD-dependent oxidoreductase structurally related to DAO; it is from one or more of these that eukaryotic DAOs must have evolved (figure 5). Archaea do not possess a eukaryotic-like DAO activity but rather a bacterial-like D-amino acid dehydrogenase activity (Nagata *et al.* 1999). Nor has DAO been clearly demonstrated in plants, although plant genomes do contain genes coding for structurally related proteins. A fungal DAO has been crystallized; it is a flavoprotein that has an enzymatic activity similar to that of DAOs in metazoans, with some differences in enzymatic mechanism (Pollegioni *et al.* 2002). Insects possess DAO activities in many tissues, but DAO activity in insect brain is very low (Corrigan *et al.* 1963). The existence of serine racemase activity in insect brain is unknown.

The presence of DAO enzymes in single-celled creatures allows them to grow on D-amino acid substrates, via the oxidation to achiral products. The selective advantage of being able to metabolize the D-amino acids made in bacteria has probably directed the evolution DAOs in animals. The most commonly found neutral D-amino acids in prokaryotes are alanine and proline (Bruckner *et al.* 1993). The substrate specificity of DAO varies among species, but typically the best substrates are proline, methionine and alanine. Using data that Krebs (1948) reported for pig kidney DAO, if D-proline is assigned a value of 100, the rates of oxidation of the D-isomers of methionine, phenylalanine, alanine, valine and serine are 71, 38, 36, 28 and 21, respectively. These relative rates of oxidation have been largely confirmed for the DAOs of various mammalian tissues and species (Neims *et al.* 1966; De Marchi & Johnston 1969; Gaunt & de Duve 1976). Non-mammalian species have a similar profile, and sometimes D-serine is oxidized even less efficiently than in mammals

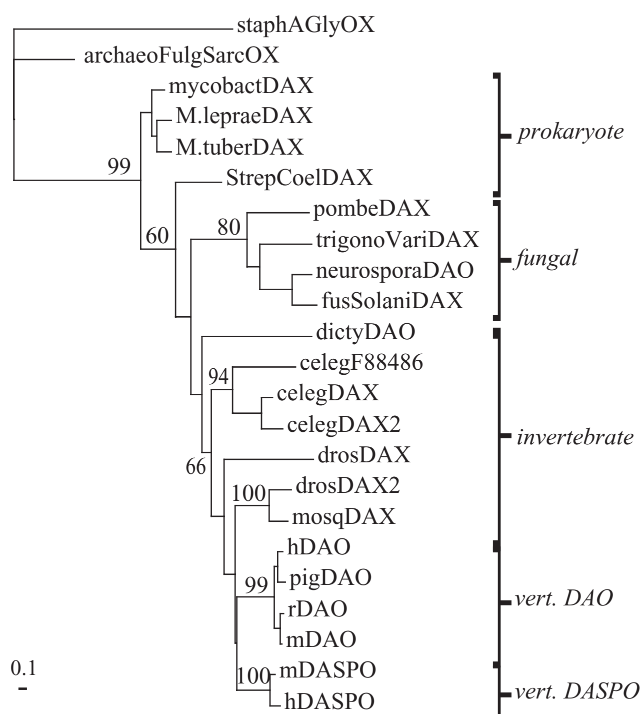

Figure 5. ClustalX neighbour-joining tree depicting the evolutionary relationships among flavin-dependent DAO enzymes and their relatives. The bacterial DAO enzymes are related to bacterial sarcosine oxidase and glycine oxidase, shown on this tree as outgroups. Eukaryotes have evolved two members of the DAO family: DAO, which oxidizes neutral D-amino acids, and DASPO, which oxidizes the acidic D-amino acid aspartate. DAX, DAO family member of uncharacterized substrate specificity. Numbers at the tree branches indicate bootstrap support (100 replications). Scale bar, 0.1 substitutions per site.

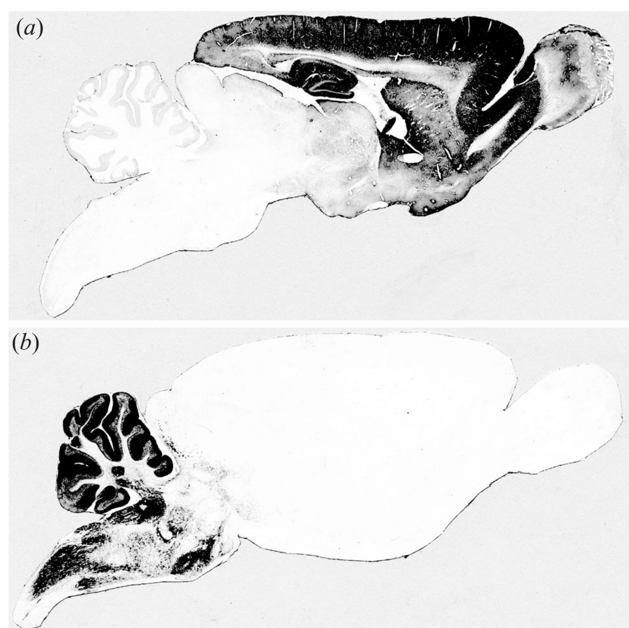

Figure 6. Contrasting localizations of endogenous D-serine and DAO in adult rat brain. (*a*) D-Ser was visualized with an antibody that recognizes glutaraldehyde-fixed D-serine. (*b*) DAO was visualized in fixed brain slices by enzyme histochemistry.

(Auclair 1959; Sarower *et al.* 2003). No DAO activity yet reported prefers D-serine over other neutral D-amino acid substrates, suggesting that the primordial substrate preference was not for the specific metabolism of D-serine, but rather to destroy and/or use other D-amino acids from bacteria. Vertebrates have one gene coding for DAO, which is a soluble enzyme containing a C-terminal motif that targets the enzyme to peroxisomes, whose lumens have an alkaline pH that is optimal for DAO enzymatic activity (Dansen *et al.* 2000).

The acidic D-amino acids D-glutamate and D-aspartate are also abundant in bacteria but are very poor substrates for DAO, and metazoans have evolved a second DAO-related gene, D-aspartate oxidase (DASPO), to deal with this. The various DASPOs destroy D-aspartate and NMDA efficiently, D-glutamate less efficiently, but no other D-amino acids are oxidized (Van Veldhoven *et al.* 1991; Kera *et al.* 2001). One exception to this substrate preference occurs in the snail, where D-glutamate is preferred (Parveen *et al.* 2001). It is perhaps surprising that D-aspartate is usually the preferred substrate, because D-glutamate is much more abundant in bacteria (Bruckner *et al.* 1993). However, in eukaryotes D-aspartate is overall the most frequently encountered free D-amino acid and D-glutamate is less common (Hashimoto & Oka 1997; Nagata *et al.* 1999; Kera *et al.* 2001). These observations suggest that eukaryotes evolved DASPO much more recently than DAO, probably as an elaboration of the DAO structure, perhaps via gene duplication. DAO (human chromosome 12q24) and DASPO (chromosome 6q21) share 30% sequence identity and comprise a small family in vertebrate genomes (figure 5); see www.ensembl.org/Homo_sapiens/familyview?family=ENSF00000001055. Most lower eukaryotes also possess two DAO-family genes, although the substrate preferences of the expressed proteins have not been determined. D-Serine and D-aspartate are the two major D-amino acids synthesized in substantial quantities in mammals (Hashimoto & Oka 1997), so it makes sense that a means to metabolize these molecules has been conserved and elaborated during evolution.

The contrasting maps of D-serine immunoreactivity and DAO activity in adult rat brain dramatically illustrate the influence of DAO on D-serine levels (figure 6). In a way similar to the situation in the liver and kidney, DAO in the brainstem, medulla and spinal cord keeps the D-serine levels low. In these brain regions, DAO appears exclusively in astrocytes (Horiike *et al.* 1987, 1994), so the D-serine produced in these cells rapidly enters peroxisomes and is destroyed; it is not known whether the movement of D-serine into peroxisomes is an active or passive process. The lack of D-serine staining in the midbrain regions shown in figure 6 (notably in the inferior and superior colliculi) is explained by a combination of there being low levels of serine racemase in this region (Wolosker *et al.* 1999*a*) and a reduction in sensitivity of the DAO histochemical technique owing to fixation.

By striking contrast, forebrain DAO activity is undetected and D-serine levels are very high, strongly implying that DAO is the major enzyme capable of destroying the endogenous D-serine in brain. This basic observation, that D-serine levels are the inverse of DAO levels, has been reported using a variety of methodologies and approaches.

Biochemical studies show the dramatic differences between forebrain (*ca.* 300 μg g^{-1}) and hindbrain (less than 10 μg g^{-1}) (Hashimoto *et al.* 1993*a*, 1995*a*,*b*; Nagata *et al.* 1994). Mutant mice lacking DAO have drastically increased levels of D-serine in the hindbrain, whereas levels in the forebrain remain unchanged (Nagata 1992; Hashimoto *et al.* 1993*b*). This rule also applies to endogenous D-aspartate and its oxidase DASPO in tissues (Schell *et al.* 1997*a*). In mammals, the distribution of an endogenously synthesized D-amino acid in a tissue is usually the *inverse* of its oxidase; the exceptions are the low levels of D-serine in the heart, skeletal muscle and testis; these tissues lack both DAO and serine racemase (Weimar & Neims 1977; Wolosker *et al.* 1999*a*). In the case of D-serine in brain, such a relationship between a neuroactive compound and its metabolic enzyme is contrary to how one normally thinks about neurotransmission. Acetylcholine, for example, is released near synapses rich in acetylcholinesterase and monoamine oxidase concentrates in monoaminergic pathways. By contrast, when D-serine is made in forebrain astrocytes lacking DAO, it escapes through transporters and exchangers into the extracellular spaces where it cannot be degraded, rapidly cleared (by high-affinity uptake) or swept away by the blood (as is the case with liver): and so it accumulates.

Thus the presence of very high levels of D-serine in the forebrain is due not only to high levels of serine racemase but also depends crucially on the absence of DAO. Two studies examined the generalizability of this concept among vertebrates and reached surprising conclusions with relevance to the coevolution of D-serine and the NMDA receptor (reviewed in Horiike *et al.* 2001). Working alone at Stanford in 1966, Dora Goldstein, while studying the effects of barbiturates on DAO, noted that the uneven distribution of DAO observed in mammals did not hold true for fishes and frogs (Goldstein 1966). In lower vertebrates DAO was distributed rather evenly across the various brain regions, with substantial activity in forebrain. This observation sat quietly in the literature for 28 years until Nagata *et al.* (1994) reported a comparison of D-serine levels in lower vertebrates versus mammals. Levels of D-serine in all brain regions of the carp (adults and infants), frog and chicken are very low (less than 10 nmol g^{-1} wt), whereas levels in the forebrains of the mouse, rat and bull are uniformly high (*ca.* 350 nmol g^{-1})—the inverse of the DAO distribution reported by Goldstein.

The molecular mechanism underlying the loss-of-function of DAO gene expression in the forebrain glia of mammals is unknown. When homogenates from different brain regions are mixed and assayed, the effect on DAO activity is strictly additive, which argues against the existence of an endogenous DAO inhibitor in forebrain (Weimar & Neims 1977). Rather, the loss of DAO probably occurs at the transcriptional level, and the regulation of DAO expression in brain offers an attractive area for future study. Some peroxisomal enzymes are highly inducible, so perhaps others can be fully repressed in the presence of the appropriate extracellular signals. One might speculate that mammals, with their well-developed NMDA receptor-rich forebrains, gained a selective advantage when their forebrain astrocytes ceased to destroy endogenous D-serine and instead released it in close vicinity to NMDA receptors. The evolutionary benefits of gene loss have been discussed (Olson 1999), but DAO may represent an interesting and dramatic case of brain region and cell-specific transcriptional repression driving natural selection. If so, this event has happened very recently in evolutionary terms and appears to be restricted to glia in the forebrains of mammals.

6. THE ONTOGENY OF D-AMINO ACID OXIDASE AND D-SERINE

The developmental pattern of DAO expression in brain further emphasizes the inverse relationships between D-serine and its oxidase. Although the DAO activity in liver and kidney has already attained half its adult levels in newborn rats and mice, in all brain regions it remains undetectable through the first 12 postnatal days (Weimar & Neims 1977). Correspondingly, D-serine immunoreactivity in newborn rats is intense in many areas of developing brain: spinal cord, hindbrain, midbrain, thalamus and the olfactory mitral layer (figure 7*a*(i)). The visualization of NMDA receptor subunits 2A and B in adjacent sections (figure 7*b*) with antibodies reveals substantial regional overlap of the receptor channel and its putative endogenous ligand. One important caveat relating to the images shown in figure 7 is that the NMDA receptor antibodies used for these experiments show little or no cross reactivity with NR2C and NR2D subunits in tissue sections (Petralia *et al.* 1994*a*). These two subunits are abundant during early postnatal development; when incorporated into channels with NR1, these would also require the presence of a glycine site agonist to operate (Monyer *et al.* 1994). Thus the overlap of D-serine and NMDA receptors depicted in figure 7 is probably an under-representation of the actual situation in brain.

The regional co-expression of D-serine NR2A/B persists for the first postnatal week, at which time the immunoreactivity for both appear to have moved towards the forebrain (figure 7*a*(ii), *b*(ii)). At the microscopic level, areas of intense D-serine immunoreactivity correspond to clusters of what appear to be glial cells with small, round cell bodies; these cells have not been identified immunocytochemically, so it remains possible that some are (or will become) neurons. The small D-serine cells appear to originate near the ventricles and, based on the progressive orientation of their leading processes, give the impression that they are migrating into the thalamus during postnatal week two and into the cerebral cortex during week three (M. J. Schell, unpublished observations; figure 7, white arrows). At the end of the second postnatal week, DAO expression commences in the hindbrain and cerebellum (Weimar & Neims 1977). Concomitantly, D-serine levels fall in these brain regions (Hashimoto *et al.* 1995*b*; Schell *et al.* 1997*b*), a change already visible with immunohistochemistry at day 14—just one day after DAO expression is first detected (figure 7*a*(iii)).

The delayed expression of DAO during ontogeny is conserved in the primate brain, where no activity is detected in newborns, and substantial activity does not appear in the cerebellum until postnatal week 20 (Volpe *et al.* 1970). Likewise, in human brain, DAO levels in the cerebellar hemispheres of 13–14-month-old children are 15% of adult levels (Neims *et al.* 1966). D-Serine levels

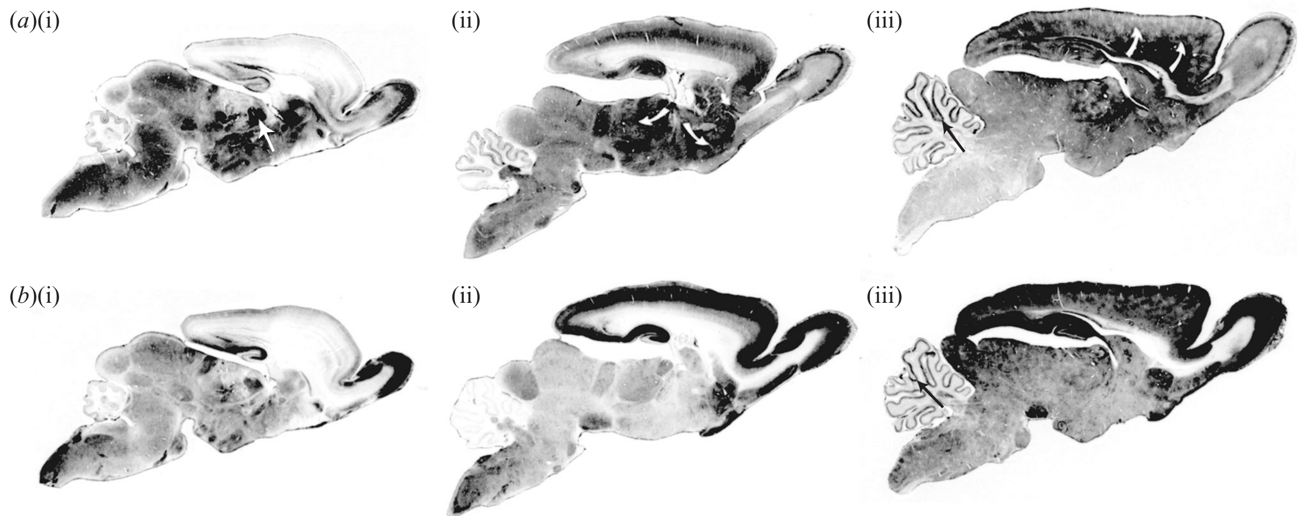

Figure 7. Co-development of D-serine and NMDA receptor regional localizations in a developing brain. (*a*) D-Serine-like immunoreactivity, and (*b*) NR2 A/B-like immunoreactivity. Postnatal age of rats (in days) is: (*a*(i) and *b*(i)) 1; (*a*(ii) and *b*(ii)) 7; and (*a*(iii) and *b*(iii)) 14. White arrows depict brain areas rich in clusters of D-serine positive glia at various ages of development. Black arrows in postnatal day 14 brains point to the transient co-localization of D-serine and NMDA receptors in the cerebellum.

range between 140 and 400 nmol g^{-1} wet weight in the prefrontal cortices of human foetuses (aged 14–40 weeks) and remain elevated in this region throughout adulthood (Hashimoto *et al.* 1993*c*). Similar to the case in other mammals, the DAO activity in the cerebral hemispheres of adult humans (55–82 years old) is undetected (Neims *et al.* 1966).

The transient expression of D-serine in the cerebellum during postnatal development is especially notable, because NMDA receptors also exhibit spatio-temporal expression in this region during the postnatal period. In the rodent cerebellum, the NR1 subunit occurs on most or all neurons at all ages of development (Petralia *et al.* 1994*b*), but the various NR2 subunits (required to form a functional channel with NR1) exhibit temporal and cell-type-specific expression (Akazawa *et al.* 1994; Monyer *et al.* 1994). For example, NMDA receptor channels occur on Purkinje neurons during the first three weeks of postnatal development but are absent from adults (Dupont *et al.* 1987; Garthwaite *et al.* 1987; Krupa & Crepel 1990). The specific NR2 subunit composition in developing Purkinje cells varies with age and may be partly species specific (Akazawa *et al.* 1994; Scherzer *et al.* 1997; Cull-Candy *et al.* 1998; Misra *et al.* 2000; Thompson *et al.* 2000). During the first postnatal week, the predominant NR2 subunit in rodent cerebellar Purkinje cells is NR2D, which exhibits very characteristically slowly-deactivating currents when incorporated into channels (Monyer *et al.* 1992; Misra *et al.* 2000). Levels of D-serine are substantial in the newborn rat cerebellum and increase during the first postnatal week (Hashimoto *et al.* 1995*b*; Schell *et al.* 1997*b*). The most intense immunoreactivity for D-serine on day 7 is located in the glia surrounding the Purkinje cells (Schell *et al.* 1997*b*), suggesting that glial D-serine released there could modulate NMDA receptors composed of NR1 and NR2D.

The developmental co-expression of D-serine and NMDA 2A/B subunits in the cerebellum has been examined in more detail (Schell *et al.* 1997*b*). During the second postnatal week, D-serine levels and NR2A/B immunoreactivity both increase, reaching a peak around age 12–14 days. Then D-serine levels begin to fall, reaching a mere 2.5% of the P12 levels by adulthood (Schell *et al.* 1997*b*). Concomitantly, NR2A/B immunoreactivity begins to fall after day 14, and in adult Purkinje cells it is no longer detected in mature adult rats. Bergmann glia, the specialized astrocytes of the cerebellar molecular layer, surround the Purkinje neuron synapses and probably saturate the Purkinje cell NMDA receptors with co-agonist while their NMDA receptor synapses develop. When NMDA receptors begin to show reduced expression in the Purkinje cells, DAO expression in the Bergmann glia rapidly becomes the highest of any brain cell (Weimar & Neims 1977; Horiike *et al.* 1987), and D-serine plummets (discussed further in Schell *et al.* 1997*b*). Interestingly, in the guinea-pig, an animal whose cerebellum develops prenatally, DAO levels begin to increase during the last prenatal week and levels are already high at birth, adding support to the idea that the delayed DAO expression in cerebellum corresponds to the end of synaptogenesis (Weimar 1977, pp. 73–185). Despite the very high levels of DAO and very low biochemical levels of D-serine in the molecular layer of adult cerebellum, D-serine is still detected in adult cerebellum by immunohistochemistry (Schell *et al.* 1995). NMDA receptor-containing synapses persist in the presynaptic terminals of adult parallel fibres (Casado *et al.* 2000) and also in basket and stellate cells (Scherzer *et al.* 1997). If D-serine is released locally near the synapses of these cells in the molecular layer, it still may be able to function as a glycine site ligand, with the high levels of DAO in the surrounding glia perhaps leading to a rapid removal and destruction of the agonist: a fate more akin to faster conventional neurotransmitters.

In the developing granule layer of the cerebellum, the various NR2 subunits also show spatio-temporal expression at the mossy fibre–granule cell synapse, with NR2A, B and C contributing to the NMDA-induced currents to various degrees at different ages (Farrant *et al.*

1994; Ebralidze *et al.* 1996; Cathala *et al.* 2000). The peak day for the ratio of NMDA to non-NMDA receptors in this region is 12 days of age, which is identical to the peak age of D-serine expression in the cerebellum (Schell *et al.* 1997*b*; Cathala *et al.* 2000). During the first three postnatal weeks, D-serine is transiently expressed in astrocyes that surround the synapses of the mossy fibres (Schell *et al.* 1997*b*; Mothet *et al.* 2000). D-Serine is not present in this region in adults when NR2C is the exclusive NR2 expressed (Farrant *et al.* 1994). Thus, similar to the case in the molecular layer, transient D-serine expression in the granule layer occurs in parallel with spatio-temporal changes in NMDA receptor expression thought to be important for the development and remodelling of synapses (Rabacchi *et al.* 1992). By contrast, in the granule cell layer of mature adults, no D-serine is detected and glycine is more likely to be the predominant glycine site agonist.

A recent physiological study has demonstrated a rapid NMDA receptor-dependent period of activity-dependent synapse elimination in the cerebellum that occurs between postnatal days 15 and 16 only (Kakizawa *et al.* 2000). When mice are injected with NMDA receptor channel blockers during this period (but not before or after), Purkinje cells become multiply innervated with climbing fibres (when normally they are only innervated by one). This observation is surprising, because the climbing fibre–Purkinje cell synapse at this age is not thought to use NMDA receptors. The conclusion of this work was that the activity of the mossy fibre–granule cell synapse, known to use NMDA receptors, was responsible for the activity-dependent synapse elimination at the climbing fibre synapse. At this age, DAO expression is rising rapidly in the glia surrounding the mossy fibre synapses; this could help orchestrate a game of synaptic musical chairs, whereby only active synapses are retained and redundant connections are pruned away. In summary, the delayed expression of DAO in the cerebellum suggests both that there is a selective advantage to delaying the expression of DAO until after postnatal week two and also an advantage to the high and persistent DAO expression in adults. In cerebellum at least, D-serine appears to act at glycine sites only during development, while glycine acts as the main or exclusive glycine site agonist during fast neurotransmission in adults.

7. A D-AMINO ACID OXIDASE LINK TO SCHIZOPHRENIA?

Over the past 15 years, substantial evidence has accumulated to suggest that schizophrenia is somehow linked to a hypofunction in the glutamatergic neurotransmitter systems in forebrain and limbic areas (reviewed in Goff & Coyle 2001). Much of this evidence stems from the observation that NMDA receptor channel-blocking drugs (phencyclidine and ketamine) cause schizophrenia-like symptoms in humans and primates and also exacerbate the symptoms of people with the disease (see, for example, Lahti *et al.* 1995). Additional evidence comes from studies of transgenic mice with reduced NMDA receptor expression; these animals exhibit social and sexual deficits that have been likened to schizophrenia, and the deficits are reversed by antipsychotic drugs (Mohn *et al.* 1999).

While antipsychotic drugs are effective at reducing the florid positive symptoms of the disease in humans (auditory hallucinations, paranoia), they do not reduce the negative symptoms (loss of affect). To counteract the presumed NMDA receptor hypofunction, a number of therapeutic approaches for schizophrenia have attempted to enhance NMDA receptor function by co-administering glycine (Javitt *et al.* 1994), D-serine (Tsai *et al.* 1998) or D-cycloserine (Cascella *et al.* 1994; Heresco-Levy *et al.* 2002) with antipsychotic drugs (reviewed in Coyle *et al.* 2002; Javitt 2002; Millan 2002). Some of the studies have reported modest success and no obvious side effects. However, the supplementations are less successful when the amino acid is co-administered with clozapine, the antipsychotic of choice for many patients (Potkin *et al.* 1999; Tsai *et al.* 1999; Evins *et al.* 2000). In the case of D-cycloserine, a partial agonist at the glycine site (Emmett *et al.* 1991), a narrow range of dosages is required to produce a beneficial effect (Goff *et al.* 1995). These therapies have yet to be reconciled with earlier work that points to possible side effects. The well-documented nephroxic effects of systemic D-serine administration have been discussed in earlier sections of this review. A simple dietary supplementation with L-serine, which has been shown to raise brain levels of D-serine when given systemically to rats (Takahashi *et al.* 1997; Hashimoto 2002), provides an alternative strategy worth investigating. D-Cycloserine was initially developed as an antibiotic to treat tuberculosis (Epstein *et al.* 1955); within a year of its being introduced, psychotropic side effects were reported (Lewis *et al.* 1957). During the subsequent decade a variety of psychotropic effects (seizures, hyperactivity, insomnia, confusion and psychosis) were reported in at least 15 studies (Simeon *et al.* 1970).

A recent genetic linkage study has implicated DAO in some forms of schizophrenia, suggesting that changes in DAO activity in these patients might influence D-serine levels and, by implication, NMDA receptor function (Chumakov *et al.* 2002). It is worth reviewing these findings in the light of the subject of this review, especially when one considers that schizophrenia might be a uniquely human disease. A 50 million base pair region on human chromosome 13 between q24 and q34 has been linked to schizophrenia in a number of studies (Lin *et al.* 1997; Blouin *et al.* 1998; Shaw *et al.* 1998; Brzustowicz *et al.* 1999; Levinson *et al.* 2000). Chumakov *et al.* (2002) focused on this region in their own linkage study, which implicated a five million base pair region at 13q34 in a French–Canadian schizophrenia population. All single nucleotide polymorphisms across this region were identified and compared in cases and controls. A 65 000 base region showed association with the disease in this population and also associated with the disease in a Russian schizophrenia population. The strengths and limitations of the approach used in this and other schizophrenia linkage studies have been discussed (Cloninger 2002; Harrison & Owen 2003).

No commonly used gene prediction programs indicate the existence of any genes in the region identified by Chumakov *et al.* (2002), suggesting that the stretch in the genome linked to schizophrenia lies in a 'gene desert'.

Nevertheless, rapid amplification of cDNA ends from human cDNA libraries identified two putative transcripts, called G72 and G30. The genes coding for these transcripts would be transcribed from opposite strands of the same DNA, and the intron–exon junctions suggested by the cDNAs do not have the typical characteristics of splice junctions in the genome. The G72 transcript was detected in amygdala, caudate nucleus, spinal cord and testis. The G72 transcript occurs in multiply spliced forms and is specific to higher primate genomes; non-human primate genomes (chimpanzee, gibbon, gorilla and rhesus monkey) are predicted to code for transcripts that are considerably shorter than those found in humans. *In vitro* transcription/translation of the G72 transcript produces a polypeptide of 153 amino acids, while similar experiments using the G30 transcript produce no protein. Antibodies against the G72 protein product label the endoplasmic reticulum and Golgi apparatus in cells overexpressing the G72 protein. These workers then carried out a yeast two-hybrid screen using the predicted G72 gene product and discovered a protein–protein interaction with DAO. When G72 protein is mixed with a 20-fold molar excess over DAO protein, DAO activity is enhanced approximately threefold over basal levels. The study proposes a model whereby the expression of G72 in schizophrenia produces an increase in DAO activity and a concomitant decrease in D-serine levels, causing NMDA receptor hypofunction. This model is also bolstered by evidence of a genetic interaction between G72 and DAO that is synergistic in some risk genotypes. Recently, the same G72/G30 region has also been associated with bipolar disorder, though the specific single nucleotide polymorphisms implicated were not the same as in the schizophrenia study (Hattori *et al.* 2003).

While the genetic associations are intriguing, the proposed biochemical mechanism is difficult to reconcile with other studies that have addressed the relationship between DAO and D-serine. D-Serine levels have been measured in postmortem schizophrenic brains and found to be not statistically different from controls (Kumashiro *et al.* 1995). This objection may be explained simply because the schizophrenia brains used in the D-serine study were not of the same aetiology as in the French–Canadian or Russian cohorts, and schizophrenia is a complex disease unlikely to be ascribed to one causative agent or mutation. Indeed, a very recent study reports a modest reduction in the levels of D-serine in the serum of schizophrenia patients (Hashimoto *et al.* 2003). The intracellular location of the putative G72/DAO protein–protein interaction remains uncertain, because G72 protein is localized to the early secretory pathway, while DAO occurs in the lumen of the peroxisome. The model suggested by Chumakov *et al.* (2002) is most difficult to reconcile with the reported distribution of DAO in the brains of mammals; DAO activity in mammals is thought to be absolutely restricted to the cerebellum, brainstem and spinal cord (Volpe *et al.* 1970), while schizophrenia involves a deficit in the prefrontal cortex and limbic system (Harrison 1999). Based on the observation that mice lacking DAO show no changes in forebrain levels of D-serine (Nagata 1992; Hashimoto *et al.* 1993*b*), DAO is not thought to regulate D-serine levels in forebrain. A brain regional gene expression study has classified DAO as a highly cerebellum-enriched transcript (Sandberg *et al.* 2000), and Western blots of mouse brain regions do not detect DAO protein in forebrain (Katagiri *et al.* 1991).

However, a very low but detectable DAO activity has been reported to occur in the forebrains of rodents more than 13 weeks old (Weimar & Neims 1977) and also in bovine (Neims *et al.* 1966) and cat (De Marchi & Johnston 1969) forebrain. Indeed, if no mechanism whatsoever exists for D-serine catabolism in the forebrain, then it is not clear why D-serine levels do not continue to increase in adulthood unless D-serine crosses the blood–brain barrier and is destroyed/excreted by the kidneys. Moreno *et al.* (1999) have reported immunohistochemical mapping of DAO in rat brain. Surprisingly, they report that DAO protein is present in all brain regions, in both neurons and glia. Because DAO protein levels in certain brain regions or in certain cells might not always equate with DAO activity, perhaps DAO is regulated by an inhibitory protein–protein interaction or covalent modification in the forebrains of mammals (but not other vertebrates) and/or by an activating interaction with G72 in human schizophrenics.

8. CONCLUSIONS

Table 2 summarizes the proposed sequence of evolutionary events leading to the appearance of the NMDA receptor glycine site and its regulation by D-serine. The approximate emergence times for the components of this signalling system are taken from recent biological clock estimates for the divergence of phyla (Kumar & Hedges 1998; Wang *et al.* 1999; Hedges *et al.* 2001); these are for comparison only, because their absolute values remain disputed (Benton & Ayala 2003). Primitive glutamate receptor channels occur in bacteria but these do not bind D-serine (Chen *et al.* 1999). Glutamate receptors also occur in plants, but these do not have the properties of NMDA receptors (Lam *et al.* 1998). NMDA receptor-like proteins definitely occur in nematodes (Brockie *et al.* 2001) and fruitflies (Cattaert & Birman 2001), but whether these receptors require a mammal-like glycine site to operate (and whether this site also recognizes D-serine) remains equivocal (figure 2). The best one can do is place the emergence of the NMDA receptor glycine site sometime after the divergence of arthropods and before the appearance of amphibians—between 1000 and 650 Myr ago.

Bacteria do not produce D-serine in large quantities, but rather produce D-alanine, D-proline and D-glutamate for their cell walls (Bruckner *et al.* 1993; figure 3). Eukaryotes, perhaps to eliminate the D-amino acids common in the prokaryotes they ingest and harbour, evolved DAO activities by elaborating on the structure of other flavin-requiring oxidases (figure 5). Mammalian B_6-dependent serine racemase, which evolved independently of the bacterial B_6-dependent alanine racemases, appears to be an example of convergent evolution, arising from a side reaction carried out by B_6-dependent enzymes related to bacterial serine and threonine dehydratases (Wolosker *et al.* 1999*a*; Mehta & Christen 2000). It is unknown when exactly serine racemase-like proteins began producing substantial quantities of D-serine, but the phylogenetically earliest known examples of substantial D-serine production in animals occur in insect larvae and annelids

Table 2. Summary of proposed sequence of events leading to the evolution of a glycine site regulated by D-serine.

component	earliest appearance	divergence (Myr ago)[a]
glutamate-gated ion channel	prokaryotes	> 2500
DAO	prokaryotes	> 2500
NMDA-like glutamate receptors	nematodes and arthropods	1200
PLP-dependent serine racemase	annelids and arthropods	1000?
mammalian-like NMDA receptor glycine site	tetrapods	1000–650
high D-serine levels in brain	mammals	300

[a] Divergence times in millions of years before the present (Myr ago) are based on the work of Hedges and colleagues (Kumar & Hedges 1998; Wang *et al.* 1999; Hedges *et al.* 2001).

during certain stages of development (Corrigan 1969). Nothing is yet known about the D-serine levels in the insect or nematode nervous systems, which appear to lack DAO activity (Corrigan *et al.* 1963), and it remains possible that substantial D-serine is synthesized by earlier-diverging eukaryotes but gets degraded by DAO.

The lower vertebrates possess NMDA receptor channels with glycine sites identical to those found in mammals (figure 2). However, the forebrains of lower vertebrates have low D-serine levels owing to high DAO activity in all brain regions (Nagata *et al.* 1994). In these creatures the glycine site would appear to be regulated chiefly by glycine or some other unknown endogenous agonist. It is only in the mammalian forebrain that high levels of D-serine occur in glia located in close vicinity to high densities of NMDA receptors. This raises the interesting possibility that mammals have found it evolutionarily advantageous to both developmentally delay (as in the hindbrain) or totally repress (as in the forebrain) the expression of DAO to allow D-serine to accumulate to sufficient levels to occupy NMDA receptor glycine sites. The coevolution of the NMDA receptor glycine site and the high levels of D-serine in the forebrains of mammals offers an interesting case study in the field of 'phylogenetic pharmacology', a term coined more than 30 years ago (Fischer 1972).

This proposed sequence of coevolution raises a question: why would mammals bother to use D-serine for regulating neurotransmission when glycine itself can be assumed to regulate all NMDA receptor glycine sites in lower vertebrates—and also those sites on NMDA receptors in the spinal cord, brainstem and adult cerebellum of mammals? The answer may lie in the differences between glycine and D-serine synaptic dynamics. Glycine also gates inhibitory chloride channels in all vertebrates (Aprison *et al.* 1969) and has the properties of a fast neurotransmitter: it is concentrated in neurons (Campistron *et al.* 1986), released from synaptosomes by depolarizing stimuli (Mulder & Snyder 1974), sequestered by high-affinity reuptake systems (Logan & Snyder 1971) and turns over rapidly (McBride *et al.* 1973; Daly *et al.* 1976; Sato *et al.* 1991). The localizations of the two main glycine transporters in brain (Zafra *et al.* 1995; Jursky & Nelson 1996) strongly resemble localizations of glycine-like immunoreactivity (Schell *et al.* 1997*b*), suggesting that the majority of the glycine transmitter pool is sequestered inside cells. Thus the disposition and dynamics of glycine are well suited for the homeostatic and motor functions carried out in the caudal brain areas where it is enriched. Such functions must occur quickly and dependably for an organism to survive, and the neuronal mechanisms controlling these functions require a relatively small amount of plasticity once the brain has developed. Even motor learning—obviously a form a plasticity important throughout life—must occur precisely and requires small adjustments in timing and synchronization occurring over millisecond time-scales. The neurotransmission underlying these processes must be dependable, repeatable and rapid; no obvious benefit would be conferred by the extensive diffusion of a transmitter far from its site of action. Glycine—released by neurons to bind to nearby NMDA receptor glycine sites, followed by rapid uptake into cells—seems well suited for this role.

Compare these dynamics with the disposition of D-serine in the forebrain. Once D-serine is synthesized in the glial cytosol and released near synapses, it probably remains in the extracellular space for a long time, its actions limited mainly by diffusion. Higher-level thinking (logic, language, imagination) occurs predominantly in the big forebrains of higher mammals, and some of these processes require minutes, days or even weeks to complete. In humans, leaps in imagination are inevitably preceded by long periods of incubation, when various scraps of information and experience 'pollinate' neuronal activity. Perhaps the slower, less point-to-point dynamics of D-serine are more suited for this kind of brain activity. These processes have been discussed extensively in various contexts (Iversen & Goodman 1986), including the non-synaptic actions of monoamines (Beaudet & Descarries 1978; Bunin & Wightman 1999), neurotransmitter/receptor mismatches (Herkenham 1987), neuropeptides (Iversen 1984) and the diffusible neurotransmitter gases (Baranano *et al.* 2001). Such processes are the targets for virtually all useful drugs in neuropharmacology. Various names have been suggested: non-synaptic diffusion transmission (Bach-y-Rita 1993), intersynaptic diffusion (Barbour & Hausser 1997), parasynaptic diffusion (Schmitt 1984) and, perhaps most commonly, volume transmission (Agnati *et al.* 1995). This last term carries with it the implication that we must consider some difficult parameters when thinking about movements of molecules through the spatio-temporal continuum in neuropil: diffusion, geometry (fractal dimension), viscosity and tortuosity (Nicholson & Sykova 1998; Rusakov & Kullmann 1998).

Non-synaptic processes also play crucial roles during brain development. Recently, mice have been engineered with targeted disruptions in the synaptic vesicle secretion machinery (Verhage *et al.* 2000; Varoqueaux *et al.* 2002).

The neurons of these animals are synaptically silent and possess no evoked presynaptic secretion. Remarkably, their brains develop and assemble normally until birth, when the animals die owing to failure to breathe. Thus the bulk of the early anatomical and synaptic development in brain requires no fast neurotransmitter secretion whatsoever. Instead, during this time excitatory neurotransmission is controlled by non-synaptic mechanisms: synthesis, diffusion, uptake and degradation (Nedergaard *et al.* 2002). Although synaptic mechanisms obviously have increased importance after birth, non-synaptic mechanisms continue to operate and complement fast neurotransmission.

Over the past 20 years it has become clear that glia participate crucially in this orchestrated cellular activity (Laming 1989; Muller 1992; Smith 1992; Araque *et al.* 1999). To understand how the brain works, we must understand not only the activity of neurons, but also the relationship between information processing among neurons and glia (Galambos 1961). In addition to glial modulation of established neuronal circuitry, glial influences also operate during brain development, when both neurons and glia are migrating over great distances in the brain, over periods of days to weeks (Peretto *et al.* 1999; Conover *et al.* 2000; Rakic 2003; Suzuki & Goldman 2003). In this context, the D-serine released from glia as a consequence of metabolic coupling with neurons might be thought of as a component of the extracellular milieu, whose regional presence leads to an increased likelihood that NMDA receptor channels will open. In a more metaphorical sense, the evolution of D-serine and NMDA receptors in brain can be likened to the coevolution of insects and flowers, and D-serine astrocyes might be imagined to be insects that inhabit the neuropil and pollinate neuronal NMDA receptors.

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