Fabrication and use of D-serine biosensors for characterising D-serine signalling in rat brain

By

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Acknowledgements

It is not often that one has the opportunity to be self-involved so completely for such a long period. I take courage that I have survived such a test and seek pardon from my family and friends for my self-absorbed ways. Most especially my mother and father- all their hardships have borne fruit. It is for their eyes I shine.

Declaration

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

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Abstract

D-serine is a co-agonist at NMDA receptors in the brain but the study of this amino acid is restricted by current techniques. I have designed highly sensitive D-serine biosensors that permit accurate real-time recordings of D-serine in the brain in a selective manner. I demonstrate that these tools are ideal for investigating factors involved in the regulation of this amino acid and the role that D-serine plays in excitotoxic cell death mediated via NMDA receptors.

I have established that the extracellular basal concentrations of D-serine in the rat brain are heterogeneous and vary even within brain structures. This suggests that D-serine is an important regulatory constraint for NMDA receptor activation, as receptor response can only be potentiated in regions with low D-serine content. Additionally, I show that these microelectrode biosensors have the potential to be used *in vivo* to detected extracellular D-serine levels.

In addition I have observed real-time activity dependent regulation (both loss and release) of D-serine by ionotropic glutamate receptor agonists AMPA, NMDA and kainate, PAR1-agonist TFFLLRNH$_2$ and high frequency stimulation *in vitro* in a number of brain areas. A decrease in D-serine concentration is potentially neuroprotective as it suggests a reduction in NMDA receptor activation. However, D-serine release can be observed in regions where the co-agonist site of the NMDA receptor is likely to be already saturated implying an alternative function of D-serine in the brain. These findings indicate multifaceted regulation of this amino acid that is brain-region specific.

Finally, I have investigated the role of D-serine release during models of stroke (hypoxia and ischemia) and found that D-serine levels are reduced in brain regions deprived of oxygen. This is ultimately neuroprotective as it will reduce over-excitation at the NMDA receptor during these insults. In the more profound model of stroke, oxygen-glucose deprivation, D-serine is eventually released. This release precedes anoxic depolarisation and could therefore contribute to its initiation via enhanced activation of the NMDA receptor.

Abbreviations

µM- Micromolar

5-HT- Serotonin

aCSF- Artificial cerebrospinal fluid

Ag/AgCl- Silver/silver chloride

AMPA- α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

ATP- Adenosine triphosphate

Asc-1- Alanine serine cysteine transporter-1 (neuronal)

ASCT- Alanine serine cysteine transporter (glial)

BSA- Bovine serum albumin

CA1- Cornu Ammonis 1

Ca^{2+}- Calcium ions

CNS- Central nervous system

CO_2- Carbon dioxide

D-aa- D-amino acids

DAAO- D-amino acid oxidase

DAAORg- *Rhodotorula gracilis* D-amino acid oxidase

D-Ser- D-serine

E. coli- Escherichia coli

EPSC- Excitatory post synaptic current

fEPSP- Field excitatory post synaptic potential

$fEPSP_N$- NMDA- receptor mediated field excitatory postsynaptic potential

GRIP- Glutamate receptor interacting protein

Gly- Glycine

H_2O- Water

H_2O_2- Hydrogen peroxide

HPLC- High performance liquid chromatography

HRP- Horse radish peroxidise

LOD- Limit of detection

L-ser- L-serine

LTD- Long term depression

LTP- Long term potentiation

mGlu- Metabotropic glutamate receptors

mM- Milimolar

mRNA- Messenger ribose nucleic acid

nA- Nanoamps

NMDA- N-methyl-D-aspartate

nNOS- Neuronal nitric oxide synthetase

NO- Nitric oxide

PAR-1- Protease activated receptor type 1

PICK-1- Protein interacting with C kinase 1

PIP2- Phosphatidylinositol 4, 5-bisphosphate

PBS- Phosphate buffer solution

SR- Serine racemase

Chapter 1: Introduction

1.1 Glutamate receptors and the brain

1.1.1 Ionotropic glutamate receptors

Glutamate receptors are essential for excitatory neurotransmission in the brain. These are sub-categorised into two groups: the ionotropic glutamate channels that participate in fast synaptic transmission and the metabotropic glutamate receptors, which mediate slower, long-lasting and more diverse post-synaptic actions. The NMDA receptor is a subtype of the ionotropic glutamate receptor family; other members include AMPA and kainate receptors. Often NMDA and AMPA receptors co-exist at the synapse and mediate the bulk of fast excitatory synaptic transmission (figure 1). Kainate receptors also exist throughout the brain but their function is not clearly understood.

AMPA-gated channels are permeable to monovalent cations and evoke depolarisation in the post-synaptic membrane. NMDA receptors are also cation-selective but they differ from AMPA receptors in a number of ways. At the resting potential (-70mV), a voltage-dependent blockade by extracellular Mg^{2+} prevents ion permeation. Depolarisation removes the Mg^{2+}-block so that the NMDA channel is permeable to Na^+ and Ca^{2+}. It is through this Ca^{2+} influx that NMDA receptors mediate a number of important secondary events including synaptic events associated with development, cognitive function, alcohol dependence and excitotoxicity.
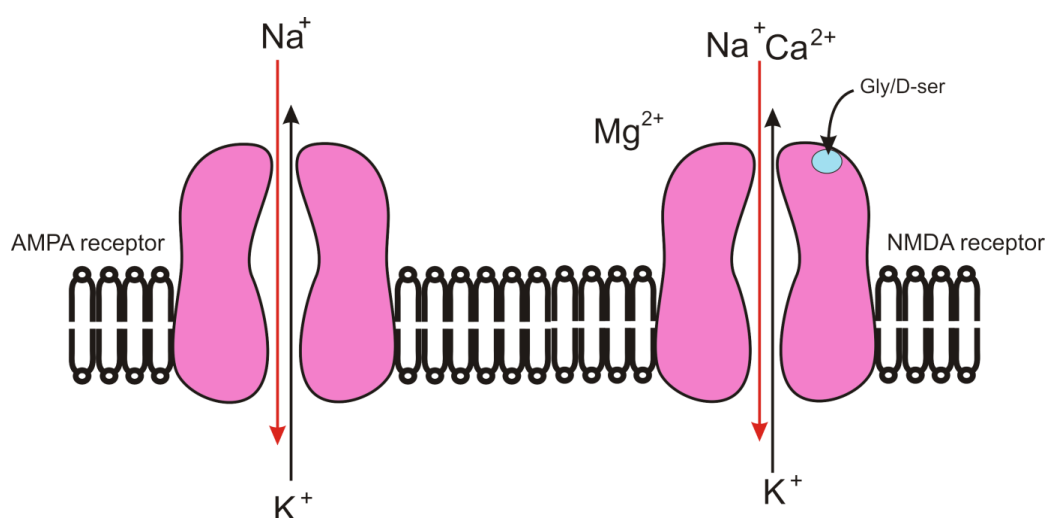
Figure 1: NMDA and AMPA receptors are often co-expressed at the synapse and both channels participate in synaptic transmission. AMPA receptors mediate fast synaptic transmission while NMDA receptors have a slower kinetics.

1.1.2   NMDA receptor Subunits and pharmacological differences

Identified by a unique set of pharmacological and functional properties, NMDA receptors exist as tetramers composed of NR1, NR2 and/or NR3 subunits (Dingledine *et al.*, 1999). There are 8 variants of the NR1 subunit generated by alternative splicing from a single gene. Four genes encode for known NR2 subunits (NR2A-D) and two genes have been identified that expressed the NR3 subunits (A and B). Co-expression of at least one NR1subunit, (where usually glycine/D-serine binds) and one NR2 subtypes (containing the glutamate binding site) is required for a functional NMDA receptor complex. Although the consensus is that receptors exist as tetramers incorporating two NR1 and two NR2 subunits of the same or different subtypes (Dingledine *et al.*, 1999), cells expressing NR3 are thought to express ternary NR1/NR2/NR3 tetrameric complexes (Sasaki *et al.*, 2002).

The subunit composition (especially NR2 and NR3 subunit variance) endows distinct pharmacological characteristics in the NMDA receptor complex. Thus NMDA channels differ in their kinetic properties, sensitivity to ligands, permeability to divalent ions and their interactions with intracellular proteins (Cull-Candy *et al.*, 2001; Chen & Roche, 2007). Since different subunit compositions of NMDA receptors have distinctive localisation and expression patterns, the functional regulation and role of this channel has been attributed to numerous central nervous system events. It may be that heterogeneity in NMDA receptor subunit composition is the basis for regulation of NMDA receptor function (Laurie *et al.*, 1997).

1.1.2.1 NR1 subunit

The NR1 subunit is an essential subunit, found ubiquitously throughout the brain and during development. Splicing of a single gene at exons 5, 21, and 22 generates 8 NR1 protein variants. The extracellular N-terminal domain (encoded by exon 5) can modulate the pharmacological properties of NMDA receptors and is the site of binding for the co-agonist, shown in the figure 2 (Traynelis *et al.*, 1998; Rumbaugh *et al.*, 2000). The intracellular C-terminal domain (encoded by exon 21 and 22) regulates protein-protein interactions, receptor trafficking, and NR1 phosphorylation (Ehlers *et al.*, 1996; Ehlers *et al.*, 1998; Standley *et al.*, 2000). For example, Tingley et al (1997) show that phosphorylation within exon 21 by PKC at residues S890 and S896 has differing consequence. Phosphorylation of S890 disrupts the clustering of

the NR1 subunit (Tingley *et al.*, 1997) while phosphorylation of S896 alone has no effect on NR1 clustering, instead phosphorylation of S896 together with PKA phosphorylation of S897 are required to increase NMDA receptor surface expression (Scott *et al.*, 2001).
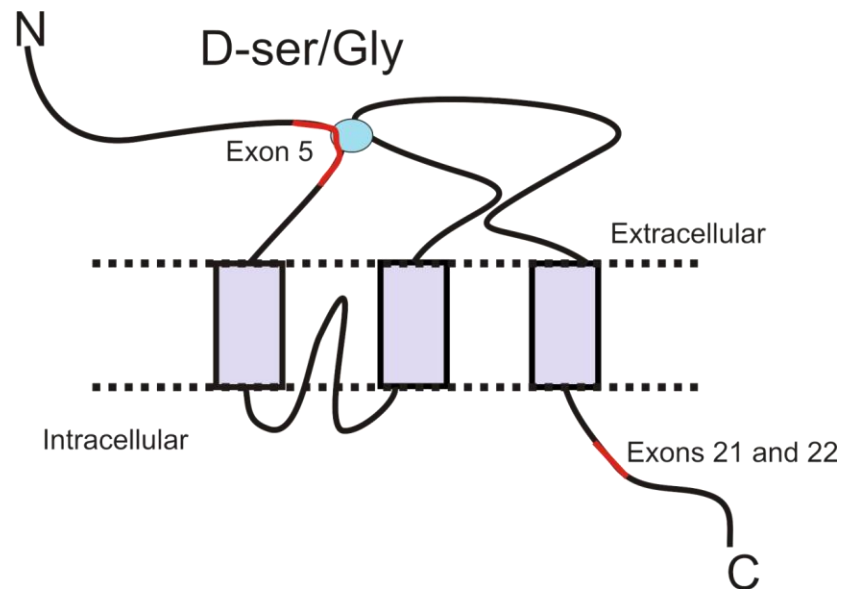
Figure 2: Structure of the NR1 subunit. D-serine/glycine (blue circle) bind in a binding pocket encoded by exon 5, while exons 21 and 22 encode an intracellular binding site.

Hence, the different splice variants of the NR1 subunits may all have distinct features endowed by particular sequence sites. These then contribute to the range of physiological function that NMDA receptors participate in. In addition, binding by a ligand (D-serine or glycine) on this subunit, does alter the affinity of glutamate binding on the NR2 subunit (Fadda *et al.*, 1988).

1.1.2.2 NR2 subunit

Although NR2 subunits are also necessary components of NMDA receptor complexes, unlike NR1, each NR2 subunit confers distinct channel properties that differentially affect synaptic NMDA receptor function. The expression pattern of the NR2 subunit is developmentally regulated; for instance the expression pattern of NR2A is up-regulated and NR2B expression is down-regulated with age. This shows concurrent decrease in NR2B and increase in NR2A expression as neurons mature (Cull-Candy *et al.*, 2001). This may be a critical regulation of the central nervous

system since the NR2A subunit containing receptors display fast kinetics with 100 ms deactivation time constant while receptors with NR2B subunits show slower deactivation time constant, approximately 250 ms (Cull-Candy & Leszkiewicz, 2004). The outcome of this will be a decrease in decay time of the NMDA receptor-mediated currents, with increased levels of NR2A expressions. NR2A containing NMDA receptors are generally confined to synaptic sites while NR2B containing NMDA receptors are found most at extrasynaptic sites (Li *et al.*, 1998; Stocca & Vicini, 1998; Tovar & Westbrook, 1999; Li *et al.*, 2002). This confers functional differences between NMDA receptors containing the NR2A and NR2B subunits include noticeable increases in endocytosis by NR2A-containg receptors and higher surface mobility in NR2B-containing receptors (Roche *et al.*, 2001; Lavezzari *et al.*, 2004; Scott *et al.*, 2004).

The NR2C subunit is restricted primarily to the cerebellum and is expressed later in development. Channels possessing this subunit show low conductance openings exhibiting specific kinetics and low sensitivity to magnesium. This receptor contains phosphorylation sites targeted by a variety of kinases: PKA, PKB and PKC (Cheng *et al.*, 2005; Chen *et al.*, 2006).

NR2D is predominantly expressed early in development and is localised mainly in thalamic and hypothalamic nuclei, hippocampus and in the brain stem, suggesting a role of this subunit only in early development (Monyer *et al.*, 1994). NR2D-containing NMDA receptors are restricted to the thalamus and sub-thalamic nuclei in adult brain, indicating some functional relevance here (Williams, 1995; Wenzel *et al.*, 1996). In particular increased expression of this subunit after LTP events on the postsynaptic membrane has been described; with suggestions that there is a NR2A and NR2D subunit switch (Erreger *et al.*, 2007; Harney *et al.*, 2008).

1.1.2.3 NR3 subunit

The NR3A subunit is widely distributed early in development whereas NR3B is restricted primarily to motor neurones (Ciabarra *et al.*, 1995; Sucher *et al.*, 1995). The NR3A-containing NMDA receptor shows low conductance, modest permeability to Ca^{2+} and can pass current at hyperpolarised potentials in the presence of Mg^{2+}-blockade (Tong *et al.*, 2008; Henson *et al.*, 2010). There is some evidence that the NR3A subunit is neuroprotective due to the low conductance to Ca^{2+};

cultured neurones prepared from NR3A-knockout mice displayed greater sensitivity to excitotoxic stress applied under ischemic, hypoxic and acute NMDA receptor activation. Over-expressions of the NR3A subunit by mutagenesis showed increased resistance to cell damage under these conditions (Nakanishi *et al.*, 2009). Interestingly, while glutamate activates triheteromeric NMDA receptors composed of NR1/NR2/NR3A subunits, glycine is sufficient to activate diheteromeric NR1/NR3A-containing receptors (Henson *et al.*, 2010). At these channels D-serine acts as a partial antagonist rather than a full agonist.

Nishi and colleagues described a novel protein that showed highest similarity (51%) to the NR3A subunit, consisting of 1003 amino acids encoded by at least 9 exons. This was termed NR3B. NMDA-receptors containing this subunit also showed suppression of glutamate induced current (similar to NR3A) with evidence that it is an important regulatory subunit controlling NMDA receptor transmission in motoneurons (Nishi *et al.*, 2001). The phenomenon of excitatory glycine channels (usually this is an inhibitory neurotransmitter) is seen with co-assembly of either of the NR3 subunits with NR1, these channels that are unaffected by NMDA or glutamate and inhibited by D-serine. These receptors are permeable to calcium and resistant to Mg^{2+} as well as other known NMDA receptor antagonists (Chatterton *et al.*, 2002).

1.1.3 Summary

The subunit composition alone communicates the complexity and variation that exists in NMDA receptors and their properties. Properties of this channel determine the site of expression, the stage in development in which these are expressed and signalling events partaken in. Additional complexity is conveyed by the postsynaptic protein interactions and signalling cascades associated with the activation of this channel: the postsynaptic density, figure 3. Numerous proteins contribute to an NMDA receptor event, many of which have yet to be fully described. Its activity potentially has vast consequences through signalling cascades in adjacent cells (figure 3). It is a wonder that any CNS event can be attributed to 'the NMDA receptor' since such diversity exists in its composition, performance and behaviour.
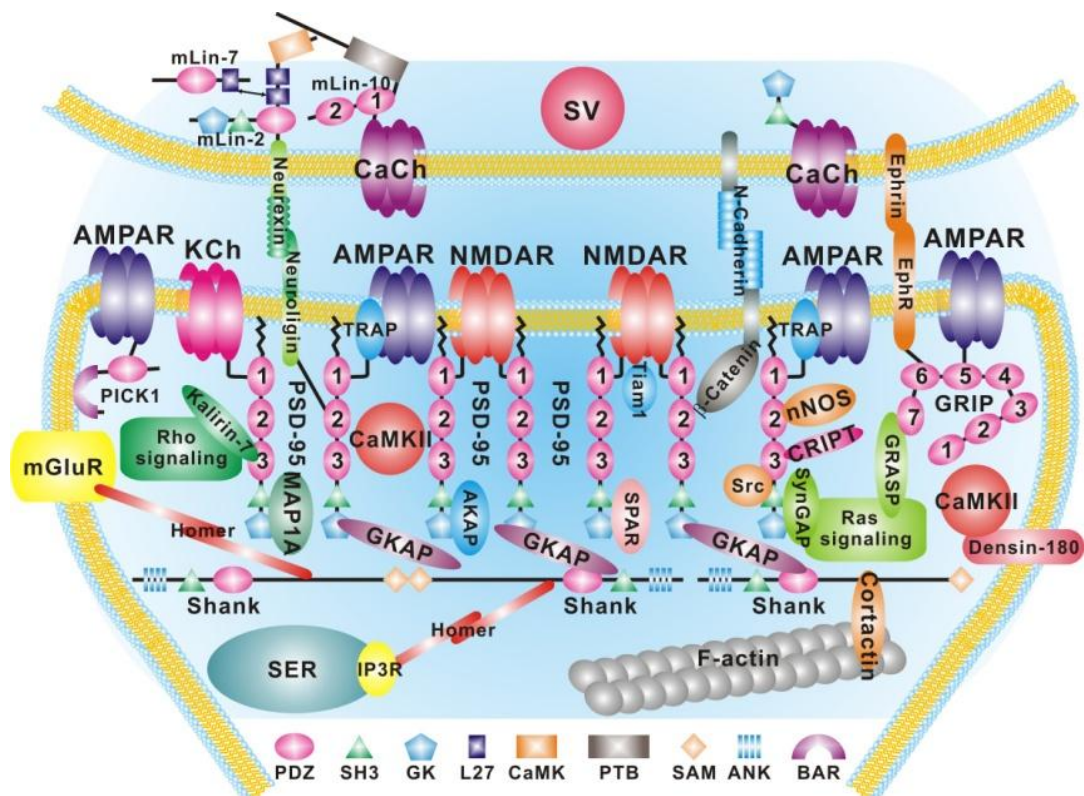
Figure 3: Activation of NMDA receptors can potentially regulate numerous signalling cascades and events; the subunit composition contributes the different pathways regulated by the NMDA receptors (Feng & Zhang, 2009).

1.2 NMDA receptor co-agonist site and D-serine

The co-agonist site is essential for NMDA receptor function. This is emphasised in studies using transgenic mice that have reduced affinity for glycine/D-serine on the NR1 subunit. Severe reductions in affinity for a co-agonist (86-fold, K483Q) cause the animals to die within a few days after birth (due to failure to feed). Mice with a less serious (5-fold, D481N) reduction in affinity survive but show deficits in spatial learning and altered anxiety-related behaviour (Kew *et al.*, 2000; Ballard *et al.*, 2002).

1.2.1 The NMDA receptor co-agonists

The excitatory effects of D-serine had been established as early as 1961. While investigating the actions of amino-acids related to GABA and glutamate on the spinal cord of toad, Curtis et al report that D-forms of optically active excitants (or depressants) were always stronger than the corresponding L form and this was true for serine also (Curtis *et al.*, 1961). However, it has taken near 40 years to show that this excitatory effect of D-serine occurs through the NMDA receptor. The slowness

in recognising the role of D-serine was partly due the lack of understanding of the dual-agonist requirements of NMDA receptor (initially) and then more so because D-amino acids were not believed to participate in CNS processes.

The requirement of an agonist, in addition to glutamate, at the NMDA receptors was first discovered in the late 1980s. Johnson and Ascher (1987) demonstrated that NMDA receptor response could be potentiated by a small, heat stable factor: glycine (Johnson & Ascher, 1987). Figure 4 illustrates that glycine and glutamate together potentiate NMDA receptor activity much more than as single agonists. A number of other amino acids were also analysed, including alanine and serine, (though it is not mentioned whether D-isomers were tested); these were found to have little effect on NMDA receptor whole cell current.
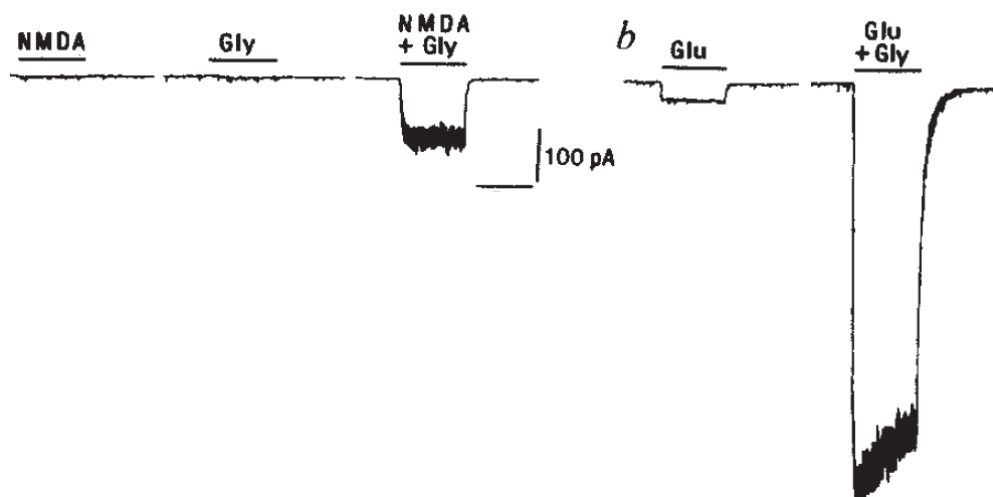
Figure 4: Glycine can potentiate NMDA receptor whole cell current in cortical mouse embryos (Johnson & Ascher, 1987).

Later that year it was confirmed that glycine did not bind the site targeted by NMDA and glutamate on this receptor, though it could regulate binding kinetics of this site. Reynolds et al used 3H-labeled MK-801 (which binds a site in the channel), to show that glycine binding increased the affinity of the glutamate binding site but that D-serine was more effective at this (Reynolds *et al.*, 1987). Also NMDA-dependent increase in Ca^{2+} influx was higher with (30μm) D-serine compared with the same concentration of glycine in primary cultures of mouse straital neurons as measured by a Ca^{2+}-sensitive fluorescent dye fura-2. This is can be an indirect measure for NMDA receptor potentiation and is supported by *in vivo* reports of intracerebellar injections of D-serine eliciting cycling GMP increase in a dose-dependent manner in mice (through changes in intracellular Ca^{2+} and NOS activation) (Wood *et al.*, 1989).

Fadda et al (1988) report valuable evidence, that co-agonist binding directly modulates the glutamate recognition site in membranes preparations from rat forebrain. The result of glycine binding to the co-agonist site caused an increase in the affinity of glutamate binding without affecting the density of the binding site. D-serine was found to induce a similar effect, however, with a higher affinity than that of glycine, table 1.1 (Fadda *et al.*, 1988).

	K_d (nM)	B_{max} (pmol/mg)	
Control	140 ± 12	3.2 ± 0.21	n=14
Glycine (5μM)	99 ± 10	3.1 ± 0.19	n=7
D-serine (5μM)	72 ± 11	3.2 ± 0.26	n=6

Table 1.1: Glycine and D-serine receptor binding kinetics carried out in rat brain; control was 1mM NMDA and data is given as mean ±SEM (Fadda *et al.*, 1988).

Kleckner and Dingledine (1988) confirmed the unique requirement of two agonists (supposedly glycine and glutamate) for ion influx to occur in NMDA receptors expressed in Xenopus oocytes (Kleckner & Dingledine, 1988). The term 'glycine site' of the NMDA receptor was coined, with half-maximal response reached with 670nM glycine. But in contrast to the findings of Johnson and Ascher, it was noted that 'D-serine was nearly as effective as glycine (90± 5.9%) when compared at 3 mM and 98% as effective at higher concentrations.

In hindsight, it is difficult to perceive how the role of D-serine at the NMDA receptor could have been overlooked. Although, collectively, some of the earliest studies supporting the actions of glycine also provide strong evidence for the actions of D-serine in the same function. Since D-amino acids were considered 'unnatural' these physiological observations were ignored, in fact it is fortunate that D-serine was considered a glycine mimic and used at all. Paradoxically, these earlier studies now form the basis for the excitatory functions of D-serine in the brain.

In the 1990s the presence of D-serine in the brain was slowly realised. It was the enzyme involved in the breakdown of D-serine, which provided the first clues for the presence of D-serine in brain tissue (Nagata *et al.*, 1989; Nagata, 1992). D-amino

acid oxidase knockout mice showed 3 times the amount of D-serine compared to controls (32.7±9.5, n=5; knockout: 96.9±10.7noml/g, n=7). The likelihood of these significantly high levels of D-serine in the brain resulting from ingestion of bacteria were low, since mice isolated in a 'germ-free' environment showed the same levels of brain D-serine. Furthermore, crossover through the blood-brain-barrier is relatively poor, since levels of D-serine in plasma were lower compared to some regions of the brain (Man & Bada, 1987; Nagata *et al.*, 1994; Dunlop & Neidle, 1997). Hashimoto et al detected free D-serine content of the body using HPLC in rats and found the vast majority this was localised to the brain. Additionally there was heterogeneous distribution of this amino acid, with highest levels detected in the cortex, straitum and hippocampus. D-serine concentrations persisted from birth to 86 postnatal weeks (Hashimoto *et al.*, 1993b). Localisation and postnatal changes in D-serine expression in the brain were found to closely resemble the NMDA receptor (Schell *et al.*, 1997). But there was still strong scepticism about the function of a D-amino acid at such a crucial receptor in the brain. There was as yet no direct evidence for the role of D-serine as a co-agonist; perplexing questions about its synthesis, breakdown and release remained unanswered; while glycine had long been established as a NMDA receptor 'co-agonist'.

An endogenous source of D-serine was soon discovered in the brain in the form of a racemase (Wolosker *et al.*, 1999a; Wolosker *et al.*, 1999b). Serine racemase was able to synthesise D-serine from the L-isomer and was found to be expressed most highly in astrocytes, highlighting a new point of controversy. Glial cells were 'ennobled' with the task of regulating neurotransmission and synaptic activity through the release of this amino acid. But the most direct evidence for the role of D-serine as a NMDA receptor co-agonist came from studies using DAAO to deplete D-serine levels in cells or in vitro and monitoring the effect of this on channel activity (Mothet *et al.*, 2000; Yang *et al.*, 2003; Panatier *et al.*, 2006). Mothet et al (2000) show that the removal of D-serine reduces NMDA receptor mediated transmission, using whole cell patch-clamp recordings, while exogenous applications allow complete recovery (Mothet *et al.*, 2000). Since DAAO is not thought to act on glycine, only the reduction in D-serine levels caused a reduced NMDA receptor activity. When glycine levels were reduced (using glycine oxidase) in the hypothalamus of rats, it had little effect NMDA receptor activity (figure 5).
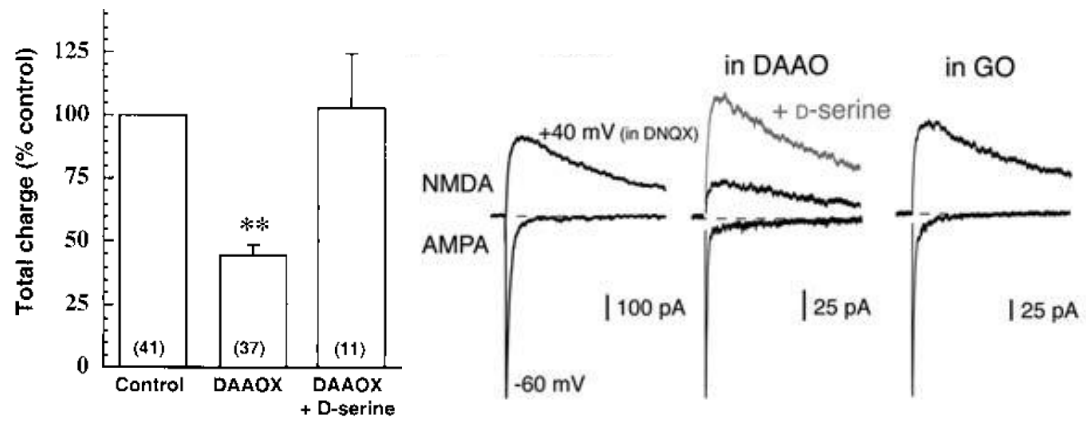
Figure 5: Removal of D-serine reduced NMDA-receptor mediated transmission, but this fully recovers with applications of D-serine (Mothet *et al.*, 2000). Glycine oxidase application on the other hand has little effect on the NMDA receptor mediated current (Panatier *et al.*, 2006).

This strategy has been criticised due to the low affinity of DAAO (Km= 30mM) and poor activity of the commercially available DAAO. Improvements in this have led to the use of D-serine deaminase, which is highly selective for D-serine (Km=0.1mM). The removal of D–serine with this enzyme hindered D-serine associated neurotoxicity and cell death (Shleper *et al.*, 2005; Kartvelishvily *et al.*, 2006). D-serine transporters were also discovered, which provided a mechanism by which D-serine action at the postsynaptic neurone could be terminated, in regions of low DAAO expression.

D-serine is now recognised as physiological ligand at the NMDA receptor co-agonist site, mediating several NMDA receptor-dependent processes. It increases the receptors affinity for glutamate, decreases its desensitisation and promotes NMDA receptor turnover by internalisation (Fadda *et al.*, 1988; Lerma *et al.*, 1990). Recent studies have implicated D-serine signalling in cognitive function, cell migration and neurotoxicity (Wolosker, 2007). But glycine can also act as a NMDA receptor co-agonist and has been implicated in a number of NMDA-receptor related functions. Whether the co-agonist site has specific brain functions has not been fully explored, but localisation studies have shown region-specific expression of D-serine and glycine, co-agonist preference also varies depending on NMDA receptor composition, as discussed previously and in some rare cases opposing roles of glycine and D-serine are observed in the same channels (eg. NR1-NR3A/B receptor).

1.2.2 Co-agonist localisation

The co-agonist site is found on the NR1 subunit of NMDA receptors, the expression of which is ubiquitous throughout the brain since this subunit is required for all functionally active receptors. Glycine and D-serine localisation studies on the other hand have revealed distinct patterns. But glycine does not have a specific role at the NMDA receptor in the brain, unlike D-serine. So that high glycine content may not be an indicator of NMDA receptor associated function. However, D-serine is not thought to have any other function in the brain apart from its co-agonist role hence, it may be expected that this amino acid is found highest in regions of high NMDA receptor expression.

Schell et al (1995) were the first to use specific antibodies for D-serine conjugated to glutaraldehyde to investigate D-serine localisation. Highest immunoreactivity was found in gray matter of the cortex, hippocampus and amygdala, this correlated closely with immunoreactivity of the glycine co-agonist site as visualised by autoradiography and are inversely correlated to the presence of D-amino acid oxidase (Schell *et al.*, 1995). D-serine studies found that its distribution in the brain was heterogeneous, highest levels were found in the developing cerebellum while in adult most D-serine was found in the forebrain and cerebellum, at 8 weeks (Hashimoto *et al.*, 1993b; Schell *et al.*, 1997). However, substantial variation in D-serine concentration is seen during development. In the rat prefrontal cortex, D-serine levels peak at gestational week 14 and then decline rapidly, suggesting involvement in the regulation of NMDA receptors during development (Hashimoto *et al.*, 1993b). Similarly in the periphery D-serine levels are high on the day of birth but shortly thereafter fall to very low levels (Hashimoto & Oka, 1997).
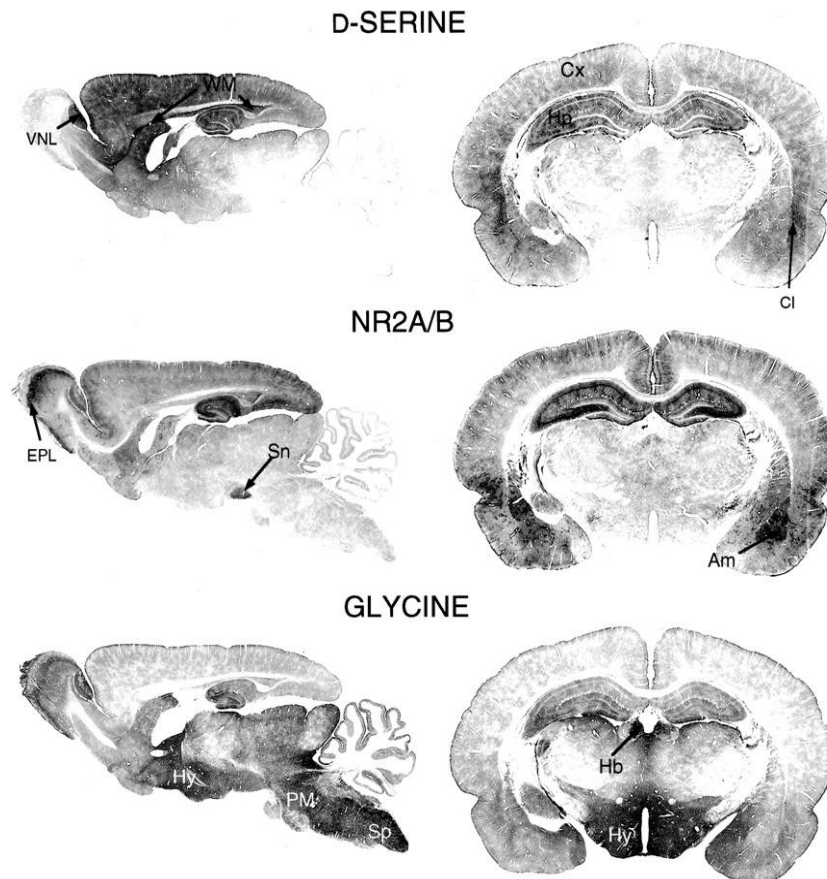
Figure 6: D-serine localisation patterns closely resemble the NR2A and NR2B subunits, with highest expression in the cortex and the hippocampus; while glycine localisation is highest in the hypothalamus in the rat brain (Schell *et al.*, 1997).

By contrast glycine is found in high concentrations in the hindbrain, the adult cerebellum and olfactory bulb, with some similarity to NR2A/B subunit distribution (Schell *et al.*, 1995). In the cerebellum, dense staining is associated with the deep cerebella nuclei, whereas the granular and molecular layers are only moderately stained (Baer *et al.*, 2009). Only slight overlap is observed between glycine and D-serine, with expression patterns of the NR2A/B subunits matching expression of D-serine more closely (figure 6). This localisation maybe an indicator specific function, for example, in the cerebellum D-serine is involved in cell migration, a function which occurs very early in development, this may explain the high levels of D-serine observed in young animals, while in the adult cerebellum, higher levels of glycine are seen. This change in co-agonist expression may also be an indicator of a change in NMDA receptor subunit composition, since subunit expression is modified with maturity.

1.2.3 Co-agonist site affinity and specificity

While investigating agonist-induced current response of cloned NMDA receptors in *Xenospus* oocytes, Matsui et al show D-serine has three to four times higher affinity than glycine for the NMDA receptor glycine site (Matsui *et al.*, 1995) while Chen et al (2008) do not find significant difference in the affinity for either of the co-agonists (Chen *et al.*, 2008). Although the affinity of glycine and D-serine vary for different NMDA receptor subunits, as shown in figure 7, the general preference order is NR2D>NR2C>NR2B>NR2A for both co-agonists (Matsui *et al.*, 1995; Woodward *et al.*, 1995). But generally glycine levels in the brain are higher than D-serine levels, although the two distinct agonists have a specific expression patterns that are not similar. Additionally, glycine in the synaptic cleft is subject to a powerful uptake system: the glycine transporters GlyT1 and 2 (Chen *et al.*, 2003; Betz *et al.*, 2006; Betz & Laube, 2006). D-serine which is also a potent agonist at this site is not taken up by glycine transporters and only low affinity transporters of this amino acid are known (Javitt *et al.*, 2002; Ribeiro *et al.*, 2002), so there may even be higher levels of D-serine in the extracellular space than glycine.

	NR2A	NR2B	NR2C	NR2D
Matsui et al	D-ser (0.32μM) Gly (0.97μM)	D-ser (0.26 μM) Gly (0.84 μM)	D-ser (0.21 μM) Gly (0.76 μM)	D-ser (0.17 μM) Gly (0.56 μM)
Chen et al	D-ser (1.27μM) Gly (1.31μM)	D-ser (0.65 μM) Gly (0.72 μM)	D-ser (0.32 μM) Gly (0.34 μM)	D-ser (0.16 μM) Gly (0.13 μM)

Figure 7: The affinity of glycine and D-serine for different NMDA receptors differs with subunit composition. The highest potency of D-serine and glycine is seen at the NR2D-containing and the lowest at the NR2A-containing NMDA receptors. Brackets contain the EC50 values for recombinantly expressed NMDA receptors in *Xenopus* oocytes (Matsui *et al.*, 1995; Chen *et al.*, 2008).

Additionally since agonist binding at the NR1 subunit is dependent on a series of hydrogen bonds to side-chain and main-chain atoms as well as water molecules, it has been suggested that D-serine binds more tightly to the ligand-binding pocket in comparison with glycine as a result of 3 additional hydrogen bonds (at Thr518, Asp732 and Ser688) and displacement of a water molecule (Furukawa & Gouaux, 2003).

This may be linked to increased affinity for glutamate by receptors binding to D-serine described earlier and higher influx of Ca^{2+} ion compared to glycine, while little difference in maximal strength was observed in NMDA receptor response using 3mM D-serine and glycine.

1.2.4 Is the co-agonist site saturated?

For a long time it was believed that the glycine binding site was constantly saturated as a result of very high concentrations of glycine (and D-serine) found in cerebrospinal fluid (Ferraro & Hare, 1985) and a number of studies showed that exogenous applications of (up to 100μM) glycine/D-serine had no modulatory effect on NMDA receptor function, as shown in figure 8 (Ahmadi *et al.*, 2003; Billups & Attwell, 2003). Contradictory to this, other researchers show that application of exogenous glycine/D-serine does increase NMDA-receptor response (Thiels *et al.*, 1992; Mothet *et al.*, 2000; Hayashi *et al.*, 2006). In fact *in vitro* studies in cultured neurones (Johnson & Ascher, 1987), hippocampus slices and *in vivo* studies in rat cerebellum by Salt et al all argue in favour of the glycine site not being fully saturated (Salt, 1989; Furukawa & Gouaux, 2003; Furukawa *et al.*, 2005).
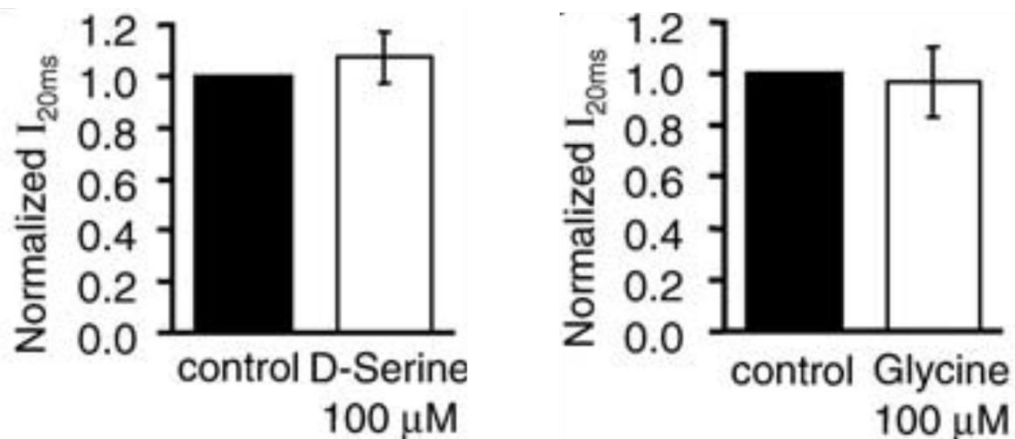
Figure 8: Glycine and D-serine do not potentiate EPSC at the mossy fibre to granule cell synapses in rat brain; NMDA receptor current amplitude was measured 20ms after the peak of the EPSC (Billups & Attwell, 2003).

The grounds for this rigorous interest in determining the extent of saturation of the glycine site is to determine the physiological importance of the co-agonist site. The extent of saturation is a measure of NMDA receptor activity i.e. the level of Ca^{2+} influx and subsequent triggering of signalling cascades. If the co-agonist site is fully saturated then maximum channel potentiation occurs at all times, and significant

alterations in co-agonist concentration will be required to alter signalling cascades or CNS events. But if the site is not fully saturated then the co-agonist concentration is a rate-limiting factor in NMDA receptor activity hence modulatory mechanisms must be in place to augment co-agonist concentration as required. At non-saturating levels, potentially small changes in co-agonist concentration can alter NMDA receptor events significantly, this may be important in cases of abnormal NMDA receptor activity. Also there may be a link between the co-agonist saturation state and its participation in a particular function in the brain. For example, it has been noted that there is a difference in saturation states between pre- and posysynaptic NMDA receptors (Kew *et al.*, 1998). The majority of post-synaptic NMDA receptors are composed of NR1-NR2A while presynaptic receptors are NR1-NR2B (Sjostrom et al. 2003; Woodhall et al. 2001; Yang et al. 2006). The EC_{50} of glycine and of D-serine for NR1-NR2A receptors are known to be higher than those for NR1-NR2B. Thus a concentration of glycine or D-serine will be saturating for the (presynaptic) NR2B-containing receptors and not saturating for the (postsynaptic) NR2A-containing receptors.

1.3   D-serine metabolism in the central nervous system

1.3.1   D-serine synthesis

D-serine synthesis, release and breakdown mechanisms in the brain have yet to be fully described. Following the discovery of large amount of D-serine in the brain, a search began for a plausible mechanism by which it could be generated, especially since levels of D-serine in the brain were higher than kidney and liver. This search was pacified by the discovery of a racemase able to synthesise D-serine from L-serine the mammalian brain (Wolosker *et al.*, 1999a; Wolosker *et al.*, 1999b). Immunostaining experiments localised this 340amino acid protein to regions of high D-serine content: the cortex, hippocampus and amygdala (Wolosker *et al.*, 1999a; Stevens *et al.*, 2003; Xia *et al.*, 2004). SR is thought to contribute to 90% of brain D-serine *in vivo*, as detected from SR knockout mice, suggesting this enzyme is the major source of D-serine in the brain (Inoue *et al.*, 2008).

Further study of SR revealed that racemisation of L-serine is influenced by divalent cations (Mg^{2+} and $Ca^{2+)}$, co-factors GRIP (proteins glutamate receptor interacting protein), PICK1, and PLP (pyridoxal 5′-phosphate) and cell energy metabolism,

especially ATP levels (figure 9). Increased SR activity results in D-serine release by mechanisms not fully understood (Cook *et al.*, 2002; De Miranda *et al.*, 2002; Neidle & Dunlop, 2002; Foltyn *et al.*, 2005; Kim, PM *et al.*, 2005) .

But the regulators of SR also partake in central physiological events not directly related to D-serine, in the brain. PICK1 for example interacts with a number of glutamate receptors including GluA2 and GluA3 AMPA receptor subunits, the GluK1 and GluK2 subunits of kainate receptors and the mGlu7 receptor via their C-terminal PDZ binding domains (Dev *et al.*, 1999; Dev *et al.*, 2000; Hirbec *et al.*, 2003). Unsurprisingly then, PICK1 has multiple effects in neurones including roles in the insertion of AMPA receptors, internalisation of kainate receptors and regulation of AMPA receptor subunit composition (Daw *et al.*, 2000; Terashima *et al.*, 2004).
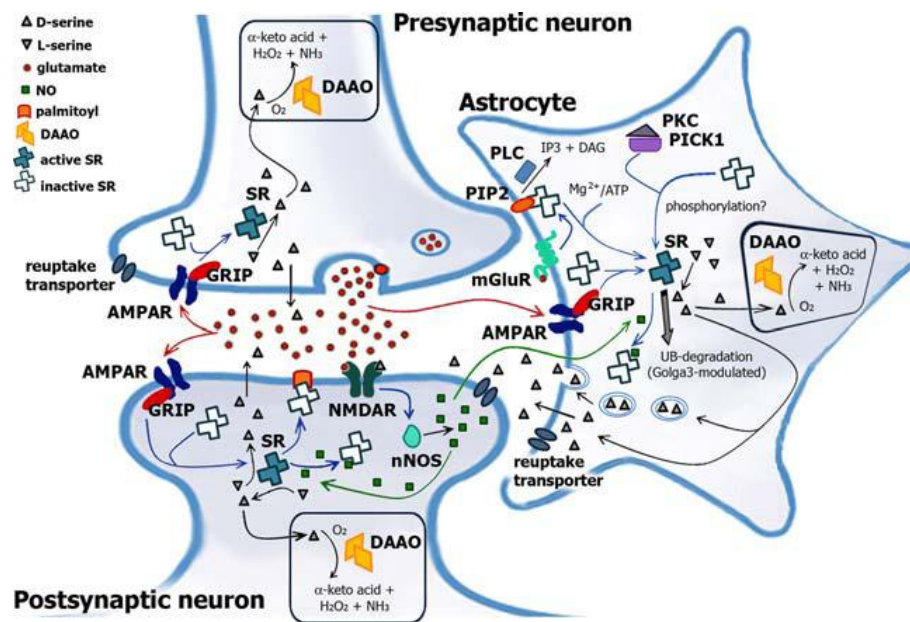
Figure 9: Serine racemase activity is regulated by many different signalling molecules and cascades including positive regulators GRIP, PICK1 and down-regulators such as PIP2 and NO. A (Pollegioni & Sacchi, 2010).

Intriguingly, D-serine synthesis is coupled with pyruvate generation: as many as 3 molecules for every D-serine molecule, as shown in figure 10 (Panizzutti *et al.*, 2001; De Miranda *et al.*, 2002). Pyruvate can be utilised in energy metabolism and also acts as a neuro-protectant in animal models of stroke, also protecting cells against oxidative damage and zinc neurotoxicity (Desagher *et al.*, 1997). Thus SR-derived pyruvate is likely to play important roles in astrocytes.
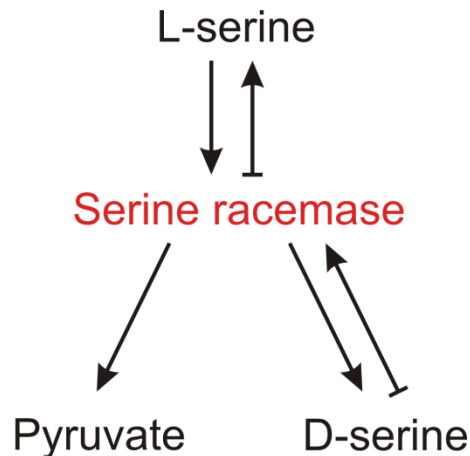
Figure 10: Reaction scheme for serine racemase. Both D-serine and pyruvate are made from L-serine but D-serine can also act as a substrate for SR, to make L-serine.

It is not clear whether SR-derived pyruvate is eventually exported to neurones and capable of altering their ability to deal with NMDA receptor activation promoted by SR-derived D-serine (De Miranda *et al.*, 2002). Pyruvate can enter the krebs cycle via different pathways, promoting ATP synthesis or being converted into lactate by the enzyme lactate dehydrogenase (LDH). If pyruvate gains access to the Krebs cycle, the ATP obtained closes a positive metabolic cycle, stimulating SR, which originally synthesized the pyruvate.

1.3.2   D-serine synthesis in neurones and glia

The growing interest in the role of D-serine at the NMDA receptor formed the basis for the tripartite synapse model, which suggests the function of astrocytic processes at the synapses, in addition to the pre- and postsynaptic neurones (Araque *et al.*, 1999). SR was initially only localised to astrocytes. Restricted to type-II protoplasmic astrocytes found in close proximity to synapses, SR provided a ready-source of D-serine at the post-synaptic neurone (and the NMDA receptor). D-serine and other gliotransmitters (chemical transmitters synthesised by glia; ATP and glutamate included) are thought to be involved in glial regulation of synaptic transmission and neuronal activity (Araque *et al.*, 1999; Haydon, 2001; Haydon *et al.*, 2009). That is to say glia, a 'house-keeping' cell type largely ignored for decades have a vital function in processes of the CNS (Fellin *et al.*, 2006a; Fellin *et al.*, 2006b).

However, like glutamate and ATP, it has now been suggested that D-serine can also be synthesised by neurones. Sensitive immunohistochemical methods with specific antibodies showed that SR was contained in astrocytes as well as some neurone populations such as pyramidal neurones in the cerebral cortex and some glutamatergic neurons (Yasuda *et al.*, 2001; Williams *et al.*, 2006). Puyal et al (2006) provide further evidence of presence of SR in neurones and further changes in D-serine levels and distribution during postnatal development. In particular, evidence for a glial-to-neuronal switch in the vestibular nuclei was described. This may explain the apparent contradiction seen in the initial studies localising SR to only glial cells (Puyal *et al.*, 2006). Significant levels of SR mRNA and protein were also found in primary neuronal cultures in the cerebral cortex, striatum and hippocampus, with suggestions that neuronally derived D-serine could contribute to NMDA receptor activation in cortical neuronal cultures (Kartvelishvily *et al.*, 2006; Yoshikawa *et al.*, 2007). But Miya et al 2008, while investigating SR distribution in the brain showed that SR expression predominates in many types of neurones in the cerebral cortex, hippocampal CA1 region and cerebeller Purkinje cells. Double immunoflourescent staining revealed that SR signals co-localised with a neuron-specific nuclear protein but not with the astrocytic markers glial fibrillary acid protein and 3-phosphoglycerate dehydrogenase (Miya *et al.*, 2008).

1.3.3 D-serine release

SR appears to be intrinsically involved in the control of D-serine levels in the brain. It is influenced by a number of co-factors and co-enzymes that may also be utilised in the metabolism of D-serine in the brain. Glial cells contain the enzyme SR and these cells use changes in intracellular levels of calcium to communicate with neighbouring cells or to perform any action. Furthermore, calcium is required for full activity of SR and D-serine release. Release of D-serine has been detected in the brain with a number of stimuli including non-ionotropic receptor agonists AMPA and kainate, as well as with compounds that augment intracellular calcium such as ionophore A123187 (Cook *et al.*, 2002; Kim, PM *et al.*, 2005; Mothet *et al.*, 2005). In this context, it has been suggested that synaptically released glutamate might stimulate glia to release D-serine, which in turn enhances NMDA responses.

The underlying signalling events involved in D-serine release have yet to be fully ascertained. Mothet et al (2005) suggest that D-serine release is dependent on intracellular calcium changes as well as extracellular calcium, the removal of which inhibited release. Interestingly, D-serine release also was markedly reduced by concanamycin A, a vacuolar-type H(+)-ATPase inhibitor, indicating a role for the vesicular proton gradient in the transmitter storage/release (Mothet *et al.*, 2005). Although Wako et al find little evidence for vesicular storage of D-serine, based on the lack of structural evidence for D-serine containing vesicles (Wako *et al.*, 1995). D-serine may also be released into the extracellular space by known amino acid transporters in the brain. A glial-expressed Na^+-dependent transporter, with low affinity and low specificity for D-serine (ASCT-2) has been described. Transport is coupled with movement of neutral amino acids, so that D-serine transport is inhibited by L-amino acids and addition of D-serine to cell cultures elicited robust efflux of pre-loaded D-serine (Ribeiro *et al.*, 2002).

1.3.4   D-serine removal/metabolism

1.3.4.1 DAAO

D-amino acid oxidase (DAAO) is able to metabolise D-serine as follows: D-serine + H2O$\rightarrow$ hydroxypyruvate + H_2O_2 + NH_3 and is found in highest concentrations in liver and kidney but is also abundant in the brain (Huynh *et al.*, 1985). Early studies localised DAAO to Bergmann glial cells and other astrocytes but not neurones or oligiodendrytes (Horiike *et al.*, 1987; Hashimoto & Oka, 1997) where it is thought contribute to the removal of D-serine from the extracellular space and hence the termination of the electrophysiological function of D-serine at the NMDA receptor. D-serine has a half-life of approximately 16 hours (Foltyn *et al.*, 2005), to which DAAO is likely to be a contributing factor at least in hindbrain areas and the cerebellum where highest levels of this enzyme are found, even in neuronal populations (Moreno *et al.*, 1999). The highest D-serine concentration was also found in these areas in DAAO knockout mice, while D-serine levels in forebrain regions remained unchanged (Hamase *et al.*, 2005). The CNS concentration of D-serine seems to be inversely correlated with the distribution of DAAO (Nagata, 1992). This distribution appears 3 weeks after birth in rats and is likely to be a

consequence of brain-structure specific increases in DAAO expression (Hashimoto *et al.*, 1993a). It is unclear what factor(s) trigger DAAO activity in the brain, but interestingly nitric oxide (NO) can increase activity of this enzyme while also inhibiting SR activity. If both DAAO and SR exist in the same cell, it is likely that NO released as a result of ionotropic glutamate receptor activity could reduce D-serine synthesis and release, while at the same time increasing its oxidation.

1.3.4.2 Serine Racemase

D-serine may also be removed by SR, which is expressed in the brain with a similar distribution to that of D-serine. Either pharmacological inhibition of this enzyme, which lead to reduced extracellular D-serine by an unknown mechanism or through SR-αβ-eliminase activity of SR, where D-serine is metabolised to L-serine (Panizzutti *et al.*, 2001; Foltyn *et al.*, 2005).

1.3.4.3 D-serine transporters

Transporters of D-serine are also likely to play an active role in its removal from the synaptic cleft. To date, the transport system most associated with serine has been reported to be system ASC, although uptake of D-serine may also occur through system L (Sershen & Lajtha, 1979). In C6 cells, the D-serine uptake system showed broad substrate specificity and higher affinity for L-serine than for D-serine (Hayashi *et al.*, 1997). This is expressed in glia and serine transport is Na^+-dependent transporter, with low affinity and low specificity for D-serine (ASCT-1). Transport is coupled with movement of neutral amino acids, so that D-serine transport is inhibited by L-amino acids and addition of D-serine to cell cultures elicited robust efflux of pre-loaded D-serine (Ribeiro *et al.*, 2002). A neutral amino acid transporter with much higher affinity for D-serine (and L-serine) has since been discovered which is expressed at the pre-synaptic terminals, dendrites and somata of neurones in cortex, cerebellum and hippocampus (Asc-1) (Javitt *et al.*, 2002; Matsuo *et al.*, 2004). Due to such high expression in the brain, and higher affinity for D-serine, the Asc-1 transporter may be an alternative mechanism for enzymatic removal of D-serine in regions where DAAO is absent.

1.4 D-serine function in the CNS

As a NMDA receptor co-agonist D-serine has been associated with a number of brain functions as well as neuropatholgies.

1.4.1 Role of D-serine in cell migration

NMDA receptors are essential in granule cell migration in the developing cerebellum (Hatten, 1999). It is thought that glutamate release by Bergmann glia promotes the mobility of granule cells through stimulation of NMDA receptors. These cells also express SR during cerebellar development and the highest levels of D-serine release correspond to the most intense cell migration period, p14, after which a reduced D-serine release is observed (Boehning & Snyder, 2003). Inhibition of SR or DAAO treatment of mice cerebellar slices blocks cell migration, by reducing calcium influx from NMDA receptor activation. DAAO treatment reduces granule cell migration by 60%; this is reversible with D-serine applications, which restores cell migration. An inhibitor of DAAO (sodium benzoate) also reverses the effects of DAAO on cell migration. Inhibitors of SR (Et-phan and Met-phan) also markedly inhibited granule cell migration as a result of reduced intracellular calcium (Kim, PM *et al.*, 2005).

1.4.2 Role of D-serine plasticity

The ability of excitatory synapses to undergo long lasting changes in the efficacy of basal synaptic transmission is thought to be the underlying mechanism for learning and memory (Bliss & Collingridge, 1993). NMDA receptor activation is necessary for the formation and consolidation of LTP (Collingridge *et al.*, 1983). Ca^{2+} influx via the NMDA receptor triggers the subsequent and persistent changes in the expression of AMPA receptors (this is expressed as long term potentiation (LTP) or long term depression (LTD). The degree of activity of NMDA receptors is determined in part by extracellular Mg^{2+} and the co-agonist, D-serine and glycine (Liu, L *et al.*, 2004). Yang et al showed that the removal of D-serine from the extracellular space markedly compromised the induction of LTP, an effect that could be reversed with supplements of D-serine (Yang *et al.*, 2003).

1.4.3 D-serine and Schizophrenia

Schizophrenia is a complex mental disorder that has also been linked to dysfunctional NMDA receptor activity. Blocking NMDA receptors (e.g. with ketamine) caused schizophrenia-like symptoms in primates, as a result of hypofunction in this channel (Lahti & Tamminga, 1995). Additional studies of transgenic mice with reduced NMDA receptor expression also show correlative social and sexual behaviour to that seen in patients with schizophrenia. Pharmacological approaches targeted the modulatory glycine site, to boost NMDA receptor activity in a selective manner in the brain. Oral administration of D-serine was found to be most effective at lower doses (30mg/day compared to 800mg of glycine/day), as it crosses the blood brain barrier easier than glycine. Additionally, glycine can also affect inhibitory synapses of the brain stem and spinal cord by activating strychnine sensitive receptors. D-serine was well tolerated by patients and efficient in improving schizophrenic symptoms (Levy *et al.*, 2005).

Recently some studies have even suggested a genetic link between D-serine and schizophrenia. Gene G72 was found on chromosome 13, located between q24 and q34 has been associated with schizophrenia (Lin *et al.*, 1997; Blouin *et al.*, 1998; Shaw *et al.*, 1998). The translational product of this is able to interact with DAAO by protein-protein interactions, increasing the activity of the enzyme three-fold *in vitro* (Chumakov *et al.*, 2002). This increased metabolism of D-serine supposedly reduced its availability at the NMDA receptor. Librie et al (2010) studied two mutagenic mice lines to find evidence for this model. A mouse with a mutation in the NR1 subunit (D481N) showed deficits in sociability, prolonged latent inhibition, enhanced startle reactivity and impaired spatial memory but a mouse with hypofunctional DAAO (G181R) mutation had elevated brain levels of D-serine, but alone it did not affect performance in the behavioural measures. Mice containing both of these mutations displayed improvement in social approach and spatial memory retention, as well as a partial normalisation of startle responses; hence increased D-serine availability resulting from reduced DAAO activity corrects the performance of mice with deficient NMDA receptor glycine site activation (Labrie *et al.*, 2010).

These observations would suggest that glutamate neurotransmission via the NMDA receptor may be decreased in Schizophrenia, as a result of reduce availability of D-serine at the modulatory glycine site but no direct evidence of this has so far been uncovered. D-serine concentrations in the post-mortem brain tissue from schizophrenia patients are significantly altered for example. However, gene expression and biochemical activities of the enzyme DAAO were found to be elevated in cerebellum (Kapoor *et al.*, 2006).

1.4.4 Alzheimer's disease

A study using D-serine deaminase demonstrated that D-serine, but not glycine mediates NMDA receptor-elicited cell death in hippocampal slices (Shleper *et al.*, 2005). In Alzheimer's disease, a neurodegenerative disorder, pathogenesis is recognised by the formation of neurotic plaques, the major component of which is the inflammatory activation of microglia due amyloid β-peptide (αβ) (Barger & Basile, 2001; Butterfield & Boyd-Kimball, 2004). This leads to neuronal death, as a result of over-excitation of the NMDA receptor. Increased NMDA receptor activity has been found in the brain of individuals affected by AD and blockers of NMDA (eg. Memantine) are neuroprotective (Lipton, 2004). The stimulant of NMDA receptor-mediated neurodegeneration may be D-serine, which has been shown to be released by αβ in cultured microglia cells (Wu *et al.*, 2004).

Increased expression of both SR protein and mRNA has been found in microglia exposed to αβ, as well as increased D-serine concentrations, indicating a dual-action of this peptide. A susceptible region, the activator protein-1 (AP-1) binding sequence located in the first intron of the SR gene, may be targeted by αβ, where binding increases its transcription rate (Wu & Barger, 2004). Additionally αβ can regulate SR post-transcriptionally by causing increases in the microglial Ca^{2+} levels which up-regulates the enzymatic activity (Cook *et al.*, 2002). Additional supporting evidence has been found in patients with AD, showing increased levels of SR activity in the hippocampus (Wu *et al.*, 2004). Interestingly, while examining AD patients with high anxiety and low anxiety symptoms, it was found that binding affinity to the modulatory glycine site was significantly higher in high anxiety patients, and these showed reduced NR2A density (Tsang *et al.*, 2008).

1.4.5 DAAO in brain physiology

DAAO activity has been identified in a number of organisms and most recently in human brain. As early as 1987, before even the localisation of D-serine the brain, DAAO was localised in rat cerebellum, particularly to astrocytes and Bergmann glia cells (Horiike *et al.*, 1987). Moreno et al set out to investigate this localisation further with affinity-purified polyclonal antibody (Moreno *et al.*, 1999). They confirmed earlier investigations and found highest staining in hindbrain regions, with highest levels in Bergmann glia of rat brain but also found expression in forebrain regions, in cerebral cortical and hippocampal neurones. Physiological importance in brain D-serine metabolism was investigated once this D-serine was localised to the brain. Knockout mice showed increased levels of D-serine in hind brain regions (Almond *et al.*, 2006; Hamase *et al.*, 2006).

In cultured cells over-expressing DAAO, D-serine treatment led to apoptosis (by hydrogen peroxide production); cell death was partially blocked by DAAO inhibitors (Park *et al.*, 2006). Taking advantage of the fact that H_2O_2 is a strong oxidant that induces apoptosis of tumour cells in vitro, Fang et al conjugated DAAO with polyethylene glycol (DAAO-PEG) and administered this to tumour-bearing mice. The administration of D-proline resulted in significant suppression of tumour growth, by generating H_2O_2 (Fang *et al.*, 2002). Attempts to use DAAO as an evaluative assay for tumour activity found highest activity in rat kidney, 8 times higher compared to rat liver while in tumour cells, activity was considerably less (Sasamura *et al.*, 2002).

1.5 Techniques used to study D-serine in the brain

1.5.1 Chromatography

D-serine had been shown to potentiate the NMDA receptor-mediated response (Fadda *et al.*, 1988; Kleckner & Dingledine, 1988; Wood *et al.*, 1989). This may have motivated researchers to look for D-serine in the brain at a time when the overwhelming attitudes confined D-amino acids to bacteria and lower organisms. The earliest investigations focused on enantioselective techniques to determine D-serine (and other free D-amino acids) which rely on high-performance liquid

chromatography (HPLC). Total amino acid content of brain tissue is extracted and samples are derivatized to fluorescent diastereomers with o-phthalaldehyde and a chiral thiol (e.g. N-acetylcysteine) and subjected to HPLC. Gas chromatography–mass spectrometry analysis and capillary electrophoresis are also used for the determination of D- and L-amino acids. Although these methods are exceptionally sensitive and can be used for the quantitative determination of various D- and L-amino acids simultaneously, they are time consuming, and require proficiency and expensive equipment.

Hashimoto et al were the first to detect significant levels of D-serine in rat brain using both HPLC and gas chromatography (Hashimoto *et al.*, 1992a; Hashimoto *et al.*, 1992b). The D-serine levels were a third of L-serine levels, and the highest of any D-amino acid found in the CNS. Hamase et al (1997) used a similar technique, involving flourogenic derivatization of each amino acid, isolation of each amino acid by reverse HPLC, followed by enantiomeric separation with Pirkle-type chiral stationary phases (Hamase *et al.*, 1997). It was established that D-serine was found in various regions of the brain, in a non-uniform manner, with highest D-serine levels in forebrain and trace amounts in the cerebellum. As well as looking at D-serine, they found that D-alanine was only found in the pituitary gland of both male and female rats, while D-leucine was detected in trace amounts in the pineal glands and hippocampus (Zhao *et al.*, 2004; Miyoshi *et al.*, 2009).

The most recent developments in this method include linkage of liquid chromatography with tandem mass spectrometry (Song *et al.*, 2008). D-serine levels were determined as a peak and percentage D-serine was calculated as D-serine/ (L-serine + D-serine). Changes in D-serine concentrations were followed during development, to determine involvement of D-serine in CNS maturation. Song et al showed that D-serine levels increase from 5% in prenatal rats to 14% in a 90-day old rat. Highest levels were found in the cortex, striatum and hippocampus, confirming earlier results.

While the presence of D-serine in the brain can be confirmed by these techniques, and developmental changes followed; the localisation patterns, metabolism and functional significance at the NMDA receptor is impossible to determine. Perhaps the major problem with this technique is the need to homogenise the brain cells for

further analysis; this disrupts all cellular networks so that differentiation between extracellular and intracellular brain D-serine content is impossible. Also the localisation pattern is lost completely, which may have provided clues about site of D-serine synthesis, function and metabolism. Confining brain D-serine to neurones or glia identifies the site of its synthesis, so that investigations for endogenous sources of D-serine can be focused and factors influencing its metabolism ascertained. Hence, chromatography techniques although very useful in confirming the presence of D-serine in the brain, are not practical for exploration of the functional role of D-serine in the brain; with added drawbacks in high running costs, excessive consumption of samples and time-consuming nature of this system.

1.5.2   Antibody staining: where is D-serine localised in the brain?

Perhaps realising the limits of HPLC and gas chromatography in investigating D-serine function in the brain Schell et al developed a stereo-selective antibody for D-serine, which overcomes at least two difficulties (Schell *et al.*, 1995). First, the localisation patters in the brain can be clarified: D-serine was enriched in the rostral cerebral cortex, hippocampus, anterior olfactory nuclei, corpus striatum, and amygdala; much less was apparent in the caudal part of the brain, including the adult cerebellum, where glycine immunostaining is more prevalent (Schell *et al.*, 1995; Schell *et al.*, 1997). This confirmed earlier studies using chromatography techniques described above. In fact the localisation pattern of D-serine when compared to localisation of the NR2A/B subunits of the NMDA receptor was found to correlate significantly. This study is a recognised landmark in changing the prevailing attitudes towards acceptance of D-serine as a co-agonist at the NMDA receptor, though not without recognition of past pioneers, upon whose efforts this work was built.

Secondly, and somewhat controversially, D-serine was localised to glia cells. In particular protoplasmic astrocytes, which ensheathe synapses signifying a role of glial in NMDA receptor-mediated neurotransmission (Schell *et al.*, 1997). This formed the basis of the tripartite synapse models, that suggests glia are activate players in neurotransmission and synaptic activity (Araque *et al.*, 1999). Moreover, it was assumed that the source of D-serine in the brain was also glia, a point confirmed

by the discovery of SR in astrocytes (Wolosker *et al.*, 1999a; Wolosker *et al.*, 1999b).

However, recent studies show that in fact D-serine and SR was also present in neurones as well as glia, though some studies confined SR only to neurones (Yasuda *et al.*, 2001; Williams *et al.*, 2006; Yoshikawa *et al.*, 2007). Williams et al raised antibodies optimised to formaldehyde-fixation, to show that D-serine was concentrated into vesicle-like compartments, rather than distributed uniformly across the cytoplasm. Subsequent dual immunofluorescence for glutamate and D-serine revealed D-serine in a subset of glutamatergic neurons, particularly in brainstem regions and in the olfactory bulbs (Williams *et al.*, 2006). While Yoshikawa et al (2007) used *in situ* hybridization based on tyramide signal amplification for detection SR mRNA, showing its presence in cortical and hippocampal neurones (Yoshikawa *et al.*, 2007). Considerations have been given to a glia to neuron switch of the SR enzyme, which may overcome the supposed contradiction in the results of these studies (Puyal *et al.*, 2006). But two contradictory outcomes are seen with antibody staining suggesting poor selectivity of antibody. Earlier antibodies (Schell *et al.*, 1995) showed some response to L-serine and glycine also while Williams et al show only D-serine is detected (Williams *et al.*, 2006).

Nevertheless the spatial localisation of D-serine by immunohistochemical studies greatly advanced our understanding of the role of this amino acid in the CNS. A physical relationship between NMDA receptors and D-serine was recognized with insight into the source of brain D-serine. However, as with HPLC, antibody techniques are time-consuming, expensive and do not allow study of living brain tissue. Additionally, monitoring changes in D-serine levels in real-time is still not feasible, hindering progress in determining likely factors that control D-serine signalling. This technique also doesn't allow extracellular brain D-serine concentrations to be examined, so the saturation state of the modulatory glycine site of NMDA receptors remains unexplored.

1.5.3 Enzymatic tools and capillary dialysis

An enzymatic assay involving DAAO allowed quantitative monitoring of D-serine in the brain. The enzyme breaks down D-serine to hydroxyl pyruvate, which when coupled with lactate dehydrogenase or amplex red, allows indirect monitoring of

changes in D-serine using a spectrometer. Hence, study in living brain tissue is possible. The use of this enzymatic assay with capillary electrophoresis, overcomes another milestone in D-serine research. In particular it allows extracellular D-serine levels to be defined and activity-dependent changes to be monitored from dialysate.

Capillary electrophoresis has advantages not associated with HPLC and immunohistochemical studies, it has high separation efficiency, instrument simplicity, minimal operation costs and compatibility with a small sample volume (Layu, A 1999). Bowser et al monitored dialysate collected from the rat striatum using micro-dialysis capillary electrophoresis (Ciriacks & Bowser, 2004; O'Brien & Bowser, 2006). Dialysate was analyzed every 12.5 seconds using the online instrument. This system allowed not only basal concentrations to be ascertained ($8 \pm 2\mu M$) but D-serine release was also observed (with aCSF containing high-K^+ and kainic acid). The microdialysis-CE-LIF instrument was able to monitor this enzymatic reaction as it proceeded over a period of 60 min, *in vitro*.

More recently a D-serine dehydratase was purified from *Saccharomyces cerevisiae*, Dsd1p specifically acts on D-serine. D-Threonine also serves as a substrate, but the efficiency is 3% of that with D-serine. The D-serine content is determined using a lactate dehydrogenase coupling method, and a change in absorbance at 340nm wavelength was measured as a measure of D-serine change. The urinary D-serine concentration of a lab member was found to be 243 ± 7.0 l μM (mean $\pm$ SD), similar to that observed by HPLC analyses (239 ± 9.8 μM)(Ito *et al.*, 2007).

1.5.4 Enzyme biosensors

Enzyme based systems and biosensors are becoming increasingly important as analytical devices for use in the central nervous system, as they allow transient events occurring in the second to second time frame, to be captured. Current directions in D-serine study point towards biosensor systems incorporating DAAO, as a biological sensing element on a microelectrode probe. This probe can be directly inserted into tissue both *in vitro* and *in vivo* allowing detection of D-serine with a high resolution, accuracy and stability.

To summarise then, current tools allow localisation of brain D-serine (antibody staining), quantitative measurements of this amino acid (HPLC and enzymatic

assays), and activity-dependent changes can be monitored in living tissue (capillary dialysis and enzymatic assays). But these techniques are often difficult, expensive and time-consuming to use. In light of this an ideal tool for studying D-serine may be defined as one which allows quantitative D-serine measurements, directly in brain tissue, while allowing manipulations with pharmacological drugs to be observed in real-time. It also needs to be cheap, easy to use and allows fast detection of D-serine in various models of the brain. The aim of this study is to create such a tool.

1.6 Brain Models used to study D-serine

1.6.1 Acutely isolated cells

Cultured cells are an incredibly versatile model for exploring cellular processing and signalling in neurones and these have been utilised to determine signalling mechanism of D-serine in NMDA receptor-associated pathologies, factors influencing D-serine enzymes (SR and DAAO) and role of D-serine transporters. However this model does have disadvantages: the preparation process is long, typically 12-15 days, therefore there is increased chance of morphological and genetic changes occurring, which may not be representative of brain physiology. Also the tightly packed network structure of neurones and glia as seen in *in vitro* or *in vivo* models is completely lost, so signalling mechanisms may differ from the *in vivo* brain.

Nevertheless, cultured cells are oft-used as a first step towards investigating a principle. Since cultured cells can be segregated into neuronal or glial populations, a number of researchers have used single cell-type populations to localise D-serine, SR and D-serine release to neurones or glial cell types (Schell *et al.*, 1995; Cook *et al.*, 2002; Mothet *et al.*, 2005). One of the earliest example of this is the rat cortical glial culture based experiment used by Schell et al, which were pre-loaded with radio-labelled D-[^{3}H]serine. Exposure to glutamate-related drugs caused release of radioactivity into the medium, D-[^{3}H]serine could be released by non-NMDA agonist AMPA and kainate (Schell *et al.*, 1995). The presence and release of D-serine from glia was supported by immunohistochemical studies and repeated by a number of groups (Schell *et al.*, 1997; Mothet *et al.*, 2000). But Kartvelishvily et al use the same conception to challenge this hypothesis; showing that D-serine can also be released from neuronal cultures, by the agonists AMPA, NMDA and kainate

(Kartvelishvily *et al.*, 2006). The presence of SR mRNA and protein was also localised to hippocampal and cortical neurones (Yoshikawa *et al.*, 2007; Miya *et al.*, 2008).

Cultured cell studies have also allowed for detailed investigations of factors influencing SR activity; these are numerous and display complex regulation of basal D-serine levels (Panizzutti *et al.*, 2001; De Miranda *et al.*, 2002; Neidle & Dunlop, 2002). Balan et al (2009) isolated membrane-bound SR from rat brain and used cultured neurone to investigate regulatory dynamics of this. They found that translocation was blocked by a palmitoylation inhibitor indicating that membrane binding is mediated by fatty acid acylation of SR (Balan *et al.*, 2009). This exemplifies the ease with which the environment of cultured cells can be manipulated to explore brain mechanisms. In a brain slice for example, this process would have been much more complex, with the need to break up cells and isolate neurones specifically after drug application.

Ribeiro et al (2002) investigated the presence of ASCT type transporter in primary astrocyte cultures, which can uptake D-serine in a sodium-dependent manner, though with low affinity and specificity. Several small amino acids were able to inhibit D-serine uptake and addition of D-serine to cells elicited robust efflux of intracellular L-serine (Ribeiro *et al.*, 2002). Again the same problem as above is faced; the need to isolate astrocytes.

The role of D-serine in cell migration was investigated using cultured cells, where the removal of D-serine by degradation and pharmacological inhibition of SR, both impede migration, while D-serine activates this process (Kim, PM *et al.*, 2005). Here cultured cells were used to investigate the principle while further detailed study was carried out *in vitro* to confirm these findings. Similarly, many other functional roles were attributed to D-serine through investigations employing cultured cells as a brain model. Mothet et al (2002) showed that degradation of D-serine from hippocampal neurones by DAAO treatment reduces NMDA-mediated currents (Mothet *et al.*, 2000). Hence this amino acid may be of import in the many NMDA receptor mediated functions in the brain. Evidence for its role in learning and memory was investigated in cultured neurones, which showed diminished LTP when treated with DAAO (Yang *et al.*, 2003). While the role of D-serine in cell death and neurotoxicity

has also been implicated, in neuropathological models of Alzheimer's (Wu *et al.*, 2004).

1.6.2   Organotypic brain slices

A step up from using cultured cells is to use brain slices grown as slice cultures; these have the advantage of fairly intact neuronal morphology and network connections lacking in cell cultures (Zimmer & Gahwiler, 1984). But structural and physiological changes can still occur as a result of long incubation periods in artificial buffers (12-15 days). Shleper et al investigated neurotoxic effects of both glycine and D-serine using organotypic hippocampal slices. D-serine was found to play a dominant role and its removal by D-serine deaminase, abolished NMDA-elicited neurotoxicity (Shleper *et al.*, 2005). This study is very difficult to carry out *in vitro*, since slices were cultured in media containing D-serine, its subsequent enzymatic removal indicated a role of D-serine in neurotoxicity. The only way to achieve similar down-regulation of D-serine would be to use SR-knockout mice or to use pharmacological inhibitors of SR. Although SR-knockout mice are available, some D-serine may still be present in the tissue as a result of its transport across the blood brain barrier and often the SR-down regulation is rarely complete.

1.6.3   *In vitro* brain slices

The *in vitro* brain slice allows some of the flexibility in experiment that cultured cells and slices have while structurally being a much closer representation of the brain. The vast majority of the neuronal and glial networks remain intact, and neurotransmission can be monitored to determine the state of a slice. Additionally, brain slices are typically prepared on the day of use, so any physical changes or artefacts that can occur in cultured cells/ organotypic slices maybe largely avoided.

O'Brien used a microdialysis probe in mouse cortical brain slices, to investigate conditions under which D-serine can be released. Analyte measurements were made every 20-27s. Stimulation with high potassium induced increased release of D-serine. Kainic acid (KA) induced D-serine release, but this release was not blocked by CNQX, suggesting that AMPA/KA receptors do not mediate D-serine release. Application of L-serine, the precursor of D-serine, resulted in increased extracellular D-serine concentrations (O'Brien & Bowser, 2006). Although, this tool for

measuring D-serine change is slow, the use of the *in vitro* model potentially allows factors controlling brain D-serine signalling to be identified.

1.6.4 *In vivo* brain

Very few examples exist for D-serine investigations *in vivo*, perhaps because of the many hindrances in using this model including strict licensing laws and regulations, and presence of anaesthetics that may alter electrophysiology. But this is considered the best brain model because complete intact cell structure is maintained along with natural milieu of circulating hormones and factors.

Surprisingly, many examples of *in vivo* study of D-serine were carried out at a time before the role of D-serine in the brain was established. In the late 1980s *in vivo* experiments showed that D-serine, acting via the NMDA modulatory glycine site potentiated the ongoing neuronal activity through the NMDA receptor complex (Wood *et al.*, 1989; Rao *et al.*, 1990). Direct cerebellar injections of D-serine elicited dose-dependent increases in cerebellar cyclic GMP (levels were monitored via a radioimmunoassay), in mice. This is a well-characterised secondary messenger modulated by the NMDA receptor complex and reflects the ongoing stimulation of NOS and NO activity resulting from channel activation. Glycine was also found to increase cGMP levels, though with a shorter-time course, possibly resulting from active uptake mechanisms. These studies provide strong evidence that the co-agonist site of NMDA receptors is not saturated *in vivo*.

Thiels et el (1992) set out to investigate the role of the modulatory glycine site in LTP at the commissural-CA1 synapses in anesthetized rats. Administration of the specific glycine site antagonist 7-chlorokynurenic acid and a general NMDA receptor antagonist (D-2-amino-5-phosphonovaleric acid), significantly attenuated or completely blocked the development of long-term potentiation. However, when 7-chlorokynurenic acid was infused together with D-serine (1 mM), long-term potentiation developed that was comparable to that observed in control animals. Intrahippocampal administration of D-serine alone was associated with slightly greater magnitude of long-term potentiation than observed in control animals (Thiels *et al.*, 1992). When published this study was taken as evidence for the importance for glycine in LTP, since D-serine was commonly used as an inert glycine analogue. Now that endogenous brain D-serine is well recognized, these experiments highlight

an important role of D-serine in LTP, which is supported by many recent studies (Yang *et al.*, 2003; Zhang *et al.*, 2008).

More recent explorations of brain D-serine *in vivo* have examined basal concentrations of this amino acid, through microdialysis, to determine the saturation of the modulatory glycine site (Hashimoto *et al.*, 1995; Matsui *et al.*, 1995). SR KO mice also allow the study of D-serine in *in vivo*. Inoue et al (2008) show that NMDA-induced excitotoxic lesions were significantly smaller in SR KO mice (which had 10% of normal D-serine content) than in controls (Inoue *et al.*, 2008).

1.6.5 Humans

D-serine in humans has been studied in association with the neuropathalogical disease schizophrenia which affects approximately 1% of the world's population, with close to 10% of patients committing suicide. NMDA receptor hypofunction (as a result of reduced D-serine levels) is thought to be a contributing factor to the pathophysiology of this disease. Studies have shown NMDA receptor antagonists (phencyclidine and ketamine) induce SZ-like symptoms in healthy volunteers and exacerbate psychosis in SZ patients.

A small study (n=132) found that D-serine (and glycine) is effective in reducing negative symptoms of schizophrenia, but little change is observed in positive symptoms (Tuominen *et al.*, 2005). Bendikov et al investigated the amino acid content of cerebrospinal fluid in SZ patients and normal volunteers (n=12), a 25% decrease in D-serine levels and D/L-serine ratio was observed; in serum also D-serine levels were reduced, although in post mortem CSF from SZ patients, little change was observed (Bendikov *et al.*, 2007). Tsai et al analysed 26 studies with a total of 800 subjects revealing that D-serine (glycine and partial co-agonist sarcosine) significantly improved negative, positive and general psychopathological symptoms, concurring with studies by Heresco-Levy et al (2005), although not in conjunction with clozapine treatment (Tsai *et al.*, 1998; Heresco-Levy *et al.*, 2005; Lane *et al.*, 2005). But Lane et al carried out a 6-week double-blind, placebo-controlled trial of add on treatment with D-serine in 60 patients with chronic schizophrenia, finding little significant difference between this and placebo (Lane *et al.*, 2005).

A number of investigations have used post-mortem tissue to study the significance of D-serine in schizophrenia. Normal DAAO immunoreactivity was abundant in glia, especially Bergmann glia, of the cerebellum, whereas in the cortex, hippocampus and substantia nigra, it is predominantly neuronal (Verrall *et al.*, 2007). This is altered in SZ, higher level of DAO expression was observed in schizophrenic choroid plexus epithelial cells than that in non-schizophrenic cases (Ono *et al.*, 2009). Elevated DAAO protein and mRNA expression was found in the cerebellum of SZ brain, with a two-fold increase in the activity of this enzyme seen the cortex (study group size, n=15) (Kapoor *et al.*, 2006; Madeira *et al.*, 2008).

Steffek et al (used Western blot analysis) find increased expression of astrocytic SR, in the hippocampus of SZ patients but not in the cortex using Western blot analysis (Steffek *et al.*, 2006). Conversely, reduced SR protein expression is seen in the cortex (39%) and hippocampus (21%) but no change was seen in the cerebellum (Bendikov *et al.*, 2007; Verrall *et al.*, 2007).

There are obvious limitations to using humans as test subjects. Where useful information has been found is through post-mortem study of diseased brain. The aim of studies in cell culture, *in vitro* and *in vivo* brain is to ultimately find a means for correcting dysfunctional events in the human brain. In the case of D-serine and NMDA-associated neuropathologies, only in the last two decades has D-serine function in the brain been considered and its innovative use for treatments is praise-worthy.

To summarise, although various models for studying D-serine events in the brain have been employed, the ultimate aim of each researcher has been to study this molecule in an environment closely resembling the human brain. Various acceptable models do exist and these have been employed to gather tremendous knowledge about D-serine signalling events in the brain, which has pioneered treatment of schizophrenia for example.

1.6.6 The future in brain D-serine study: D-serine biosensors

Enzyme based amperometric biosensors are becoming increasingly important analytical devices for use in the central nervous system. They have major advantages over traditional analytical tools including ease of use, fast response time,

exceptionally selective response for an analyte, (thereby removing the need for prior separation by HPLC) and these biosensors are often small in size which minimises tissue damage *in vitro* and *in vivo*. Upon detection of the analyte, an electrochemical signal is recorded at the electrode surface as a change in current at either fixed or varying potentials, allowing transient events occurring in the second to second time frame, to be captured in real-time.

But there are potential problems in using enzyme based sensors. Enzymes removed from their natural environment tend to lose their activity, and thus limit the lifetime of a sensor. Furthermore, enzyme stability is reduced by the harsh environment of the matrices encompassing the enzyme and the very toxic environment of the brain itself. Selectivity issues may also arise; although the enzyme may be specific, many species in the brain are electro-active (for example serotonin and ascorbic acid) and can be oxidized at the electrode surface at low potentials (amperometric sensors are used at +500mV) giving false-positive signals. Continuing research aims to overcome these factors with stabilising matrices and use of screening layers against interferences.

There is an increasing trend towards the development of sensors for a number of brain neurotransmitters and in particular D-serine. Biosensors for D-serine have always used DAAO, as a biological sensing element on a microelectrode probe. The performance criterion for such a device for direct use in tissue both *in vitro* and *in vivo* includes selective detection of D-serine with a high resolution, and with accuracy. The stability is also an important factor, since an experiment may last anywhere between an hour (*in vitro*) to days (*in vivo*). Although, the basic materials for biosensor technology have been available for over a decade, initial advances in making a sensor for D-serine were slow. Some of the earliest D-serine sensors were in fact biosensors of all D-amino acids (Guilbault & Hrabankova, 1971). There was great interest by the food and beverage industry for an easy, cheap and reliable method for detection of D-amino acids, as these indicated the presence of bacterial contamination (Gandolfi et al 1992; Gandalfi et al 1994). This launched interested in fabrication of a D-amino acid biosensors, which forms the basis for current techniques used to make D-serine sensors. Two factors in particular have been central to limiting initial enthusiasms for this tool: the deposition techniques which

are used to immobilise the DAAO enzyme and the source of DAAO, which differ in stability and affinity for D-serine as a substrate.

1.7 D-serine biosensor fabrication

1.7.1 Detection principles

$$\text{D-serine} + H_2O + O_2 \xrightarrow{\text{D-amino acid oxidase}} \text{2-oxo-3-hydroxypropionate} + NH_3 + H_2O_2$$
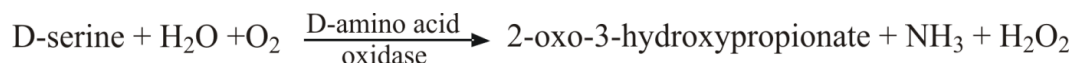
Biosensors formed from DAAO are based on a variety of detection principles. As can be seen from the equation, the deaminiation of an amino acid, in this case D-serine, results in the production of NH_3, and H_2O_2 while O_2 is required for a reaction to occur. Indirect measurements of D-amino acid concentrations and DAAO activity have been based on the change in detection in all three of these factors.

1.7.1.1 H₂O₂

$$H_2O_2 \xrightarrow[+500mV]{\text{Pt.}} O_2 + 2e^-$$
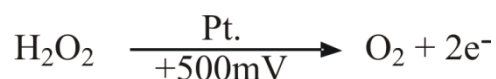
The vast majority of D-serine and D-amino acid biosensors detect H_2O_2, which is a by-product of metabolism of D-amino acids, as indirect measure of activity or amino acid concentration (Mikkelsen & Rechnitz, 1989; Wcislo *et al.*, 2007; Pernot *et al.*, 2008; Zain *et al.*, 2010). The hydrogen peroxide can then be detected amperometrically by a (platinum, gold or carbon) electrode polarized to 500–700 mV relative to an Ag/AgCl reference. O_2 is involved in electron transfer to the electrode surface in the form of H_2O_2. At the surface, peroxide is oxidized to regenerate O_2. Because the oxidation of peroxide requires high potentials, at which many molecules will oxidize such as ascorbate, 5HT, dopamine and urate.

1.7.1.2 Oxygen detection

Inaba et al (2003) combined DAAO with pyruvate oxidase to make a sensor which indirectly measured the fermentation rate, by detecting the amount of oxygen consumed. An oxygen electrode consisting of a platinum cathode, a lead anode, an alkaline electrolyte (KOH) and an oxygen permeable Teflon membrane was used. Since DAAO action on D-alanine releases pyruvate, which is subsequently oxidised

by pyruvate oxidase, D-alanine was taken as an indirect measure of the amount of oxygen consumed in the second enzyme reaction, calculated as a difference in signal between two oxygen electrodes (Inaba *et al.*, 2003).

1.7.1.3 Ammonium ions

A thin layer of DAAO was placed on top of a monovalent cationic electrode, which detected ammonium ions. The probe was found to be a suitable assay for a number of D-amino acids including D-phenylalanine, D-alanine, D-valine, D-methionine, D-leucine, D-norleucine, D-isoleucine and asparagines (Guilbault & Hrabankova, 1971).

1.7.2   Source of D-amino acid oxidase

Hans Krebs noted that D-amino acids could be rapidly deaminated when incubated with fresh slices of rat kidney and liver, while naturally occurring l-isomers were also catalysed. He showed that the factor involved in action on non-naturally occurring amino acids could be extracted from fresh or dry tissue (while the enzyme acting on l-isomers was inhibited by the purification steps). This was the D-amino acid oxidase (Krebs, 1935). A prototype of the oxidase class of flavoproteins, DAAO was found to catalyse the oxidative deamination of non-acidic D-amino acids to their corresponding α-ketoacids. The most preferred substrates of this enzyme include amino acids with small hydrophobic side chains, followed by those bearing polar, aromatic and basic groups (Pollegioni *et al.*, 1992). As a stable homodimer with tightly bound flavin adenine nucleotide (FAD) molecule, this 40kDa protein, can be used as a means to analyse D-amino acids in various body regions. In the brain this enzyme becomes more selective due to the narrow expression of D-amino acids here. To date only one amino acid is shown to be present in significant amounts in brain tissue, upon which the DAAO acts: D-serine. Thus this enzyme can be used to make a biosensor which will selectivity detect D-serine.

DAAO from a number of sources has since been discovered including humans, porcine kidney, *Trigonopsis variabilis, Rhodotorula gracilis, Candida boidinii* and *Fusarium solani*. These enzymes differ in stability, substrate preference and specificity as well as in binding site kinetics. Unfortunately, direct comparisons of the kinetic parameters among these DAAOs is not feasible since published data has

been collected using different techniques and under different experimental conditions. However, only the mammalian source of the enzyme is commercially available, and easy methods for expression and purification of other DAAOs have only in the last 5-10years become available. Some of the earliest protocols for example, for DAAORg purification lasted 4-5 days, with native expression of the protein in yeast, followed by separation with ammonium sulphate, DEAE-sepharose and Mono S columns. As seen with native protein purifications, the yield was poor (Pilone Simonetta *et al.*, 1989a; Pollegioni & Pilone, 1992). This purification step was greatly improved by the purification of cDNA from the yeast, which was inserted into a plasmid to be expressed in *E. coli*. The protein was found to catalytically active and soluble, with much greater yield of protein (Pollegioni *et al.*, 1997). But the greatest advancement came in the form a histidine tag, which could be encoded onto the protein C-terminal, allowing a single-step purification using a nickel column (Molla *et al.*, 1998).

DAAORg has a number of unique qualities, including highly efficient catalysis and tight binding with the coenzyme FAD, that make it more efficient at oxidising D-amino acids (and D-serine) compared to other sources of the enzyme (Pilone Simonetta *et al.*, 1989b; Pollegioni *et al.*, 2002). Unsurprisingly, the turnover numbers (with D-Ala as substrate) determined are highest for DAAORg 345 s-1, compared to DAAOTv and DAAOPk, 52.5 and 12.7 s-1 respectively (Porter *et al.*, 1977; Pollegioni *et al.*, 1992; Tishkov & Khoronenkova, 2005). Furthermore, it is more stable in an immobilised form and can best withstand changes in temperature and pH when compared to enzyme from *T. variabilis* and porcine kidney (Pilone Simonetta *et al.*, 1989b; Pollegioni *et al.*, 2002; Pollegioni *et al.*, 2004). It is believed that the specific presence of a 23-residue C-terminal loop (βF5-βF6), is responsible for correct dimeric formation, that is accounts for higher stability of DAAORg (Pollegioni *et al.*, 2002). A further peculiar feature of the DAAORg structure is the absence of a loop acting as an active site 'lid'. In fact, in the mammalian enzyme, the conformational change of this 'lid' (loop βI5-βI6) allows the substrate/product exchange at the active site: Indeed, the dissociation of the product from the enzyme is the rate-limiting step in catalysis (Porter *et al.*, 1977). In DAAORg, the active site entrance is only partially hindered by the flexible side chain of a tyrosine (Tyr238), resulting in a faster exchange and a more efficient enzyme (see above).

The commercially available DAAOPk was widely used in assays and for fabrications of biosensors, but it showed low stability, and a low turnover number. D-serine biosensors formed from this enzyme were not sensitive enough for used in the brain due to the low detection limits, in the 100μM range; slow response and poor stability (Johansson *et al.*, 1993; Jianzhong *et al.*, 1994). But a surprising paper published recently has utilised this enzyme to make a sensor that is by far the most sophisticated biosensor made using DAAOpk. Sensitivity was reported to be $61\pm 7\mu A$ mM^{-1} cm^{-2}, LOD of 20nM and very fast response (Zain *et al.*, 2010), suggesting that although the enzyme is a contributing factor to the features of a biosensor, other dynamics are also involved. But a biosensor made using the same cross-linking method and DAAORg enzyme shows enhanced features, including a LOD of 16μM (theoretical) and sensitivity of $89\pm 33\mu A$ mM^{-1} cm^{-2} (Pernot *et al.*, 2008).

Conclusively, then, a limiting factor in making a D-serine biosensor sensitive enough for use in the brain has been the availability of a more stable and active form of DAAO. Although, even now, no other source of DAAO is commercially available, the advances in expression and purification technology make other sources of DAAOs more accessible, leading to better D-serine biosensors.

1.7.3   Enzyme entrapment techniques

The early 1990s saw an increase in reports on the development of biosensors using DAAO coupled to electrochemical transducers for the direct, rapid detection of D-amino acids in solution. These were based on a variety of detection methods, enzyme entrapment protocols and assembly materials. The deposition is often responsible for the micro-environment surrounding the enzyme, so it is important for biosensor stability and hence its uses; the assembly material can determine the shape of a biosensor while certain electrochemical method are limited to specific uses.

1.7.3.1 Adsorption and cross-linking

Enzyme layers on an electrode surface can be made by mixing the DAAO enzyme with carrier proteins such as BSA. Exposure to glutaraldehyde causes cross-linking to occur which entraps the enzyme to form a working biosensor. Disadvantages of this technique are that coating is not uniform, and often difficult on small surface as with microelectrodes and glutaraldehyde often diminishes enzyme activity.

Nevertheless the vast majority of D-serine biosensors to date have used this technique (Mikkelsen & Rechnitz, 1989; Pernot *et al.*, 2008; Zain *et al.*, 2010). Jianzhong et al (1994) designed a fibre optic system which selectively detected H_2O_2. HRP was immobilised on bovine albumin matrix with glutaraldehyde. Although there is a long delay in response (5minutes), DAAO was incorporated into this system to detect D-amino acids successfully (Jianzhong *et al.*, 1994). Additionally the enzyme DAAO and LAAO were immobilised with glutaraldehyde on a three-electrode biosensor for the purpose of detecting total L- and D-amino acid concentration in dairy products. This device successfully some D-amino acids, with an LOD of 0.15mM and results compared favourably with a standard photometric amino acid test and was used to monitor milk ageing effects (Sarkar *et al.*, 1999). Improvement to this design were made later, which saw immobilisation of DAAO on a graphite working electrode of a screen-printed strip modified with Prussian blue and nafion layers, with an extra polymer layer for screening against interferences. Cross-linking of DAAO was achieved the same way, using glutaraldehyde /BSA; the LOD however as greatly improved: 1µM, with linear range of 5-200µM for D-alanine (Wcislo *et al.*, 2007). These sensors were useful in monitoring D-amino acid contents of milk, fruit juices and other foods but they did not meet the specifications for use in the brain, to monitor D-serine.

But Pernot et al (2008) and Zain et al (2010) utilise this very technique to fabrication of D-serine biosensors which are sensitive, with fast response times and relatively stable. Some recordings have also been made in the brain, showing increased D-serine levels with intraveneous injections of D-serine (Pernot *et al.*, 2008; Zain *et al.*, 2010). This suggests that glutaraldehyde/BSA cross-linking methods can be utilised to make useable biosensors.

1.7.3.2 Electropolymers

The use of electropolymers for biosensor fabrication has advantages over the above technique in that the formation of a layer can be controlled by the duration and magnitude of the applied voltage. Thin uniform layers can be achieved with self-limiting polymers such as polyphenol and polytyramine. Polypyrrole and polythiophen allow formation of thicker layers, allowing greater amounts of protein to be entrapped. Pernot et al (2008) use poly-m-phenylenediamine for deposition and

as a screening layer, which ensures that electro active species do not interfere with the biosensor response. 87% selective response for D-serine was observed (Pernot *et al.*, 2008).

Zain et al (2010) also use this method to provide a screening layer with poly-ortho-phenylenediamine with subsequent glutaraldehyde/BSA technique for entrapment of DAAOpk (Zain *et al.*, 2010). Although this method can be used in the fabrication of biosensors, to date, D-serine or D-amino acid biosensors have not been made this way. Generally biosensors made this way have poor sensitivity due to the severe chemical environment of electropolymers. Also the polymerisation technique itself may result in free radicals, which can degrade the enzyme.

1.7.3.3 Sol-gel methods

The sol-gel process is a chemical technique for synthesising a silicate matrix around a biomolecule. Ellerby et al (1992) have shown that sol-gels can be used to entrap enzymes in a porous glass layer, the micro-environment within which can be easily controlled (Ellerby *et al.*, 1992). The process of making silicate matrix requires a number of steps including hydrolysis of sol-gels under acidic conditions, with subsequent water and alcohol condensation that forms a 3D matrix. Entrapment of multiple enzymes and co-factors is permitted with the enzyme remaining relatively stable, with full retention of catalytic activity. This process has been fully utilised by Dale et el to fabricate biosensors for number of brain signalling molecules including ATP, Glutamate, Adenosine, Acetylcholine and lactate (Llaudet *et al.*, 2003; Dale *et al.*, 2005; Llaudet *et al.*, 2005; Tian *et al.*, 2009). This method has not been employed for the purpose of D-serine biosensor fabrication so far but has great potential. This project aims to utilise this method for the fabrication of D-serine biosensors encompassing DAAORg.

1.7.4   Assembly material
1.7.4.1 Carbon/graphite based biosensors

Carbon paste was often preferred over ordinary solid electrodes, as it allowed easy modification of the entire bulk of material, detection surface can be renewed with sanding and many options are available as to the required shape or size. This is also a cheap material with relatively good conductivity for amperometric biosensors. Of the

earliest attempts to immobilise DAAO with HRP in carbon paste showed restricted sensitivity and stability (Kacanikil et al 1993, 1994), slight improvements were made by Johansson et al, who showed made a sensor with the detection limit of 5µM for hydrogen peroxide (Johansson *et al.*, 1993). This sensor was active for a number of D-amino acids including D-serine.

But for a D-serine biosensor for use in the brain, an assembly small in size, perhaps with a pin tip is required, which minimises damage with insertion into tissue. Carbon/graphite based sensors are disadvantaged in that they do not allow this. However, for large electrodes, for use in D-amino acid detection for example, these may be more useful.

1.7.4.2 Platinum biosensors

Platinum microelectrodes offer the most conductive surface for biosensors and can be shaped to form electrodes with small diameters and length. For example Pernot et al use a microelectrode that is 25micron in diameter and 125µm in length (Pernot *et al.*, 2008). Certainly for the purpose of use in the brain, this is the most suitable material, although it can be costly.

1.8 Aims

- The aim of this study is to design a D-serine biosensor and characterise its performance against current devices.
- To use D-serine biosensors in *vitro* and *in vivo* brain models to investigate extracellular D-serine levels in regions of the brain to determine NMDA receptor co-agonist site occupancy.
- D-serine biosensors will be used to analyse activity dependent changes in extracellular D-serine levels in the rat brain as a result of:
    - Ionotropic glutamate receptor activity
    - Glial-specific activation
    - High frequency stimulation
- D-serine biosensors will be used to assess the role of D-serine in NMDA-receptor mediated cell damage during hypoxia and ischemia, as models of stroke.

Chapter 2: Fabrication and characterization of D-serine biosensors

2.1 Abstract

The amino acid D-serine is vital in the central nervous system because of its role as a co-agonist at the NMDA (N-methyl D-aspartate) receptor. This signalling molecule has been linked to many physiological functions in the brain as well as pathological states including Alzheimer's and Schizophrenia. The study of D-serine is a specific way to examine NMDA receptor function in the brain, indirectly. But current tools to sense this amino acid are limited by high running costs, excessive consumption of samples and the time-consuming nature of the systems.

Biosensors for D-serine offer an alternative approach. Highly sensitive and selective real-time measurements of D-serine can be made in the second-to-second time frame in intact brain tissue. We have designed and made D-serine biosensors using a unique sol-gel electrochemical deposition method, entrapping D-amino acid oxidase (from *Rhodotorula gracilis)* within a porous biolayer on a platinum surface. These micoelectrodes have a sensitivity of $200\pm 15\mu A$ mM^{-1} cm^{-2} and a lower detection limit of 25nM, with a working stability of 40 days. Response upon D-serine detection is within seconds and use of a poly-1, 3 phenylenediamine screen ensures over 90% selective response for D-serine, which is improved further with the use of a null sensor. This novel tool provides an exciting way to study the functional role of D-serine in the CNS.

2.2 Introduction

Significant levels of D-serine were localised to the brain in the 1990s showing D-serine distribution paralleled that of NMDA receptors (Hashimoto *et al.*, 1993a; Schell *et al.*, 1995; Schell *et al.*, 1997). Over the next decade the role of D-serine as a co-agonist of this channel came to be recognised and dysfunctional D-serine signalling is associated with a number of neuropathies including schizophrenia and Alzheimer's (Krystal & D'Souza, 1998; Wu *et al.*, 2004). However, the tools for studying this amino-acid remain limited, with antibody staining being the main technique used for determining D-serine distribution in the brain and HPLC and capillary based assay systems used to measure the concentrations of this signalling molecule (Ciriacks & Bowser, 2004; O'Brien & Bowser, 2006). Biosensors for D-serine combine both of these features; offering real-time output combined with good spatial and temporal resolution, without the draw backs of complex procedures and high running cost of traditional methods. Continuous real-time measurements of D-serine can be made in the second-by-second time frame which has the potential to revolutionise the way in which D-serine signalling is studied.

D-serine microelectrode biosensors are made using the enzyme D-amino acid oxidase (DAAO), which oxidises D-serine to form hydroxypyruvate, and by-products H_2O_2 and NH_3, as shown the scheme below. DAAO activity is ubiquitous in microorganisms where it is involved in catabolic utilisation of exogenous D-amino acids for growth and detoxification. But it is now thought to be present in many species including humans where it is localised to kidney, liver and brain (Neims *et al.*, 1966; Kawazoe *et al.*, 2007).

$$\text{D-serine} + H_2O + O_2 \xrightarrow{\text{D-amino acid oxidase}} \text{2-oxo-3-hydroxypropionate} + NH_3 + H_2O_2$$
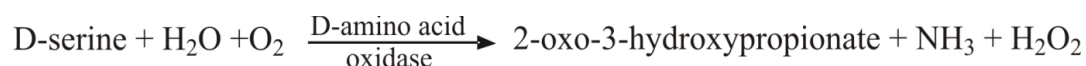
Scheme 1: D-amino acid oxidase reaction scheme with D-serine as a substrate

Enzyme biosensor systems may suffer from limited operational lifetimes. Both the properties of the enzyme and method of biosensor fabrication influence the operational stability. In this study DAAO from the yeast *R. gracilis* (DAAORg) was selected due to highly efficient catalysis rate and tight binding of the FAD cofactor, which confers stability and faster turnover rate compared to other sources of DAAO (Pilone Simonetta *et al.*, 1989b; Pollegioni *et al.*, 2002). Furthermore, DAAORg is

more stable in an immobilised form and can best withstand changes in temperature and pH when compared to enzyme from *T. variabilis* and porcine kidney (Pilone Simonetta *et al.*, 1989b; Pollegioni *et al.*, 2002; Pollegioni *et al.*, 2004). However, DAAORg is not commercially available but a simple expression and purification protocol is available that gives high enzyme yield (Pollegioni *et al.*, 1997). For D-serine biosensor fabrication the sol-gel electrochemical deposition technique was employed; this is a highly favourable method for forming stable enzyme biolayers (Dale *et al.*, 2005; Llaudet *et al.*, 2005). It is the H_2O_2 generated in the enzyme reaction (scheme 1) which can be detected electrochemically through oxidisation of the platinum microelectrode. The potentiostatic detection of enzymatically generated H_2O_2 thus acts as a measure of D-serine. The microelectrode biosensor gives a quantifiable measurement of D-serine in different brain regions, allowing temporal and spatial properties of this signalling molecule to be established.

2.3 Methods

2.3.1   Synthetic Gene expression and DAAO purification

The pET system provides a very powerful technique for the cloning and expression of recombinant enzymes in *E. coli* and was selected because of its ease of use, versatility and rapid expression. The DAAORg gene was ordered from Genscript in a pET28 vector conferring kanamycin resistance and a 6 x histidine tail. The cDNA codon sequence was optimised for expression in bacteria but the amino acid sequence remained unchanged. The optimisation process ensured that protein synthesis was not limited by rare amino acid codons, which may not exist in large amounts in bacteria but may be abundant in the native yeast.

The DAAO*R*g vector insert and subsequent protein synthesis provided an uncomplicated means of purifying this enzyme which was commercially unavailable. Temperature and growth conditions were optimised to yield high expression of protein and purification of DAAO*Rg* was facilitated by the presence of a histidine tag that was utilised by way of affinity chromatography purification technology using a 5ml nickel-column.

The DAAO*Rg*/pET 28 construct was used to transform BL21 (DE3) Rosetta cells which were cultured overnight in Luria broth medium (1L contained 10g bacto-

tryptone, 5g yeast extract and 10g NaCl) at 37ºC supplemented with 50mg ml-1 kanamycin, and 35mg ml-1 chloroamphenicol to confer selectivity for cells containing the DAAORg plasmid insert (Bertani, 1951). Cells were cultured for 8hours before induction with 1mM isopropylthiogalactoside (IPTG), in order to begin protein synthesis overnight at 293 K at 30°C; prior to harvesting the cells by centrifugation at 6000g for 20min. The cell pellet was then re-suspended in 50mM Tris/HCl pH 8.0, 2µM FAD, 2 µM pepstatin, 0.2mM PMSF and 5% glycerol before sonication and clarification of the extract by centrifugation at 50 000g for 45min.

The clarified extract was applied to a 5 ml HisTrap Hp column (using a GE Pharmacia AKTA purifier), pre-equilibrated with 50mM Tris/HCl pH 8.0, 0.3M NaCl, 5% glycerol, 2.5mM imidazole (buffer A). Plots of A254nm against volume provide a useful elution profile for a column, since nearly all proteins absorb light at wavelength because of their content of the aromatic amino acids tryptophan and tyrosine. The column was washed again extensively with buffer A, to remove weakly bound protein and bound-enzyme was eluted with a linear increasing gradient of imidazole from 2.5 to 50mM (Buffer B). Under these conditions, the DAAORg protein bound with strong affinity and this single step yielded only one peak (Marked X, on figure 1) implying purification of a single protein. The Ni-column is loaded with clarified extract (mixture of all native *E. coli* proteins and DAAORg) and non-histidine-tagged proteins are washed away. Only bound protein with a strong affinity for the column (i.e. a 6 histidine-tag) remains on the column until the linkage is broken by the presence of imidazole.
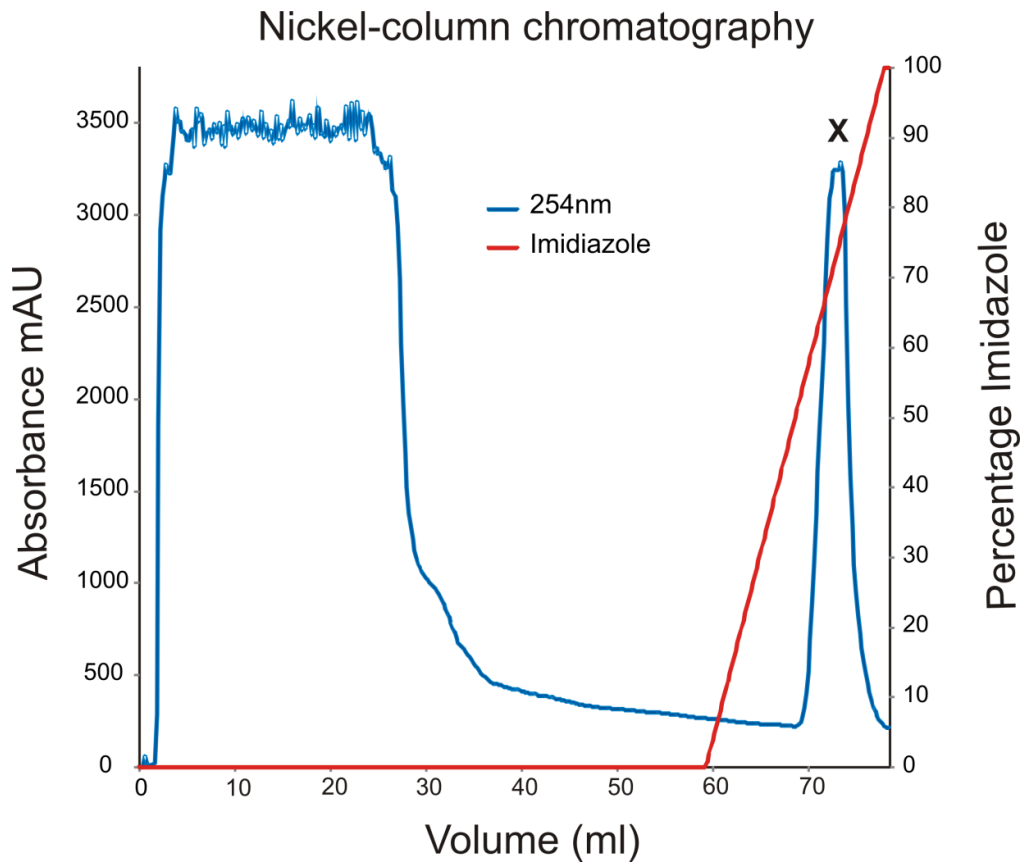
Figure 1: Affinity chromatography trace of DAAORg. Protein is loaded onto the column, a rise in wavelength 254nm is observed (blue line, only histidine tagged protein remains bound and DAAORg is eluted (X) with increasing imidazole concentration (red line) up to 200mM, with protein eluted at 60-70%.

A SDS-gel confirmed the presence of a 40kDA protein eluted from the nickel-column with imidazole as shown in figure 2 (although DAAO*Rg* is known to exist as a dimer in the native form, only a monomer is detected by the SDS-gel).
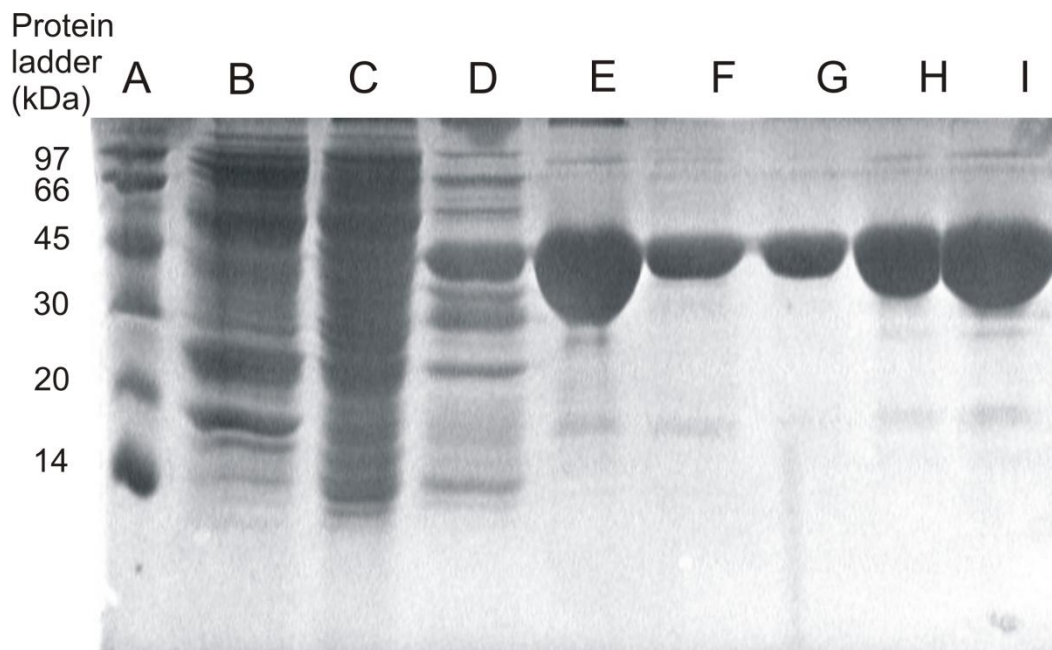
Figure 2: SDS gel of proteins eluted by Nickel-chromatography. SDS-gel of DAAORg purified using a 5ml Nickel column; lanes A-C show unbound protein being washed off before the imidazole elution step; lanes E-I (Peak marked X in figure 1) indicate relatively pure DAAORg enzyme eluted upon application of 200mM imidazole.

2.3.2   Determination of protein concentration

In order to determine the concentration of the protein purified, using nickel-column chromatography, a BioRad assay system was used, 10µl of protein solution was added to 790µl H_2O and 200µl BioRad reagent in a cuvette, mixed and the absorbance measured at 595nm using Pharmacia Biotech UltraSpec 2000. For protein solutions that gave a reading outside of the linear range of the assay (above 0.6 at 595nm), solutions were diluted and the reading repeated. Protein concentration was calculated to be 114mg mL^{-1} with an extinction coefficient assumed to be 1.0 cm^{-1} mg^{-1} mL.

2.3.3   Enzymatic assay

To further confirm the presence of D-amino acid oxidase, an enzyme assay was devised; DAAO*Rg* is able to oxidise D-serine into hydroxypropionate with by products, ammonia and H_2O_2, as shown in the scheme 2 below. In the presence of horseradish peroxidise, H_2O_2 reacts with amplex red in a 1:1 stoichiometry to produce the red-fluorescent oxidation compound resorufin which can be detected at wavelength 555nm (Zhou et al 1997).
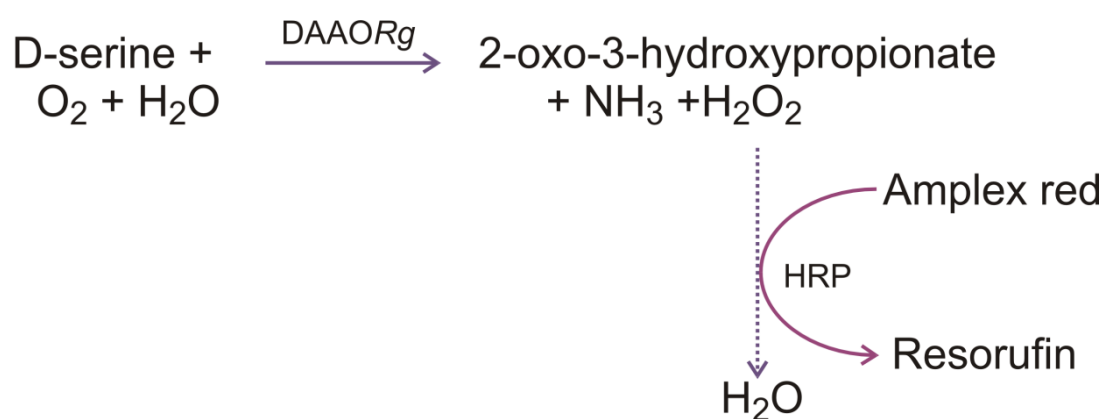
Scheme 2: Enzymatic reaction scheme to determine DAAORg activity

The spectrometer trace of figure 3 shows an increase in absorbance upon D-serine detection (an indirect measure of D-serine oxidised by DAAORg and H_2O_2 production) confirming the successful expression and purification of DAAO*Rg*. This change in absorbance can be converted into μMols/min of enzyme activity (or units) with resorufin extinction coefficient of 54000 M^{-1}. With further concentration by centrifugation, the total activity per mL was calculated as 800 μMols/min or 800 units, with a specific activity of 7units/mg. Specific activity was determined by dividing the total enzyme activity (800μMols/min) by the total amount of protein (114mg/mL).
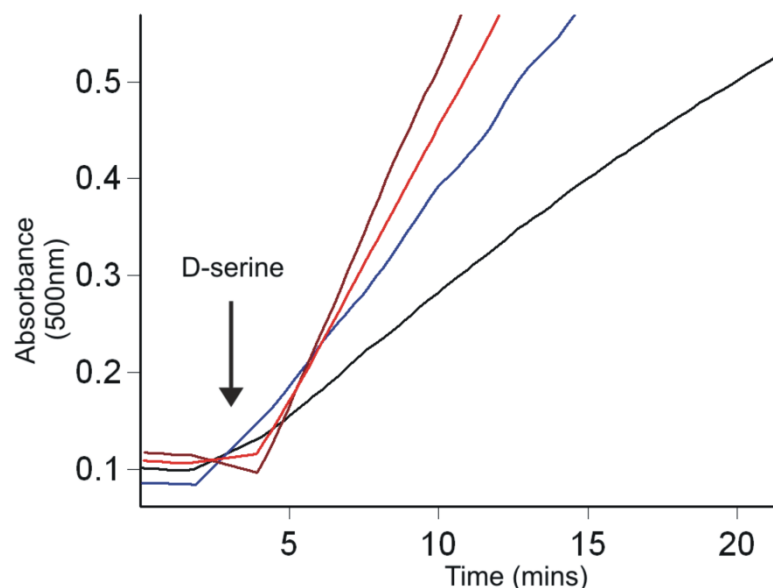
Figure 3: Assay to determine DAAORg enzyme activity. Change in absorbance at 555nm wavelength is used to determine enzyme activity (scheme 2). The rate at which DAAORg oxidises D-serine is determined with the addition of 0.2mM (black); 10mM (blue); 15mM (red); and 30mM (purple) D-serine. It is the formation of resorufin that is observed as an increase in absorbance in this trace.

2.3.4 Protein storage

DAAORg protein was stored at -20°C and stability was analysed using the amplex red assay for activity (mentioned in section 2.3.3). Protein was stored in 50mM Tris/HCl pH 7.5, 50mM lactitol, 10% glycerol, 5mM EDTA and 5μM FAD. The presence of exogenous FAD is known to protect against enzyme inactivation (Pollegioni *et al.*, 2004). Aliquots were made and stored -20°C, and regularly tested for activity using the assay mentioned above. The enzyme is stable under these conditions for over 10months and can also be freeze-dried and stored at -20°C for up to 2 month without enzyme activity being affected.

2.3.5 D-serine Biosensor fabrication

The chemical technique used to make D-serine biosensors involves the electrochemical deposition of sol-gels and enzyme onto a platinum electrode, first described by Dale *et a*l is an unsurpassed method for enzyme-biosensor fabrication. Using this method DAAO*Rg* was entrapped onto a conductive surface to form a robust and porous biolayer around a platinum microelectrode of varying sizes, further detailed in table 2.1. This variation in size is useful in experiments requiring different biosensor properties, for example in the case of *in vivo* and *in vitro* studies,

the biosensor of choice is likely to be 0.5mm to minimise damage of the brain upon insertion and background noise; whereas in cultured cell experiments the greater the surface, the more cells the biosensor will be in contact with, therefore more cells will be sampled with a 2mm biosensor.

	Assembly 1	Assembly 2	Assembly 3
Surface area	3.97×10^{-4} cm^2	8.05×10^{-4} cm^2	3.22×10^{-3} cm^2
Diameter	25µm	50µm	50µm
Length	0.5mm	0.5mm	2mm

Table 2.1: D-serine biosensors are available in various sizes. The surface area of a microelectrode assembly represents the area able to detect D-serine, allowing specific design of sensors for particular use.

Before the enzyme biolayer was deposited, the platinum surface was treated with 1,3 phenylenediamine by cycling the Pt. between +200mV and 800mV in the solution, as previously detailed (Llaudet *et al.*, 2005). This methodology was found to be successful in minimising background current and also acts as a barrier against electro-active species making contact with the platinum surface directly, thus improving selectivity. These include serotonin and ascorbic acid which can be oxidised at the platinum surface to give false-positive results (as shown in figure 4). Hydrogen peroxide, the agent detected at the platinum surface which acts as an indirect measure of D-serine presence, is still able to pass through the screening layer to be oxidised at the platinum.
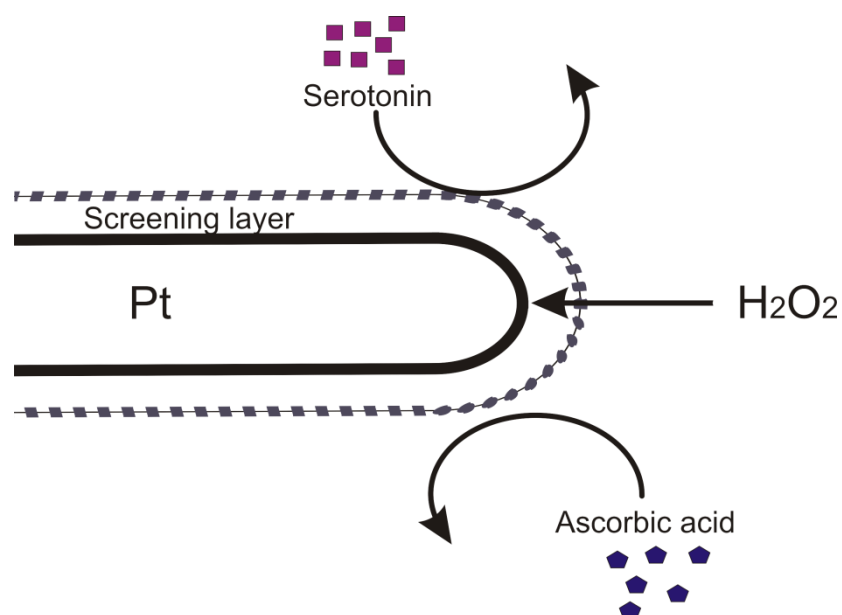
Figure 4: Screening of platinum surface to reduce interference. The platinum sensor is coated with poly-1, 3-phenylenediamine, screening against electro-active species such as serotonin and ascorbic acid but hydrogen peroxide is still able to pass through the screening layer to be oxidised at the platinum surface.

An enzyme biolayer is then deposited, the composition and properties of the enzyme-mix used were altered to maximise the retention of enzymatic activity within the potentially harmful environment in the layer, by altering ionic properties and the hydrophobicity of the final matrix and incorporation of stabilisers (Llaudet *et al.*, 2005). Many different compositions within the sol-gel biolayer were tried and tested before a stable environment within the matrix for DAAO*Rg* enzyme was determined. In brief, a number of hydrolysed silanes were combined with 50mM Tris/HCl buffer pH 7.4, 5M glycerol, 5M thioglycerol, 0.5M NaCl and 100% PEG 400; 10µl of this was dissolved with 7 units of previously purified DAAO*Rg* to form an enzyme-sol-gel mix, which was used to fill a small 1cm glass capillary (Llaudet et al. 2003). A pre-treated sensor assembly (coated with poly-1,3 phenylenediamine as detailed above) was introduced into one end of the capillary and an Ag/AgCl reference electrode was inserted into the other end, as shown in figure 5. A current of -1.3mV was applied for 30s using a potentiostat (model AEW-2) from Sycopel Scientific for electrochemical deposition onto the platinum wire of the biosensor assembly. The electrode was removed and stored in 50mM PBS and 5µM FAD, overnight and a second coating with poly-1, 3-phenylenediamine was applied to maximise screening against possible interference.
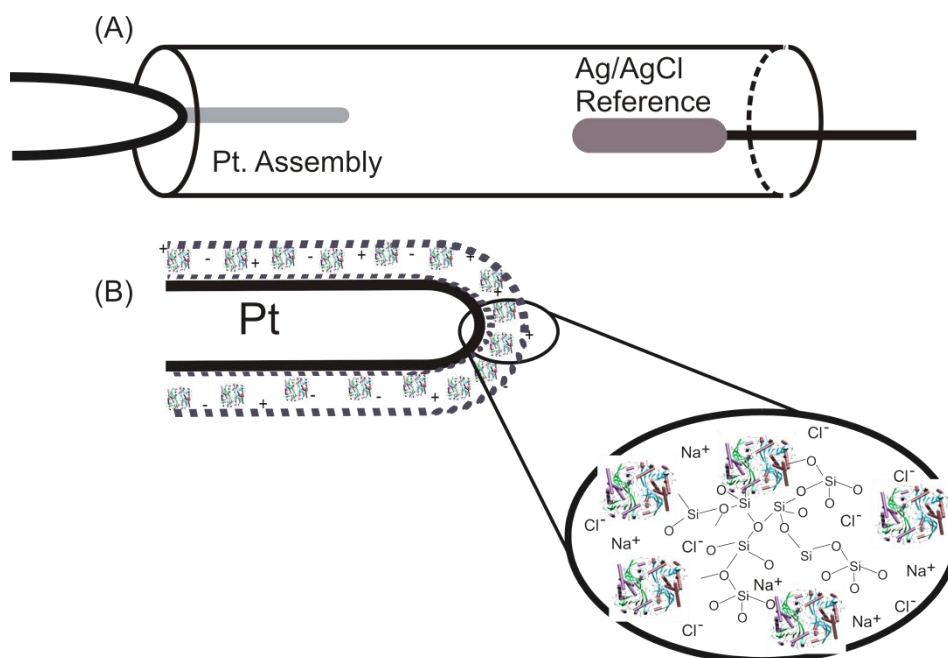
Figure 5: Electrochemical deposition of DAAORg on a platinum microelectrode assembly. A D-serine biosensor a reference and platinum assembly are introduced into a glass capillary filled with enzyme and sol-gel mix before an electrical current is applied to deposit a sol-gel/enzyme biolayer onto the Pt. surface (A). Enlarged depiction of DAAORg enzyme entrapped within a sol-gel matrix (B).

The sensors were operated at +500mV relative to a Ag/AgCl electrode to detect oxidation of H_2O_2 on the electrode surface but at such a high potential a number of other species is also oxidised. Biosensors were tested in 20µM serotonin and 100µm ascorbic acid to assess the selectivity of the sensor.

2.4 Results

D-amino acid oxidase is able to utilise a variety of D-amino acids (D-aa), except acidic D-amino acids. For many DAAO enzymes, the non-polar amino acids are the preferred substrates followed by polar and basic amino acids. Table 2.2 below gives the affinity for D-serine as a substrate of a variety of DAAO enzymes, though conditions used for determining the Km vary slightly making direct comparison difficult. Following the expression and purification of DAAORg, I measured the Michaelis-Menten kinetics of this enzyme. As shown by figure 6, the Km determined is 4.17mM, at pH 8.0, 25°C using a Lineweaver-Burke plot, a contrast from previous data, which may be due to a different assay conditions used.

R. gracilis	**Km = 13.7mM (Boselli *et al.*, 2002)**
Sus Scrofa	Km = 12.7 (Bakke *et al.*, 2006)
Trigonopsis varaibillis	Km = 25mM (Kubicek-Pranz & Rohr, 1985)
Candida boidini	Km = 33.7mM (Yurimoto *et al.*, 2001)

Table 2.2: Km of DAAO enzyme for D-serine substrate from various sources
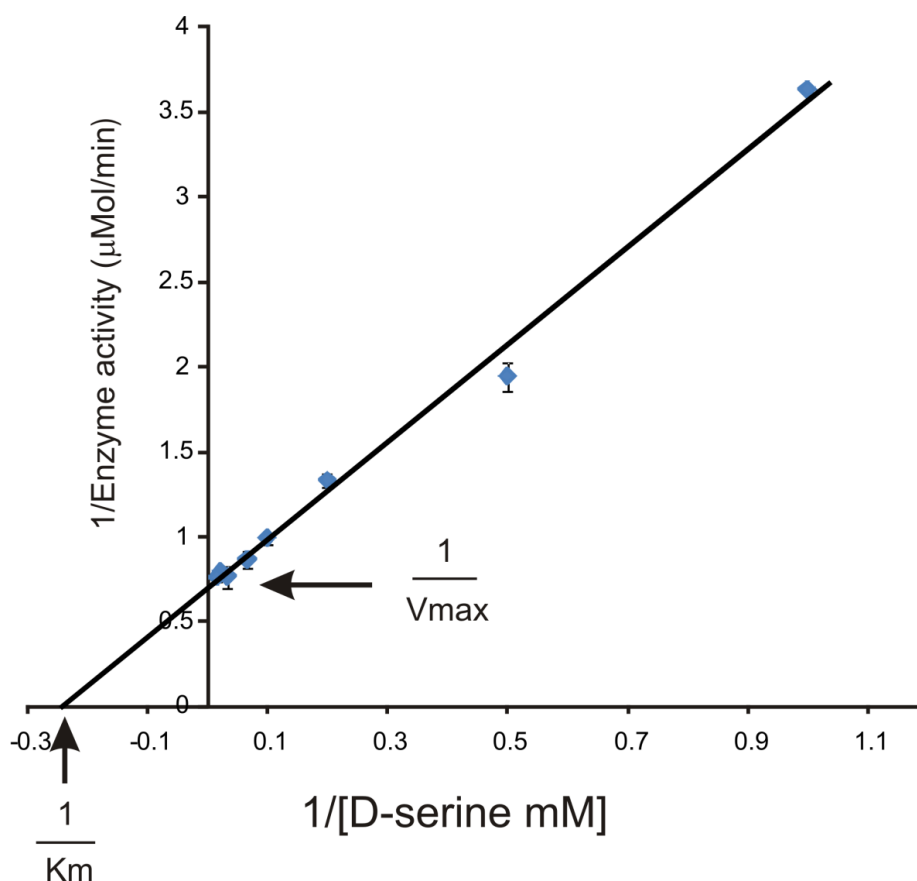
Figure 6: Enzymatic rate and kinetics of free-DAAORg. The K_m is determined as 4.17mM from a Lineweaver-Burke plot and Vmax is achieved at 1.45µMol/mM/minute.

The Km of an enzyme will have influence on the sensitivity of the biosensor, in this case, the lowered Km is likely to be useful for the fabrication of a more sensitive biosensors but a very low Km can also mean that the linear response range is significantly reduced. In this case, the lower Km, has not affected the linear response of the biosensor within the required range of use, sensors are linear up to above the expected physiological D-serine levels in the brain.

2.4.1 Sensitivity

The principal challenge in designing D-serine biosensors for *in vitro* and *in vivo* study of D-serine signalling is adequate performance in sensitivity, speed of response and limit of detection. It is essential that the biosensors are able to detect very minute amounts of D-serine, quickly in the brain, in a linear manner. The sensitivity of the 0.5mm (with 50μm diameter) biosensors to D-serine was $200\pm15\mu A \ mM^{-1} \ cm^{-2}$ (n=9). They responded rapidly, within seconds of detecting the amino acid in solution, as shown in figure 7 which shows the response curve of a 0.5mm biosensor, with a diameter of 50μm.
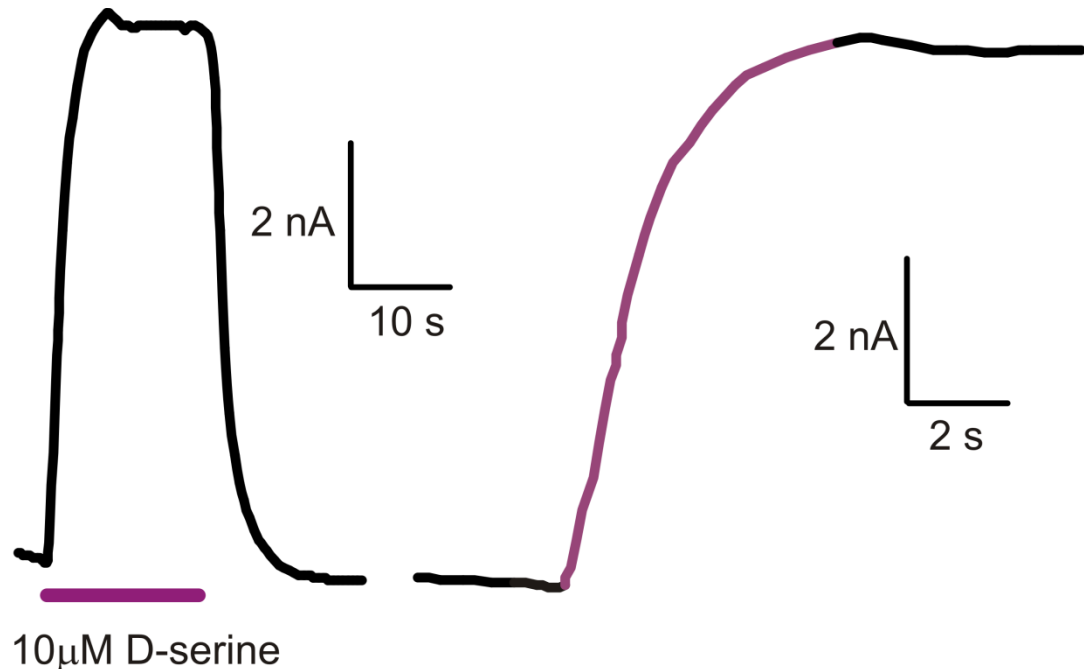
Figure 7: Response curves of a 0.5mm D-serine biosensor to 10μM D-serine. In a simple flowing system, the response of the biosensor was recorded to injection of 10μM D-serine. A 10-90% response rise-time was estimated to <3s and the sensitivity of $200\pm15\mu A \ mM^{-1} \ cm^{-2}$ when normalised to the surface area.

The limit of detection (LOD) and linear range of a biosensor are a function of entrapment process and the recognition element (in this case DAAO). It is essential that the response is linear; this was tested from 0.2µM to 200µM D-serine concentrations and was linear; only concentrations from 0.5 to 10µM (n=3) are shown in figure 8. The size of the biosensor does not impact the sensitivity as this is normalised to surface area, to allow comparison of data obtained from different biosensors.
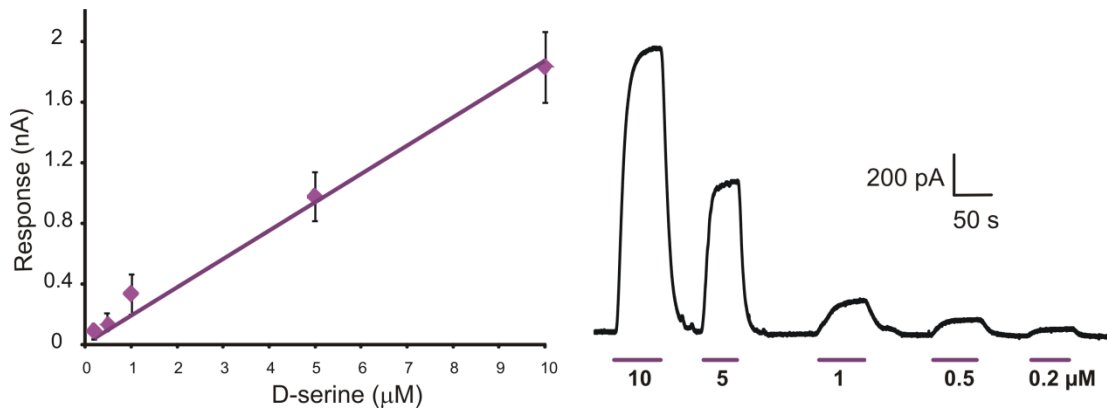
Figure 8: Linear response of a 0.5mm D-serine biosensor to expected physiological concentrations of D-serine. The response of D-serine biosensors is linear from 0.2nM to 10µM, which is within the physiological range expected in the brain; further studies indicate that the response is linear up to 200µM, though these levels of D-serine are unlikely to be in the brain under a physiological state.

The LOD of the D-serine biosensors is 25nM (figure 9), although the levels of D-serine in the brain are expected to exceed this significantly. This is a feature of the stable environment in which the enzyme is entrapped within the bilayer, achieved by sol-gel deposition.
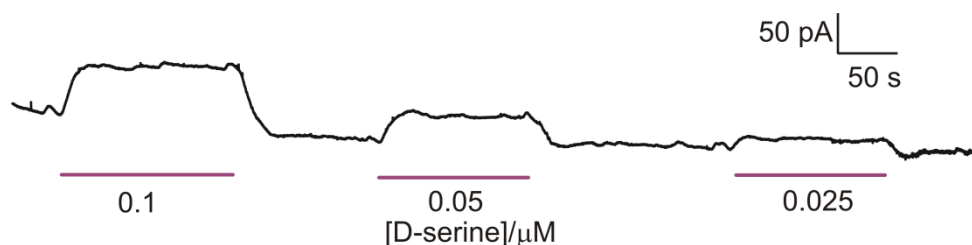
Figure 9: Limits of detection for D-serine biosensors. D-serine biosensors were tested against 0.1µM, 0.05 and 0.025µM D-serine, the sensors are highly sensitive and when used in the brain they will be able to detect even very minute amounts of D-serine allowing a more detailed study of this chemical transmitter in the brain.

Although O_2 is necessary in the enzymatic scheme, there appears to be little need for a continuous supply of this for oxidation of D-serine, as demonstrated by figure 10. The response of the sensor to D-serine was tested at physiological buffer saturated with 95% O_2 / 5% CO_2 before switching to a buffer saturated with 95% N_2 / 5% CO_2. For 4 sensors tested, the mean reduction observed in the current was 4.5 ± 1.0% of the total response to D-serine. The reasons for this apparent insensitivity may be that oxidation of the peroxide on the Pt surface will regenerate O_2, which can then be utilised by the enzymes within the thin sensing layer.
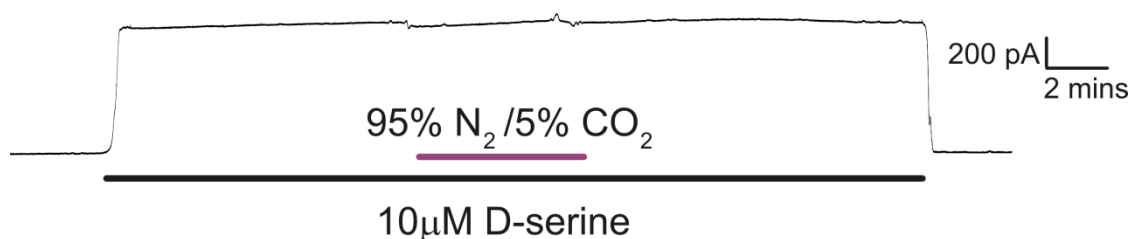
Figure 10: oxygen sensivity of D-serine biosensors. A 10µM solution of D-serine was applied in physiological buffer solution saturated with 95%O_2 / 5%CO_2 (black bar), before being switched to D-serine in solutions saturated with 95% N_2 / 5% CO_2 (purple bar) and back again. Only a very small reduction in sensor current is observed.

Additionally, in order to compare the Km of free-DAAORg and that entrapped within a sol-gel bilayer kinetics data was gathered. The Km of free DAAORg (4.17mM) has been lowered (to 2.67mM) by the entrapment of the enzyme; suggesting that the protein is in a stable environment within the biolayer (figure 11). This could be as a result of a permanent conformational change, due to the fabrication process; or a steric effect that orientates the enzyme in such a way that D-serine binding to DAAORg is favoured.
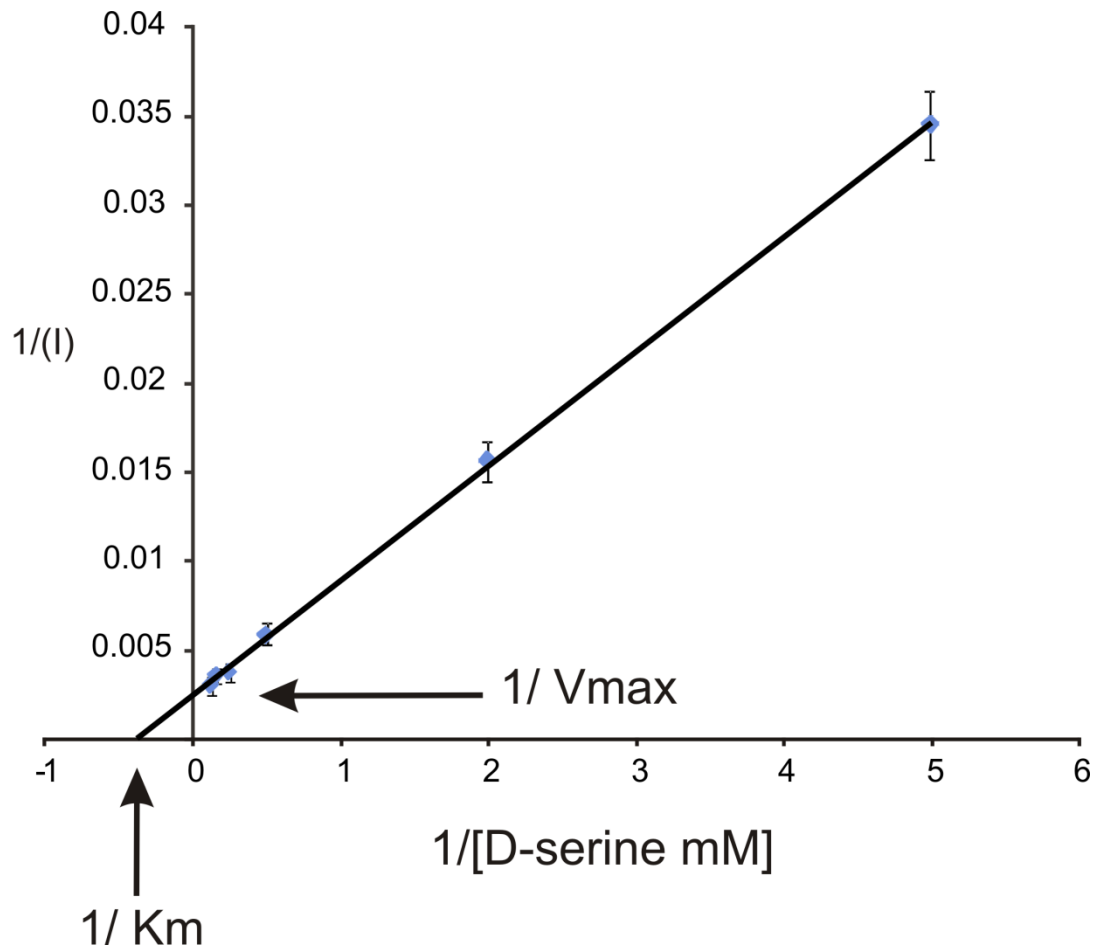
Figure 11: Sensivity of D-serine biosensors in a bilayer on Pt. The K_m is 2.67mM and Vmax is achieved at 417nA/mM min^{-1}.

2.4.2 <u>pH</u>

The optimum pH for the enzymes DAAO from *Rhodotorula gracilis* is between 8.0 and 9.0. The physiological pH that the biosensors will be tested and used at is pH 7.4± 0.2, any considerable deviance from this likely to alter D-serine response with pH. Below neutral pH the response of the biosensors is reduced considerably, as observed in figure 12. Here the biosensors were tested between pH 6.4 and pH 8.0, around physiological pH, small excursions of pH will not markedly alter the sensitivity of the biosensor. Additionally the Pt surface was shown by Llaudet et al (2005) to be sensitive to changes in pH, therefore a null was used to eliminate any changes observed in current by subtracting the null biosensor response from the D-serine signal (Llaudet *et al.*, 2005).
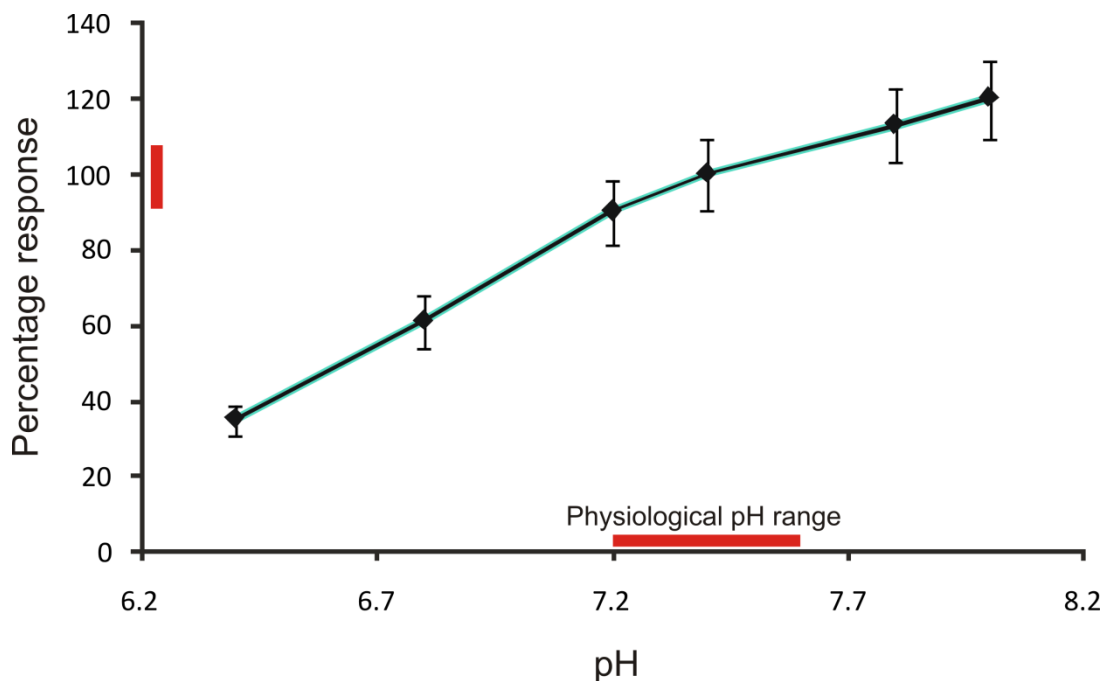
Figure 12: Response of D-serine biosensors to varying pH levels. The response of D-serine biosensors was analysed at varying pH levels between pH 6.2 to pH 8.0. The biosensor response is not altered very much by physiological changes in pH (pH7.2±0.2) shown by red bar on the x-axis, with 90% response at pH 7.2 and 106% response at pH 7.6, shown by the red bar on the y-axis.

2.4.3   Selectivity

The selectivity of a biosensor is vital for its function. Both *in vivo* and *in vitro* experiments rely on the biosensor only detecting the required analyte; therefore the biosensors are 'screened' against a number of potential interferences. H_2O_2 is oxidised at the platinum electrode to give an indirect measure of the presence of D-serine but a high-applied potential (+500mV) is required, this creates potential problems as number of other electro-active species can also be oxidised and thus give false-positive results. This is a universal challenge of electrochemical biosensors but various screening methods have been devised to sufficiently screen against artefacts. One method is to utilize a selective screening layer that allows permeation of H_2O_2, but not larger interfering molecules. Polyphenol, polyresoucinol and poly-1, 3-phenylenediamine have all been previously used. It is the latter we find to be the most effective at screening out interference.

A poly-1, 3-phenylenediamine screen can selectively reduce interference against agents that directly make contact with the platinum wire: that is ascorbic acid (AA,

100µM tested); serotonin (5HT, 20µM tested) as well as L-serine, glycine and D-aspartate which are amino acids present in the brain that may interact with the DAAO enzyme; 10µM solutions of these amino acids were made and tested, as shown in figure 13. The highest likely concentrations of interferences present in the brain were tested. Table 2.3 shows relative response of interferences normalised against the D-serine response.
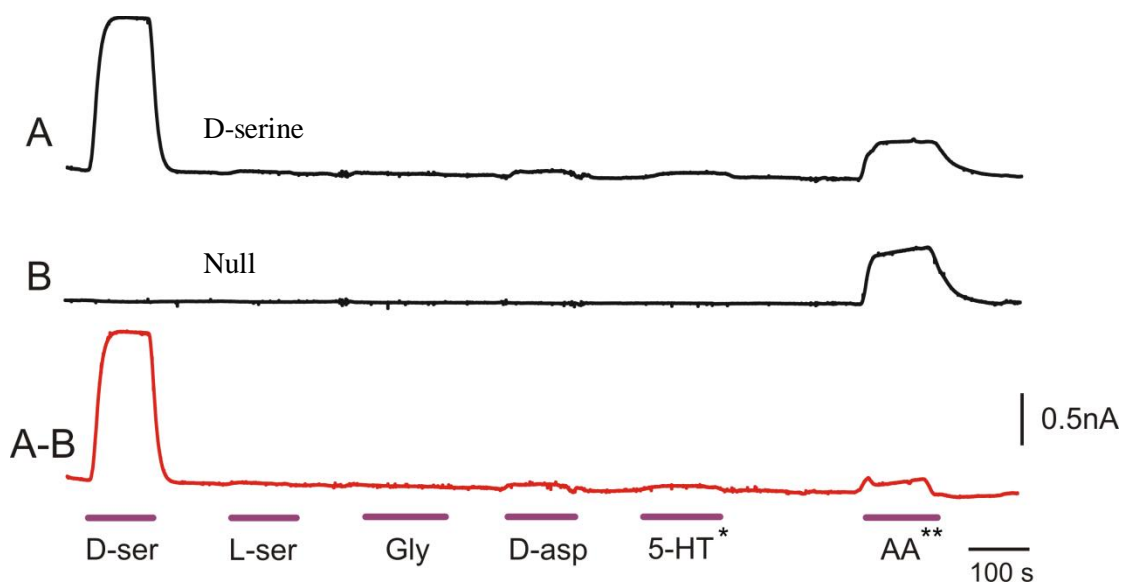
Figure 13: D-serine biosensor selectivity against potential interference The D-serine biosensor (a) is used alongside a null sensor (b) which were tested against 10µM D/L-serine (L-ser), glycine (Gly), D-aspartate (D-asp); 20µM serotonin (5HT*) is also screened against well by both the null and D-serine biosensor while 100µM ascorbic acid (AA**) does give a signal, which is minimised upon subtraction of the null trace from the D-serine signal. By using a null sensor virtually all non-enzymatic signals are removed from a trace.

	Response (nA)	Percentage response
10µM D-serine	1.48± 0.10	100%
10µM L-serine	0.01± 0.00	0.5%
10µM Glycine	0.02± 0.00	1.2%
10µM D-aspartate	0.03± 0.00	1.8%
100µM Ascorbic acid	0.11± 0.01	7.2%
20µM Serotonin/5HT	0.03± 0.00	2.0%

Table 2.3: Selective response of D-serine biosensors to a number of interferences at the predicted levels found in brain (n=7).

We found that re-applying a DAB layer onto the biosensor following dry storage improves selectivity and this significantly prolonged the working-life of the biosensors.

2.4.4   Stability

Dried biosensors retain over 85% sensitivity for up to 7 weeks, although, sensors can be used for longer depending on the experimental procedure and required sensitivity (Figure 14). Wet storage has been found to be as adequate; with biosensors retaining full sensitivity for 10days.
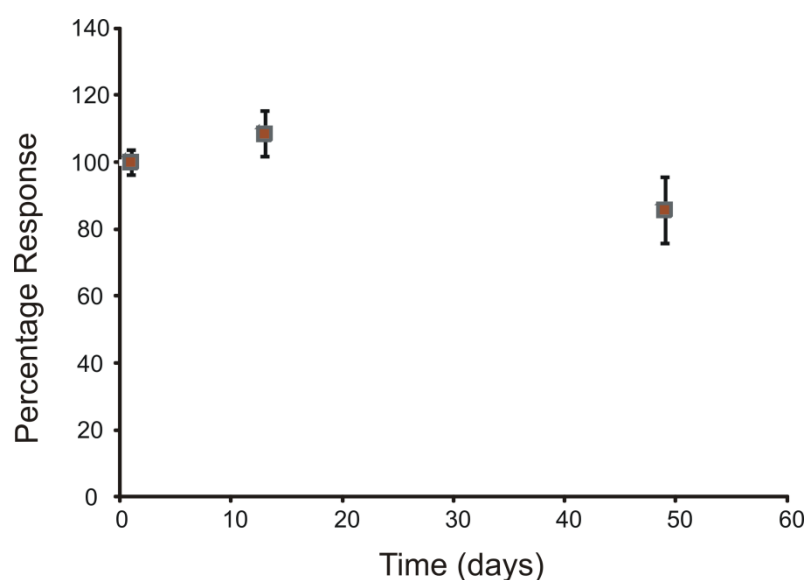
Figure 14: D-serine biosensor stability. The D-serine biosensor is stable for over 50days, initial response was taken before the sensors were dried, a procedure which appears to stabilise the response (n=4).

2.5 Discussions

Although, the first biosensors were made nearly 50 years ago initial advances in making a sensor for D-serine were slow. The major hindrances to date have been the source of the DAAO enzyme, entrapment techniques and the microelectrode assembly materials. Here, we have combined a highly favourable enzyme entrapment technique (sol-gel), with the most stable and sensitive DAAO enzyme (DAAORg) to make Pt. microelectrode sensors. This has resulted in D-serine biosensors which are far superior to other similarly designed D-serine biosensors. In fact two recently published findings conducted in parallel to this study give point of

comparison (Pernot *et al.*, 2008; Zain *et al.*, 2010). Pernot et al (2008), use DAAORg with elctropolymer/ glutaldehyde entrapment technique to make D-serine biosensor, while Zain et al (2010) use a porcine kidney DAAO (DAAOPk) enzyme with the same method of deposition. Both sensors were made on Pt. wire and only differ in source of enzyme and/or entrapment technique employed. Comparison of the sensitivity, limit of detection and stability of these biosensors with our sensor allows assessment of the fabrication method and the enzymes utilised.

The sensitivity of a biosensor is an essential feature and closely associated with this is the limit of detection. Two comparisons can be made here, first a comparison of the source of DAAO (DAAORg vs. DAAOPk) and secondly the enzyme entrapment techniques (sol-gel vs. glutaraldehyde). Pernot et al use DAAORg in biosensor fabrication while Zain et al (2010) use DAAOPk, both employing the same enzyme entrapment techniques. However, the DAAORg enzyme proves to be superior and gives a more sensitive biosensor ($89\pm 33\mu A$ mM^{-1} cm^{-2}) compared to DAAOPk biosensor ($61\pm 7\mu A$ mM^{-1} cm^{-2}) with a slightly better resolution (16nM to 20nM), although this is theoretical and based on low background noise (about 0.05pA; signal/noise=3 for their biosensor), which results from the very small biosensor designed. The difference in LOD is not likely to alter the applicability of the biosensor, since levels of D-serine are thought to be in micromolar range in the brain. Surprisingly, biosensor stability, which is partly due to the enzyme and in part the deposition technique, is slightly better with DAAOPk (6 months) compared to DAAORg (5 months).

When the same source of enzyme (DAAORg) was used to make our biosensor and that of Pernot et al (2008); the techniques used to entrap DAAORg onto the platinum wire could be compared. The biosensors in this study are appreciably superior in sensitivity ($200\pm 15\mu A$ mM^{-1} cm^{-2} to $89\pm 33\mu A$ mM^{-1} cm^{-2}); as a result of the sol-gel electrochemical deposition technique used while Pernot et al (2008) employed glutaldehyde entrapment of the enzyme. The method of enzyme-entrapment involving sol-gel electrochemical deposition has proven to be a more effective way of making enzyme biolayers at the platinum surface. However, this may have been at the cost of the stability, which begins to reduce after 2 months, although this is not the sensor working life limit, compared to 5 months stability for the sensor designed

by Pernot et al (2008). This may be important in commercialisation of the biosensor but for use in the brain it is less of an issue. Perhaps a better means of assessment in stability would have been the stability of the biosensor with *in vitro* or *in vivo* use. This data is unfortunately not available for the other two biosensors, though we have re-used sensors for up to 3 days for *in vitro* experiments. These findings are summarized in table 2.4.

The selectivity is another important feature of a biosensor to be used in the central nervous system. Often, the case is that 100% specific response is not achieved, it is recognised that biosensor systems based on peroxide carry a price, as a result of the relatively high potential (and additionally here the wide substrate range of DAAORg). Screening layers with electropolymers were employed in all 3 biosensor designs, though Zain et al use nafion in addition to poly-m-phenylenediamine. Interference from a number of sources was tested but data from Zain et al is not sufficient to make a direct comparison of selectivity. As may be expected, since similar methods of screening were used here and by Pernot et al, both biosensors give similarly selective response for D-serine (over 88 to 87% respectively). Moreover, in both cases null biosensors are used in addition to the D-serine biosensors to ensure that non-specific signals are subtracted from the D-serine trace.

	Our sensor	(Pernot *et al.*, 2008)	(Zain *et al.*, 2010)
Sensitivity	$200 \pm 15 \mu A\ mM^{-1}\ cm^{-2}$	$89 \pm 33 \mu A\ mM^{-1}\ cm^{-2}$	$61 \pm 7 \mu A\ mM^{-1}\ cm^{-2}$
LOD	25nM	16nM (theoretical)	20nM
Selectivity	88%	87%	Not given
Stability	2 months	5 months	6 months

Table 2.4: Comparisons in sensitivity, selectivity, stability and limit of detection of our D-serine biosensors compared to current D-serine biosensors.

The growing interest in D-serine biosensors for use in the brain indicates that there is a real need for a tool able to measure this amino acid directly, perhaps in the hope of resolving some of the questions surrounding the signalling of this molecule. A D-serine biosensor makes possible real-time measurements so that for the first time activity dependent D-serine release may be observed in the brain. Furthermore, accurate numerical data regarding the levels of D-serine found in different brain regions may give much insight into the occupancy of the glycine site of NMDA receptors, over which there is much debate. Drug targeting and altering of dysfunctional D-serine signalling through this site offers numerous possibilities for therapeutics but key to this is establishing the dynamics of D-serine in the central nervous system. Biosensors for D-serine offer an exciting and novel way of studying this amino acid in the whole brain. In additional these biosensors can be employed as diagnostic tools in detecting dysfunction levels of D-serine/D-amino acid in patients. For example, in schizophrenic patients, where D-serine levels are shown to be lower in patients, a device that can give instantaneous numerical readings may be beneficial in speeding up treatment and even allow treatments to be followed in patients.

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Chapter 3: Use of D-serine biosensors to investigate extracellular D-serine tone *in vitro*

3.1 Abstract

The binding of a co-agonist at the glycine site is a prerequisite for activation of postsynaptic NMDA receptors. Whether this site is fully saturated or not is hotly debated. We have used D-serine biosensors to study extracellular concentrations of D-serine in the cortex, hippocampus and cerebellum *in vitro*, to investigate the potential for excitation of the NMDA receptor.

Surprisingly, there exists much variation in the extracellular levels of D-serine throughout the brain and even within one structure. In the case of the hippocampus the greatest difference in concentrations is observed between *stratum pyramidale* ($1.1 \pm 0.3\mu M$; n=18) and *stratum radiatum* layers ($0.4 \pm 0.1\mu M$, n=14). D-serine levels detected in the molecular layer of cerebellum ($0.3\pm 0.1\mu M$, n=14) were five times lower compared to the granule cell layer ($1.64\pm 0.5\mu M$, n=18). In the cortex D-serine concentrations were averaged at $1.0\pm0.2\mu M$, median $0.9\mu M$ (n=14).

NMDA receptors composed of the NR2C/NR2D subunits are (90%) saturated at approximately $0.6\mu M$ D-serine and those containing NR2A/NR2B subunits are saturated at about $1\mu M$ D-serine. As a result in *s. pyramidale* region (largely expressing NR2A/NR2B) and the granule cell layer of cerebellum (expressing NR2C), NMDA receptors are fully saturated. But in *s. radiatum* and the molecular layer of cerebellum, the levels of D-serine are significantly lower; hence the glycine site is not saturated. Rationally NMDA receptor response can be potentiated in these low D-serine regions by applications of extracellular D-serine while in regions of high D-serine concentrations receptor potentiation is unlikely since the glycine site is fully saturated. Corroborative patch-clamp data confirms that the NMDA receptor response can be increased in *s. radiatum* but not in *s. pyramidale*. Additionally, D-serine release can be stimulated by an astrocytic peptide agonist of protease-activated receptor 1 (TFLLRNH$_2$ $6\mu M$), which is present largely on astrocytes, that can potentiate NMDA receptor response in *s. radiatum*. Hence NMDA receptor activity can be modulated by D-serine in a specific manner, placing D-serine at the very centre of signalling events in the central nervous system.

3.2 **Introduction**

The NMDA receptor is unique amongst the glutamate receptor family in its requirement of two agonists (glutamate and a co-agonist) to function. Early studies attributed the co-agonist role to glycine (Johnson & Ascher, 1987) but another more unusual candidate has since come to light: D-serine. Initially only used as a glycine mimic, D-serine often proved to be a more potent co-agonist at the NMDA receptor (Kleckner & Dingledine, 1988; Wood *et al.*, 1989). However, the physiological relevance of this was only appreciated after the discovery of significant levels of D-serine in the brain (Hashimoto *et al.*, 1992a). Antibody staining of D-serine and its synthesising enzyme, serine racemase (SR) co-localized to regions of high NMDA receptor expression, confirming a central role of D-serine in the activity of this channel (Hashimoto *et al.*, 1993b; Schell *et al.*, 1995; Wolosker *et al.*, 1999a).

However, since glycine and later D-serine levels in aCSF were found to be very high, it was generally assumed that the co-agonist site is always saturated and hence physiologically silent (Ferraro & Hare, 1985; Billups & Attwell, 2003). D-serine levels range between 6.5-8μM in the brain according to capillary dialysis studies; while glycine levels are slightly higher at 7-10μM (Hashimoto *et al.*, 1995; Matsui *et al.*, 1995; Hashimoto & Oka, 1997). Although detailed study of different brain regions and layers within a brain structure were not analysed systematically if these findings are correct all NMDA receptor subtypes should be fully saturated. Matsui et al showed that recombinant NMDA receptors expression in *Xenopus* oocyte are fully saturated (90% and above) at 0.6μM (NR2C/NR2D) and 1μM (NR2A/NR2B) D-serine. The levels of glycine required for similar saturation are 3-4 times higher (Matsui *et al.*, 1995). Nevertheless these saturating concentrations are significantly below the amount of D-serine and glycine thought to be present in the brain.

But numerous studies have shown that exogenously applied D-serine and glycine can in fact potentiate NMDA receptor response, endowing physiological importance to this site as a regulatory mechanism of NMDA receptors (Thiels *et al.*, 1992; Nilsson *et al.*, 1997; Panatier *et al.*, 2006). This signifies that the co-agonist sites of NMDA receptors at the synapses are not fully saturated. A number factors could possible contribute to the apparently ambiguous results: the transporters of D-serine and glycine, variation in affinity of different NMDA receptor isotypes for a co-agonist

(as a result of the NR2 subunit) and errors in experimental design of studies used to measure glycine and D-serine levels.

Although extracellular levels of glycine and D-serine are found to be very high, this doesn't necessarily indicate that the same concentrations are present at the synapse. This is certainly so for glycine, which is subject to a powerful uptake system; the GlyT1 and 2 transporters have very high affinity for glycine; in the rat hippocampus GlyT1 has a Km of 0.06µM. D-serine on the other hand has two comparatively low affinity transporters, the ASC-1 transporter Km is 67µM while the ASCT2 transporter has a Km of 1mM D-serine (Yamamoto *et al.*, 2004; Shao *et al.*, 2009). Implications of this are that of the two co-agonists, the more likely co-agonist to be present at the synapse or extracellular space will be D-serine. Additionally, it is well-known that the NR2 subunit of NMDA receptors largely dictates the distinct functional properties of this channel. The four NR2 (A-D) subunits endow varying agonist potencies, deactivation time courses, open probabilities, single channel conductance, as well as sensitivities to Mg^{2+} and extracellular modulators. The affinity for D-serine is generally higher compared to glycine for different NMDA receptor composites implying a preference for D-serine even if glycine was present at high levels (Matsui *et al.*, 1995). Hence D-serine concentrations are likely to be a closer representative of the saturation state of NMDA receptor compared to glycine concentrations.

Currently, investigators are largely dependent on HPLC analysis coupled with capillary dialysis studies to determine D-serine levels in the brain (Ciriacks & Bowser, 2004; O'Brien & Bowser, 2006). The major disadvantage of HPLC technique is the need to homogenise tissue or disrupt cellular networks in order to collect lysate for further analysis; this is often from the whole brain or a brain structure. This disturbance of the cellular network may alter D-serine physiology. Capillary dialysis probe are slightly better in that they can be inserted into a particular brain region but these are often large and needs to be embedded into the brain for up to a week before recordings can be made. Over this time period the cells surrounding the probe alter morphologically to accommodate the probe, hence, the recordings made may not be representative of the normal brain.

D-serine biosensors offer vast advances over these techniques for determining D-serine levels in the brain, without the disadvantages associated with traditional techniques. The probe is small in size to minimise cell damage and recordings are made within seconds and the cellular structure remains intact. Three key areas of the brain were selected to determine D-serine levels: cortex, hippocampus and cerebellum using D-serine biosensors *in vitro* in order to determine the extent of saturation at the co-agonist site of the NMDA receptor. Due to efficient design of the D-serine biosensor, recordings can be made from different cell layers within a brain structure, so investigations of the different layers of the hippocampus (*s. oriens, s. pyramidale, s. radiatum and s. lacunosum molecular*) and cerebellum (granule and molecular layers) will be carried out to ensure that D-serine levels are uniform throughout a structure of the brain as has been assumed by current studies. The extracellular concentration of D-serine in the brain will reveal the extent of saturation at the co-agonist site of NMDA receptors, and the extent by which NMDA receptors can be potentiated.

3.3 Methods

3.3.1 <u>Biosensor fabrications</u>

The chemical technique used to make D-serine biosensors involves the electrochemical deposition of sol-gels and DAAORg onto a platinum electrode, as described in chapter 2 (Llaudet *et al.*, 2005). The DAAO*Rg* enzyme catalyses the following reaction:

D-serine + H$_2$O + O$_2$ $\rightarrow$ Hydroxypropionate + H$_2$O$_2$ + NH$_3$

The H$_2$O$_2$ produced is oxidised at the platinum electrode giving an electrical signal that can be observed. Screening against interferences is achieved with 1, 3 phenylenediamine and the sensors were operated at +500mV relative for Ag/AgCl to detect oxidation of H$_2$O$_2$ on the electrode surface. Only those biosensors demonstrating 75% selectivity for D-serine and of over 150μA mM^{-1} cm^{-2} sensitivity were used, along with a null sensor in all experiments. All test solutions, silanes and reagents were obtained from Sigma-Aldrich.

3.3.2 Slice preparations

Male Sprague-Dawley rats aged 12-21 days were sacrificed by cervical dislocation in accordance with schedule 1 of the UK Government Animals (scientific procedures) Act 1986. The brain was removed and placed in artificial cerebrospinal fluid (aCSF) at 4°C before 500µm horizontal slices were cut with a Vibrotome as previously described (Dale *et al.*, 2000). Slices were placed in an incubation chamber in aCSF continuously oxygenated (with 95% oxygen/ 5% carbon dioxide) at room temperature for 1hr before use. The composition of aCSF is as follows: NaCl 124mM; KCl 3mM; CaCl 2mM; NaH_2CO_3 26mM; NaH_2PO_4 1.25mM; D-glucose 10mM; MgSO4 1mM; pH 7.4 with 95% oxygen and 5% carbon dioxide.

A single slice was transferred to a recording chamber, fully submerged with oxygenated aCSF and profused at 8mL/min (33-34°C). A Deuostat interfaced to PC by an A to D converter board was used and an Ag/AgCl was used as a pseudo-reference electrode. D-serine biosensors of 0.5mm length and 50µm diameter are used in this study unless otherwise stated. In all cases a null biosensor was used (a sensors without enzyme in the biolayer) to ensure accuracy of recordings and placed as close to the D-serine biosensor as possible, as shown in figure 1.
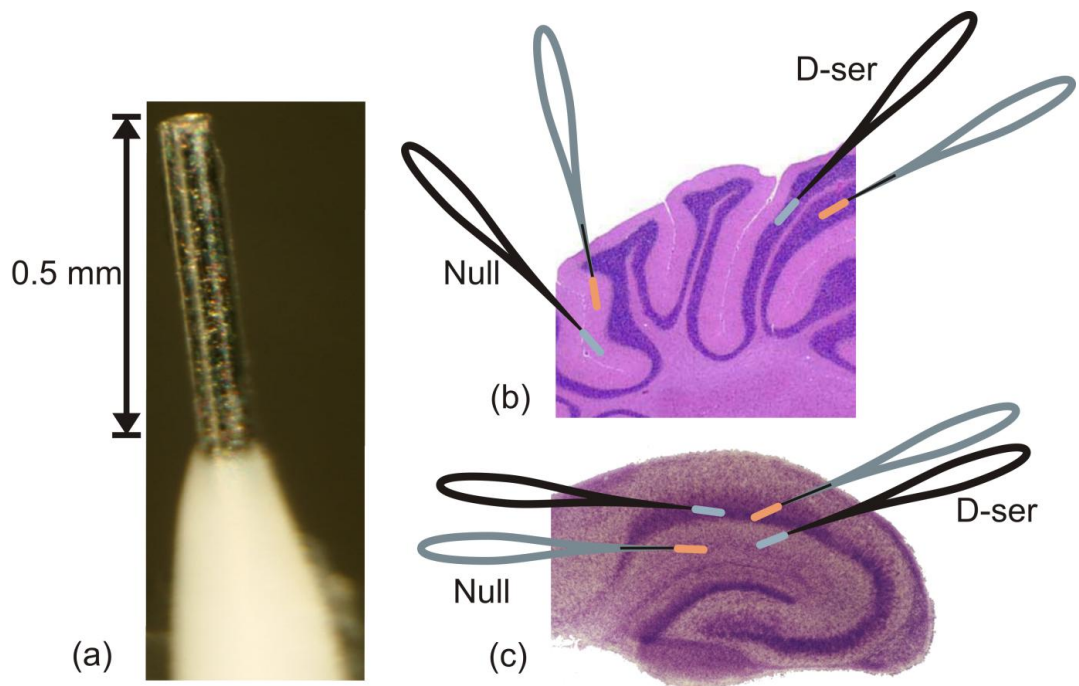
Figure 1: D-serine biosensor and null sensor placements in cerebellum and hippocampus. (a) D-serine biosensor 0.5cm in length and 50µm in diameter (b) cerebellum slice 500µm thickness, null and D-serine biosensors are placed into the granule cell layer (dark blue staining) and molecular layer (pink stained region). Null sensors are placed as close to D-serine biosensors as possible. (c) D-serine and respective nulls are placed into *s. pyramidale* (dark stain) and *s. radiatum* (light stain).http://www.biocell-interface.com/bcimages/hippocampus_b.jpg. http://www.deltagen.com/target/histologyatlas/atlas_files/nervous/cerebellum_brainstem_2x.htm

3.3.3   Determining extracellular basal D-serine levels in the brain

To determine the extracellular basal D-serine levels (or tone) slices were submerged with aCSF in a perfusion chamber before the pre-calibrated D-serine biosensors were inserted into a selected brain region. These are then allowed to stabilise for 20 minutes to permit recovery of damage done upon insertion and in order for any D-serine that has been released as a result of damage to be washed away. Biosensors were then removed from the slice, before being calibrated with 10µM D-serine. The difference between D-serine levels detected in the slice and those when the sensor is not in the slice is termed the tone or basal levels of D-serine in the selected brain region (Figure 2). This give a numerical value for the amount of D-serine in the area studied and the process was repeated in slices from different male SD rats within the 12-21 day age-range. Data was also gathered by similar means for the cerebellum
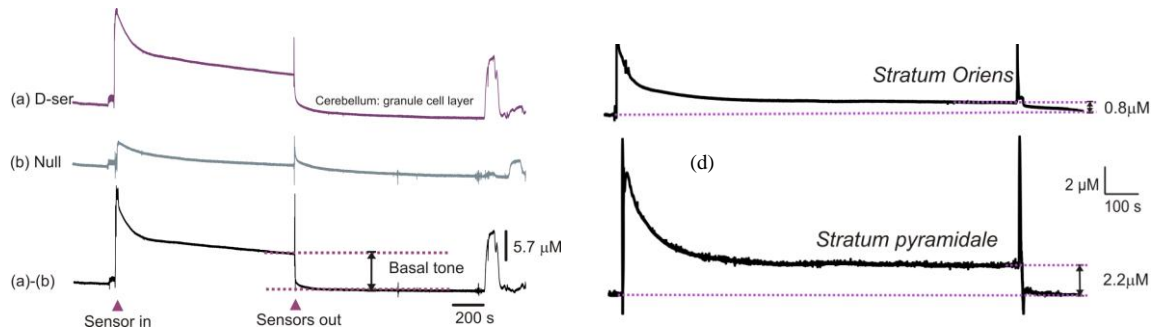
Figure 2: Basal D-serine concentrations are determined subtracting the signal from the null sensor from the D-serine trace. D-serine and null sensors are placed closed together into the granule cell layer of the cerebellum, and are removed from the slice after 20 minutes. The signal from the null sensor (b) is subtracted from the D-serine trace (a) and basal levels determined using a calibration of 10µM D-serine. 10µM 5HT is also applied to test interference. Examples of D-serine tone traces from *s. oriens* and *s. pyramidale* of the hippocampus are also shown (d).

3.3.4   Statistical analysis

D-serine levels in the hippocampus and cerebellum are presented as a cumulative probability distribution. Data from the hippocampal regions was analysed with the Kruskal-Wallis test. Pair-wise comparisons between 2 regions of the hippocampus were then made using the Kolmogrov-Smirnov test, to decide if samples are significantly different. Since each measurement represents a real-time measurement of extracellular D-serine levels in a particular region, and often the data was not normally distributed we have used median values as well as the mean to represent the basal D-serine levels. The median is not distorted by outliers while the mean is altered.

3.4 Results

3.4.1   Extracellular D-serine levels in the hippocampus

D-serine biosensors permitted detection of D-serine with minimal damage, greater accuracy and speed, allowing differentiation of cell layers within a brain structure which was previously not possible. In the hippocampus this has lead to surprising results (figure 3). D-serine levels were not uniformly distributed but instead varied significantly from cell layer to cell layer (Kruskal-Wallis test $P=0.042$, $n=62$). The

greatest difference in extracellular D-serine levels was seen between *s. pyramidale* 1.1 ± 0.3μM (median: 0.8μM, n=18) where the highest amounts of D-serine are found and *s. radiatum* 0.4 ± 0.1μM (median: 0.3μM, n=14) where the least amount of D-serine is observed (Kolmogorov-Smirov test p=0.021, n=32). In *s. lacinosum moleculare* and in *s. oriens* similar amounts of D-serine were detected: 0.7 ± 0.1μM (median: 0.5μM, n=15) and 0.6 ± 0.2μM (median: 0.3μM n=15) respectively. Figure 4 shows a cumulative probability plot of the tone data gathered from the hippocampus, with average tone levels of D-serine (in μM) labelling the relevant cell layers. Little statistical difference was observed between the *s. radiatum* and *s. lacunosum moleculare* (K-S test p=0.34, n=29) but basal D-serine tone in *s. oriens* and *s. pyramidale* is more statistically likely to be different (K-S test p=0.08, n=33). The lack of statistical significance may be due to error in sensor placement. D-serine levels in the dentate gyrus were also measured 0.6 ±0.1μM (n=14).
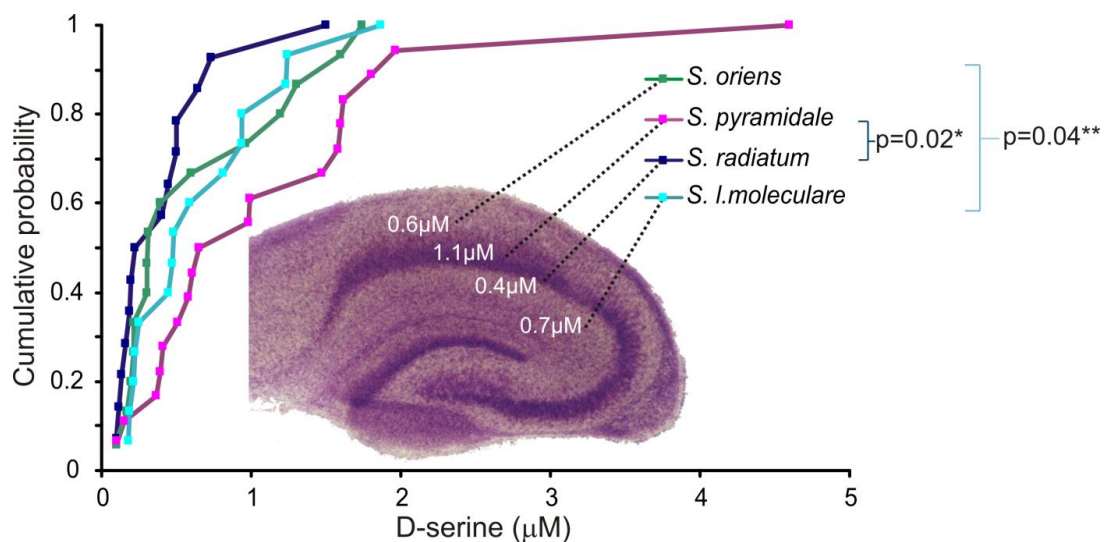
Figure 3: Basal D-serine levels in different layers of the hippocampus of rat brain Indicates the basal levels of D-serine detected in S. oriens, S .pyramidale, S. radiatum, and the *S. lacinosum moleculare* of the hippocampus of SD rats. Mean levels of D-serine are given for each layer in μM, with statistical analysis *Kolmogorov-Smirnov test **Kruskal-Wallis test.

3.4.2 Extracellular D-serine levels in the cerebellum

In the cerebellum, D-serine biosensors were used measure basal D-serine levels in the granule cell layer and the molecular layer. Significant differences were observed in the cerebellum, as shown in figure 4, where highest levels of D-serine were found in the granule cell layer 1.64 ± 0.51μM (n=18) and significantly less D-serine was found in the molecular layer 0.31 ± 0.05μM, n=14 (K-S p=0.042, n=32).
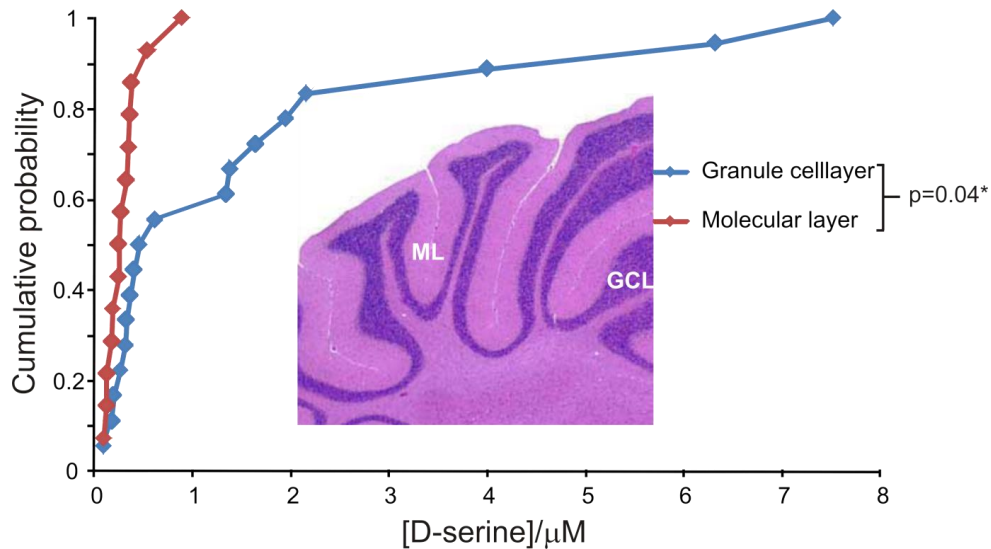
Figure 4: Basal D-serine levels in the cerebellum of rat brain. Displays the basal levels of D-serine detected in the granule cell layer and the molecular layers of the cerebellum of SD rats.*p value for the Kolmogorov-Smirnov test to determine significant difference between the two data sets.

The data obtained from the granule cell layer was rather variable. The median (0.5µM) is out by a factor of 3 from the mean. This may be explained by sensor misplacement and/or the age of the rats (D-serine levels are found to be higher in younger animals). For this latter reason, data points were plotted against the age of the animal, as shown in figure 5. However, no clear correlation with age was apparent. In fact if the cumulative probability figure is analysed closely, the data for the granule cell layer appears to composed of two distinct distributions
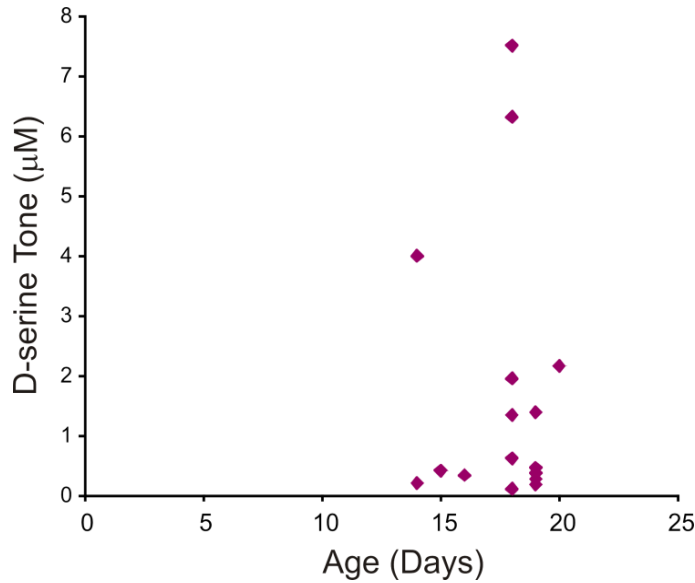
Figure 5: Scatter distribution of age and D-serine tone in the granule cell layer of cerebellum. D-serine levels in the cerebellum vary considerably but that variation is not as a result of age, as shown here. No relationship exists between the levels of D-serine detected in the cerebellum and the age of the animal from which the slice was derived.

Sensor placement may be the contributing factor to the differing distributions. The cerebellum alveoli, when cut on the horizontal plane give a slice where granule cell layer width varies. In some areas (figure 6, labelled 1) the granule cell layer is small in width. Here the layer is composed of fewer cells and if sensor is placed here, both the tone from the granule cell layer and the molecular layer will be recorded by the microelectrodes. Median M_1 correlates exactly with the median for the molecular layer, 0.3μM. Compared to regions where the width of the granule cell layer is much larger (labelled 2, figure 6); the sensor is likely to be only recording basal D-serine tone from the granule cells.
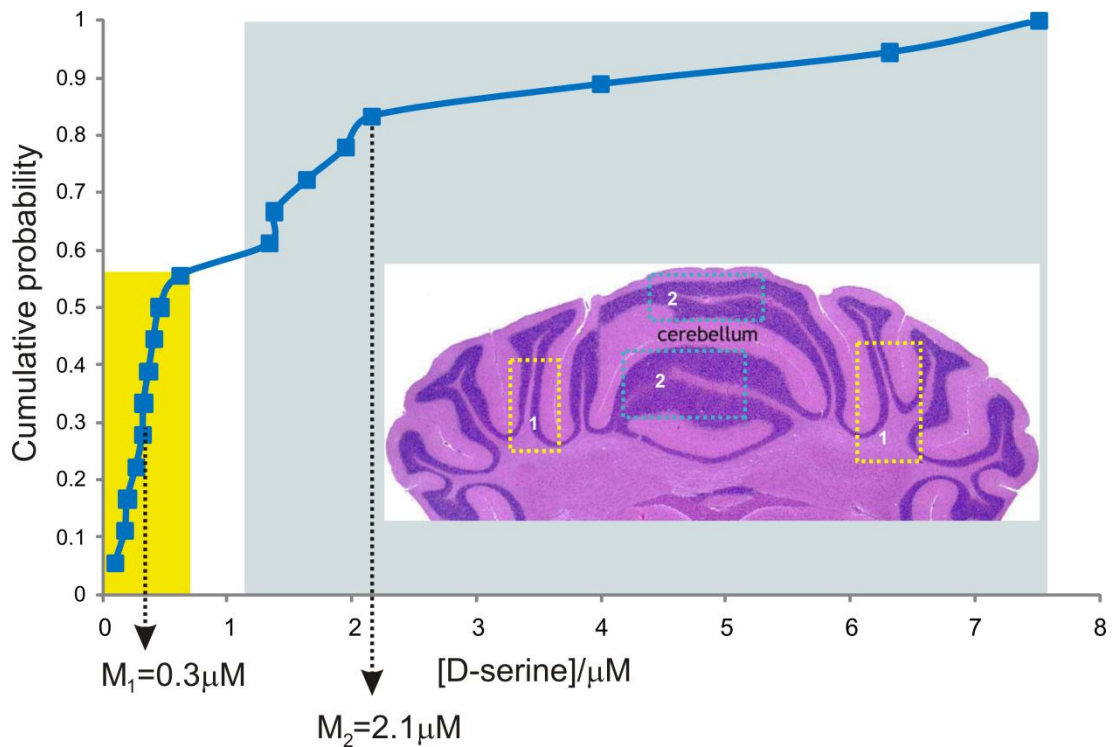
Figure 6: A single median/mean is misleading for the probability distributions seen in the granule cell layer. There appear to exist two distinction distributions, with a median of 0.3μM (M_1) and 2.1μM (M_2). It is likely that M_1 represents recordings from regions where the granule cell layer width is small, while M2 recordings are from regions labelled 2, here the granule cell population is much larger.

In light of this, M_2 may be a better representation of the basal tone observed in the cerebellum granule cell layer, 2.1μM.

3.4.3   Extracellular D-serine levels in the cortex

In the cortex extracellular D-serine concentrations were 0.99 ±0.19μM (n=14). Due to the limited resolution of steromicroscopes used, it was not possible to differentiate between cortical layers, although it is likely in light of the data gathered from the hippocampus and the cerebellum that basal D-serine tone will differ in the 6 cortical layers.   Further investigation is required to determine this variation which can also explain the wide range of D-serine concentrations observed from the cortex, with levels varying from 0.3μM to 2.5μM.   Figure 7 shows the cumulative probability plot of this data, along with median, upper and lower quartiles.
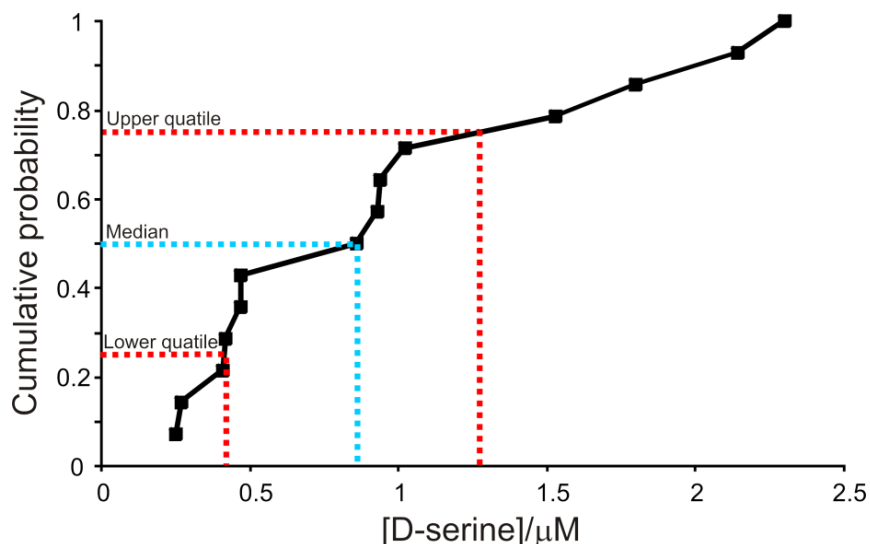
Figure 7: D-serine levels in the cortex of rat brain. Cumulative probability graph showing basal D-serine tone detected in the cortex in various layers. Median D-serine levels in the cortex are 0.9μM.

The levels of D-serine detected in the various brain structures and regions within are summarized in table 1; both mean and median values are given. Henceforth, only the median values will be used to conduct functional significance of the levels of D-serine detected although the mean values can also be justifiably used.

		Median (μM)	Mean (μM)
Hippocampus	*S. oriens*	0.3	0.6±0.1
	S. pyramidale	0.8	1.1±0.1
	S. radiatum	0.3	0.4±0.1
	S. lacunosum-moleculare	0.5	0.7±0.1
	Dentate gyrus (GCL)	0.4	0.6±0.1
Cerebellum	Cerebellum (ML)	0.3	0.3±0.1
	Cerebellum (GCL)	0.3, 2.1**	1.5±0.5**
Cortex	Cortex	0.9	1.0±0.2

Table 3.1: Median and mean D-serine concentrations in various rat brain regions. D-serine levels in the brain vary in the different structures of the brain and also within these brain regions. **The difference in median and mean is the greatest in the granule cell layer compared to any other region.

3.4.4   PAR1 induced D-serine release

Protease activated receptor-1(PAR1) is abundantly expressed on astrocytes and is activated by proteolytic cleavage by brain serine proteases under physiological

conditions (Wang *et al.*, 2002; Wang *et al.*, 2006). Activation of this receptor causes an increase in intracellular calcium levels that has the potential to cause D-serine release from cells synthesising this amino acid. It is possible to determine whether D-serine release can be evoked by a glial-specific stimulus and whether release of D-serine correlates with existing basal tone. Although D-serine has been localised to neurones as well as glia, the PAR-1 receptor agonist $TFLLRNH_2$ activates PAR-1 receptors specifically which are found on glial cells. As observed in figure 8, extensive D-serine can be evoked by this agonist in *s. pyramidale* (0.71±0.2μM, n=9). The tone in this region is high (median: 0.8μM).

In *s. radiatum* however, D-serine release as a result of PAR-1 receptor activation is significantly less and infrequent (0.30±0.1μM, n=5). In this region the existing basal tone is the lowest seen in the hippocampus (median-0.3μM).
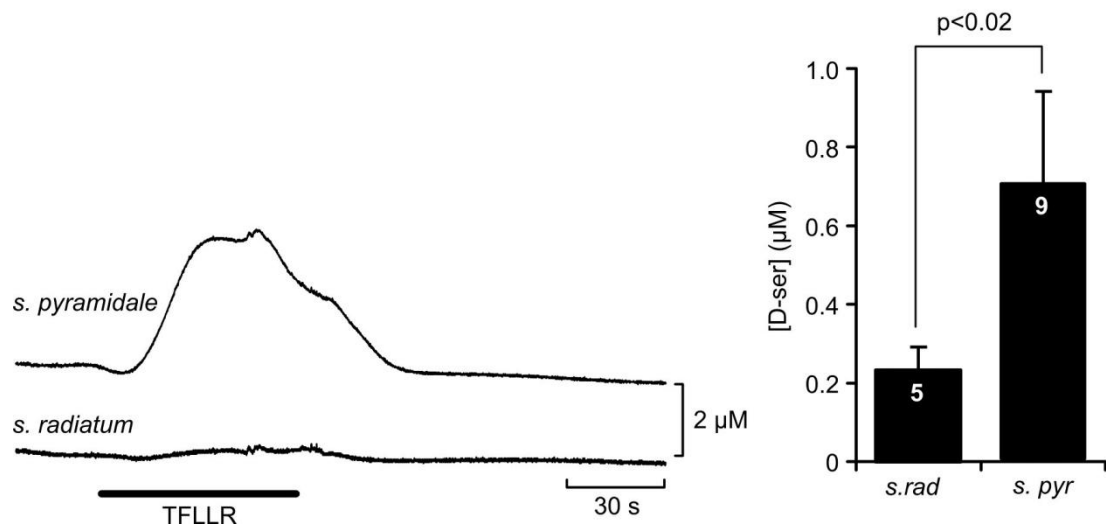
Figure 8: PAR1 activation can cause D-serine release in *S. pyramidale* and *s. radiatum*. D-serine biosensors were placed in the hippocampus before 6μM PAR-1 agonist TFLLRN was applied (black bar). D-serine release in *s. pyramidale* was significantly higher compared to *s. radiatum* as shown in biosensor traces on the left (T-test P<0.02) and on the right the bar graph shows the concentrations of D-serine released in the two respective regions.

4.0 Discussion

D-serine biosensors have been used in this study to gain novel insight into basal D-serine levels in the brain. These findings can be used to determine NMDA receptor co-agonist site saturation states. We have also shown novel release of D-serine as a result of a glial-specific agonist, $TFLLRNH_2$.

In order to determine the significance of D-serine tone in the brain, data regarding glycine site occupancy levels was take from a detailed study carried out by Matsui et al. Saturation levels of recombinant NMDA receptors (NR2A-D) expressed in *Xenopus* oocytes were explored. The characteristics of these receptors were found to be typical of neuronal NMDA receptors (antagonised by AP5, Mg^{2+}), authenticating the data gathered (Matsui *et al.*, 1995). Using data from this study, we have calculated dose response concentration curves for the 4 NMDA receptor variants tested, using the Hill equation ($I=I_{max}[D\text{-serine}]^n/EC_{50} + [D\text{-serine}]^n$) where I_{max} is the maximal response, EC_{50} is the D-serine concentration yielding response one-half of the I_{max} and n is the Hill coefficient, shown in figure 9.
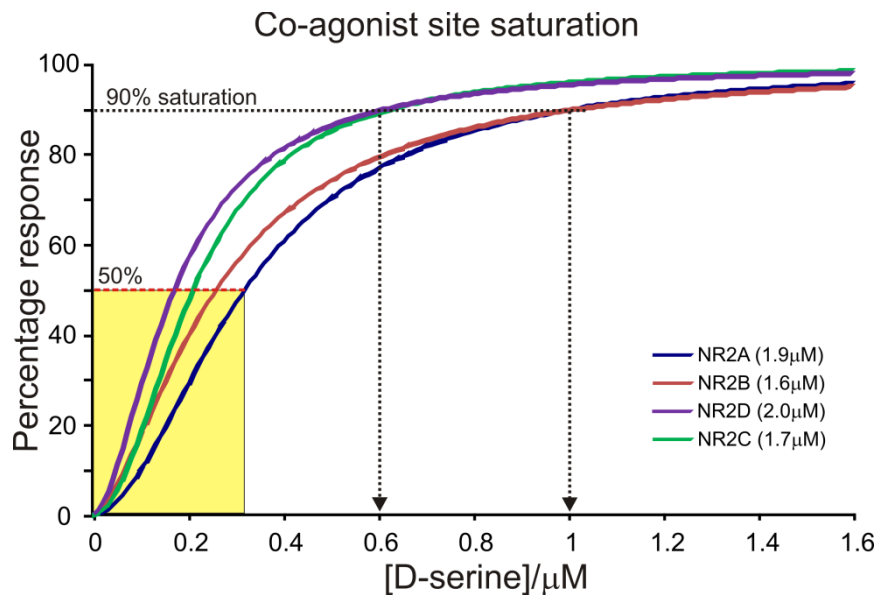
Figure 9: Saturation of the co-agonist site of NMDA receptors based on NMDA receptor on the hill coefficient from data collected by Matsui et al (1995). NR2C/D containing NMDA receptors have a higher affinity for D-serine than NR2A/B-receptors (brackets contain the hill coefficient values collected). Saturation (90%) is achieved at 0.6µM and 1µM for NR2C/D and NR2A/B containing NMDA receptors.

According to the dose-response curves, receptors containing NR2A and NR2B subunits require higher levels of D-serine for 90% response (in the region of 1µM), compared to NMDA receptors containing NR2C and NR2D subunits (approximately 0.6µM) (Matsui *et al.*, 1995). Half-maximal or 50% saturation is achieved between 0.17µM to 0.32µM D-serine for NR2D to NR2A containing receptors. Implications of this are that in regions of fixed extracellular D-serine levels, the occupancy levels at the co-agonist site will vary with the NR2 subunit composition. Receptors

composed of NR2A and NR2B subunits will be less saturated compared to those composed of NR2C and NR2D subtypes, in the same region. This has important functional consequences since, it has been proposed that different variants of the NMDA receptors (particularly NR2A and NR2B) are linked to different intracellular cascades and participate in different functions in synaptic plasticity and pathological conditions (Liu, XB *et al.*, 2004; Kim, MJ *et al.*, 2005). Therefore, pharmacological agents manipulating D-serine related signalling cascades will have varying influences, depending on the NR2 composition as NMDA receptor populations.

This data can be used to determine the extent of NMDA receptor excitation in the various regions of the hippocampus, cerebellum and cortex using the extracellular tone measurements made here using D-serine biosensors. Extracellular tone is denoted by the median levels of D-serine concentration detected in each brain area; this is used to determine the degree of saturation at the co-agonist site by D-serine.

3.4.5 Exploration of the saturation state of NMDA receptor co-agonist site in the hippocampus

The NMDA receptor subtypes most highly expressed in the hippocampus are the NR2A and NR2B containing channels. Although, the intensity of the expression of each subunit is thought to change with development; at P12 (animals in this study were P12-21) the levels of NR2A are found to rise rapidly to reach adult levels by P22 while NR2B protein levels begin to decline rapidly to undetectable levels by P22 (Wang *et al.*, 1995). Functionally this means that NMDA receptor potentiation declines slightly, with increased NR2A-containing NMDA receptor expression, as more D-serine is required to achieve the activation state of NR2B-containing NMDA receptors. Incidentally, NR2A-NMDA receptors display faster kinetics with a 100ms deactivation time constant while NR2B-channels show slower deactivation time constant, approximately 250ms resulting in reduced decay time (Cull-Candy & Leszkiewicz, 2004).

Lowest levels of D-serine are detected in the s. *radiatum* and s. *oriens* (medians 0.31 µM each). At these levels according to the dose-response curves NR2A/B-NMDA receptors (and depending on glycine levels), the occupancy levels vary over approximately 45-55%. NR2A/B receptors localised in the s. *oriens* and s. *radiatum* regions can therefore be potentiated by extracellular D-serine, by as much as 50%.
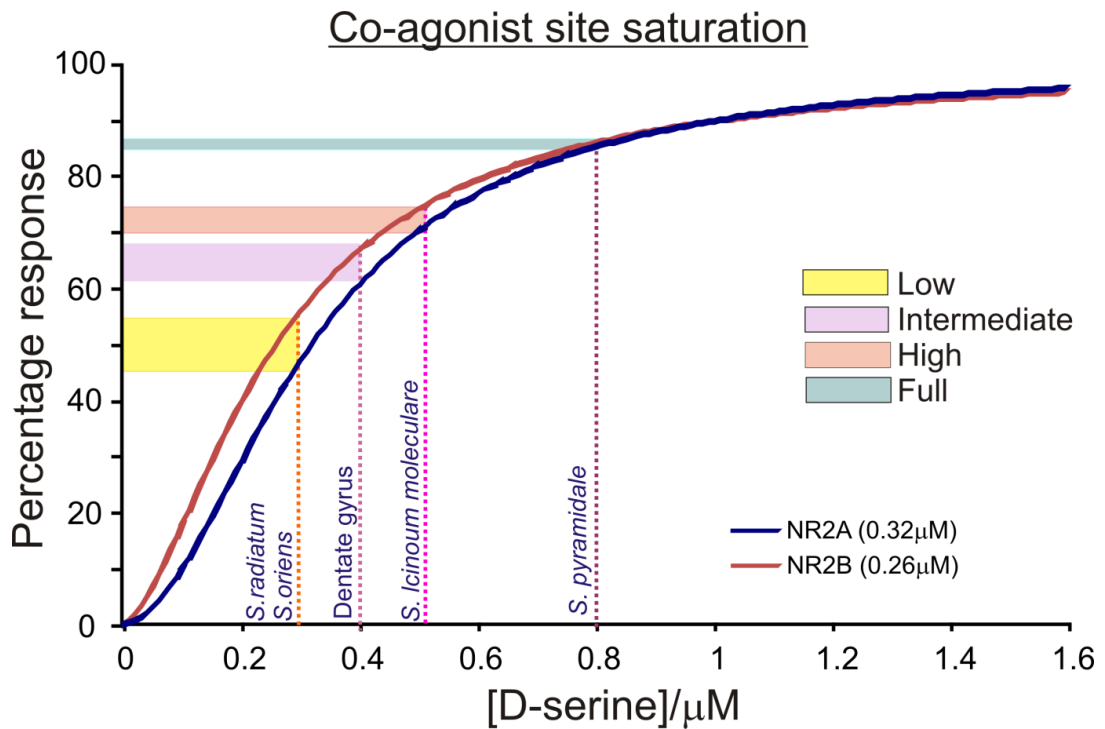
Figure 10: NMDA receptor co-agonist site saturation in the hippocampus. Regions within the hippocampus differ in the levels of D-serine, which denotes differing excitation ability of NMDA receptors. Levels of D-serine in *s. radiatum* and *s. oriens* are low (45-55%), here NMDA receptor response can be potentiated while in *s. pyramidale* the response is almost fully saturated (87%).

D-serine concentrations in dentate gyrus indicate that NMDA receptor saturation is below 60% (intermediate). The region is one the few areas of the brain that continues to incorporate granule neurons during adulthood, a process influenced by excitatory amino acids through the NMDA receptor (Schlessinger *et al.*, 1975). It is likely that NMDA receptor response can be potentiated by as much as 40% here. In *s. lacunosum-molecular* region, NMDA receptors occupancy is between 70-75% due to higher D-serine availability, therefore the NMDA receptor response is likely to only be potentiated by 20-30% with increased availability of D-serine or glycine. Levels of D-serine in the *s. pyramidale* are such that the NMDA receptor occupancy is almost 90%, making it unlikely that receptor response can be potentiated.

If the D-serine release induced by glial agonist TFLLRNH$_2$ is considered in this context, then in *s. radiatum* NMDA receptor response is potentiated by approximately 20% as a result of 0.3±0.1μM D-serine release while in *s. pyramidale*, the effect of the D-serine (0.7±0.2μM) on NMDA receptor activation is likely to be call as saturation levels are already 85% and above. It is interesting that highest D-

serine release by this glial specific stimulus is observed in a region where the tone is already high, perhaps indicating a relationship between components which maintain the steady-state D-serine release and activity dependent D-serine release. But D-serine release in areas previously saturated may be indicative of another function for D-serine release, which is independent of NMDA receptors. PAR-1 is thought to regulate astrocyte proliferation and increased expression of this receptor in astrocytes has been shown to protect neurones from toxicity (Ishida *et al.*, 2006). But the protective effect of PARs depends on the extent of injury or thrombin concentration, with high levels leading to neurodegeneration and cell death.

3.4.6   Co-agonist site occupancy in the Cerebellum

The mRNA of all the subunits (NR2A-D) has been localised to the cerebellum. The molecular layer is composed of Purkinje cell dendrites and Bergmann glia processes. The levels of D-serine are not saturating in this layer and receptor response can be potentiated by 20-55% depending on receptor subunit composition. This also means that depending on the glycine tone due to the low availability of the co-agonist D-serine, NMDA receptors in this region are purposely maintained at low potentiation states. Thus D-serine, as well as glutamate and membrane depolarisation need to be regulated for the activation of this channel.

The granule cell layer of the cerebellum, where the highest levels of NR2C receptor subtypes are found, has high levels of extracellular D-serine (median 2.1µM). Under these conditions most NMDA receptors will be fully saturated and so will be unaffected by changes in D-serine concentration. Studies by Attwell and colleagues examining changes in the NMDA receptor component of the synaptic current at the rat cerebellar mossy fibre to granule cell synapse under applications of glycine and D-serine support these findings. Applications of up to 100µM D-serine or glycine had little effect on the synaptic current (Billups & Attwell, 2003). NMDA receptors in this region are maintained fully saturated at the co-agonist site, so that other factors limit NMDA receptor potentiation ability.
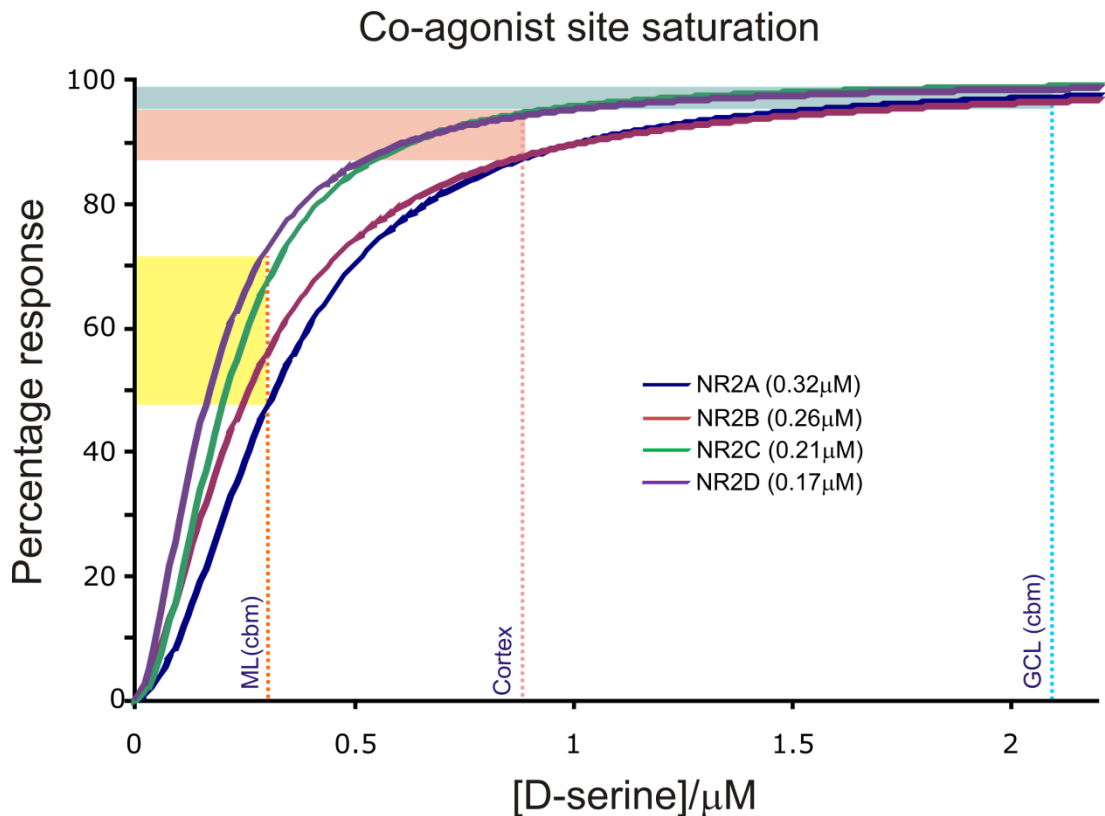
Figure 11: Saturation of the co-agonist site of NMDA receptors in the cerebellum and cortex. The dose-response curve of a NMDA receptor varies depending on the NR2 (A-D) subunit composition. Here levels of extracellular D-serine detected in ML (molecular layer, cerebellum), GCL (granule cell layer, cerebellum) and cortex are plotted.

If D-serine levels vary throughout the brain and even within a single a structure, it stands to reason that extracellular concentrations are maintained by regulatory mechanisms. Whether this involves increased uptake of D-serine or a reduction in D-serine synthesis/release has yet to be established. The mRNA distribution of SR correlates with the data gathered in this study, the highest level of the D-serine synthesising enzyme are found in the granule cell layer of the cerebellum and *s. pyramidale* of hippocampus. Expression in the cortex is more wide spread, maybe suggesting that D-serine levels in the different cortical layers are not distinct from each other (figure 12).
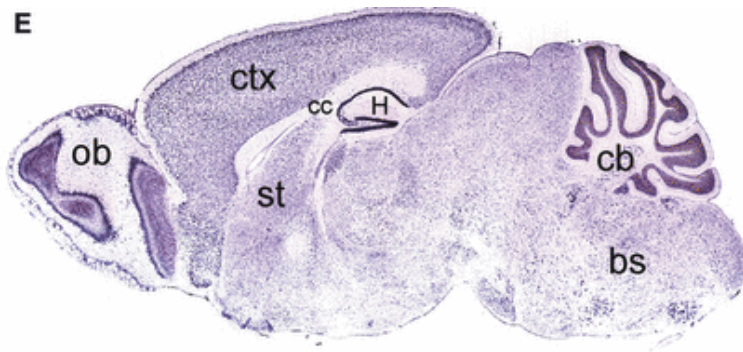
Figure 12: Antibody staining for serine racemase mRNA shows highest distribution in the hippocampus and granule layer of the cerebellum of mouse brain, these are the regions where the highest concentrations of D-serine were recorded using D-serine biosensors (Allen brain atlas).

A number of factors can potentially be involved in regulating extracellular levels of D-serine, but incredibly the regulation of this amino acid must also vary within a single brain region to maintain the diversity in D-serine concentrations that is observed in the hippocampus for example. Figure 13 shows a model whereby extracellular basal D-serine levels are maintained and the saturation state of the co-agonist of NMDA receptors and hence potentiation ability of this channel determined.
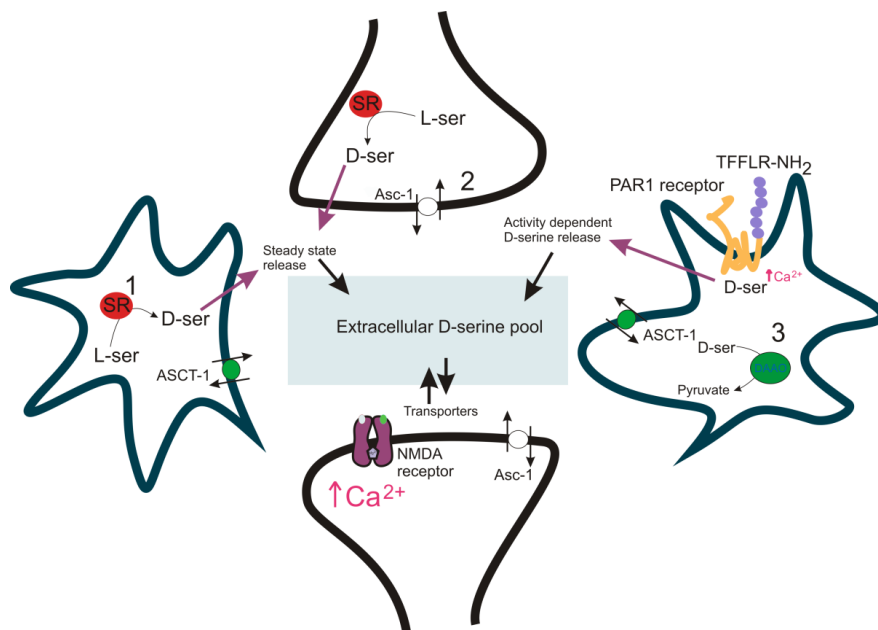
Figure 13: Factors contributing to extracellular D-serine basal levels. D-serine steady-state and activity dependent release and D-serine transporters increase extracellular pool of D-serine while removal of D-serine can only occur by re-uptake by transporters with subsequent breakdown of D-serine by DAAO.

Chapter 4: Use of D-serine biosensors *in vivo*

4.1 Introduction

D-serine biosensor technology has the potential to revolutionise the way in which D-serine signalling is studied. The *in vivo* model is the closest physiological replica for the living brain but also the most difficult environment for use of enzyme-based biosensors; with enzyme stability and non-selective interference posing the biggest problems. The practicability of D-serine biosensor use *in vivo* was explored. Some limitations in the design of the micro-electrode assembly and screening layer were apparent but D-serine biosensors can be used to detect changes in D-serine.

4.2 Method

In vivo data was gathered as part of collaboration with Dr. Matt Jones of Bristol University. Rats were anaesthetized with sodium pentobarbitone (60mg/kg) and received a subcutaneous injection of atropine sulphate to attenuate mucosal secretions. Rats were then placed in stereotaxic frame and secured with atraumatic ear bars coated with a topical local anaesthetic (Xylocaine). Absence of limb withdrawal corneal reflexes and lack of whisking were taken as evidence of unconscious state. Core body temperature was maintained at 37°C through the use of a homeotheric blanket controlled by a probe measuring rectal temperature.

In all experiments a craniotomy exposed the cortex directly above the hippocampus in both the left and right hemisphere and a second craniotomy exposed the dorsal surface of the cerebellum. The dura was removed and the brain surface was periodically irrigated with saline before a calibrated D-serine and a null biosensor were inserted into the brain (Figure 1). Sensors were allowed to stabilise for 10 minutes in the cortex before being inserted further to make recordings from the hippocampus, then these were removed from the brain completely for calibration in a separate perfusion chamber, at room temperature.
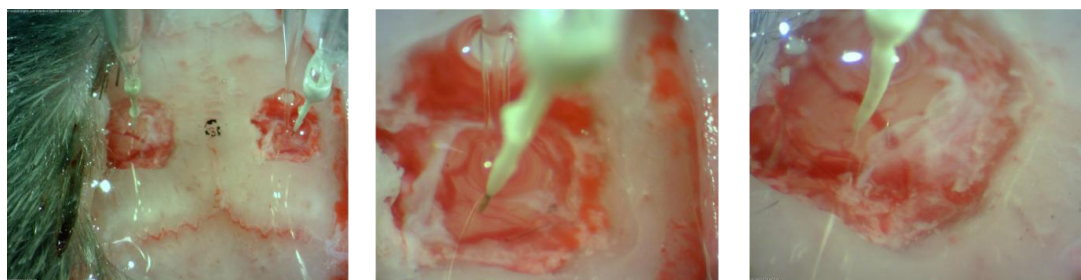
Figure 1: Use of D-serine biosensors *in vivo*. Sensors were placed directly above the area of interest, above the brain tissue (of rats aged approximately 6 weeks) but beneath a saline solution before insertions of 0.5mm to reach the cortex and further insertion of 2mm for the hippocampus.

Once the electrophysiological experiments were complete, the brain was removed and fixed in 4% paraformaldehyde (PFA) in 0.1M PBS pH 7.4 overnight. Before sectioning, tissue was treated with 30% sucrose in PBS pH 7.4 for 24hours at 4°C and then embedded in cryo-M-Bed (Bright instruments). 20µm free-floating saggital sections were made and mounted on frosted slides, in order to image the path of the D-serine biosensors, into the brain.

4.3 Results

Extracellular D-serine levels were determined by inserting a D-serine biosensor into the cortex initially, directly above the CA1 region of the hippocampus (figure 2). Following measurements in the cortex (allowing 10minutes for stabilisation of the signal), the biosensor was inserted further, into the hippocampus and allowed to stabilise before making a calibration. Figure 2 shows cryostat sections from the hippocampus and cortex. Inflammation of the regions where the sensors were inserted shows much damage. A null sensor was also used, which ensured that any non-enzymatic interference could be detected.
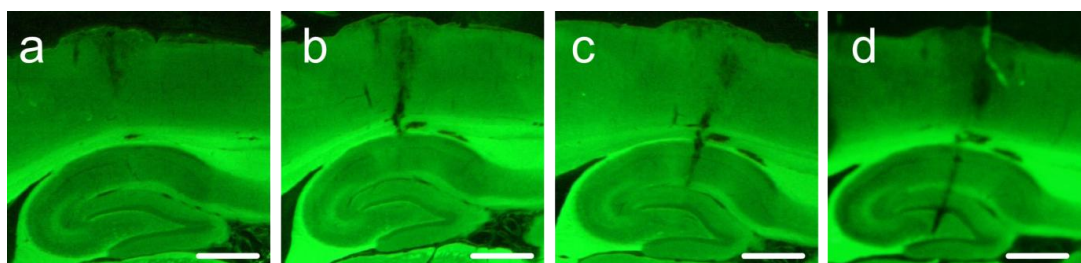
Figure 2: Progression of D-serine biosensor into the hippocampus. D-serine sensor is inserted into the cortex (a), following a stable recording it is inserted further until the hippocampus is reached (b, c, and d); the white bar represents a scale of 1mm.

Since the biosensors need to be calibrated immediately after the experiment, a test-chamber was devised but sensors could only be calibrated at room temperature. As a result the calibration (of 10µM D-serine) was smaller, since there is some dependency of the enzyme on temperature (Pilone Simonetta *et al.*, 1989b; Pernot *et al.*, 2008). Figure 3 shows a trace of D-serine and respective null sensors allowed to calibrate in the cortex, before being inserted further into the hippocampus. Sensors are then removed from tissue and re-calibrated. Similarly D-serine measurements were made in the cerebellum, though sensors were only inserted 1.5mm into the tissue. Differentiations between the granule cell layer and the molecular layer could not be made, as done in *in vitro* studies.

The resulting *in vivo* D-serine tone is much higher than the levels detected *in vitro* (Figure 4 and table 4.1) Also the hippocampus and cortex measurements are not statistically different (P=0.21, T-test). A number of factors may have contributed to this including age of rats (these rats were approximately 6 weeks old while in vitro measurements were from p14-21 days), experimental constrictions (sensors ideally need to calibrated at 34-37°C) and limitations of the *in vivo* model for detected basal tone within brain regions- discrimination of different hippocampal or cerebellum layers is extremely difficult since the different layers cannot be visualised.
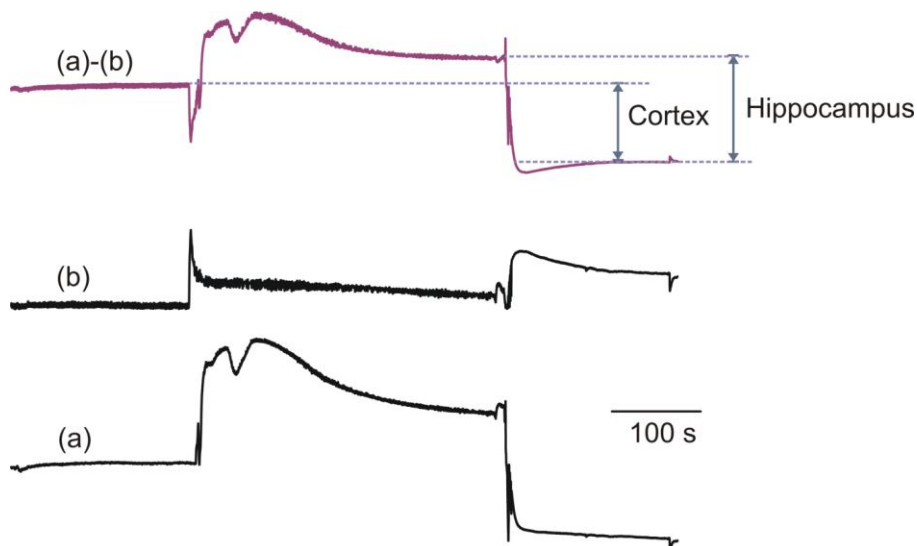
Figure 3: *In vivo* measurements of D-serine tone in the hippocampus and cortex. A trace of D-serine sensor is inserted into the cortex, following stabilisation of current; the sensor is inserted further until the hippocampus before being removed from the brain entirely.

Though, this may be a real *in vitro* to *in vivo* difference in D-serine concentrations, further refinement of D-serine biosensors and experimental technique is required to confirm any conclusions. However, in a preparation of rat brain homogenate D-serine concentrations have been shown to range from 10μM in the cerebellum to 400μM in cerebral cortex, possibly supporting these *in vivo* findings (Nagata *et al.*, 1994). Still, with refinement of D-serine sensor design damage in the cortex may be minimised upon insertion into the hippocampus, and with smaller sensors, it may be possible to place both null and D-serine biosensors in closer proximity. However, it is unlikely that the changes in D-serine levels within a single structure of the brain can be observed in *in vivo*, since the length of the sensors is greater than the different layers of the hippocampus for example.
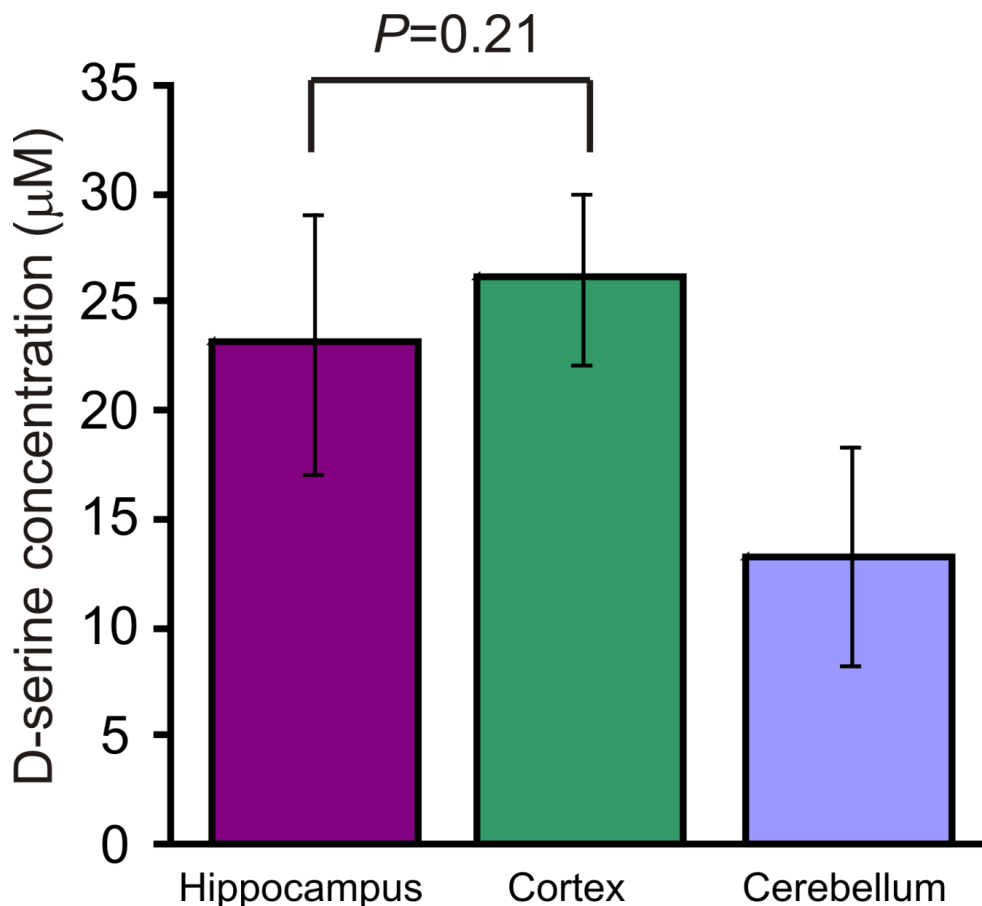
Figure 4: Basal D-serine concentrations are high in the hippocampus and cortex but these are not significantly different from each other (T-test P=0.21). D-serine concentration in the cerebellum is lower and also agrees more closely to the *in vitro* findings shown in chapter 3.

Brain region	Tone
Hippocampus	23.1±6.4µM, n=4
Cortex	26.1±3.5µM, n=4
Cerebellum	13.3±5.1µM, n=4

Table 4.1: *In vivo* **D-serine measurements in the brain**

4.1 Discussion

A number of issues arose with *in vivo* use of D-serine biosensors, which were not faced with *in vitro* use which highlighted the need for improvement in sensor design and use.

(1) Screening against non-specific interference

D-serine biosensors and null sensors are coated with poly-DAB to screen against non-specific interferences such as ascorbic acid and serotonin. For *in vitro* use this is a proficient barrier against non-specific signals but in *in vivo*, D-serine biosensors lost both sensitivity and selectivity much faster than with in vitro use, where sensors could be continually used for a few days. The major problem is the harsh *in vivo* environment, where both factors contributing to enzyme instability and increased number of interferences are present. For this reason new screening methods have been devised.

(2) Microelectrode size: diameter and length

Although *in vitro*, the sensor is only inserted into tissue of 500µm thickness, *in vivo*, the biosensor travels up to 2.5mm into the brain, to the reach the hippocampus. The current microelectrode are 50µm in diameter for the first 0.5mm and then significantly increase in width, causing much of the tissue damage observed in the cortex (figure 2). However, changes can be easily made to the biosensor design. An ideal assembly will have a 3mm length, of diameter 50µM or smaller for easy insertion into tissue. The mass damage observed in the cortex may have prevented clear reading in the hippocampus, where *in vitro* levels of D-serine are generally lower than those detected in the cortex. No significant difference was observed between D-serine levels in the hippocampus and cortex *in vivo*.

(3) It is not possible to made recording from sub-regions within the hippocampus or cerebellum

Biosensor use *in vitro* allow for measurements to be made within different structures of the brain, as in the case of the four layers of the hippocampus. However *in vivo*, this was difficult to do with accuracy and routinely, as there was no way of visualising the sensor path into the brain. Cyrostat sectioning allowed for arbitory judgements to be made regarding the placements of the sensors. Figure 3 shows clear changes in D-serine levels in the cortex and hippocampus but changes in D-serine levels within the hippocampus couldn't be made as the sensing area of the biosensor is larger than the defined cell layers (which is approximately 70μm in length). Therefore *in vivo* only whole structure measurements can be made, missing out possibly vital differences within a structure. Similarly in the cerebellum the changes observed in basal D-serine levels in the granule cell layer and the molecular layer were not seen. Incidentally, the D-serine concentration detected *in vivo* were most similar to those observed *in vitro* for the cerebellum, this is likely due to less damage in tissue as sensors were not inserted so deep into tissue, 1mm compared to 2.5mm in the hippocampus/ cortex.

(4) Calibration chamber

Sensors need to be calibrated before and after a recording, *in vitro*; this is usually done by applying a known amount of D-serine in the circulating aCSF. However, for *in vivo* experiments a separate chamber was devised but the sensors could not be calibrated at the temperature that they were used at (33-37oC), instead calibrations were made at room temperature. A small but noticeable reduction in the response of the sensor can occur. If the calibration is lower then, the basal D-serine tone detects is magnified. This may account for in part the higher levels of D-serine detected in *in vivo* compared to *in vitro*, though age of animals may also be a large factor.

Chapter 5: Activity dependent modulation of D-serine release in rat brain

5.1 Abstract

AMPA, kainate and NMDA receptors form the ionotropic glutamate receptor family; these channels mediate the majority of excitatory brain function. D-serine is a major co-agonist of the NMDA receptor, the binding of which is prerequisite for activation. Here we use D-serine biosensors to analyze the mechanisms of glutamate receptor evoked D-serine release and determine the functional implications of these changes on the NMDA receptor. We have systematically explored different regions of the brain and find surprising heterogeneity of mechanism.

We show that D-serine release can be evoked by the glutamate receptor agonists 5µM AMPA (+0.5±0.1µM, n=9), 20µM NMDA (+1.1±0.3µM, n=9) and 12.5µM kainate (+1.1±0.4µM, n=9) in the cortex, where levels of D-serine are already saturating. This release follows an increase in intracellular calcium levels in astrocytes, as observed by a Rhod2 signal and is diminished significantly by the removal of extracellular calcium. Two regions in the hippocampus were also explored. In *s. pyramidale* all 3 ionotropic agonists can evoke both loss and release of extracellular D-serine indicating the existence of two opposing mechanisms. In *s. radiatum* AMPA and NMDA caused a loss in extracellular D-serine: but loss with NMDA (-0.4±0.1µM, n=11) is twice that seen with AMPA (-0.2 ± 0.0µM, n=14), while with kainate both release and loss of extracellular D-serine is observed. In the context of the existing basal tone, the loss of D-serine has the potential to significantly diminish NMDA receptor activity, while the release occurs in regions where the NMDA receptor co-agonist site is already saturated; hence less likely to have functional significance for NMDA receptor activation.

During a LTP inducing tetanus (100Hz, 200ms), a process involving the ionotropic glutamate receptors NMDA and AMPA heavily, in CA1 of hippocampus D-serine is released (0.73±0.35µM, n=5) but this D-serine release occurs several seconds after the tetanus.

5.2 Introduction

Glutamate, the major excitatory neurotransmitter of the brain acts via ligand gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors. Activation of these channels is responsible for excitatory synaptic transmission and mechanisms underlying learning and memory (LTP and LTD). The NMDA channel is unique among these receptors with regards to a voltage-dependent block by Mg^{2+} and permeability to Ca^{2+}, both features are key to the physiological role of NMDA receptors in learning and memory (Collingridge *et al.*, 1983; Oliver *et al.*, 1990b). D-serine is an additional ligand to glutamate, which acts at the modulatory glycine site of the NMDA receptor; its binding is necessary for activation (Fadda *et al.*, 1988; Wolosker *et al.*, 1999b). Predictably it has been linked to multiple roles in the CNS, including modulating glutamatergic synapses (Wolosker *et al.*, 1999a); and action as a motility signal to promote development and maturation of brain cells (Kim, PM *et al.*, 2005). In excess D-serine has been shown to promote cell death, through over-excitation of the NMDA receptor (Aschner *et al.*, 1999). Thus there is a need to identify the factors involved in manipulating D-serine metabolism (release, uptake and breakdown) in the brain.

The D-serine synthesising enzyme serine racemase (SR) appears to be central to the regulation of extracellular D-serine levels. First discovered in the late 1990s, this enzyme is known to be present in both in glia and neurones (Wolosker *et al.*, 1999a; Wolosker *et al.*, 1999b; Kartvelishvily *et al.*, 2006). Under physiological conditions the conversion of L-serine to D-serine predominates (V_{max}: 5µmol/mg/h; K_m: 9.8mM), while a surprising reverse reaction of D-serine to L-serine can also occur (V_{max}: 22µ mol/mg/h; K_m: 60mM). Due to the much higher Km value in the direction of D- to L-serine, the enzyme should predominantly make D-serine but the rate of the reverse reaction is much faster when it does occur. Further study of SR revealed that racemisation of L-serine could be regulated by a number of divalent cations including Mg^{2+} and Ca^{2+}, as well as ATP and co-proteins GRIP (proteins glutamate receptor interacting protein), protein interacting with C-kinase (PICK1) and PLP (pyridoxal 5′-phosphate) all of which influence the racemisation of L-serine to D-serine and basal D-serine concentrations. The exact mechanisms by which SR regulation results in altered extracellular D-serine levels is not fully understood

(Wolosker *et al.*, 1999b; Kim, PM *et al.*, 2005; Fujii *et al.*, 2006). Stimulation of this enzyme by the co-factors leads to D-serine release in the brain and removal of co-factors of SR causes a loss in D-serine levels in the extracellular space (Cook *et al.*, 2002).

The ionotropic glutamate agonists alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate all cause D-serine release from cortical neuronal cultures, where SR is over-expressed (Kartvelishvily *et al.*, 2006). Similar patterns of release are observed in glial cultures for AMPA receptor activation but NMDA receptor activation is known to reduce extracellular D-serine levels (Kim, PM *et al.*, 2005; Mustafa *et al.*, 2007). Both of these alterations result from modification of SR. AMPA receptor activation causes the dislocation of glutamate receptor interacting protein (GRIP), which when free can bind SR to trigger D-serine synthesis (Kim, PM *et al.*, 2005). NMDA receptor activity on the other hand inhibits SR through S-nitrosylation of the enzyme and results in reduced D-serine release in cell cultures (Mustafa *et al.*, 2007). Kainate and ionophore A23187 can also cause D-serine release by boosting SR activity through increased intracellular calcium which results in as much as 21μM D-serine release from glial cultures (Cook et al 2002). As may be expected the removal of calcium results in inhibition of D-serine release (Mothet *et al.*, 2005).

By far the main concern with these findings is their limitation to cultured cells. *In vitro* or *in vivo* brain models are much closer representations of the brain in the sense of the tightly packed cellular structure and physiological milieu. A limitation in the technology used to monitor D-serine levels is also a major contributor to many of the questions relating to D-serine signalling in the brain remaining unanswered. Real-time D-serine detection in intact brain tissue is not possible with HPLC and very difficult with capillary dialysis. D-serine biosensors provide significant advances over these techniques allowing both speedy detection of D-serine (in the second timeframe) and sensitive measurements in a selective manner.

Here we use D-serine biosensors to determine the influence of ionotropic glutamate receptor agonists on D-serine signalling in the brain and ultimately NMDA receptor excitability. Recordings were made from the cortex and 2 regions with the hippocampus (*s. radiatum* and *s. pyramidale*) and the influence of calcium (both

intracellular and extracellular) on D-serine metabolism will be explored. Since all of the ionotropic glutamate receptors contribute to learning and memory, the role of D-serine in long term potentiation (LTP) will be examined.

5.3 Methods

5.3.1   *In vitro* slice preparation

Slice preparations are as described in chapter 3. Male Sprague-Dawley rats aged 12-21 days were used and 500μm horizontal hippocampus/cortex slices were prepared (Dale *et al.*, 2000). D-serine biosensors of 0.5mm length and 50μm diameter were used in all studies, inserted into the slice, allowed to stabilise for 20 minutes before application of drugs. In all cases a null biosensor was used (a sensor without enzyme in the bilayer) to ensure accuracy of recordings.

5.3.2   Regions of interest: hippocampus and cortex

Recordings were made in the cortex, *s. radiatum* and *s. pyramidale* of hippocampus. The regions were purposely selected to represent functionally and structurally different areas of the brain, in the context of D-serine tone (Chapter 3). In the cortex, basal D-serine concentrations are saturating at the modulatory glycine site, this region can be compared with *s. pyramidale* of the hippocampus, which also has high D-serine levels. *S. radiatum* on the other hand has lower levels of D-serine and the modulatory glycine site is not fully saturated. Hence comparisons can be made between two regions within a single structure (*s. radiatum* and *s. pyramidale*), two areas containing high levels of D-serine (*s. pyramidale* and cortex) and areas of low D-serine tone vs. high D-serine tone (*s. pyramidale* vs. *s. radiatum* and *s. radiatum* vs. cortex) for a more detailed investigation.

5.3.3   Synaptic transmission and ionotropic glutamate receptor agonist
            concentrations

Field EPSPs (fEPSPs) were evoked by stimulating the Schaffer collateral commissural pathway with small impulses (150μA) at 15s intervals and subsequently recorded from *s. radiatum* of CA1. As well as monitoring synaptic communication, the fEPSP allowed assessment of the healthiness of a slice and the reversibility of

drug treatment. The concentrations of AMPA-5µM, NMDA-20µM and kainate-12.5µM used, are maximal concentrations for which the fEPSP fully recovers and non-physiological D-serine release as a result of cell death is avoided. At higher concentrations of AMPA (10µM) and kainate (25µM) some cell death occurs (Figure 1). The physiological effect of which is failure of the fEPSP to return to the initial strength, as can be seen for 10µM AMPA and 25µM kainate. Data from Arias et al also confirms that 10µM AMPA (and 30µM NMDA) is toxic to the slice *in vitro* (Arias *et al.*, 1999).
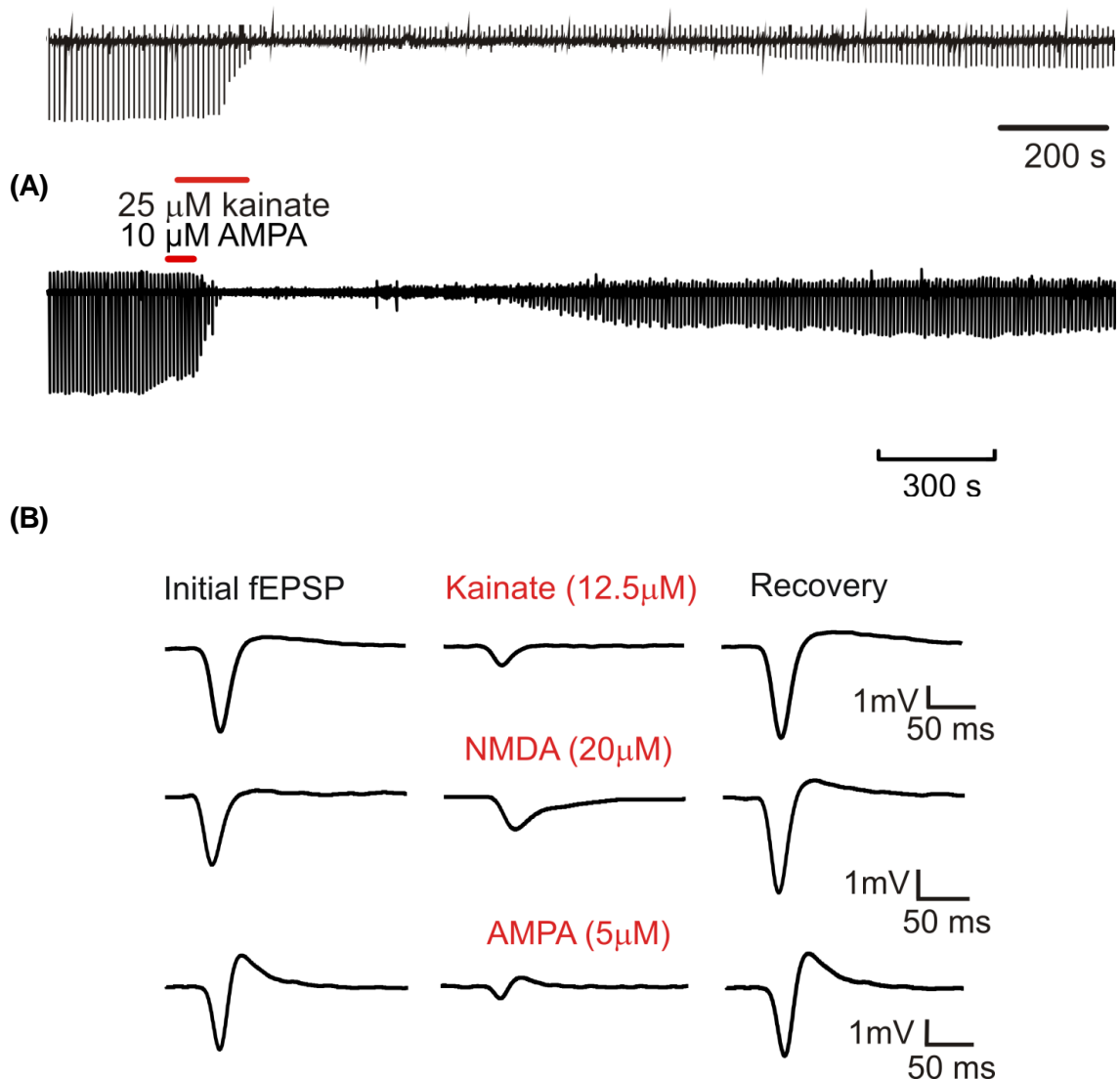
Figure 1: 10µm AMPA and 25µM kainate is toxic to the slice *in vitro*. At these higher concentrations of agonist AMPA and kainate, the fEPSP does not fully recover, a sign of irreversible toxic changes in the slice as result of exposure to drugs (A). Part B displays single examples of fEPSPs observed intially, in the presence of non-damaging concentrations of AMPA, NMDA and kainate and also the recovery of synaptic transmission after these drugs are washed out.

5.3.4   Analysis of recordings

Traces from D-serine biosensors were analysed as shown in figure 2. A null sensor (not containing DAAO enzyme) is used alongside a D-serine (or glutamate) biosensor. The subtraction of the null trace from the D-serine (or glutamate) trace is used to represent the actual change observed in the slice. The red bar represents the application of a drug in all traces and the release or loss of a signal is taken as the difference between the initial levels minus the peak of any change that occurs (loss or release), shown by the arrows in figure 2. All data was sampled at 10 kHz and filtered at 1Hz and 3kHz using a A/D board. The acquisition software Signal was used for all data acquisition.
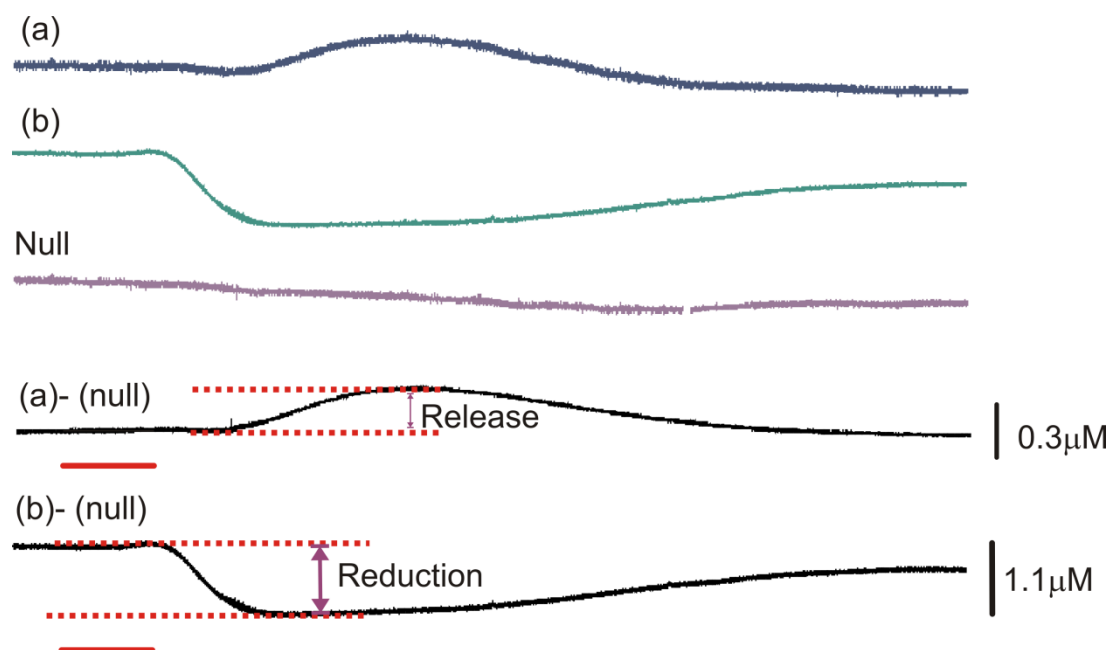
Figure 2: Null sensor traces are subtracted from the D-serine trace to visualise an actual change observed with the biosensors. The null biosensor trace is subtracted (a) and (b) to give the 'actual' trace to be used for further analysis. Release in neurotransmitter is taken as the difference between the initial basal levels and the peak in rise observed. Loss similarly is the difference between the initial concentrations and the greatest level of reduction seen.

Once the release or loss has been calculated, additional information can be gathered regarding the kinetics of the changes that occur. For example, the time taken for the loss or release to occur and the time taken for steady levels to be reached, may not the same. (x) Represents the time taken for the change to peak, while (y) represents the time taken for the D-serine (or glutamate) levels to return to baseline (red dotted

line). This may provide information about mechanism kinetics involved in restoring baseline levels of a neurotransmitter.
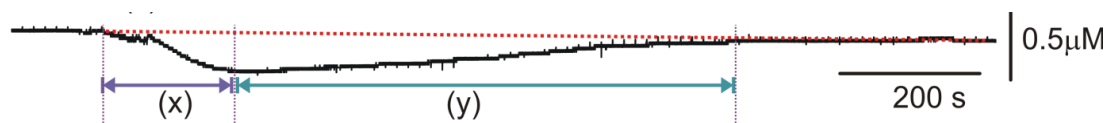
Figure 3: Kinetics of changes observed in D-serine. The time taken for the change to peak from the initial levels has been denoted (x), while the time taken for these levels to return to initial levels has been denoted (y). This indicates that mechanisms enabling change x are faster than those initiating change y.

5.3.5 Rhod2 staining

Hippocampal slices of 200μm thickness were prepared as described above. Rhod2 was prepared by dissolving 50μg of the dye in 2μl of dimethylsulfoxide and 440μl distilled water at room temperature. Slices were transferred to a small slice chamber filled with this solution, and continually oxygenated. After an incubation of 40 minutes slices were washed in normal aCSF and contained in this solution in the dark until required. Rhod2 was excited at 540± 10nm and emission was measured at 610nm wavelength from a number of cells around the biosensor.

5.4 Results

D-serine can be released by ionotropic glutamate agonists AMPA, NMDA and kainate in neuronal culture studies from cells over-expressing SR (Kartvelishvily *et al.*, 2006). Here we test these findings in normal *in vitro* slices using the D-serine biosensor to measure real-time changes in D-serine levels in the hippocampus and cortex with bath applications of these agonists.

5.4.1 Does acute AMPA receptor activation alter D-serine levels?

AMPA receptors along with NMDA receptors mediate the bulk of fast excitatory synaptic transmission in the brain and activation of this channel has been shown to cause D-serine release, both neuronal and from glial cultures (Kim, PM *et al.*, 2005; Kartvelishvily *et al.*, 2006). In agreement with these studies, D-serine is released in the cortex as a result of acute AMPA receptor activation *in vitro*, +0.5±0.1μM, n=9. The recovery of the fEPSP however occurs much later than the recovery in D-serine

concentrations, possibly as a result of simultaneous reductions in glutamate levels, which recover much more slowly.

However, we report a surprising heterogeneous response of D-serine to acute AMPA receptor activation in the hippocampus. Both release of D-serine and reduction in concentration can result from acute AMPA receptor activation, indicating presence of 2 different mechanisms. In *s. pyramidale* of hippocampus release of D-serine is observed and in a couple of cases a reduction in D-serine concentrations also occurs, the overall change in D-serine levels observed is positive +0.2 ±0.1μM, n=9. An example trace is shown in figure 4b. Simultaneous recordings of glutamate were also made and levels reduced with acute AMPA receptor activation. D-serine release is observed within seconds of AMPA application, with peak levels coinciding with lowest concentrations of glutamate. In *s. radiatum* however, D-serine levels are reduced much more so, with a negative overall change -0.19 ± 0.0μM, n=14. The reduction in fEPSP is also observed, which recovers as D-serine levels begin to return to initial basal concentrations (figure 4c). This decrease occurs at a much faster rate (110±12s, n=14) compared to the eventual recovery in D-serine levels (364±49s, n=14), suggesting that the signalling mechanisms responsible for the loss of D-serine are much faster than the signalling pathway responsible for the recovery in D-serine levels.

Figure 4: Acute AMPA receptor activation can trigger a reduction in extracellular D-serine and D-serine release in different brain regions. Red bar represents an application of 5µM AMPA for 1 minute in two areas of the hippocampus: *s. radiatum* and *s. pyramidale* and the cortex. fEPSP was recorded in *s. radiatum* in all cases and can be observed on the microelectrode. (a) D-serine is released in the cortex (b) D-serine release and simultaneous reductions in glutamate are seen in *s. pyramidale* (c) D-serine levels are reduced with AMPA receptor activation in *s. radiatum* (d) change in D-serine concentration is plotted as a cumulative probability graph.

The cumulative probability plot of this data best highlights the diversity in changes that occur in D-serine levels as a result of acute AMPA receptor activation. To answer the question set at the beginning of this section: can AMPA receptor activation alter extracellular D-serine concentrations? The answer most definitely is yes but what is more interesting is that this change is not uniform throughout the brain or even within a single brain region implying the activation of more than one signalling cascades. Release of D-serine is observed in *s. pyramidale* and cortex while in the *s. radiatum* D-serine levels decrease. In *s. radiatum* (blue line) the action of AMPA resulted in a decrease in D-serine concentration while the majority of the data points in *s. pyramidale* (red line) are positive, showing D-serine release. There is one occasion when release is observed in s. *radiatum* and a loss of D-serine levels is seen in *s. pyramidale*. This may be an anomaly due to sensor placement, since the physical difference between *s. radiatum* and *s. pyramidale* is not much greater than the diameter of the D-serine microelectrode (50µm).

We explored the link between D-serine release and changes in intracellular calcium levels. In the cortex, D-serine release may be linked to an increase in intracellular calcium in astrocytes, as observed by a Rhod 2 calcium signal. An increase in calcium is observed prior to D-serine release, as shown in figure 5.
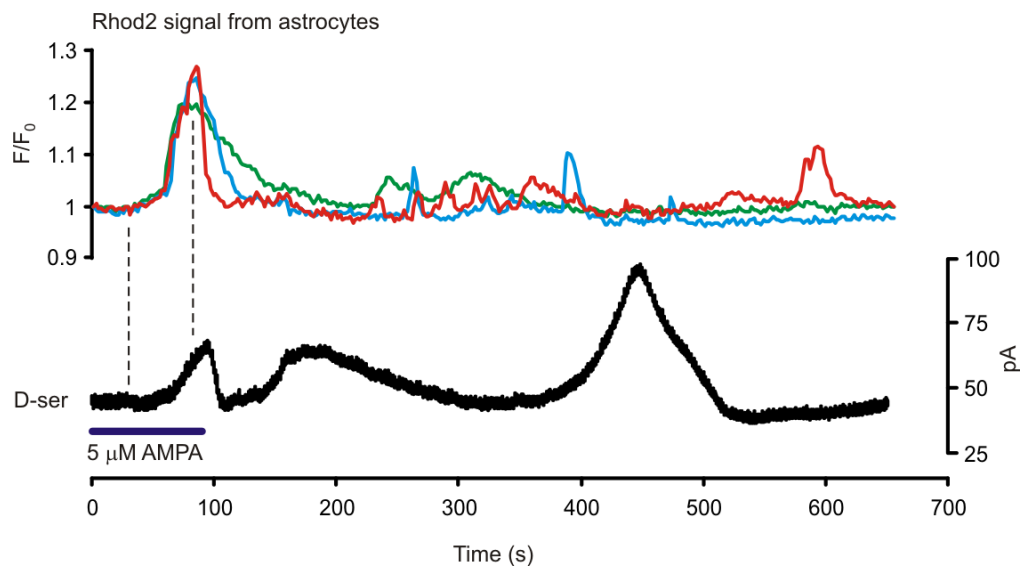
Figure 5: AMPA receptor activation causes D-serine release through a change in intracellular calcium as observed by a Rhod2 signal. D-serine biosensors were used in conjunction with histo-chemical staining techniques to investigate whether D-serine release precedes changes in calcium. Bath application of AMPA (blue bar) causes an increase in intracellular calcium levels in astrocytes as observed by the Rhod 2 signal. Release of D-serine precedes this.

However, when attempts were made to boost intracellular calcium using pharmacological compounds Adenosine-5'-γ-thiotriphosphate (γ-ATP), methylthioadenosine 5'-diphosphate (MeS-ADP), and ionophore A23187 little change in D-serine concentrations was seen. Cook et al have shown that as much as 21μM D-serine can be released from primary astrocyte cultures using ionophore A23187, with suggestions that direct binding of calcium to SR, increases the synthesis of D-serine and its release into the extracellular space (Cook *et al.*, 2002). We saw little evidence of this in our *in vitro* brain slice model (n=7). Although, the pharmacological effects of the drug can be observed by the increase in the fEPSP (figure 6), as a result of increased intracellular calcium. Little change in the fEPSP is observed with the addition of 1.5μM of ionophore A23187, suggesting that 500nM effectively saturates the physiological effect of this compound.
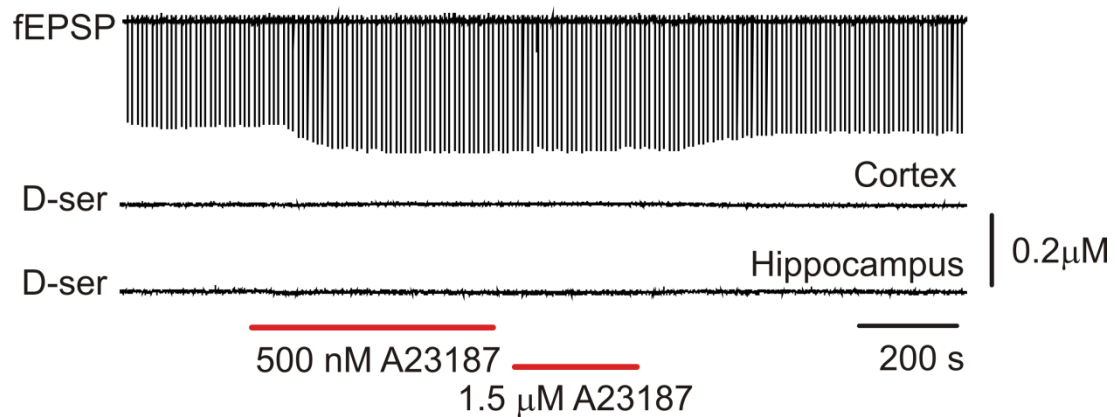
Figure 6: Increasing intracellular Calcium with A23187 does not alter D-serine levels in the hippocampus and cortex. *Application of 500nM and 1.5μM of the calcium augmenting compound A23178 does not cause D-serine release in vitro. However, the pharmacological effect of the drug is almost instant, with the field EPSP increasing as a result of increased calcium levels. Fields recordings were made in the hippocampus with simultaneous biosensors recordings from the cortex and hippocampus.*

MeS-ADP (60 μM), an agonist at $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors and γ-ATP (100μM), an analogue of ATP can both boost intracellular calcium levels. Although, the pharmacological effects of these drugs can be observed on synaptic transmission, little change in D-serine levels was observed in the hippocampus and cortex with MeS-ADP (n=7) or γ-ATP (n=5).

Hence, although changes in intracellular calcium are important to D-serine release with acute AMPA receptor activation we find little evidence for D-serine release

triggered by non-specific amplifications of intracellular calcium by pharmacological compounds *in vitro*.

5.4.2 Does acute NMDA receptor activation alter extracellular D-serine concentrations?

The agonist NMDA (20µM) can be used to distinguish the NMDA receptor from other ionotropic glutamate receptors. It is proposed that NMDA receptor activation can reduce D-serine levels in the cultured cells, by down-regulation of the D-serine synthesising enzyme SR though S-nitrosylation (Mustafa *et al.*, 2007). Our findings present a more complex and multifaceted series of events triggered upon acute NMDA receptor activation *in vitro*, as observed by D-serine biosensors.

In the cortex, D-serine is released with acute activation of NMDA receptors, $+1.1 \pm 0.3\mu M$ (n=9). D-serine levels rise swiftly, upon the NMDA agonist reaching the slice, with a peak observed as the fEPSP dimishes (figure 7a). Following this D-serine levels fall and evventually return to the initial concentrations. The released D-serine concentrations are greater than those observed for AMPA receptors (4a), which indicates that NMDA receptor activation is a greater stimulus for D-serine release mechanism in the cortex.

In the hippocampus, acute activation of NMDA receptors interestingly had two different consequences on the extracellular D-serine concentration in *s. pyramidale*. Both loss of D-serine is seen and D-serine release (7d, red line). Overall, a loss in D-serine levels is seen $-0.33 \pm 0.2\mu M$ (n=9) with a bath application of NMDA and an immediate loss of synaptic transmission also occurs. Both mechanisms (for loss and release) are present in *s. pyramidale* and they can be singly triggered by NMDA to give opposing D-serine signals. Of note also is the D-serine release is observed with acute AMPA receptor activation in this region, compared with the loss seen with NMDA receptor activation. Since we confirmed above that two mechanisms are present in the hippocampus, one to regulate deficits in D-serine concentration and another to cause D-serine release, this variation in the responses seen with AMPA and NMDA agonist may indicate the preferential activation of the D-serine reducing mechanism by NMDA and D-serine release mechanism by AMPA.
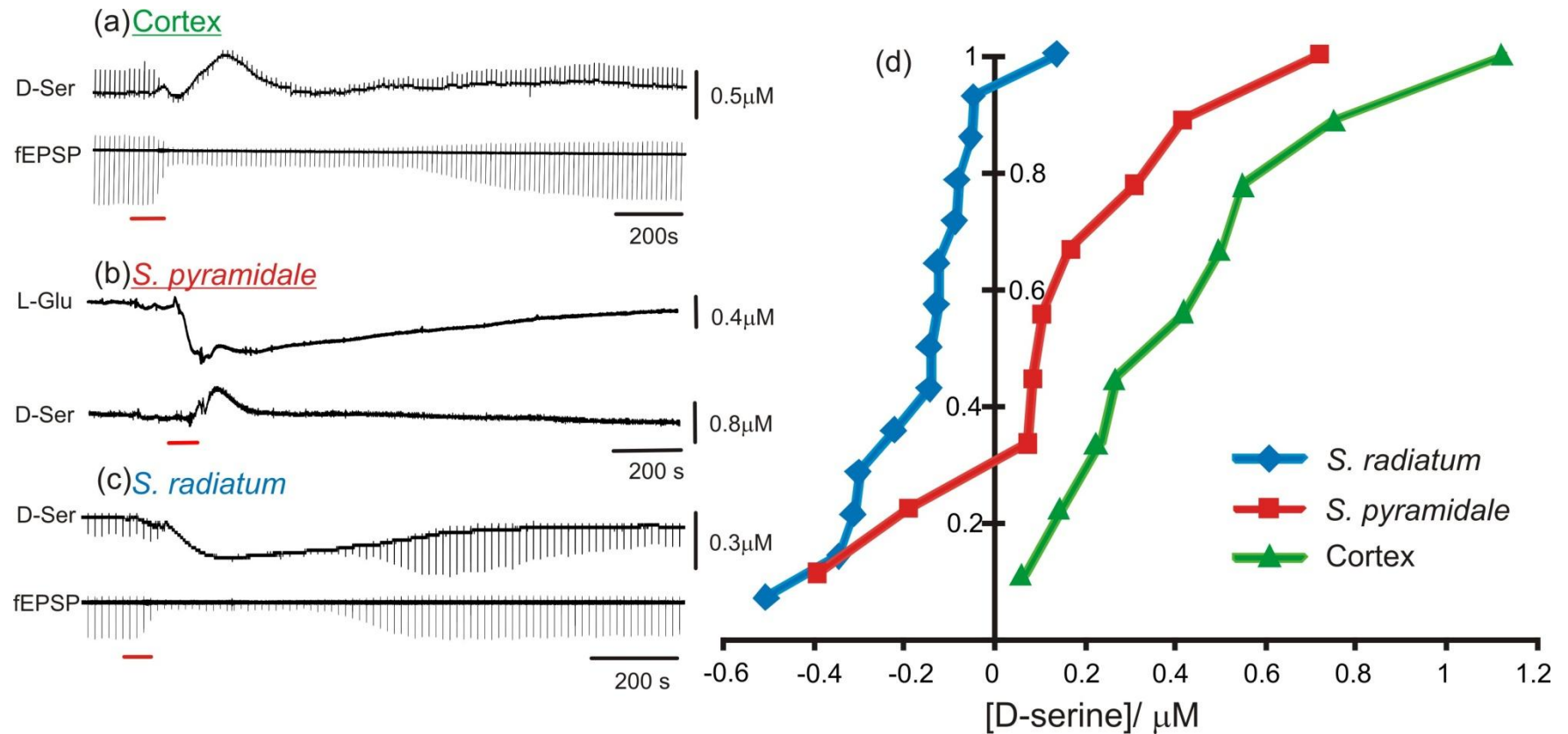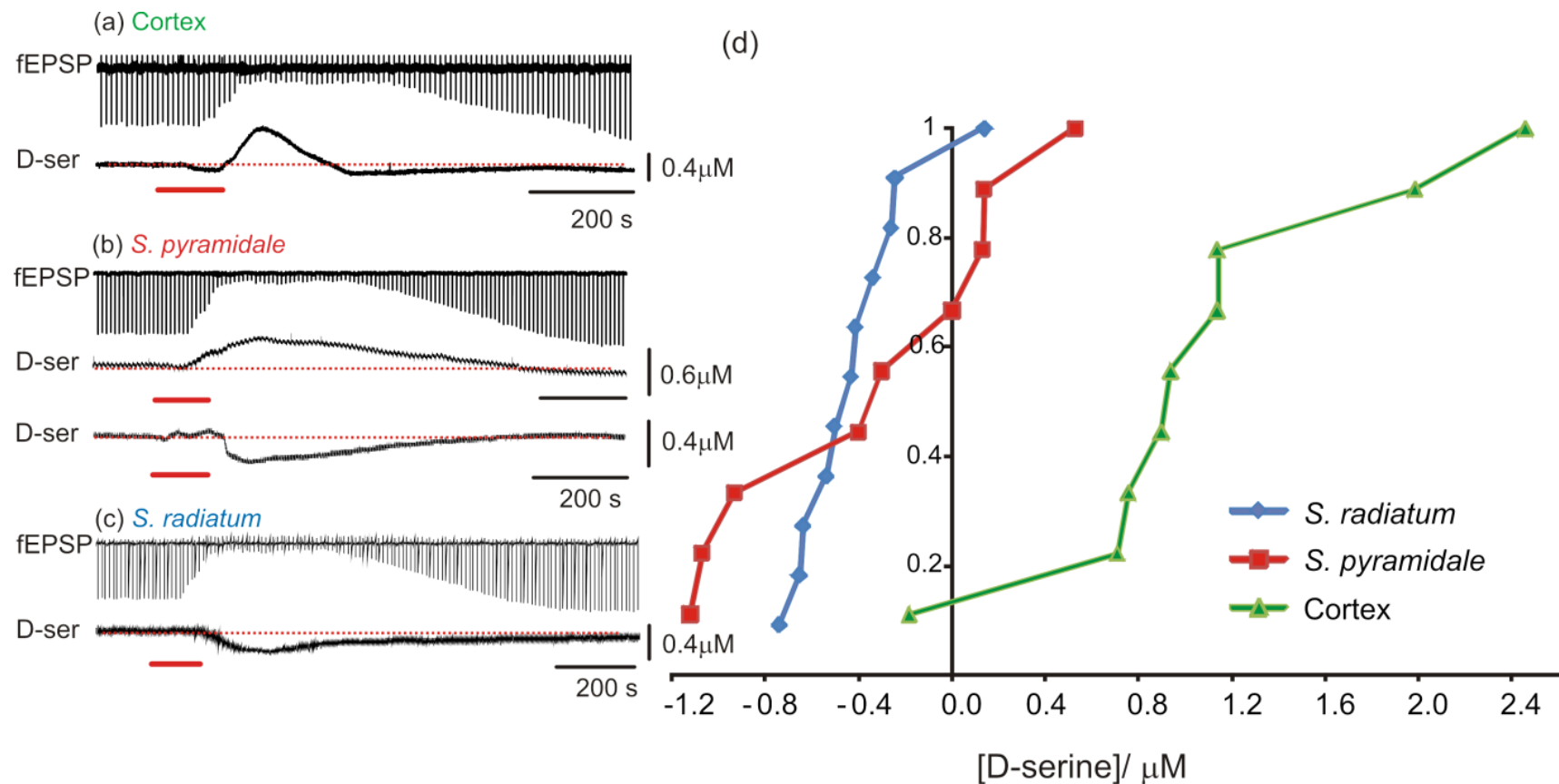
Figure 7: Acute NMDA receptor activation can trigger a reduction in extracellular D-serine and D-serine release in different brain regions. Red bar represents an application of 20μM NMDA for 2 minutes in two areas of the hippocampus: *s. radiatum* and *s. pyramidale* and the cortex. fEPSP was recorded in *s. radiatum* in all cases and can be observed on the microelectrode. (a) D-serine is released in the cortex (b) D-serine release and reduction in D-serine levels are seen in *s. pyramidale* (c) D-serine levels are reduced with NMDA receptor activation in *s. radiatum* (d) change in D-serine concentration is plotted as a cumulative probability graph.

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In *s. radiatum* (as with AMPA receptor activation) acute NMDA receptor activation causes a loss in D-serine levels (-0.42 ±0.07μM (n=11), as seen in figure 7c. This reduction in D-serine occurs in a similar pattern to that observed in figure 4c, above, for AMPA receptor activation. It may be that acute AMPA and NMDA receptor activation triggers the same mechanism, resulting in the loss of D-serine observed in both cases. Additionally, as seen with AMPA the fall from base levels it much faster (144±16s, n=11) compared to the slow recovery in D-serine levels (480±39, n=11). Synaptic transmission is terminated as observed by the fall in the fEPSP, similar to that seen under acute AMPA receptor activation.

The cumulative probability plot (7d) shows that D-serine levels can be altered by acute NMDA receptor activation. More D-serine release and more severe reduction in D-serine concentrations are triggered with NMDA compared with AMPA in the cortex and *s. radiatum*. This may be an indicator of acute NMDA receptor activation being a superior stimulus than AMPA receptor activation. But NMDA receptors preferentially cause loss in D-serine observed in *s. pyramidale* while AMPA receptors preferentially stimulate D-serine release. The functional consequence of extracellular changes in D-serine triggered by acute AMPA and NMDA receptor activation on the modulatory glycine site will also vary.

5.4.3   Does acute kainate receptor activation alter extracellular D-serine levels?

Kainate receptor activation alters D-serine levels in a similar pattern to that observed with NMDA and AMPA. Bath application of 12.5μM kainate for 2 minutes caused D-serine release in the cortex, +1.1 ± 0.4μM, n=9 (figure 8a). In *s. pyramidale* a loss of D-serine was observed, -0.16±0.1μM, n=9 (figure 8b), similar to that seen for NMDA, a few experiments showed D-serine release under the same conditions. In *s. radiatum*, both loss and release of D-serine has been seen with the activation of kainate receptors, so that the average change is very small, +0.02±0.1μM, n=9 (figure 8c). Although the median and mean data in *s. radiatum* shows little change, in fact, the extent of release and loss is similar to that seen in *s. pyramidale* (figure 8d).
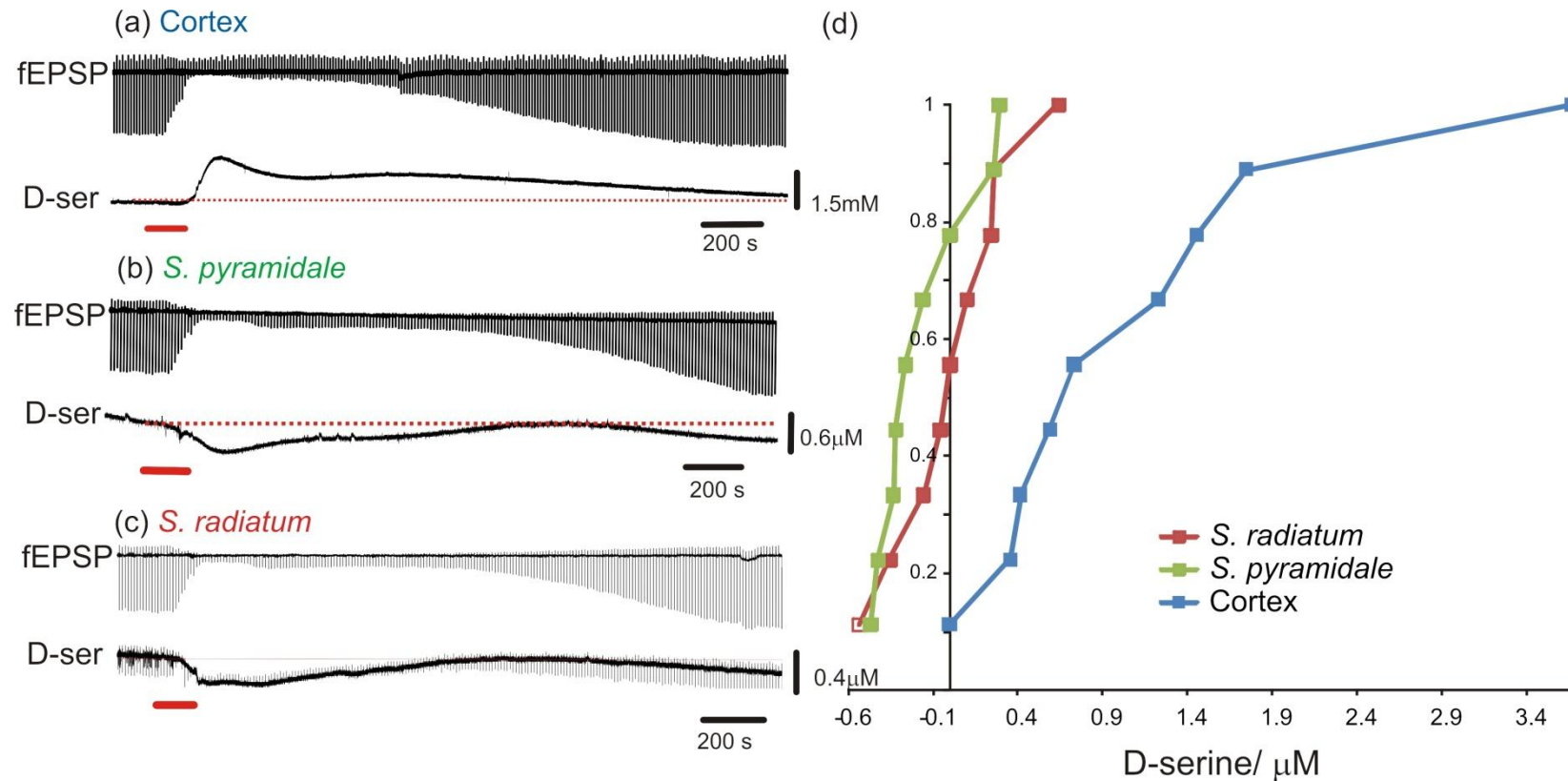
Figure 8: Acute kainate receptor activation can trigger a reduction in extracellular D-serine and D-serine release in different brain regions. Red bar represents an application of 12.5μM kainate for 2 minutes in two areas of the hippocampus: *s. radiatum* and *s. pyramidale* and the cortex. fEPSP was recorded in *s. radiatum* in all cases and can be observed on the microelectrode. (a) D-serine is released in the cortex (b) D-serine reduction is seen in *s. pyramidale* (c) D-serine release and loss is observed in *s. radiatum* (d) change in D-serine concentration are plotted for all three brain regions as a cumulative probability graph.

Acute kainate receptor activity alters D-serine levels in the brain differently depending on the brain region studied and is certainly more complicated than has been previously suggested by studies conducted in cultured cells. The release of D-serine in the cortex is similar to that observed with NMDA, indicating similar abilities of the two agonists in stimulating the D-serine release mechanism. In *s. pyramidale* the reduction in D-serine is less compared with NMDA, signifying that NMDA stimulates the reduction mechanism more so than kainate.

5.4.4 Can extracellular alterations in Ca^{2+} ions alter D-serine signalling mechanisms?

Intracellular and extracellular changes in Ca^{2+} can play a role in a wide variety of processes in the brain. Locally Ca^{2+} signalling controls neurotransmitter release, while globally it is used to regulate synaptic strength and accomplish postsynaptic processing. In glia Ca^{2+} ions are used to convey long-range signalling by means of propagating Ca^{2+} waves and control the release of glio-transmitters (Verkhratsky *et al.*, 2009). Hence it is not surprising that a number of studies have shown that D-serine can be released by agents that augment intracellular calcium, though we have not found any evidence for this here (Mothet *et al.*, 2005). But since the enzyme SR requires Ca^{2+} as a co-factor and it is intrinsically involved in the D-serine release, by unknown mechanisms (Mothet *et al.*, 2005), we considered the Ca^{2+}-dependence of the changes in D-serine levels induced by the ionotropic agonists AMPA and NMDA. Figure 9 shows an example trace, calcium free aCSF (with EGTA, a calcium-chealator) was applied to the slice, the immediate effect of which is termination of synaptic transmission (fEPSP). This is applied for 20-25 minutes to ensure full removal of calcium from the slice, before 5µM AMPA in calcium-free aCSF is applied to the slice for 1 minute (and in the case of NMDA, 20µM, for 2 minutes). This causes a reduction in D-serine levels occurs in *s. pyramidale*. Calcium is washed back in after 20 minutes and full recovery in fEPSP strength is observed. This indicates no lasting effect of the removal of calcium and acute AMPA (or NMDA) receptor activation on synaptic transmission.
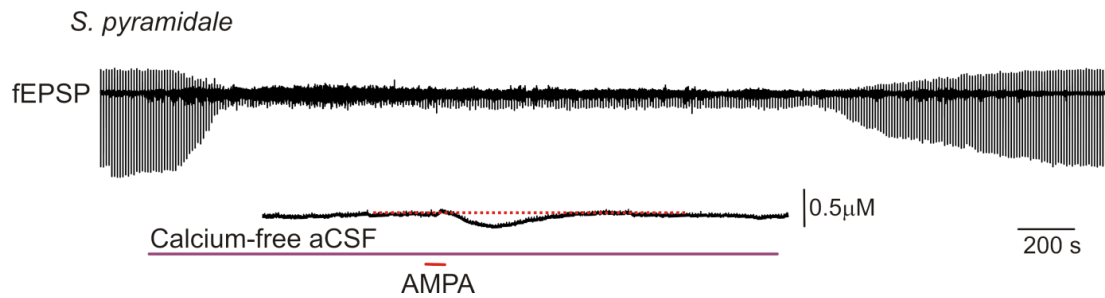
Figure 9: AMPA response in calcium-free aCSF in *s. radiatum* and *s. pyramidale*. AMPA still causes a loss of D-serine in calcium free aCSF in both areas of the hippocampus. The fEPSP is lost in calcium free-aCSF but this recovers fully with the removal of calcium-free aCSF.

Cumulative probability plots of the data gathered show clearly some dependence on extracellular calcium by the mechanisms regulating D-serine signalling triggered by AMPA receptor activation but not in all regions. D-serine signalling under calcium-free conditions in *s. radiatum* remains unaffected as shown in figure 10, with the average change of $-0.2\pm 0.0\mu M$, n=7, compared to $-0.2\pm0.0\mu M$, n=9 seen with the presence of calcium.

But much of the D-serine release observed in *s. pyramidale* ($+0.2\pm0.1\mu M$) is changed instead to a reduction ($-0.2\pm0.1\mu M$, n=7), suggestive of a central role of calcium in determining whether D-serine release occurs or loss. In the cortex, D-serine release ($0.5\pm0.1\mu M$) is severely diminished ($+0.15\pm0.07\mu M$, n=7) and is some cases no change in D-serine levels occurs at all. Earlier studies showed that an intracellular rise in calcium in the cortex precedes D-serine release, as a result of AMPA receptor activation; we now confirm that both intracellular and extracellular calcium is important in the release of D-serine from the cortex. To summarise, the mechanisms that evoke D-serine release as a result of AMPA receptor activation depend on extracellular calcium while the loss of D-serine occurs by a mechanism independent of extracellular calcium.
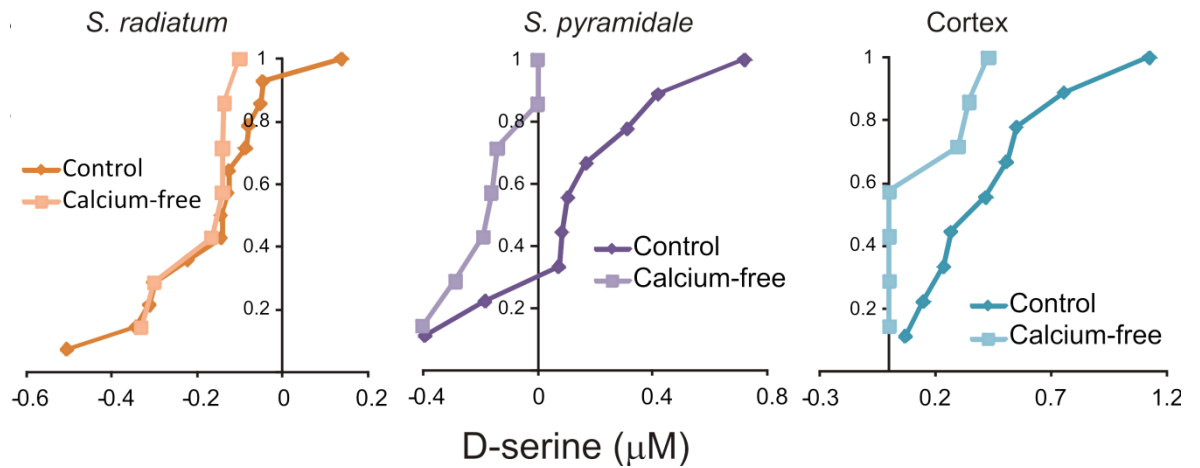
Figure 10: AMPA dependent D-serine signalling is calcium-sensitive in *s. pyramidale* and the cortex. Cumulative probability traces show that under calcium free conditions AMPA receptor dependent D-serine release is reduced in the cortex and s, pyramidale, while the little change is observed in s. radiatum.

Under calcium-free/acute NMDA receptor activation, D-serine release in *s. radiatum* ($-0.42\pm0.07\mu M$) is severely affected, with much of the reduction in D-serine no longer observed ($-0.12\pm0.04\mu M$, n=5). Signalling in *s. pyramidale* appears to be largely unaffected as can be seen in figure 11, while the average signalling changes of $-0.3\pm0.2\mu M$ in the presence of calcium is reduced slightly to $-0.1\pm0.2\mu M$, n=5, under calcium free conditions However, much of the D-serine release ($+1.1\pm0.3\mu M$) observed with NMDA in the cortex is lost, instead loss of D-serine in the cortex occurs ($-0.1\pm0.2\mu M$, n=8). This data is summarised in Table 5.1.
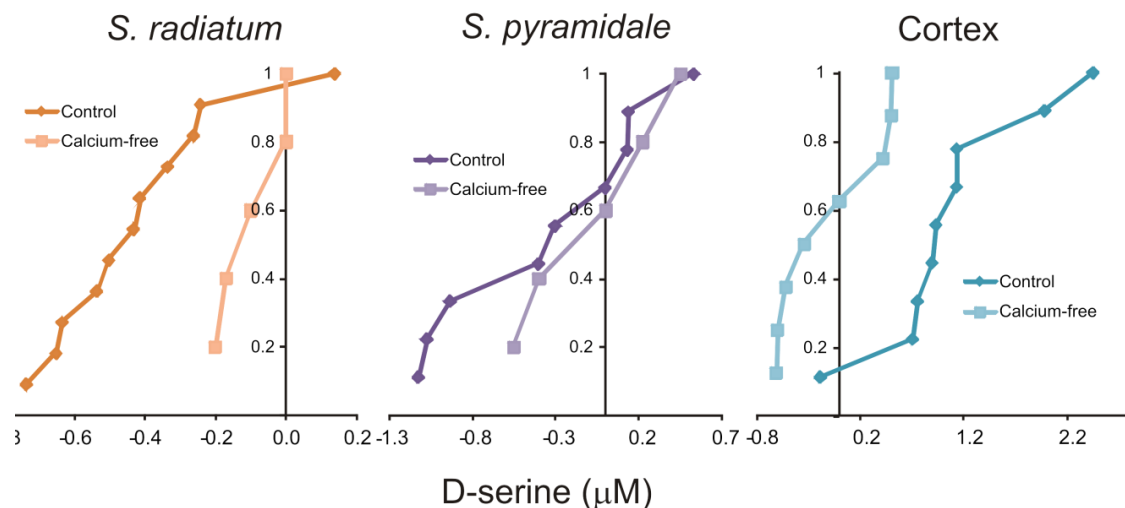
Figure 11: NMDA dependent D-serine signalling is calcium-sensitive in *s. radiatum* and the cortex. Cumulative probability traces show that under calcium free conditions NMDA receptor dependent D-serine release is reduced, while the reduction in s. radiatum is less. In s. pyramidale very little change occurs.

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	Presence of calcium	Calcium-free
s. radiatum-AMPA	-0.2±0.0µM	-0.2±0.0µM, n=7
s. pyramidale-AMPA	+0.2±0.1µM	-0.2±0.1µM, n=7
Cortex-AMPA	+0.5±0.1µM	+0.15±0.1µM, n=7
s. radiatum-NMDA	-0.42±0.1µM	-0.12±0.0µM, n=5
s. pyramidale-NMDA	-0.3±0.2µM	-0.1±0.2µM, n=5
Cortex-NMDA	+1.1±0.3µM	-0.1±0.2µM, n=8

Table 5.1: Regulation of D-serine by acute NMDA and AMPA receptors is dependent on extracellular calcium in some regions of the brain.

5.4.5 D-serine and LTP

Since NMDA receptors play a vital function in the mediation of LTP/LTD, the underlying mechanisms of learning and memory, and D-serine is a required co-agonist at the NMDA receptor, we explored the changes induced in D-serine level during LTP. The slope of fEPSP was measured for 15 minutes before high frequency stimulation (HFS) was used to induce LTP. Ten trains of four 100Hz pulses each, separated by 100ms formed the tetanus applied (a theta burst), this caused an increase in fEPSP amplitude that was long-lasting (LTP), as shown in figure 12.
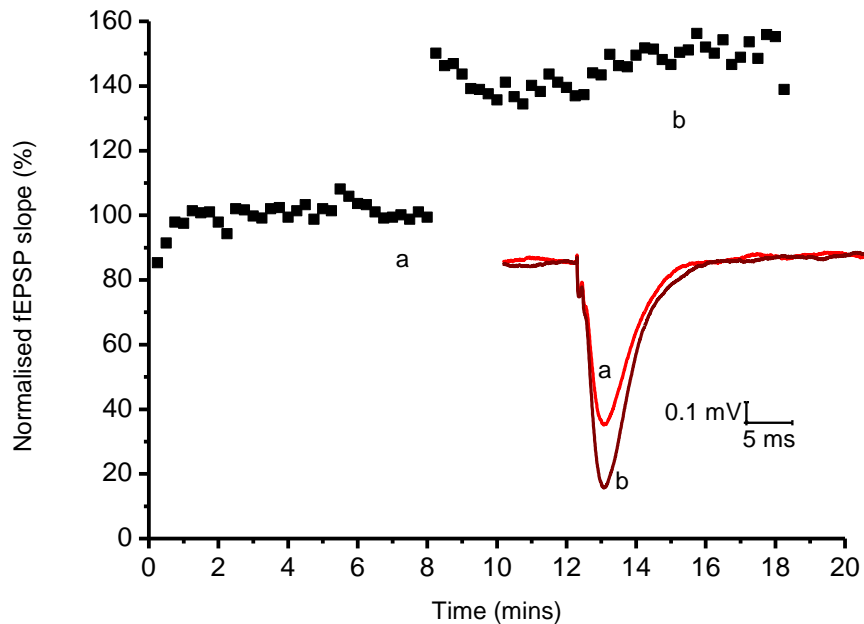
Figure 12: LTP can be achieved with high frequency stimulation. Time course of the normalised fEPSP and the spike in fEPSP amplitude induced with high frequency stimulation.

HFS induced D-serine release (0.73±0.4µM, n=5). Recordings were made in two regions of the hippocampus, and D-serine release lasts for 74±9 seconds (n=5) after the tetanus was applied. The release of D-serine appears to be too slow to partake in the LTP spike resulting from HFS but it may increase NMDA receptor activity in certain hippocampal sub-regions, such as *s. radiatum* where levels of D-serine are non-saturating.
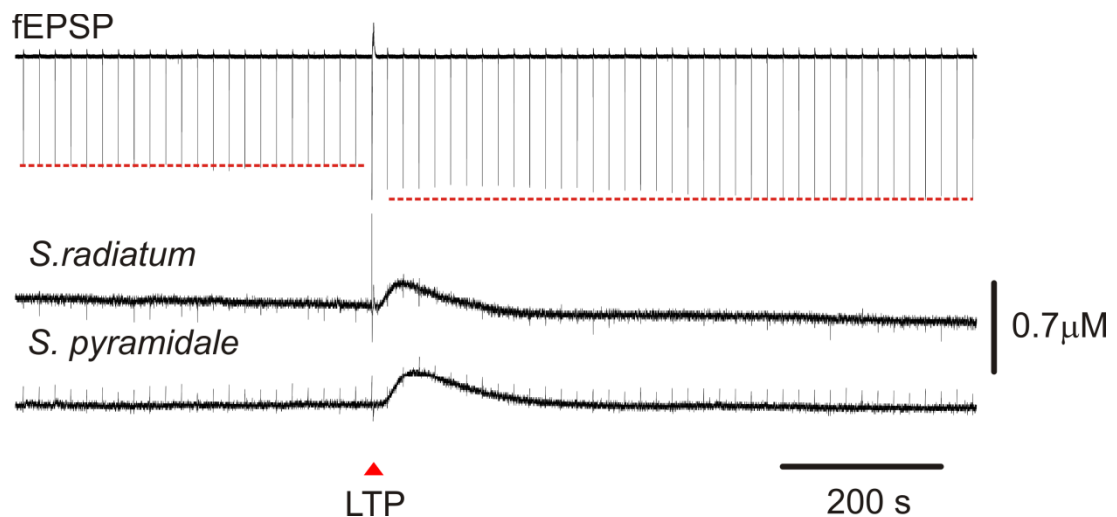
Figure 13: During LTP D-serine is released in the hippocampus, *s. radiatum* and *s. pyramidale*. The highest levels of D-serine release are observed on sensors placed closest to the stimulating electrode which was placed in CA *s. radiatum*.

5.5 Discussion

Changes in D-serine levels have been studied using various techniques, but D-serine biosensors provide perhaps the most advanced means by which this chemical transmitter can be studied in the brain. Here, we use these microelectrodes to observe activity dependent D-serine changes simultaneously from different brain regions and within a single structure in real-time. We report the most unexpected findings. Our data highlights the need for a revision of current methods used to study D-serine in the brain.

5.5.1 Ionotropic glutamate receptors influence D-serine concentration

We report the presence of multifaceted signalling events following inductions with ionotropic glutamate receptor agonists. Unlike previous studies which describe release of D-serine (with AMPA and kainate) or loss (with NMDA). Both release and loss of extracellular D-serine can be induced by ionotropic agonists in *s.*

pyramidale while only release is evoked in the cortex with all 3 agonists and overall a loss in D-serine concentrations is seen in *s. radiatum* with AMPA and NMDA. Figure 14 presents a cumulative probability plot of the different regions studied and regulatory changes brought on by the 3 ionotropic agonists. Since a change in D-serine concentration is likely to affect NMDA receptor potentiation ability, it can be concluded that both AMPA and kainate receptor activation can influence NMDA receptors, through the regulation of D-serine. NMDA receptors also appear to regulate D-serine concentrations; this could indicate the presence of negative feedback system in the hippocampus and a positive feedback mechanism in the cortex by which NMDA receptor events are controlled by the availability of D-serine.
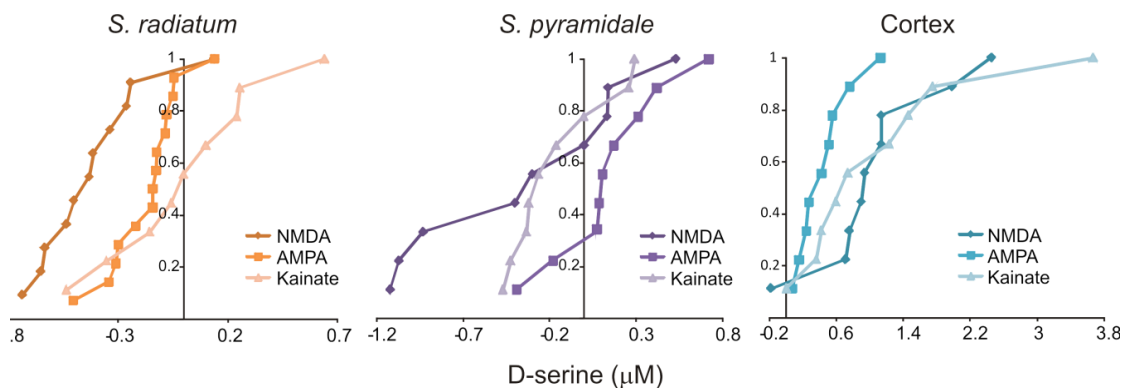
Figure 14: Changes in extracellular D-serine observed with ionotropic agonists in *s. radiatum*, *s. pyramidale* and cortex.

Since binding at the co-agonist site of NMDA receptors is necessary for activation, rationally it may be expected that increased D-serine release will potentiate NMDA receptor activity, while reduced D-serine levels could diminish receptor activation. But, this may not necessarily be the case in light of variations in basal D-serine levels and saturation states of the co-agonist site seen in different brain areas, as presented in chapter 3. Hence any changes in D-serine levels need to be considered in the context of the existing D-serine tone in order to determine their functional implications at the modulatory glycine site of the NMDA receptor. The regions where D-serine release is observed (cortex and *s. pyramidale*) are already saturated at NMDA glycine site (as discussed in chapter 3) and a reduction in D-serine levels occurs in where D-serine concentrations are not saturating.

For the purpose of analysis, median values will be taken to represent the whole data set as means/averages do not necessarily represent an observed event in the slice, as observed by the D-serine microelectrodes. Each data set is an actual change that occurs in the slice, so any point can be taken to determine its functional significance on NMDA receptor potentiation ability, theoretically. Often, these median values closely represent the mean change observed and will be used as an example to show the general implications of the changes in D-serine levels observed with ionotropic agonists, in the specific regions studied. Table 5.2 below gives the median changes observed, as well as being a useful measure for data that is not normally distributed, the median values also represent real-time observations; along with the D-serine tone in the different areas studied as determined in chapter 3.

	Tone (µM)	AMPA (µM)	NMDA (µM)	Kainate (µM)
S. radiatum	0.3	-0.1	-0.4	No effect
S. pyramidale	0.8	+0.1	-0.3	-0.3
Cortex	0.9	+0.4	+0.9	+0.7

Table 5.2: Median values for change detected with AMPA, NMDA and kainate, and the known D-serine tone in the regions examined.

5.5.2 Acute AMPA receptor activation can alter NMDA receptor co-agonist site saturation.

Changes in D-serine levels as a result of acute AMPA receptor activation show disparate functional significance at the NMDA receptor co-agonist site. Plotted on figure 14 are the basal D-serine levels observed in *s. pyramidale*, *s. radiatum* and cortex, with arrows showing the direction of change in occupancy levels, as a result of AMPA receptor activation.

Figure 15: Functional significance of the agonist AMPA on NMDA receptor occupancy. The co-agonist site occupancy levels are altered in a different way in the 3 regions of the brain studied. In s. *radiatum* co-agonist site occupancy decreases to 18-37% (NR2A-D respectively) for the different NR2 subunits; while the release of D-serine in the cortex and *s. pyramidale* does not alter site occupancy levels by much and hence NMDA receptor function is unlikely to be affected.

In *s. radiatum* percentage occupancy levels at the co-agonist site fall from 47-53% (for NR2A and NR2B containing NMDA receptors, most highly expressed in this region) to 18-23% respectively. This suggests a reduction in NMDA receptor excitability by a factor of 2 which under acute AMPA receptor activation may be beneficial, as over-excitation at the NMDA receptor is a well-known feature of cell death. Damage due to over-excitation of the NMDA receptor will be reduced as a result of low co-agonist availability. Structurally, *s. radiatum*, is the site of approximately 54% of all excitatory synapses and the highest density of dendritic spines and synapses of pyramidal neurones. The over-excitation of the NMDA receptor (under acute conditions) can potentially cause immense damage in the region. The reduction in D-serine levels seen here has the potential to moderate NMDA receptor activity and may be critical in the survival of the slice (i.e. neuro-protective).

In the *s. pyramidale* and cortex, D-serine is released. Almost twice as much D-serine release is observed in the cortex compared to *s. pyramidale* (+0.5±0.1μM and

+0.2±0.1μM respectively). However, in both regions this release appears to have little functional significance on the saturation levels of the glycine modulatory site. In *s. pyramidale* co-agonists site occupancy levels are almost saturated at approximately 85%, with the released D-serine altering this to 95% while in the cortex occupancy levels are increased from 90% to 95% irrespective of NR2 subunit composition of NMDA receptors. This change is unlikely to affect NMDA receptor activity by very much.

It is possible that this D-serine release is a by-product of over activity of SR, which makes 3 molecules of pyruvate for every D-serine molecule (De Miranda *et al.*, 2002; Strisovsky *et al.*, 2003). Pyruvate is a strong neuroprotectant in animal models of stroke, also protects cells agonist oxidative damage (Desagher *et al.*, 1997; Sheline *et al.*, 2000). Hence, pyruvate maybe the desired product, while D-serine synthesis and release is a by-product of increased SR activity. Additionally, the objective for this D-serine release may be for further breakdown to form pyruvate (by DAAO) or removal via the blood brain barrier. This latter pathway may account for high levels of D-serine detected in the urine of rodents and humans (Haung *et al.*, 1998; Foltyn *et al.*, 2005). Whatever, the purpose of this release, it is unlikely to have much functional significance at the NMDA receptor co-agonist site.

5.5.3   Acute NMDA receptor activation can alter NMDA receptor activity

Acute NMDA receptor activation causes loss of D-serine levels in *s. radiatum*. This loss has significant functional implications on co-agonist site occupancy levels, which are reduced from 47-53% (for NR2A and NR2B, most highly expressed here) to a low level. In fact the reduction in D-serine levels falls below the median basal levels of D-serine or the tone detected in this region (although the mean levels were found to be 0.4±0.1μM), indicating that NMDA receptor activity will be reduced significantly if not completely if acute NMDA receptor activation occurs under physiological conditions. This neuro-protective D-serine decrease will eliminate NMDA-receptor associated cell death in *s. radiatum*. The reduction in D-serine observed with NMDA (-0.43μM) is almost three times that seen for AMPA (-0.14μM). If the same mechanism is responsible for the reduction in D-serine levels, then acute NMDA receptor activation is a stronger stimulus for the activation of this
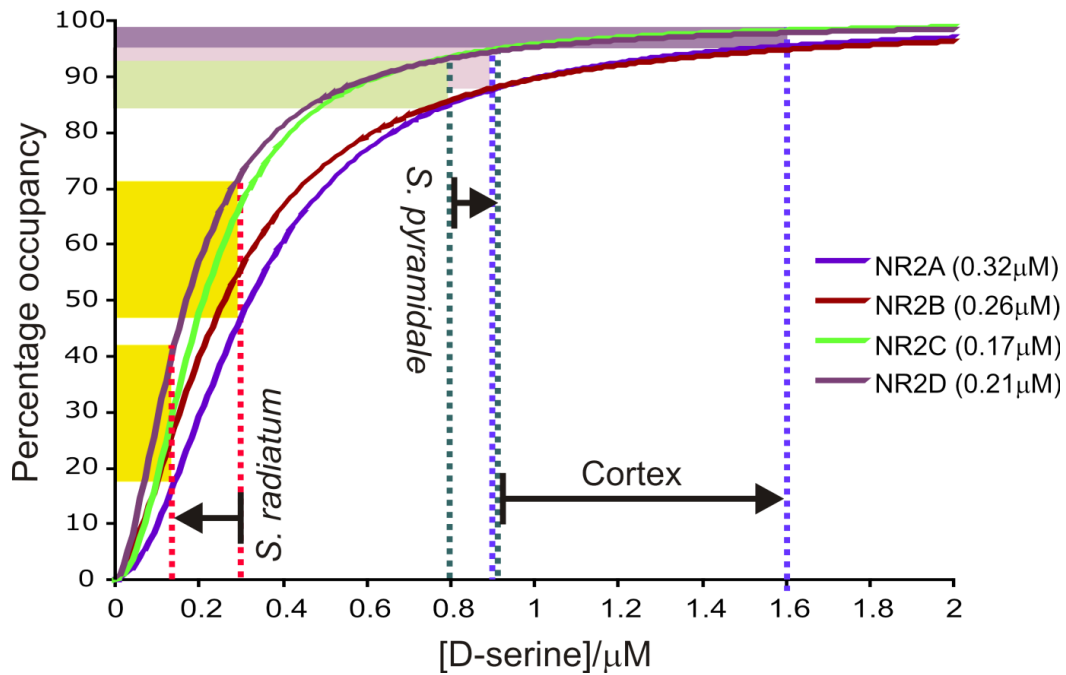
pathway.
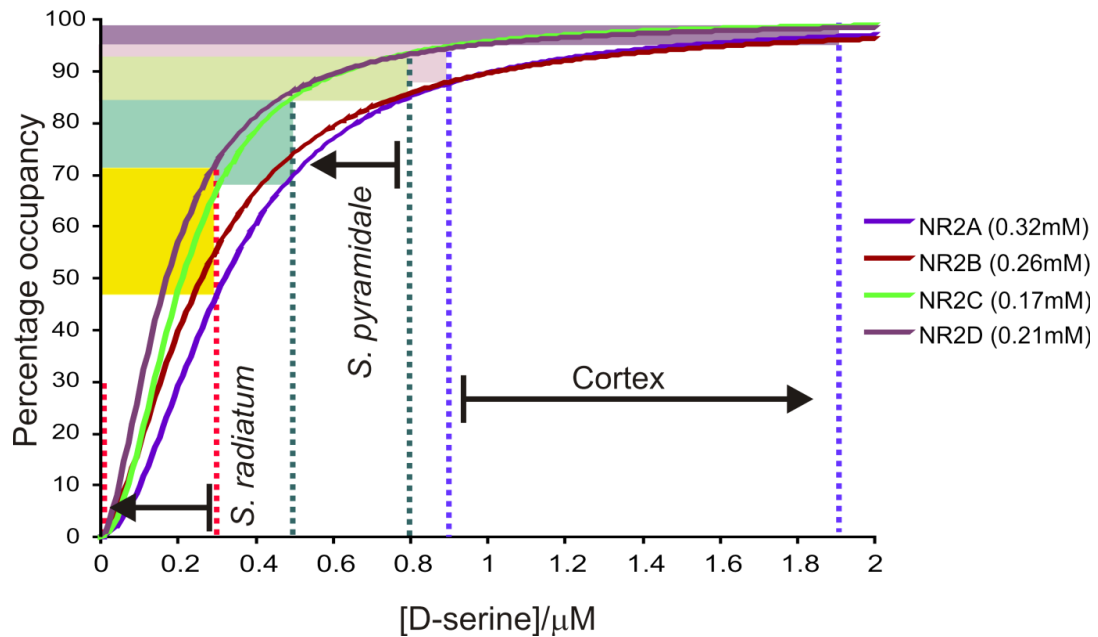
Figure 16: Functional significance of the agonist NMDA on NMDA receptor occupancy. The co-agonist site occupancy levels are altered differently in the 3 regions of the brain studied. In s. radiatum co-agonist site occupancy decreases to 0% for the different NR2 subunits; while the release of D-serine in the cortex and s. pyramidale does not alter site occupancy levels and hence NMDA receptor function is unlikely to be affected.

While overall release of D-serine is observed with acute AMPA receptor activation, under acute NMDA receptor activation an overall loss in D-serine levels occurs. This reduction in D-serine concentrations potentially has some functional consequence on NMDA receptors, reducing co-agonist site occupancy from 83% to 65%-75%. This will reduce NMDA receptor activity, by approximately 20%, which may be enough to reduce over-excitation.

The D-serine release observed in the cortex alters co-agonist site occupancy levels from 85% to 95%; NMDA receptor activation maybe potentiated slightly as a result. However, as mentioned above, the D-serine released may also be an indicator of another neuro-protective event occurring i.e. pyruvate synthesis.

5.5.4   Acute kainate receptor activation may alter NMDA receptor activity

The median data points are taken to represent a real-time physiological event; the median change in *s. radiatum* as a result of acute kainate receptor is 0μM. For this reason no effect on NMDA receptor occupancy levels is shown even though D-

serine release and loss was observed with kainate. In *s. pyramidale* D-serine loss is reduced from -0.8μM to -0.54μM; this leads to saturation levels to fall from 85% to approximately 67% for NMDA receptor composed of NR2A/B subunits. Potentially this reduces NMDA receptor activation. In the cortex, D-serine levels rise from +0.9μM to +1.6μM, increasing D-serine site occupancy from 85% to 95%. This is likely to have very little functional significance on NMDA receptor activity.
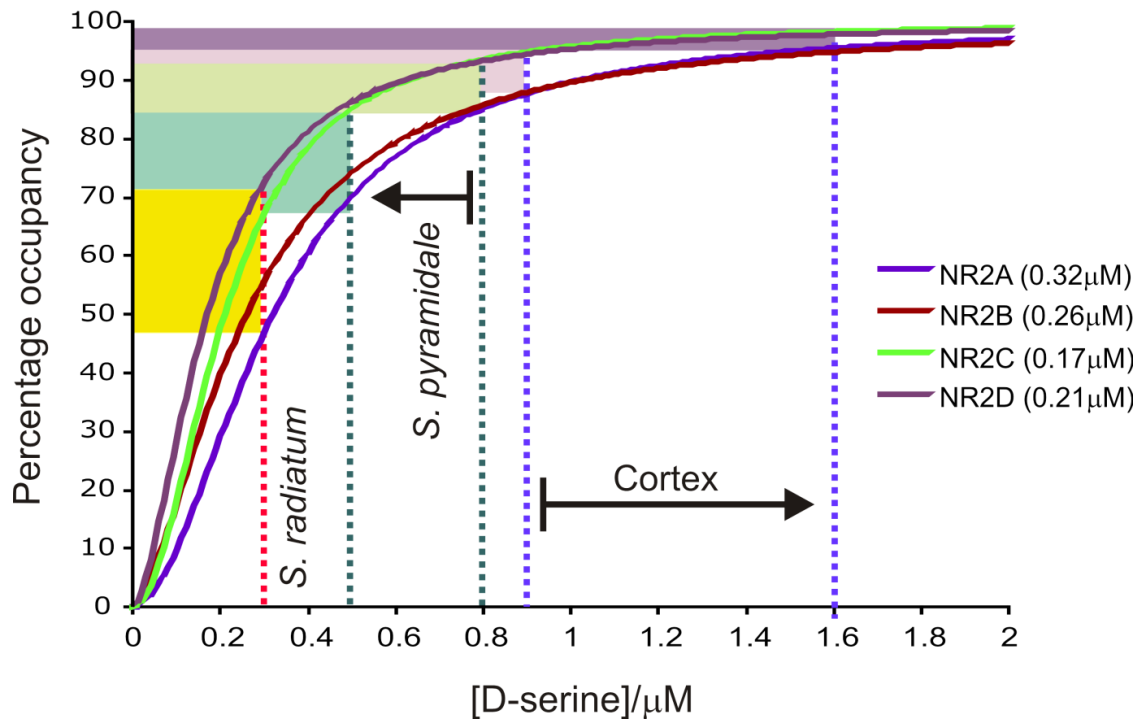
Figure 17: Functional significance of the agonist kainate on NMDA receptor occupancy. The co-agonist site occupancy levels are altered differently in the 3 regions of the brain studied. In s. radiatum no change is detected; while D-serine levels fall by -0.26μM in s. pyramidale but this only reduced glycine site occupancy levels to approximately 70%. In the cortex D-serine is released, increasing glycine site occupancy levels to 95%.

The underlying mechanisms by which D-serine release is evoked by AMPA, NMDA and kainate receptor agonists in the cortex and the loss of D-serine in *s. radiatum* occurs have yet to be determined. The latter, may potentially be a novel mechanism by which NMDA receptor-associated excitotoxic damage is avoided, while the former maybe a means of recycling D-serine (to pyruvate), or its removal by the blood brain barrier. It is plausible that this D-serine release will feed into a mechanism which results in the potentiation of NMDA receptors. Thus signalling

cascades which allow regulation of NMDA receptor action (through regulation of D-serine) by all 3 members of the ionotropic glutamate receptor family exist.

5.5.5   Calcium alters D-serine signalling mechanisms

As shown some of these signalling events are dependent on extracellular calcium and intracellular calcium changes may be involved in D-serine release in the cortex. Calcium is a known co-factor of SR; its intracellular increase can cause D-serine synthesis and release. We show that intracellular calcium change by itself does not necessarily induce D-serine release, but rather only specific signalling events achieve this, in a calcium dependent manner. For example, acute AMPA receptor activation induces D-serine release in the cortex which follows a surge in intracellular calcium but extracellular calcium is also important; its removal reduces AMPA-induced D-serine release by 66% and NMDA-induced release by 91%. In the hippocampus, loss of D-serine induced by NMDA is less in *s. radiatum* and AMPA-induced down-regulation is unaffected by the removal of calcium.

5.5.6   D-serine and LTP

Stimulation of the glycine site coupled to the NMDA receptor complex is necessary to induce LTP. Blocking the glycine site pharmacologically with an antagonist 7-chlorokynurenic acid (7-CKA) blocks LTP in hippocampal slices, an effect reversed by D-serine (Oliver *et al.*, 1990a). Hence, the role played by D-serine via NMDA receptors is essential for LTP. NMDA receptor activation during LTP results in the influx of calcium, which is a well known secondary messenger able to bind several proteins (Oliver *et al.*, 1990b). Binding of calcium to calmodulin for example, can results in alterations of signalling transduction molecules such as protein kinase A (PKA) and protein kinase C (PKC). Additionally during LTP several co-factors of the enzyme SR play an important role. PICK1 and GRIP both interact with SR (to increase D-serine synthesis) and are involved in the insertion of AMPA receptor at the synapses during LTP. GRIP for example is relieved from AMPA receptors, subsequently binds SR to increase D-serine synthesis/release. The D-serine release observed may be involved in long-lasting LTP processes and could be important for maintaining LTP in surrounding cells, since initial LTP can wane. It has been shown that blocking intracellular calcium release in an astrocyte blocks LTP in surrounding

cells (Henneberger *et al.*, 2010). It is presumed that D-serine release is blocked as a result and this diminishes LTP, applications of D-serine re-establish LTP. Here, we have been able to observed real-time changes in D-serine levels and show D-serine release may not be as necessary in establishing LTP as previously thought.

5.6 Conclusions

1. Ionotropic glutamate receptors AMPA and kainate may modulate NMDA receptor activity by regulating extracellular D-serine levels

2. NMDA receptors could self-regulate activity, by altering D-serine concentrations

3. Decreasing D-serine release reduces NMDA receptor activity and is potentially neuro-protective

4. Mechanism regulating D-serine are sensitive to extracellular calcium

Chapter 6: The role of D-serine in excitotoxic cell damage

6.1 Abstract

NMDA receptors are vital for normal brain function. But over-excitation by excessive glutamate and consequent calcium entry through this channel is a major contributor to neuronal death during stroke, epilepsy and cardiac arrest. The role of the co-agonist D-serine in permitting NMDA receptor activation under these conditions is investigated here using D-serine biosensors, which permit sensitive real-time measurements. Two *in vitro* stress models were selected: hypoxia and ischemia, to study the part D-serine plays in NMDA receptor mediated cell death.

Under hypoxic conditions D-serine levels are reduced by $-0.4 \pm 0.1\mu M$, n=12 and with ischemia the extent of reduction is initially greater $-1.2 \pm 0.4\mu M$, n=6 but D-serine levels begin to rise before anoxic depolarisation. These low D-serine concentrations may significantly reduced NMDA receptor activity by limiting co-agonist availability which will be advantageous in reducing excitotoxic damage. In fact when the NMDA receptor component was pharmacologically isolated, a reduced NMDA receptor fEPSP was observed under hypoxia (reduced to $45\pm4\%$ of initial response, n=5) while in the presence of 1mM D-serine the reduction in NMDA receptor response is significantly less (from 100% response to $68\pm6\%$, n=7; *P*=0.03, two-population t-test). The loss of D-serine observed under hypoxic and ischemic conditions diminishes NMDA response, and is a neuroprotective mechanism by which NMDA receptor mediated excitotoxicity is minimised.

6.2 Introduction

A continuous supply of blood and glucose is vital for cell survival but brain cells are more prone to irreversible damage than any other cells of the body. Unfortunately the states of hypoxia (oxygen deprivation) and ischemia (oxygen and glucose derivation) are common in many disease states such as stroke, cardiac and respiratory arrest as well as being contributing factor to neonatal brain damage and morbidity (Cortey, 1995; Martin & Wang, 2010). Together these disorders are a leading cause of neurological disability and death. In spite of this little is known about the pathogenesis of hypoxia-ischemic brain damage or why brain tissue, is so vulnerable to such insults. In particular, areas such as the hippocampal field CA1 and neocortical layers 3, 5 and 6 are characteristically destroyed after sub-maximal hypoxic-ischemia exposure (Nikonenko *et al.*, 2009).

The excitatory effects of the amino acid glutamate are well documented as a major cause of cell death after brain injury. This is usually as a result of uncontrolled activation of glutamate receptors, particularly the NMDA receptor (Choi & Rothman, 1990; Bliss & Collingridge, 1993; Monyer *et al.*, 1994). Over-excitation at this channel leads to a mass of Ca^{2+} influx that subsequently triggers signalling cascades promoting cell death. Under physiological conditions however, this channel is essential for many brain functions including synaptic plasticity-the molecular basis for learning and memory, cell migration and development (Bliss & Collingridge, 1993; Aamodt & Constantine-Paton, 1999). Maintaining this balance is one of the key reasons why specific antagonism of postsynaptic glutamate receptors has not been widely used to treat stroke patients for example, even though a variety of preparations have shown that this greatly diminishes the sensitivity of central neurones to hypoxia and ischemia (Rothman & Olney, 1986; Aamodt & Constantine-Paton, 1999).

Since the discovery of the co-agonist requirement of the NMDA receptor, a number of studies have indicated that D-serine, a major NMDA receptor co-agonist, also contributes to the excitotoxic effects of NMDA receptors (in particular in Alzheimer's disease). In view of the fact that co-agonist binding is essential, it maybe supposed that D-serine levels are increased during hypoxia and ischemia, where much cell death and injury is observed. At high concentrations D-serine will

contribute to excitation of the NMDA receptor. Conversely, reduced availability of D-serine will limit NMDA receptor activity which potentially is neuro-protective. Here we investigate the changes in extracellular D-serine levels under hypoxic and ischemic conditions in hippocampal brain slices and relate these to potential excitability of NMDA receptors. D-serine biosensors are used to make sensitive and selective real-time recordings from the hippocampus under hypoxia and ischemia, to study the role of D-serine in injury related cell death at the NMDA receptor.

6.3 Methods

6.3.1 Slice preparations

Male Sprague-Dawley rats aged 12-21 days were sacrificed by cervical dislocation in accordance with schedule 1 of the UK Government Animals (scientific procedures) Act 1986. The brain was removed and placed in artificial cerebrospinal fluid (aCSF) at 4ºC before 500µm horizontal hippocampus slices were cut with a microslicer (Vibrotome) as previously described (Dale *et al.*, 2000). Slices were placed in an incubation chamber in aCSF continuously oxygenated (with 95% oxygen/ 5% carbon dioxide) at $33^{o}C$ for 40minutes before use. The composition of aCSF is as follows: NaCl 124mM; KCl 3mM; CaCl 2mM; NaH_2CO_3 26mM; NaH_2PO_4 1.25mM; D-glucose 10mM; MgSO4 1mM; pH 7.4 with 95% oxygen and 5% carbon dioxide.

A single slice was transferred to a recording chamber, fully submerged with oxygenated aCSF and profused at 8ml/min (33-34ºC). A Duostat interfaced to PC by an A to D converter board was used and an Ag/AgCl was used as a reference electrode. A D-serine biosensor of 0.5mm length and 50µm diameter was inserted into the hippocampus (CA1 region), allowed to stabilise for 20 minutes before induction of hypoxia or ischemia. Extracellular recordings of the evoked field excitatory postsynaptic potentials (fEPSPs) were made from stratum radiatum with an aCSF-filled glass microelectrode and using a stimulating electrode bought from WPI.

6.3.2 Induction of hypoxia and ischemia

Hypoxia was induced by the substitution of normal aCSF with identical pre-equilibrated aCSF with 95% nitrogen and 5% carbon dioxide with episodes lasting for 10 minutes. Slices were exposed to a single episode as it was noticed that in the second hypoxic attack D-serine loss was less severe and the NMDA receptor component was reduced less (data not shown). Ischemia was induced by replacing normal aCSF with that containing 10mM sucrose (instead of 10mM D-glucose) and saturation with 95% nitrogen/5% carbon dioxide gases. Episodes lasted 10 minutes and severe damage of the slice was observed by loss of the fEPSPs, hence only single applications were made per slice. In order to record accurately the extracellular potential shifts associated with the anoxic depolarisation continuous DC-3kHz recordings were also made using the custom software package used to record sensor signals (Dale *et al.*, 2000).

6.3.3 D-serine and glutamate biosensors

D-serine sensors were fabricated as previously described and calibrated as described previously with 10μM D-serine and serotonin at the end of a recording. Glutamate biosensors were made using the same technique (Dale *et al.*, 2005). In all cases a null biosensor was used (a sensor without enzyme in the bilayer) to ensure accuracy of recordings. All traces shown are null subtracted. Data is expressed as mean ± SEM with n indicating the number of slices.

6.4 Results

6.4.1 Extracellular changes in D-serine during hypoxia and ischemia

Extracellular D-serine levels can determine the capacity for activation of the NMDA receptor as previously described in chapters 3 and 4. To determine how D-serine levels are altered, we placed D-serine biosensors in the CA1 of the hippocampus, the most vulnerable brain region to pathological states of hypoxia and ischemia, to determine the role D-serine in NMDA receptor mediated cell death.

D-serine levels during a 10 minute episode of hypoxia are reduced by -0.4 ± 0.1µM, n=12 (figure 1). D-serine concentrations begin to fall quickly with reduced availability of oxygen and then continue to do so until oxygen is washed back in. The fEPSP (amplitude) also falls very rapidly. Re-oxygenation causes a fast efflux of D-serine (+0.38±0.1µM, n=12), which in some cases is greater than basal levels. This transmitter release has previously been described for glutamate and adenosine, termed the post-perfusion (hypoxic/ischemic) efflux, PPE (Frenguelli *et al.*, 2003). Eventually, the concentrations of D-serine slowly return to initial concentrations (7±1 minutes, n=6) but the full recovery of the fEPSP occurs before this (5±0.3 minutes, n=6). It may be predicted that the NMDA receptor component does not recover until 7±1 minutes and this is concealed by the fast AMPA receptor currents.
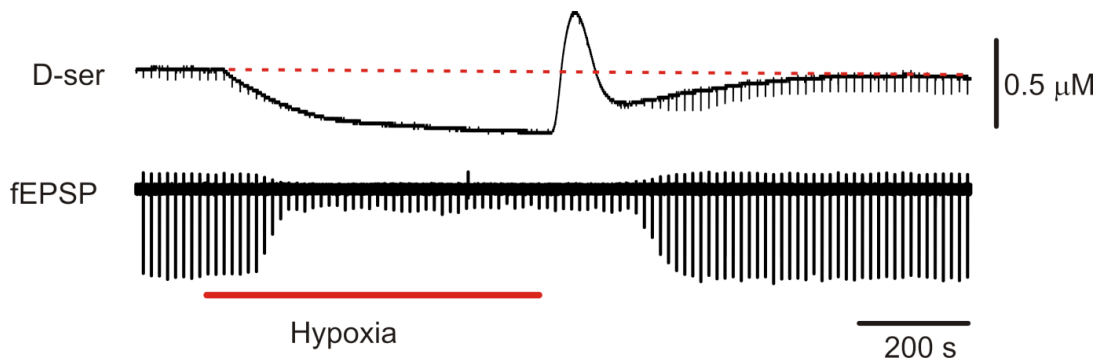
Figure 1: D-serine levels are reduced under hypoxic conditions. Re-oxygenation causes a D-serine efflux, PPE. Levels of D-serine return to baseline levels after synaptic transmission appears to have fully recovered. The null sensor trace has been subtracted from this D-serine trace.

In ischemia a similar effect on extracellular D-serine levels is observed initially, a reduction of -1.2 ± 0.4µM in D-serine concentration occurs n=6, as shown in figure 2a. As with hypoxia, as soon as ischemic aCSF is washed on, D-serine levels begin to decrease and simultaneously the fEPSP is also markedly reduced. Unlike with hypoxia however, D-serine levels appear to recover even before the ischemic aCSF is washed off, with levels rising at 4.5±0.2 minutes, n=4 into a 10 minute ischemic episode and total increase of +0.5±.01µM, n=4 is seen.
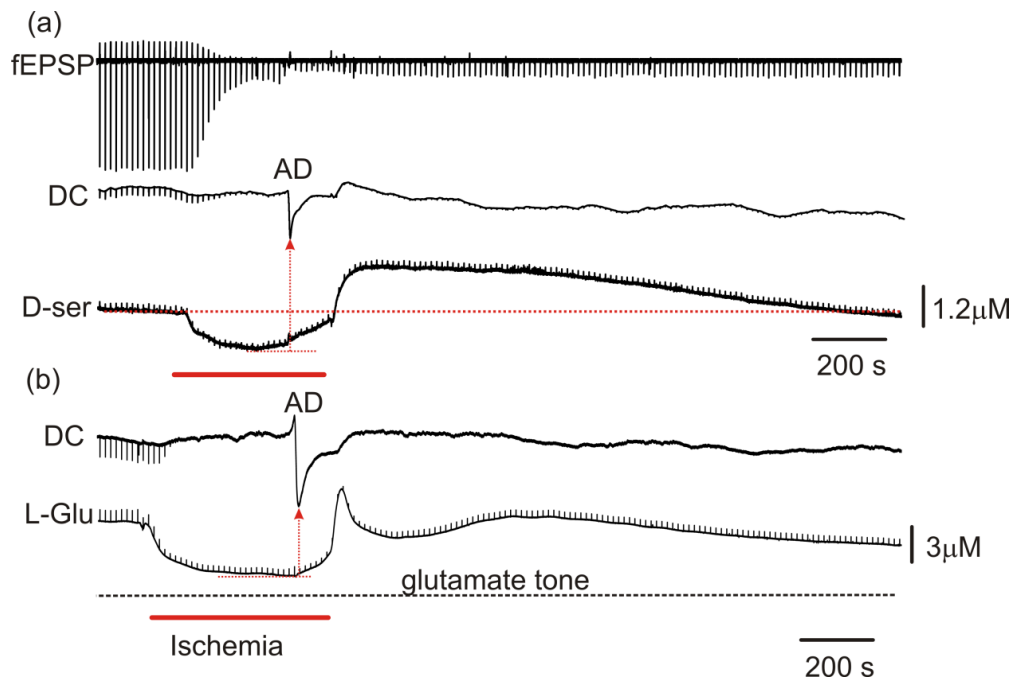
Figure 2: D-serine levels and glutamate level are reduced under ischemic conditions. D-serine and glutamate levels are initially reduced but levels of D-serine begin to rise before anoxic depolarisation while glutamate rise corresponds to AD. PPE is observed in both excitatory amino acids post-ischemia. Upon the onset of the AD a large negative deflection of the DC trace is observed.

The anoxic depolarisation (AD) is observed on the DC trace, this is a marker of cell membrane depolarisation which results from an energy deficit due to ischemia, rendering the Na+/K+ ion pump ineffective. The Na+/K+ ATPase usually maintains normal transmembrane ionic balance necessary for resting membrane potential, using up to a third of a cells energy expenditure. During ischemia, the ATP deficit disrupts ionic transmembrane balance, resulting in AD which sets in motion a series of events resulting in cell death. AD occurs at 6.1 ± 0.3 minutes into a 10 minute bout of ischemia. Upon washing a surge in D-serine levels, the PPE is observed ($+2.7\pm1\mu M$, n=4) which returns to baseline levels over a period of 21 ± 2minutes (n=4). The fEPSP doesn't recover, as seen with hypoxia most probably indicative of irrevocable cell damage so that synaptic transmission is permanently affected.

Glutamate levels are also reduced during ischemia, figure 2b, by $-3.6\pm0.3\mu M$, n=12, and a rise in glutamate is observed at 6.7 ± 0.7 minutes, n=6 of $+2.5\pm0.2\mu M$, n=7. This time at which the rise in glutamate is observed is similar to when AD occurs (6.1 ± 0.3 minutes). A PPE of $+4.3\pm0.8\mu M$, n=10 is observed post-ischemia. During ischemia the only point of difference appears to be the timing of D-serine and

glutamate release. D-serine release occurs significantly earlier (4.5±0.2 minutes, n=4) compared to glutamate release (6.7±0.7 minutes, n=6). Anoxic depolarisation occurs at 6.1±0.3 minutes (n=6) after the induction of ischemia, indicating that D-serine levels begin to rise before AD initiated cell damage (see Table 6.1 and Figure 3).
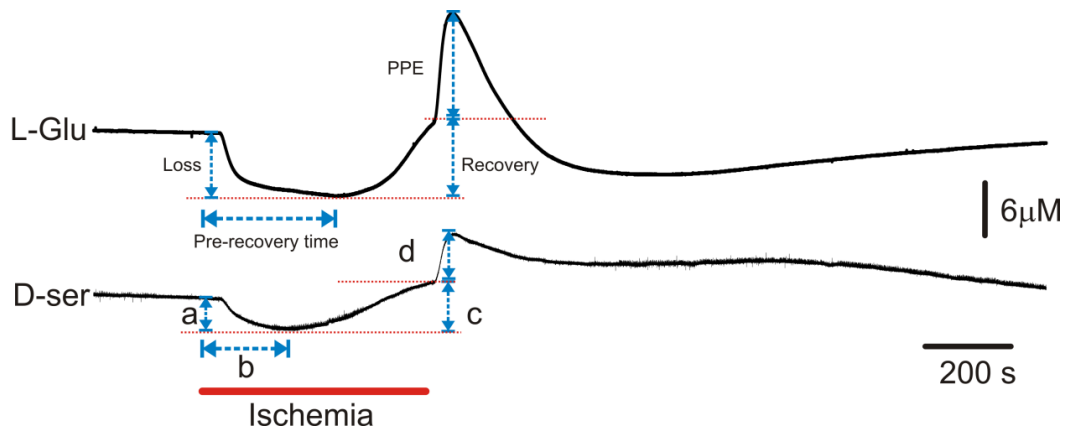
Figure 3: D-serine and glutamate levels show a very similar pattern of change under ischemic conditions. Concentrations in both amino acids are reduced initially, but a rise in D-serine concentrations is observed approximately 2 minutes before a rise in glutamate is seen. Washing in glucose and oxygen causes a rapid efflux in both neurotransmitters, which eventually returns to baseline levels.

	a- loss	b- pre-recovery time	c- release	d- PPE
D-serine	-1.2±0.4µM, n=6	274±10s, n=4	+0.5±0.1µM, n=4	+2.7±1.0µM, n=4
Glutamate	-3.6±0.3µM, n=12	402±42 s, n=6	+2.5±0.2µM, n=7	+4.3±0.8µM, n=10

Table 6.1: Changes in D-serine and glutamate levels during ischemia; a number of phases occur during ischemia, in both neurotransmitters initially a loss of concentration is observed, followed by a rise to initial resting levels and then a spike in concentration (PPE).

D-serine biosensor recordings show a clear reduction in D-serine during hypoxia and initially during ischemia. This loss of D-serine predicts that the NMDA receptor activation will be reduced, due to unavailability of D-serine during hypoxia and the initial stages of ischemia. A way to test these findings is to determine whether the NMDA receptor fEPSP (fEPSP$_N$) alters when pharmacologically isolated. Since

under ischemia synaptic transmission is lost permanently (most likely from irreparable cell damage), a change in NMDA receptor activity may be observed as a result of this rather than a cellular change resulting from ischemia-related events. For this reason, henceforth only the hypoxic model is used to investigate the role of D-serine and NMDA receptor activity in brain injury even though the ischemic model is thought to be closer representative of the physiological changes occurring in stroke. During hypoxia complete recovery in synaptic transmission is seen indicating that permanent damage as a result of cell death has not occurred.

6.4.2 Isolating the NMDA receptor component

Here the $fEPSP_N$ will be defined as the component pharmacologically isolated using CNQX at 10μM and picrotoxin (100μM) and that which is largely removed in the presence of D-AP5 (100μM), a NMDA receptor antagonist (6±1%, n=4). The $fEPSP_N$ component is approximately 22±8% (n=4) in size compared to the AMPA/NMDA fEPSP indicating that it is the AMPA receptors that make up the bulk of fEPSP amplitude (as shown in figure 4).
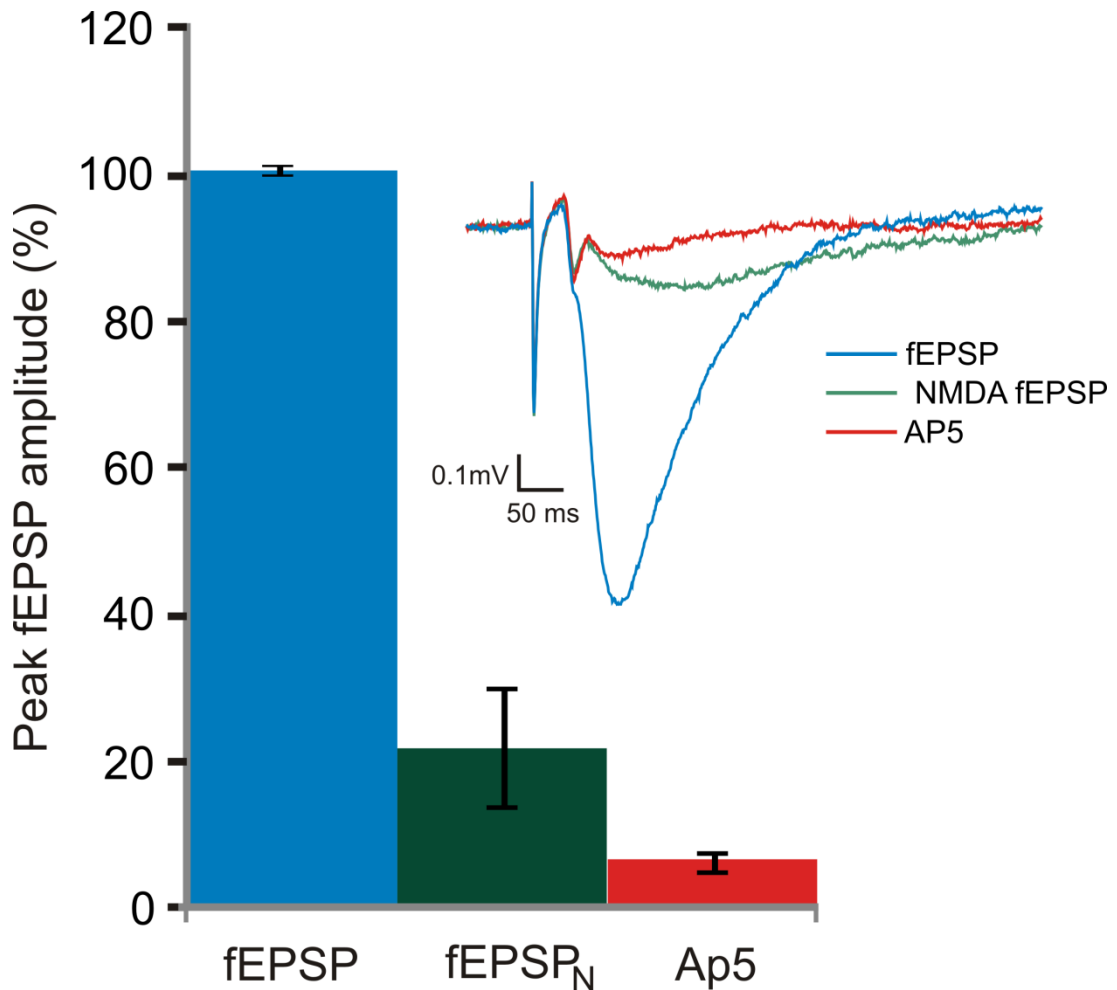
Figure 4: fEPSP$_N$ can be isolated from fEPSP pharmacologically. The NMDA receptor component is isolated using 10μM CNQX/ 100μM picrotoxin; and makes up 22±8% of full fEPSP. Synaptic transmission is reduced by 94±1% by 100μM D-AP5. Inset: an example of single superimposed EPSPs in control, in the presence of CNQX and D-AP5.

6.4.3   fEPSP$_N$ and hypoxia

Under hypoxic conditions, the NMDA receptor component of the fEPSP is reduced, as predicted by the biosensor recordings. This is shown in an example trace, figure 5. If (a) is taken as 100% of the NMDA receptor response than and the change observed under hypoxic conditions, (b), is a reduction of the fEPSP$_N$ by 45±4% (n=3) and complete recovery of synaptic transmission occurs with re-oxygenation, (c). D-serine in relation to these change are also shown. The fEPSP$_N$ diminishes as soon as D-serine levels begin to reduce. The complete recovery of fEPSP$_N$ occurs as D-serine levels approach the initial concentrations, suggesting that the D-serine is a requirement for NMDA receptor activity. Full recovery in fEPSP$_N$ is seen at 6.7±0.5

minutes, n=7, after re-oxygenation, this supports the findings seen figure 1, where D-serine levels return to initial baseline at 7 ±1 minutes, n=6.
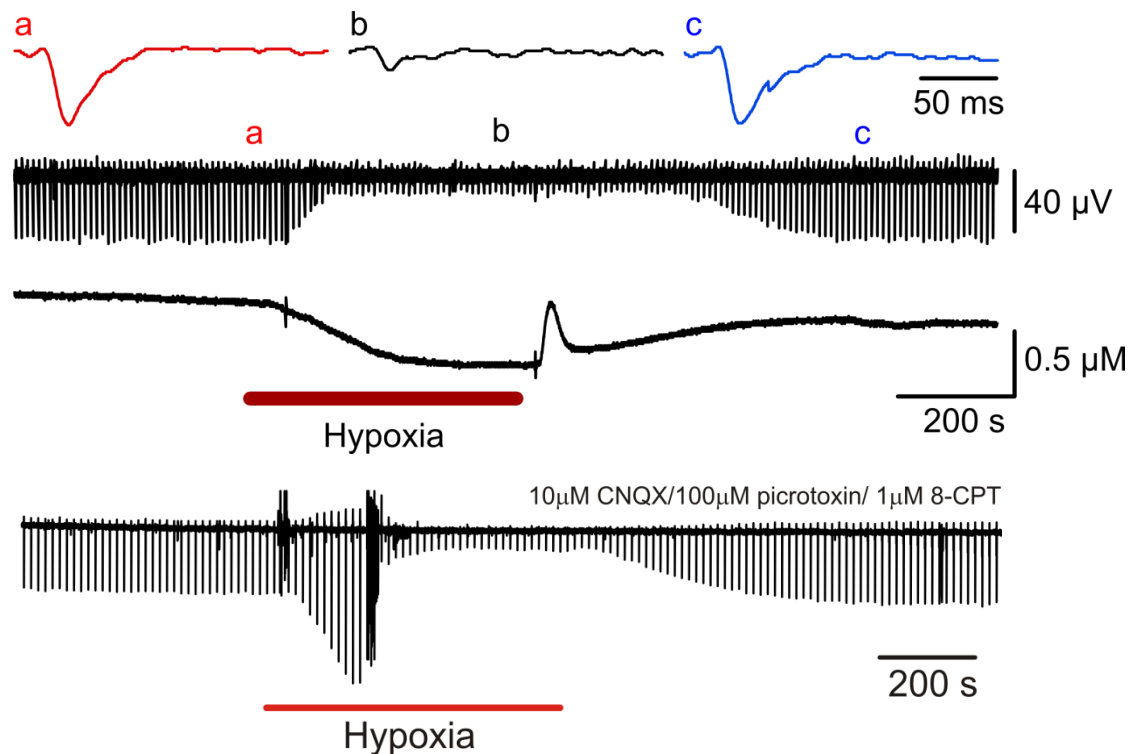
Figure 5: The isolated NMDA receptor component is reduced significantly under hypoxic conditions, both in the presence and absence of 8-CPT. D-serine levels are also reduced, and this corresponds to a reduction in NMDA receptor mediated synaptic transmission as shown in insets labelled a, b and c. In the presence of 8-CPT, an unusual rise in fEPSP amplitude is observed, the reasons for this are not clear but the $fEPSP_N$ is reduced during hypoxia and recovery is similar to that observed in the absence of 8-CPT.

A possible explanation for the reduction in $fEPSP_N$ is the presynaptic effect of adenosine. Large concentrations of adenosine release are observed during hypoxia, which in consequence reduces pre-synaptic glutamate release via A_1 receptors; this may result in reduced $fEPSP_N$ (Dale *et al.*, 2000). An antagonist 8-cyclopentyltheophylline (8-CPT) at 1μM is known to block A_1 receptors, and this is used as an additional control to ensure that $fEPSP_N$ is not altered by pre-synaptic changes. In the presence of 8-CPT the NMDA receptor component makes up 27±7%, n=4, of the total fEPSP, not significantly different from the size of $fEPSP_N$ in the absence of 8-CPT (pair-wise T-Test *P*=0.31). During hypoxia the reduction in $fEPSP_N$ observed in the presence of 8-CPT is 44±3% (n=3) of total $fEPSP_N$, as

shown in figure 6. The difference is not significant (T-test P=0.94) indicating that A_1-receptors do not affected NMDA receptor mediated synaptic transmission during hypoxia. But an unexplained potentiation of the $fEPSP_N$ is observed at the onset of hypoxia in the presence of 8-CPT which is not seen its absence (Figure 5 and 7).
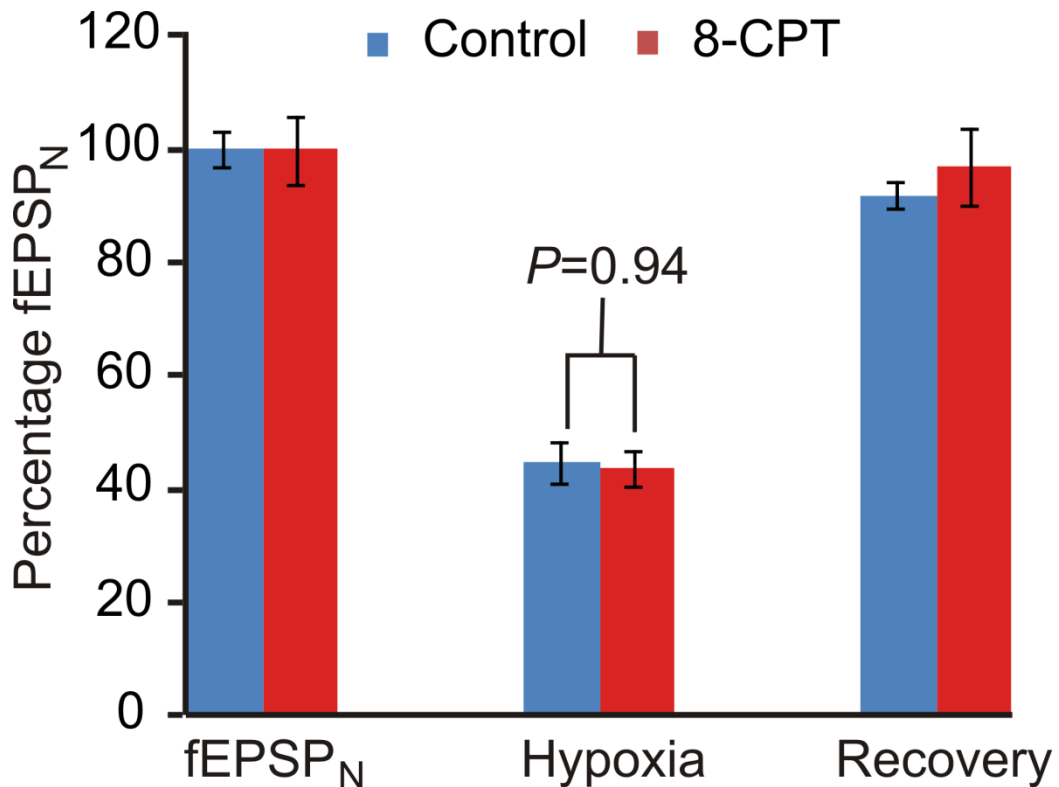
Figure 6: $fEPSP_N$ reduction during hypoxia is still observed in the present of 8-CPT. $fEPSP_N$ is defined as the component observed in the presence of 100μM picrotoxin and 10μM CNQX and which is removed by the presence of D-AP5. Here $fEPSP_N$ is shown to be not significantly affected by the presence of 8-CPT, indicating that $fEPSP_N$ reduction seen during hypoxia do not result from pre-synaptic effects mediated by adenosine release.

6.4.4 $\underline{fEPSP_N \text{ and hypoxia in the presence of D-serine}}$

This confirms that reduced availability of D-serine during hypoxia results in reduced $fEPSP_N$, as predicted by D-serine biosensor recordings (figure 1). Hence it is reasonable to assume that increased availability of D-serine is likely to compensate for the D-serine reduction and the $fEPSP_N$ will be reduced less.

To test this hypothesis experiments were repeated in the presence of 1mM D-serine. 1mM may appear to be a higher concentration than necessary but not all of this D-serine reaches the slice, as it is not all detected by the D-serine biosensors in tissue

(applications of 100µM to the slices was only detected as a 2-3µM on a sensor embedded in a slice). The D-serine may be broken down and/or taken up by transporters before it has a chance to reach the synapse. The reduction of the $fEPSP_N$ as a result of hypoxia is less in the presence of D-serine (reduced to 68±6% of total $fEPSP_N$, n=7) implying strongly that the reduction in the NMDA receptor component is due to the reduced availability of D-serine. During hypoxia, the $fEPSP_N$ is significantly less affected during hypoxia in the presence of D-serine compared to its absence (T-test, P=0.03).
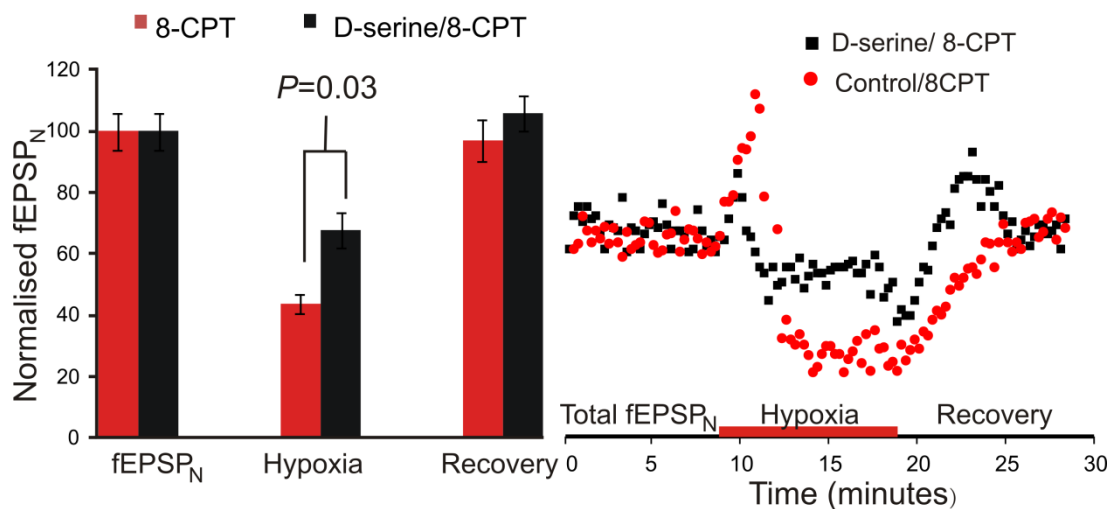
Figure 7: The normalised $fEPSP_N$ is reduced under hypoxic conditions but the reduction is less severe in the presence of excess D-serine. The bar chart shows significant affect of the presence of D-serine on $fEPSP_N$ while on the right a trace of hypoxia experiments conducted in the presence and absence of 8-CPT are shown (red bar indicates hypoxia), each circle/square indicates $EPSP_N$ amplitude.

Hence, the reduction of D-serine observed under hypoxic and ischemic conditions is an indicator of reduced NMDA receptor activity. This reduction has the potential to be highly beneficial in cases of brain injury and stroke, promoting cell survival rather than cell death. This is particularly important when the fall in glutamate concentrations is above the known basal tone for glutamate, hence, some glutamate is still available to activate NMDA receptors but it is the reduction in D-serine which potentially limits over-excitation at the NMDA channel.

6.5 Discussion

Ca^{2+} influx through the NMDA receptor can both kill neurons and promote survival under different circumstances. The extent of activation of the NMDA receptor, both

intensity and duration, determine the nature of the response, with pro-death signals requiring higher levels of activity than pro-survival pathways (Soriano & Hardingham, 2007). The factors which may influence the level of potentiation at this channel include the subunit composition, localisation (whether extra-synaptic or synaptic) and also co-factors which physically associate with the channel including the co-agonist: D-serine. Here we show that D-serine is a central component in determining the extent of NMDA receptor activation and that a reduction in the extracellular concentrations of D-serine is used as a means to lessen the activation of this channel or to promote pro-survival responses under hypoxic conditions. It is the reduced availability of D-serine which diminishes $fEPSP_N$ during hypoxia, as presence of exogenous D-serine lessens the hypoxic-$fEPSP_N$ reduction. Interestingly Bickler et al (2003) observed that NMDA receptor potentiation (as measured by intracellular calcium changes) during hypoxic episodes is lower compared to that observed under control conditions and assign this to the NR2 subunit (Bickler *et al.*, 2003). Here, we show that these observations result from reduced availability of D-serine during hypoxia, which binds the NR1 subunit but also affinity of the NR2 subunit for glutamate is altered. Hence a reduction in D-serine during hypoxia is neuro-protective, as it limits over-excitation of NMDA receptors. D-serine transport, break-down (through increasing D-amino acid oxidase activity) and reduced D-serine synthesis/release (via changes in serine racemase activity) may all contribute to achieve D-serine loss observed during hypoxia.

In the case of ischemia, the initial D-serine reduction may be neuro-protective but there is evidence that a rise in D-serine levels may contribute to anoxic depolarisation and cell death. D-serine levels rise at approximately 4.5±0.2 minutes, n= 4, into a 10 minute episode of ischemia. A study by Kirschner et al (2009) conducted using capillary dialysis techniques has showed that a 24 minute ischemic episode in acute hippocampal slices induced D-serine (2.5 fold) efflux, which parallels glutamate efflux (17 fold) similar to what we have observed. The analysis was conducted every 2 minutes and data compiled together to form the pattern of release (Kirschner *et al.*, 2009). Owing to the limited resolution of this study, the initial reduction in D-serine levels (and glutamate) is completely overlooked, and only efflux of D-serine and glutamate are seen after 10 minute of ischemia, missing out the rise in D-serine levels before the onset of anoxic depolarisation. Due to the

sensitive real-time measurements of D-serine biosensors, we have been able to follow the change in D-serine levels in the second to second time frame and show during ischemia, the rise in glutamate occurs at the same time as the anoxic depolarisation while D-serine rise precedes this event. Anoxic depolarisation signals the collapse of the ionic gradient and resting membrane potential which results in acute neuronal death due to profound depolarisation of neurones and glia. Blocking this anoxic depolarisation or the signalling events which trigger this during stroke is neuroprotective (Anderson *et al.*, 2005). The rise in D-serine occurs before anoxic depolarisation by approximately 2 minutes (Figure 2), since this is a trigger for NMDA receptor mediated cell death, it is possible that D-serine contributes to signalling events that result in anoxic depolarisation.

To conclude, the reduction in D-serine levels observed under hypoxic and initially under ischemic conditions is neuro-protective Therefore signalling mechanisms are triggered during stroke for example, which protects cells by reducing extracellular D-serine levels, as a means to diminish NMDA receptor activity. A rise in D-serine during ischemia may signal onset of cell damage. Hence signalling mechanisms involved in D-serine regulation maybe a novel target for preventing cell damage during stroke.

Chapter 7: Discussion

7.1 D-serine biosensors provide novel insight

D-serine is a specific NMDA receptor co-agonist. Its binding at this channel is a pre-requisite for the activation of the NMDA receptor complex. Hence, the study of D-serine allows for NMDA receptor activity to be indirectly monitored. This research project identified the need for a tool to study D-serine distribution in the central nervous system, in a sensitive, selective manner with real-time recordings and biosensor technology offered a solution. Biosensors for a number of neurotransmitters have been in regular laboratory use for many years and a very stable form of the enzyme DAAO (DAAORg) was recently purified. To a make a biosensor for D-serine was an obvious solution, not only to us but at least two other groups, who have since published their findings. In chapter 2, I have shown that the biosensors designed here are far superior in sensitivity as a result of the fabrication technique, compared to other published work, while selectivity and stability of these sensors are equivalent.

The small size of the microelectrodes and second-by-second D-serine detection make these ideal tools for use in brain tissue and provide much advantage over current modes of study. Perhaps the most useful feature of D-serine biosensors is that localised basal D-serine measurements in selected brain areas can be made. Previous tools employed for this purpose include HPLC assays on homogenised tissue and the use of microdialysis probes. In both cases the refinement in the detection of D-serine observed with D-serine biosensors is poor. Homogenising tissue entails complete loss of differentiation been extracellular and intracellular D-serine levels, while microdialysis probes are large (typically around 250µm diameter) and often need to be embedded in the brain for a few weeks before recordings can be made. The obvious drawbacks of which are mutations in cell morphology and physiology. Alternatively, D-serine biosensors (50µm diameter) need only to be inserted into tissue for instantaneous measurements with the additional benefit of simultaneous recordings within different regions due to the small microelectrode size. This allowed the detection of extracellular D-serine concentrations in a number of brain regions (hippocampus, cortex and cerebellum) and within substructures in these areas. This led to novel insight into the heterogeneous D-serine distributions of the brain and within brain structures, which conveys important functional regulatory role of D-serine at NMDA receptors. This detail of work cannot be as easily conducted

using traditional techniques where small samples of cerebral spinal fluid are taken via a dialysis probe and later analysed by HPLC; the entire content of extracellular D-serine form a brain structure is measured, without differentiation of sub-regions within. The enormity of this distortion is most clearly seen if the recordings made here with biosensors are pooled for each brain area, as is typical in dialysis/HPLC recordings (figure 1). The D-serine concentrations in the cortex $1.0\pm0.2\mu M$ (n=14) and cerebellum $1.0\pm0.3\mu M$ (n=32) appear to be the same and slightly less D-serine is observed in the hippocampus $0.7\pm0.1\mu M$ (n=76)
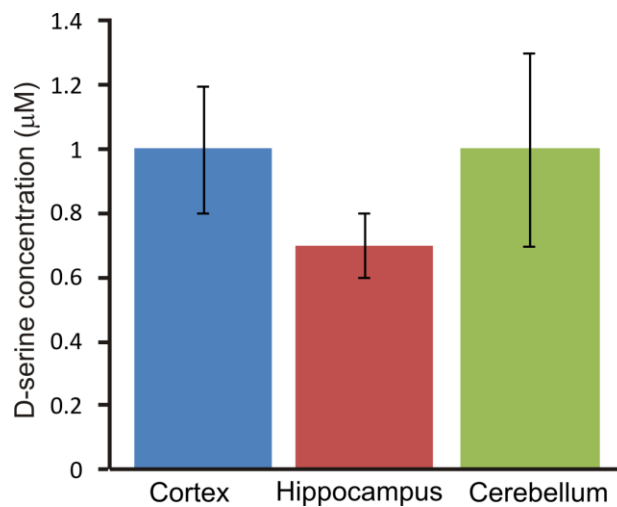
Figure 1: Total D-serine content of the hippocampus, cortex and cerebellum as measured by D-serine biosensors is misleading.

Incidentally, HPLC studies have also shown that the highest levels of D-serine are found in the pre-frontal cortex and there is less D-serine in the cerebellum (Hashimoto *et al.*, 1993b; Schell *et al.*, 1997). This data would suggest that the co-agonist site of NMDA receptors is fully saturated in the cortex and cerebellum regardless of NR2 subunit composition and in the hippocampus, the NRC/D-containing NMDA receptors are also fully saturated while NRA/B containing NMDA receptors are 80% saturated at these concentrations of D-serine. It has long been believed that the co-agonists site of NMDA receptors is fully saturated in the brain; though many studies have showed evidence that NMDA response could be potentiated with exogenous D-serine or glycine applications. By looking at D-serine concentration differences within a single brain structure as was done here, these apparently contradictory results may be reconcilable. As in the case of the hippocampus region, according to the basal D-serine tone, the co-agonist site of NMDA receptors in *s. pyramidale* is saturated ($1.1\pm0.1\mu M$) while those in *s.*

radiatum are not (0.4±0.1µM). Since the NR2A/B subunits are most highly expressed in the hippocampus and these receptors are saturated at around 1µM (chapter 3, basal tone), it may be predicted that the receptor response can be potentiated in the latter region by as much as 50-60%. Independent investigations carried out by Professor David Spanwick of Warwick University confirm these findings. The modulatory effects of D-serine on NMDA receptor whole cell current and excitatory post synaptic current (EPSC) was examined in the *s. radiatum* and *s. pyramidale* in hippocampal slices from 7 week old rats. Unsurprisingly, D-serine augmented NMDA receptor currents in *s. radiatum* but not in *s. pyramidale* (figure 2).

(a) NMDA induced currents
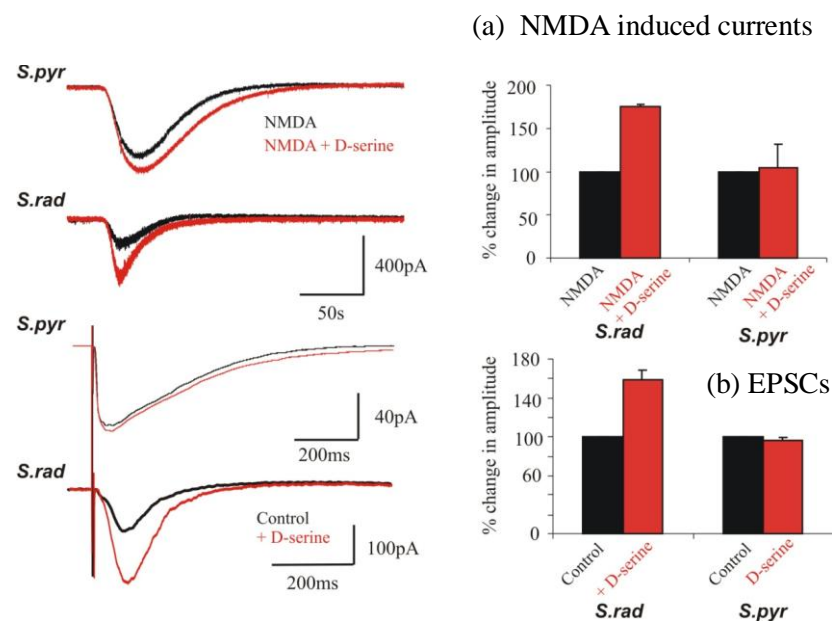
(b) EPSCs

Figure 2: Whole cell recordings from hippocampal neurons in *stratum pyramidale* and *stratum radiatum*. In pyramidale neurons of *s. pyramidale* there was little potentiation of the NMDA-evoked currents and NMDA receptor-mediated EPSCs by 100 µM D-serine. In contrast D-serine substantially potentiated both NMDA-evoked currents and NMDA receptor-mediated EPSCs in *s. radiatum*. EPSCs were evoked by stimulation of the Schaffer collaterals with a bipolar stimulating electrode. NMDA responses were evoked by brief bolus application of NMDA in the medium. Recordings were made from pyramidal neurons and interneurons in *stratum radiatum*.

The NMDA receptor EPSC is enhanced in *s. radiatum* to 159±10%, n=8 of the normalised current, n=8 (paired t-test, $P=0.006$) while in *s. pyramidale* no significant difference in amplitude is observed in the control and the presence of D-serine (paired T-test, $P=0.3$, n=7). Similarly, NMDA induced whole cell currents were augmented when D-serine was washed on in the presence of NMDA in *s. radiatum* (to 175±27%, n=5; $P=0.001$) but not in *s. pyramidale* (n=5, $P=0.46$). The

potentiation of both the response to NMDA and the NMDA EPSC with D-serine is similar to that predicted from the existing basal tone.

D-serine biosensors have provided detailed insight into extracellular basal D-serine tone, which conveys important functional information regarding NMDA receptor potentiation state. The small microelectrode size and sensitivity of the biosensors allow for measurements to be made within brain structures, this is the first step in characterising D-serine signalling in the brain.

7.2 Factors contributing to D-serine tone

Extracellular D-serine levels differ from one brain area to the next and even within a single structure, implying the existence of different factors involved in regulating D-serine release and its uptake. A major factor regulating extracellular D-serine levels is likely to be the D-serine synthesising enzyme, serine racemase. A number of studies have shown that extracellular and intracellular D-serine concentrations are altered as a result of SR activity by mechanisms not fully understood (Cook *et al.*, 2002; De Miranda *et al.*, 2002; Foltyn *et al.*, 2005). Interestingly, there is debate regarding which cells of the brain contain SR, with increasing number of immunohistochemical studies questioning the long held view that SR is only localised to astrocytes (Miya *et al.*, 2008). This is important because the cells that release D-serine in essence will be responsible for modulating NMDA receptor activity and function. SR mRNA has been detected in neuronal populations of the hippocampus, particularly to pyramidale layers of the CA1-CA3 fields and the granule cell layers of the dentate gyrus (Kartvelishvily *et al.*, 2006). Incidentally, these are the areas where using D-serine biosensors the present study has found the highest levels of D-serine in the hippocampus, suggesting that the basal D-serine tone detected may be largely synthesised in and released by neurones. However, D-serine release can also be evoked by a glial-agonist TFLLRN in *s. pyramidale*, not limiting D-serine synthesis or release only to neurones.

D-amino acid oxidase is also involved in maintaining extracellular D-serine concentrations, as elevated D-serine levels have been found in DAAO-knockout studies (Hamase *et al.*, 2005; Almond *et al.*, 2006). Moreno et al localised DAAO to both neurones and glial cells, with varying densities. Glial immmunostaining of DAAO was strongest in the caudal brainstem and cerebellar cortex, particularly in

astrocytes, Glogi-Bergmann glia and tanycytes. Hindbrain neurones were more reactive than those in the forebrain, although staining was observed in cortical and hippocampal neurons (Moreno *et al.*, 1999). Alterations of DAAO activity may be a key contributor to symptoms of schizophrenia, where activity of this enzyme is elevated by its promoter (DAAO promoter, G72). Thus, the modulation and activity of DAAO is important in maintaining correct extracellular D-serine levels in the brain.

A number of transporters of D-serine have also been found to regulate D-serine levels, especially in forebrain regions where DAAO expression is low. Astrocytes express a Na^+-dependent transporter with low affinity for D- and L-serine. In neurones (particularly on presynaptic terminals, dendrites and neuronal bodies) a Na^+-independent transporter of neutral amino acids (Asc-1) is shown to transport D-serine with high affinity. In gene knock-out studies of Asc-1, the levels of D-serine uptake was reduced by 34% and 22% in the forebrain and cerebellar synaptosomes respectively, suggesting that transporters play an essential role in removal of D-serine from the extracellular space. Potency determination of D-serine uptake showed that Asc-1 mediated rapid high affinity Na^+-independent uptake with an IC_{50} of 19μM, while the remaining uptake was mediated via a low affinity Na^+-dependent transporter with an Km of 670μM that is likely to be the glial alanine-serine-cysteine transporter 2 (ASCT2). These transporters, along with factors controlling D-serine enzymes are likely to be involved in the strict control of extracellular D-serine levels in the brain, playing a more essential role in some regions than others.

SR, DAAO and D-serine transporters are all involved in regulating extracellular D-serine concentrations and hence the saturation levels of the co-agonist site and potentiation ability of NMDA receptors. A typical pyramidal neuron has approximately 12,000μm length in dendrites and can receive as many as 30,000 excitatory synaptic inputs. Approximately 54% of all excitatory synapses contact spines in the *s. radiatum* and 36% in the basal dendrites (*s. oriens*) (Andersen *et al.*, 1966; Megias *et al.*, 2001). In the context of basal D-serine tone, 90% of excitatory synapses are in regions where NMDA receptor co-agonist site is not saturated (figure 3). In fact NMDA receptor response can be potentiated by as much as at least 50% (NR2A/B) at 27,000 synapses. This means D-serine release in *s. radiatum* and *s. oriens* has the potential to cause mass cellular damage and cell death by over-

excitation of the NMDA receptor. Even small increases in D-serine concentration can have widespread influence at excitatory synapses and NMDA receptor-dependent processes in the CNS. Reductions in D-serine may signal significantly reduced NMDA receptor-mediated excitation in CA1 neurones. It is likely then that D-serine levels are tightly regulated.
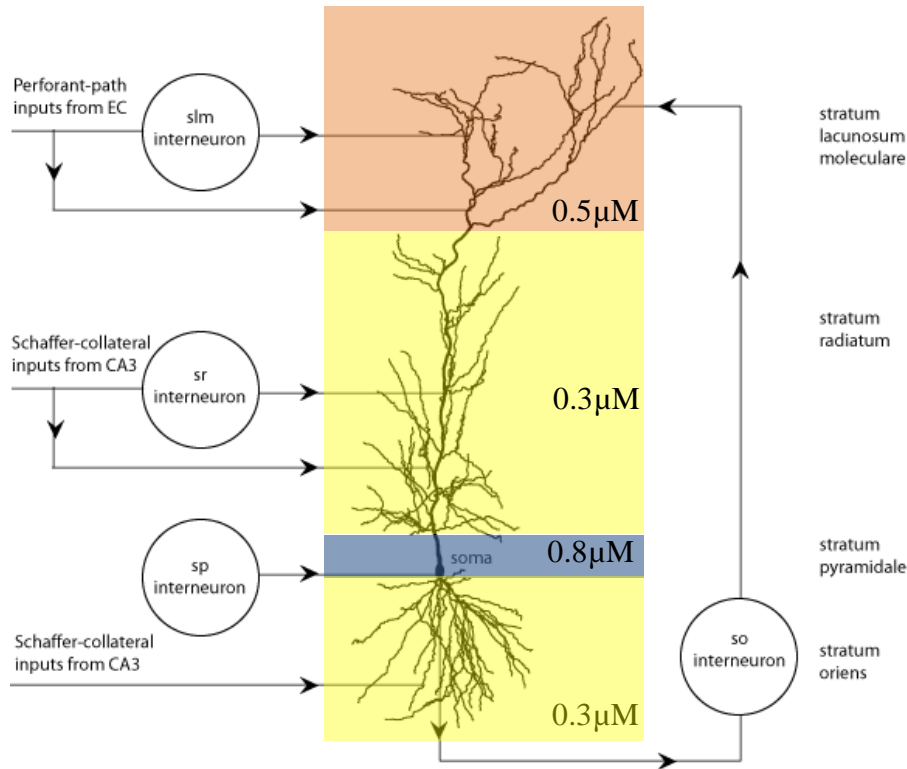
Figure 3: The vast majority of excitatory synapses on a typical CA1 pyramidale neurone are in *s. raidatum* and *s. oriens*. These regions have the lowest levels of D-serine in the hippocampus (median tone levels labelled). Orginal image taken from:http://groups.nbp.northwestern.edu/spruston/sk_models/HippocampusDB/ca1_map.htm

Notably, *s. lacunosum moleculare* can receive input from the entrihnol cortex, while *s. oriens* and *s. raditum* receive inputs from within the hippocampus (Schaffer-collateral). In the context of D-serine basal tone, excitatory inputs received from EC will involve stronger NMDA receptor response (frequency or amplitude) compared to SC inputs.

7.3 Activity dependent D-serine regulation

Alterations in D-serine levels can only be fully understood in the context of the existing basal tone and my results demonstrate the need for detailed study of D-serine signalling in the brain preferably via combination of electrophysiological and

biosensor recordings. This is because release of D-serine or slight reductions in areas of high extracellular D-serine tone are not likely to alter NMDA activity while in regions of low existing D-serine tone, slight changes can significantly alter the extent to which the NMDA receptor can be potentiated. For instance the importance of activity dependent D-serine changes cannot be fully appreciated if differentiations in basal tone within sub-layers of the hippocampus are not made. Release of D-serine with PAR1 agonist TFLLRN was much greater in *s. pyramidale* compared to *s. radiatum* but functionally, the release in *s. radiatum* is more significant. This is due to the low occupancy of NMDA receptors in *s. radiatum*; a small increase in D-serine concentration can potentiate the NMDA receptor response, while NMDA receptor response is likely to be unaffected by D-serine release in *s. pyramidale* where the receptors are almost fully saturated.

Activity dependent changes in D-serine levels were also induced by ionotropic glutamate receptor agonists. Acute activation of AMPA, NMDA and kainate receptors provoked changes in extracellular D-serine concentrations which were region-specific and most surprisingly heterogeneous even within a single structure (Table 7.1). This implies the existence of more than one signalling mechanism which is triggered by these ionotropic glutamate receptor agonists in the hippocampus while in the cortex, only release is observed.

	AMPA	NMDA	Kainate
S. radiatum	Reduction	Reduction*	Reduction/release
S. pyramidale	Release*	Reduction	Reduction/release
Cortex	Release*	Release*	Release

Table 7.1: Activity dependent regulation of D-serine by ionotropic glutamate receptor agonists; *denotes calcium sensitivity of mechanism (kainate calcium-sensitivity was not analysed).

Acute activation of AMPA, NMDA and kainate receptors evokes D-serine release in the cortex in a calcium dependent manner. SR appears to be intrinsically involved in the control of D-serine release and activation of this enzyme by co-factors (Ca^{2+}, GRIP and ATP) is known to induce release in cultured cells. Table 7.2 summarises the known regulators of SR and DAAO.

Enzyme	Positive regulators	Negative regulators
Serine Racemase	Pyridoxal 5'-phosphate, ATP, Mg^{2+}, Ca^{2+}, GRIP, PICK1	Glycine Nitric Oxide PIP2
D-amino acid oxidase	Nitric oxide DAAO promoter (G72)	

Table 7.2: Positive and Negative modulators of Serine racemase and D-amino acid oxidase.

On the presupposition that all 3 ionotropic glutamate receptor agonists augment D-serine synthesis by increasing SR activity and subsequent release via a co-factor of this enzyme, Ca^{2+} appears to be a likely candidate. The possible mechanism by which AMPA, NMDA and kainate receptors can influence intracellular Ca^{2+} levels are shown in figure 4. NMDA receptors are well-known for calcium permeability but AMPA receptor activation can also lead to increases in intracellular Ca^{2+} by increased expression of the receptors lacking the GluR2 subunit (Terashima *et al.*, 2004). Perhaps not by coincidence GluR2 insertion/expression is regulated by PICK1, a known modulator of SR (Fujii *et al.*, 2006). Kainate receptor activation can depolarise the membrane and increase intracellular calcium via the Ca^{2+}/Na^+ exchanger (Hoyt *et al.*, 1998). Additionally voltage-gated calcium channels can may be involved in increasing neuronal Ca^{2+} and cause D-serine release. If Ca^{2+} is the factor involved in D-serine release in the cortex, then certainly it makes sense that the removal of extracellular calcium profoundly reduces D-serine release evoked by ionotropic glutamate receptor agonists.

There is evidence that this D-serine release in the cortex by AMPA, NMDA and kainate agonists is of neuronal origin. Studies carried out in cortical neuronal cultures pre-treated with L-serine, show D-serine release with applications of ionotropic glutamate receptor agonists (Kartvelishvily *et al.*, 2006). Incidentally, D-serine release was blocked in the absence of Ca^{2+}, as observed here, with some evidence that release was from a cytosolic non-vesicular pool.

Nevertheless D-serine release has also been observed with AMPA receptor activation in glial cells. Studies carried out in cultured astrocytes provide evidence of AMPA receptor phosphorylation and subsequent dissociation of a bound protein GRIP (glutamate receptor interacting protein) that can bind SR directly augmenting its activity (Kim, PM *et al.*, 2005). Since glia communicate via propagation of

calcium waves, the calcium dependence of this D-serine release may support glial origin. Moreover, activation of AMPA receptors is one of the best stimuli leading to release of D-serine from the glial neutral amino acid transporter ASCT2, present on astrocytes which may contribute to the D-serine released (Ribeiro *et al.*, 2002).
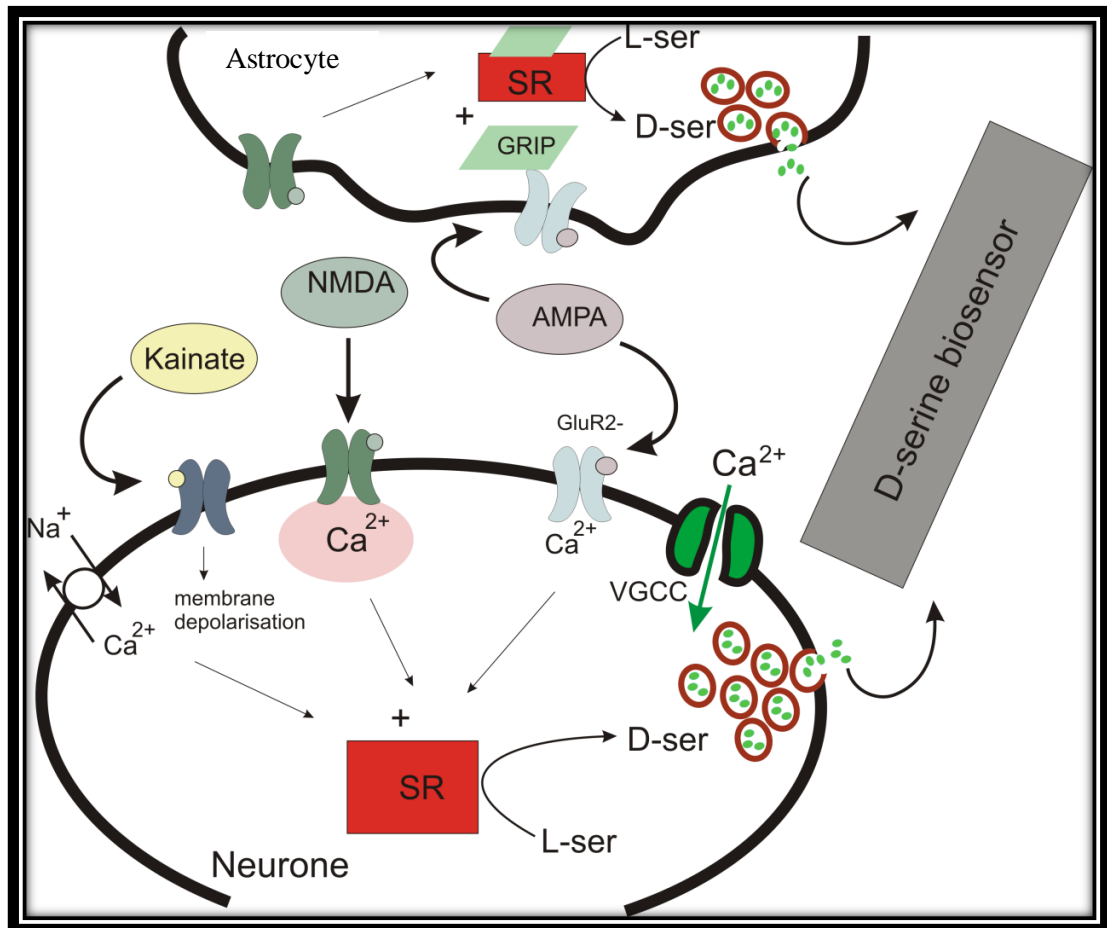
Figure 4: D-serine release mechanisms in the cortex. Membrane depolarisation gives rise to intracellular Ca^{2+} influx via number of different mechanisms, which can release D-serine stores and increase its synthesis by activating SR.

But the D-serine release in the cortex is puzzling in the context of the co-agonist site saturation state of NMDA receptors. The basal D-serine tone in the cortex is $1\pm0.2\mu M$ (mean $\pm$SEM), at these concentrations all NMDA receptor co-agonist sites should theoretically be saturated or almost fully saturated. Although it is unknown whether extracellular D-serine levels differ in the cortical layers; if there are regions where levels of D-serine are lower, then D-serine release may potentiate NMDA receptor activity.

In the hippocampus, there is evidence for the presence of two different signalling pathways that can be trigger by the same agonists. Both D-serine release and

reductions in D-serine concentrations are elicited by AMPA and kainate. In the case of kainate, both loss and release of D-serine are observed in each of the two regions of the hippocampus studied, suggesting no region-specific response in D-serine. However, with AMPA, loss of D-serine is seen in *s. radiatum* while in the majority of case D-serine release is observed in *s. pyramidale*. This is a region specific response to acute AMPA receptor activation and only the D-serine release (of *s. pyramidale*) is sensitive to the removal of extracellular calcium. D-serine loss in *s. radiatum* remains unaffected under calcium-free conditions. This suggests that the signalling events which generate the D-serine release in *s. pyramidale* are likely to be same as those observed in the cortex, where release (by AMPA, NMDA and kainate) is also affected by the removal of calcium.

But the manner in which D-serine levels can be reduced by AMPA or kainate receptor activation is difficult to grasp. Calcium independent removal of D-serine may occur via D-serine transporters (both glial and neuronal) or the cellular degradation of D-serine through α,β-elimination of water which is observed both *in vitro* and *in vivo* (Foltyn *et al.*, 2005). Mutations in the α,β-elimination property led to increased levels of D-serine in the extracellular space, suggesting that some D-serine removal occurs via this mechanism. Figure 4b, shows the manner in which D-serine reduction in a calcium independent manner can be achieved in cells.

The over-whelming response to NMDA receptor activation was a reduction in D-serine in both regions of the hippocampus (Table 7.2). Though only assumptions can be made about the mechanisms responsible for changes in D-serine observed here, a number of studies have described NMDA receptor mediated D-serine reduction in the brain, as a result of reduced SR activity (figure 4a). Balan et al (2009) show that NMDA receptor activation promotes translocation of cytosolic SR to the plasma membrane of dendrites, where PIP2 occupies the ATP binding site of SR, this dramatically reduces its activity, an effect easily reversed by the block of NMDA receptors (in primary neuronal cultures). D-serine release was diminished by 10% as a result of translocation and mutants of SR unable to bind PIP2 display a four-fold enhancement in activity (Balan *et al.*, 2009; Mustafa *et al.*, 2009). Additionally, NMDA receptor activation can inhibit SR activity through S-nitrosylation at residue C113, a physiological process reflecting the action of neuronally derived NO. This process is lost in nNOS knockout mice (Mustafa *et al.*,

2007). NO is also a known positive regulator of DAAO, perhaps simultaneous reductions in D-serine synthesis and increased degradation cause the decrease in D-serine concentrations observed with NMDA receptor activation. Additionally, D-serine loss occurs in a calcium dependent manner, and reduction in D-serine is virtually eliminated with the removal of extracellular calcium. This provides additional proof that the mechanisms responsible for D-serine loss in *s. radiatum* with AMPA are different from those activated by NMDA.
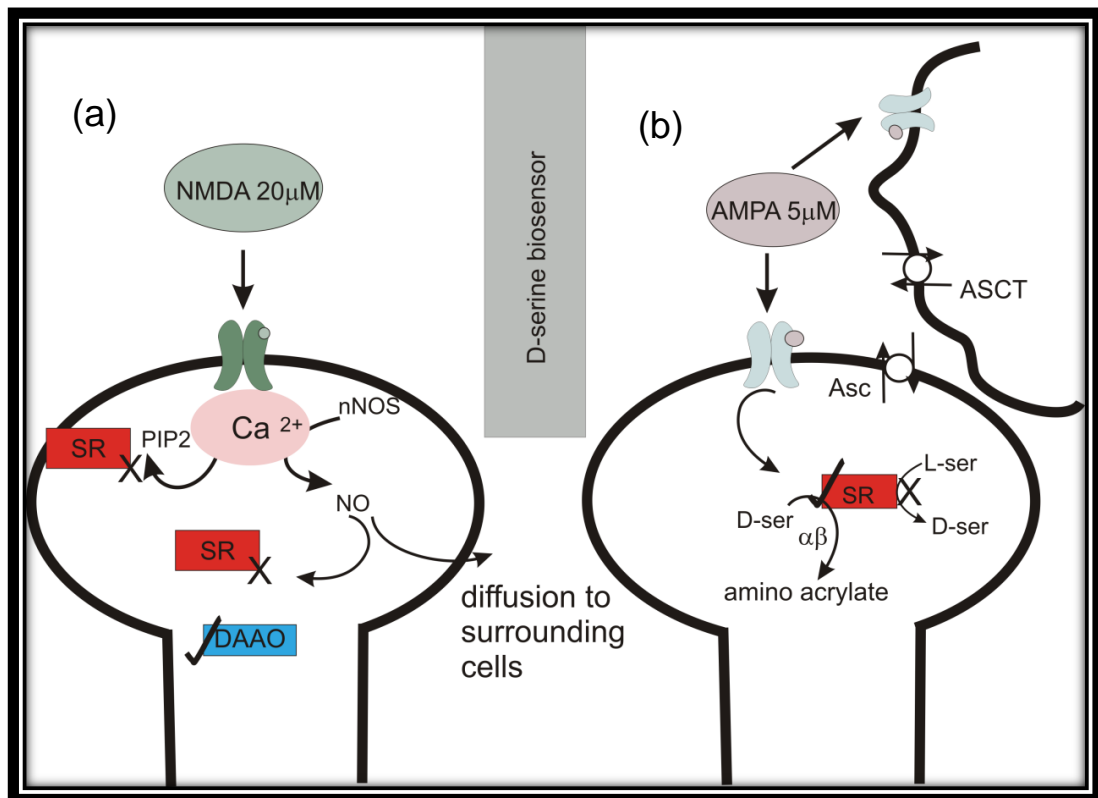
Figure 5: D-serine loss can be induced by a number of mechanisms, both calcium sensitive (a) and calcium-insensitive signals (b). A cross denotes reduction in enzyme activity or an enzymatic pathway while a tick signifies increased enzymatic activity or a pathway.

To summarise, real-time D-serine biosensor recordings have shown presence of at least 3 different signalling events which can be triggered by ionotropic glutamate receptor agonists: (1) a calcium sensitive D-serine release mechanism in the cortex and *s. pyramidale*; (2) a D-serine reducing signalling event which is calcium independent (and elicited by AMPA) and (3) a calcium-sensitive D-serine loss signal (initiated by NMDA).

Thus a single stimulus can have opposing consequences on extracellular D-serine levels in different brain regions and even within a single structure and further still, the significance of these on NMDA receptor activity can vary depending on the existing basal D-serine tone. The reduction of D-serine (calcium sensitive-NMDA and calcium-insensitive AMPA induced) significantly lowers co-agonist site saturation of NMDA receptor and its activity. Under stress conditions such as acute AMPA, NMDA and kainate receptor activation this reduction is neuroprotective. In contrast D-serine release both in *s. pyramidale* (with AMPA) and cortex does not alter NMDA receptor co-agonist occupancy and hence function. The importance of this is yet unknown.

7.4 D-serine reduction as a novel neuro-protective pathway

Stroke is a major cause of mortality and morbidity with a range of possible contributing factors including alcohol/drug abuse, smoking, poor diet, age (chances of stroke double with each decade after 55 years), gender (men are more at risk than women) and family history. Stroke is defined as a loss of brain function caused by a blockage or rupture of blood vessels. Interruption of the blood supply to the brain results in tissue hypoperfusion, hypoxia and eventual cell death. Excessive glutamate release during stroke is thought to be a major contributor of neuronal death, as mass calcium influx activates pro-death signals (Rothman & Olney, 1986).

Traditional mechanisms to prevent stroke damage have focused on reducing extracellular glutamate levels and increasing cellular adenosine levels, both of which are neuroprotective. Reductions in glutamate levels lowers AMPA, NMDA, kainate and also metabotropic glutamate receptor activation while Adenosine operates via the abundantly expressed adenosine A1 receptor and it can reduce glutamate release and hyperpolarise neurones (Fredholm *et al.*, 2005). I have shown profile of extracellular glutamate concentrations during ischemia: initially glutamate levels ate reduced followed by release at the onset of the anoxic depolarisation and detailed recordings made by Dale et al (2002) with adenosine biosensors show that during an ischemic episode adenosine release occurs almost immediately following the onset of ischemia and similarly during hypoxia (Dale *et al.*, 2000; Frenguelli *et al.*, 2003). At the anoxic depolarisation ATP release is also observed, as shown in figure 6 and PPE in both nucleotides is observed with reoxygenation.
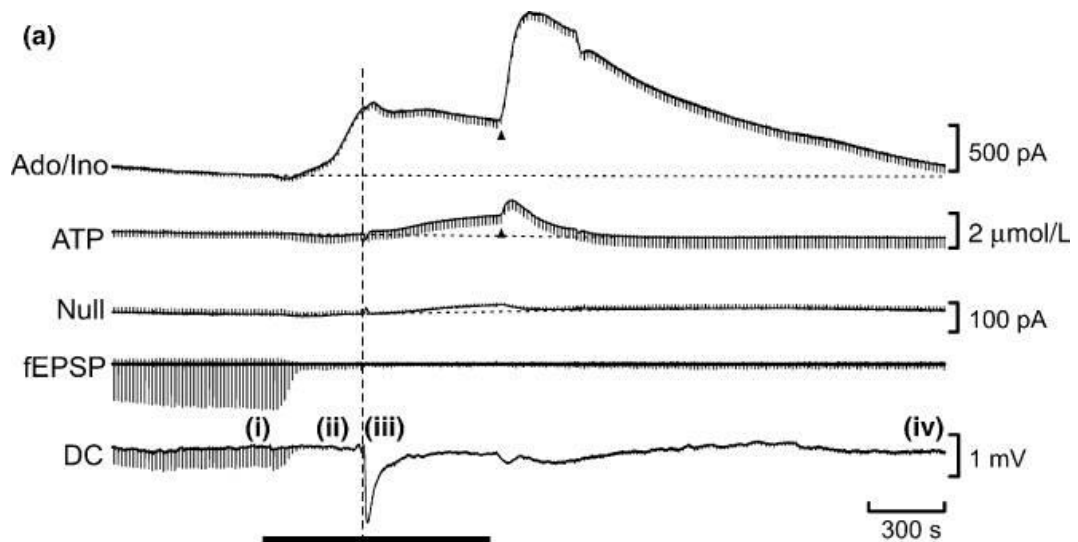
Figure 6: Adenosine release increases gradually during the in vitro **ischemic episode (black bar) then displays a surge on reoxygenation (black arrowhead). ATP release only occurs following the anoxic depolarisation (Frenguelli** *et al.***, 2003).**

Until now, it had not been possible to determine in detail the alterations in D-serine during stroke. A study recently performed using capillary dialysis to follow changes in D-serine and glutamate levels is poor in resolution compared to D-serine biosensors. Kirschner et al apply ischemia for 24 minutes, and took samples every 2 minutes to follows changes induced. Figure 7 shows these findings: the initial reduction in D-serine and glutamate levels observed here with microelectrodes are not clear but release in both transmitters occurs after 10 minutes. Additionally, instead of quantitative recordings, only percentage changes from baseline can be clearly established (Kirschner *et al.*, 2009).
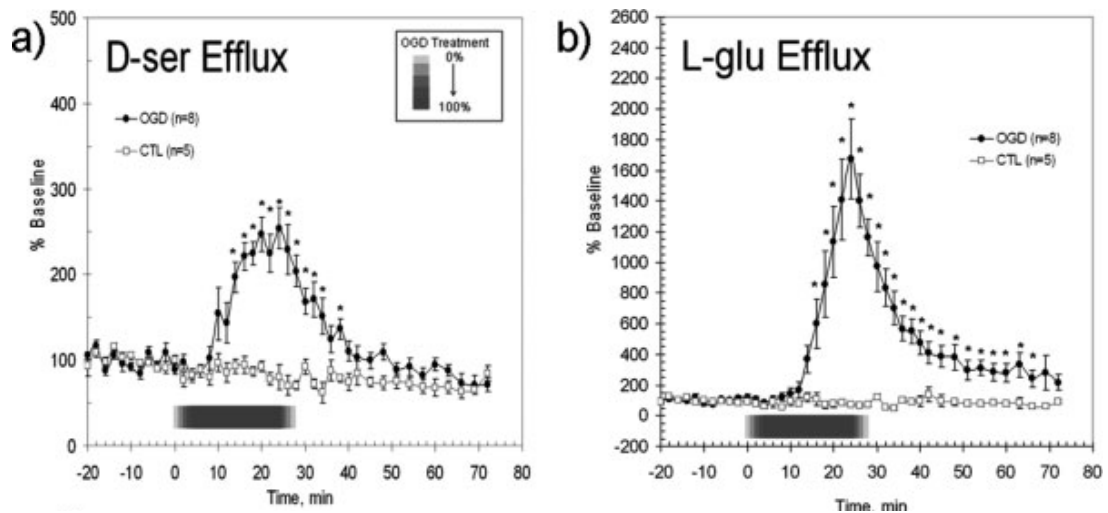
Figure 7: D-serine and glutamate efflux is observed using capillary dialysis under ischemic conditions in the hippocampus (Kirschner *et al.*, 2009).

With the use of D-serine biosensors much greater resolution of the changes that occur in D-serine levels is permitted. Two models of stroke: hypoxia and ischemia were used to analyse changes in D-serine to determine whether this co-agonist augmented NMDA receptor mediated cell death. In both models, D-serine levels are reduced (chapter 5). This reduction is neuro-protective since it signals lowered NMDA receptor activity. In fact the reduction in NMDA-receptor dependent fEPSP correlates with D-serine loss during hypoxia and addition of excess D-serine during hypoxia lessens the $fEPSP_N$ reduction. This indicates that D-serine levels are reduced to ensure cell damage due to over-excitation at the NMDA receptor does not occur. Similarly during ischemia the initial loss of D-serine is neuroprotective but unlike during hypoxia, D-serine release is observed approximately 5 minutes into oxygen-glucose deprivation. Since this is a closer model of insult and injury to cells during stroke, and NMDA receptors mediate much of the cell death that occurs during ischemia and D-serine release may initiate NMDA receptor mediated cell death. D-serine release during ischemia occurs over 2 minutes before glutamate levels rise and the onset of anoxic depolarisation occurs. Considering figure 6, adenosine release is observed immediately with ischemia and out of the two agonists for the NMDA receptor; it is D-serine which is released first. Then anoxic depolarisation occurs and both ATP and glutamate are released. It has long been believed that glutamate released is the major cause of cell damage during stroke but *in vitro* D-serine release occurs before the release of glutamate suggesting that D-serine may be a trigger which instigates excessive NMDA receptor activation leading to mass Ca^{2+} influx

which can subsequently trigger signalling cascades promoting cell death. D-serine release mechanisms, SR and DAAO may be novel targets for therapies in stroke, as these can potentially reduced D-serine release and may be important in blocking anoxic depolarisation, which protects against cell death (Anderson *et al.*, 2005). In serine racemase knockout mice, it was noted that cell damage was significantly reduced during ischemic episodes compared to controls even though increased expression and sensitivity in NMDA receptors was observed (Mustafa *et al.*, 2010). However, clinical trials with NMDA receptor glycine site antagonists licostinel and gavestinel have shown no significant difference from placebo, though the drugs are well tolerated since they are specific for NMDA receptors (Albers *et al.*, 1999; Warach *et al.*, 2006). This may be indicative of the need to minimise D-serine levels or the effect of this co-agonist at the NMDA receptor very early into an episode to reduce subsequent cell damage.

7.5 <u>Diagnostic applications for D-serine biosensors</u>

Since D-amino acids are not widely utilised by mammalian cells, the presence or absence of these may be an indicator of disruptions in normal cell function. Two specific cases where changes D-serine levels may signify physiological dysfunction are schizophrenia and Alzheimer's disease, where D-serine biosensors may be used as diagnostic tools for disease states.

7.5.1   Schizophrenia and low D-serine levels

Schizophrenia affects approximately 1% of the World's population and describes a group of psychotic disorders characterised by disturbances in thought, perception, behaviour and communication (Liddle, 1987). The negative symptoms include apathy, poor rapport, lack of spontaneity, motor retardation, blunted affect and emotional withdrawal. The positive symptoms of schizophrenia include bizarre trains of thoughts, hallucinations and delusions (Mueser & McGurk, 2004). The age of on set is early 20s in males and slightly later in females, with societal costs of the disease reaching £6.7 billion in the UK alone. Approximately 10% of affected individuals commit suicide.

There is no clear prognosis procedure which can predict physiological alterations before the onset of symptoms. NMDA receptor hypofunction is thought to contribute

to the disease, as a result of reduced availability of D-serine. The gene G72 is thought to be a genetic marker of the disease; this encodes a DAAO promoter able to increase the activity of this enzyme by three-fold (Chumakov *et al.*, 2002). The D-serine content of patients is thought to be as much as 25% less compared to control in cerebral spinal fluid (Bendikov *et al.*, 2007) and some studies have shown the effectiveness increasing patient D-serine levels (Tsai *et al.*, 1998; Heresco-Levy *et al.*, 2005; Lane *et al.*, 2005).

Changes in D-serine levels may be a biological marker for the onset of disease. D-serine biosensors can detect D-serine level and may be used in addition to existing diagnostic procedures to detected lower D-serine levels in blood, urine or CFS in patients. Additionally, the sensors may be used to follow the viability of drug treatments.

7.5.2   Alzheimer's disease and increased D-serine levels

Alzheimer's disease is thought to affect 1 in 3 individuals over 65 years of age (UK, 2009) and diagnosis of this disease is based on memory tests and brain scans. Since symptoms are slow to develop and are frequently similar to other conditions, the onset of disease is not easily recognised. Over excitation of the NMDA receptor is thought to contribute to cell death and damage, where increased D-serine release by microglia can potentially augment activity of this channel (Barger & Basile, 2001; Butterfield & Boyd-Kimball, 2004). Inflammation of microglia is caused by amyloid β-peptide (αβ), which can also bind SR (at the AP1 region) to increase D-serine synthesis and subsequent release. The D-amino acid content in CSF increases from 17.9 to 26.4nmol/ml, this change can be easily detected by D-serine biosensors. The detected of increased D-serine levels in blood, urine or CFS may be a biological marker of the disease state and D-serine biosensors may also be utilised in monitoring the success of treatments.

7.6 D-amino acid content and bacterial contamination

D-amino acids are generally restricted to bacteria and lower organisms with high concentrations of these marking the presence of bacterial contamination in dairy products including milk and cheese as well as fruit juices. The D-amino acid content of consumable products are regularly tested by the food standards agency; D-alanine

is generally taken as a indicatory of bacterial contamination. The current techniques used to detected D-amino acids are HPLC or enzymatic assays. The use of D-serine biosensors (which can also detect all non-acidic amino acids including D-alanine) is cheaper, faster, without the need for complicated equipment.

7.7 <u>Summary</u>

I have designed the most sensitive D-serine biosensors to date and shown that these tools have the ability to transform the way in which D-serine signalling in the brain is studied. I have demonstrated novel insight into D-serine distribution in the brain, and its activity dependent modulation in real-time by ionotropic agonists, a glial-specific modulator and high frequency stimulation. Additionally, I present evidence for a neuroprotective role of D-serine in stroke damage and a possible crucial function in initiating cell damage and death.

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