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Summary

Age-associated deficits in learning and memory are closely correlated with impairments of synaptic plasticity. Analysis of *N*-methyl-D-aspartate receptor (NMDAr)-dependent long-term potentiation (LTP) in CA1 hippocampal slices indicates that the glial-derived neuromodulator D-serine is required for the induction of synaptic plasticity. During aging, the content of D-serine and the expression of its synthesizing enzyme serine racemase are significantly decreased in the hippocampus. Impaired LTP and NMDAr-mediated synaptic potentials in old rats are rescued by exogenous D-serine. These results highlight the critical role of glial cells and presumably astrocytes, through the availability of D-serine, in the deficits of synaptic mechanisms of learning and memory that occur in the course of aging.

Key words: aging; astrocyte; glutamate; hippocampus; NMDA receptor; synaptic plasticity.

Introduction

Long-lasting changes in neurotransmission such as long-term potentiation (LTP) are calcium-dependent forms of synaptic plasticity considered as basic neuronal activities underlying memory formation (Martin *et al*., 2000). Aging is associated with deficits in learning and memory and with changes in the threshold and/or the magnitude of LTP (Foster, 1999; Rosenzweig & Barnes, 2003). Due to their high permeability to Ca^{2+} which triggers the activation of several intracellular signalling pathways, *N*-methyl-D-aspartate receptor (NMDAr) subtypes of glutamate receptors control the degree of synaptic efficiency (Wang & Kelly, 1996). Although impaired NMDAr activation in aged rodents (Barnes *et al*., 1997; Potier *et al*., 2000) was proposed for the deficit of synaptic plasticity (Clayton *et al*., 2002), the mechanisms involved remained elusive. In addition to glutamate, NMDAr activation requires the binding of a co-agonist at the strychnine-insensitive glycine modulatory site (Johnson & Ascher, 1987). Recent evidence indicates that the enantiomer D-serine produced in glial cells (Schell *et al*., 1995; Wolosker *et al*., 1999; Williams *et al*., 2006) is an endogenous co-agonist of NMDAr in brain areas involved in memory processing (Mothet *et al*., 2000; Yang *et al*., 2003, 2005). This raised the possibility that glial cells and particularly astrocytes, through the availability of D-serine, could be involved in the alterations of the cellular mechanisms of learning and memory that occur in the aging brain. We have examined the age-related changes in D-serine efficiency in the CA1 neuronal networks of the hippocampus which undergo well-documented alterations of synaptic plasticity in the course of aging (Foster, 1999; Rosenzweig & Barnes, 2003).

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Results

D-Serine governs long-lasting changes in synaptic transmission

Theta burst stimulation (TBS)-induced LTP (Fig. 1A) of synaptic transmission closely depended on the activation of NMDAr since it was abolished in slices pretreated with the selective antagonist D-2-amino-5-phosphonovalerate (D-APV; 50 μM) or by L689.560, a specific antagonist at the glycine modulatory site (10 μM) (Fig. 1A,C). To specify the role of D-serine in TBS-induced LTP, we incubated slices for at least 1 h with D-amino acid oxidase (DAAO), the D-serine degrading enzyme. In pre-incubated slices, the magnitude of field excitatory postsynaptic potentials (fEPSP) was not affected as compared to responses recorded in control slices (data not shown). By contrast, TBS-induced LTP was totally occluded in DAAO-treated slices (Fig. 1B,C). When saturating doses of D-serine (100 μM) were added to DAAO pre-incubated slices, LTP was restored (Fig. 1B) and the magnitude of this LTP was not significantly different to that of the potentiation recorded in control conditions (Fig. 1C). These data therefore indicate that D-serine is required for inducing TBS-induced LTP in CA1 hippocampal area.

D-Serine metabolism is impaired in aged hippocampi

We then investigated whether the tissue availability of D-serine was affected during aging. HPLC (high-performance liquid

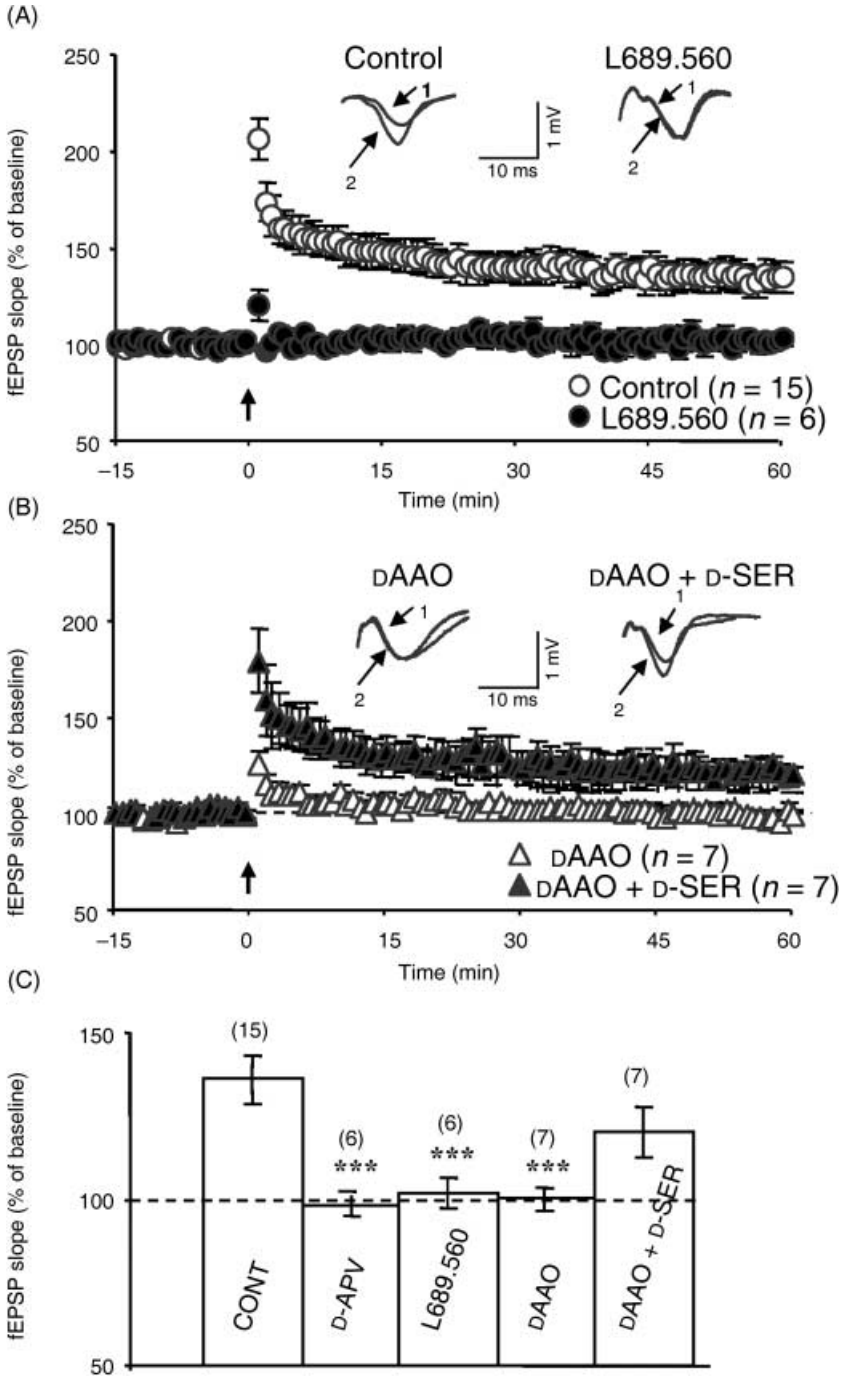

Fig. 1 Theta burst stimulation (TBS)-induced long-term potentiation (LTP) depends on D-serine in the CA1 hippocampal network. (A) Time-course of LTP induced in young rats by TBS (arrow) in control medium (open circles) and in slices supplied with the antagonist of glycine-binding site L689.560 (filled circles). (B). TBS-induced potentiation in slices pretreated with D-amino acid oxidase (DAAO) (open triangles) and in slices pretreated with DAAO and supplied with D-serine (100 μM) (filled triangles). In both (A and B), insets are superimposed examples of field excitatory postsynaptic potentials (fEPSP) recorded in each condition before (1) and 60 min after TBS (2). *n* refers to the number of recorded slices (Bar scales: 1 mV and 10 ms). (C) Bar graph illustrating the mean increase (± SEM) in fEPSP slope averaged from 45 to 60 min after TBS in each pharmacological conditions (***$P < 0.001$). The number of animals is indicated in parentheses.

chromatography) analysis revealed that D-serine was present in young hippocampus at levels of 16.0 ± 3.1 nmol mg^{-1} of protein with a D/L-serine ratio of 0.42. In tissues of aged animals, the level of D-serine dramatically decreased to 3.4 ± 1.0 nmol mg^{-1} of protein ($P < 0.01$) whereas the level of L-serine was slightly enhanced (Fig. 2A). Consequently, the D/L-serine ratio was lowered to 0.06. Interestingly, the content of glycine, another ligand for the glycine modulatory site of NMDAr, was not affected (Fig. 2A). We then checked for the expression of serine racemase which converts L- to D-serine. Both real-time quantitative polymerase chain reaction (RT-PCR) analysis (Fig. 2B, left) and semiquantitative Western blotting experiments highlighted that mRNAs (–42%, $P < 0.01$) and protein expression (–56%, $P < 0.02$) for serine racemase strongly decreased in aged hippocampi (Fig. 2C). DAAO mRNA levels were only slightly reduced (–28%, $P < 0.05$) (Fig. 2A) whereas DAAO protein levels were not affected (Fig. 2C). Age-related decrease in serine racemase expression did not reflect a general impairment of glial metabolism since we did not observe any alteration in mRNA levels for glutamine synthetase, a specific glial enzyme (data not shown). In addition, a gliosis occurs in aged rats as evidenced by the increase in the GFAP/MAP2 ratio in these animals ($P < 0.05$) (Fig. 2B, right).

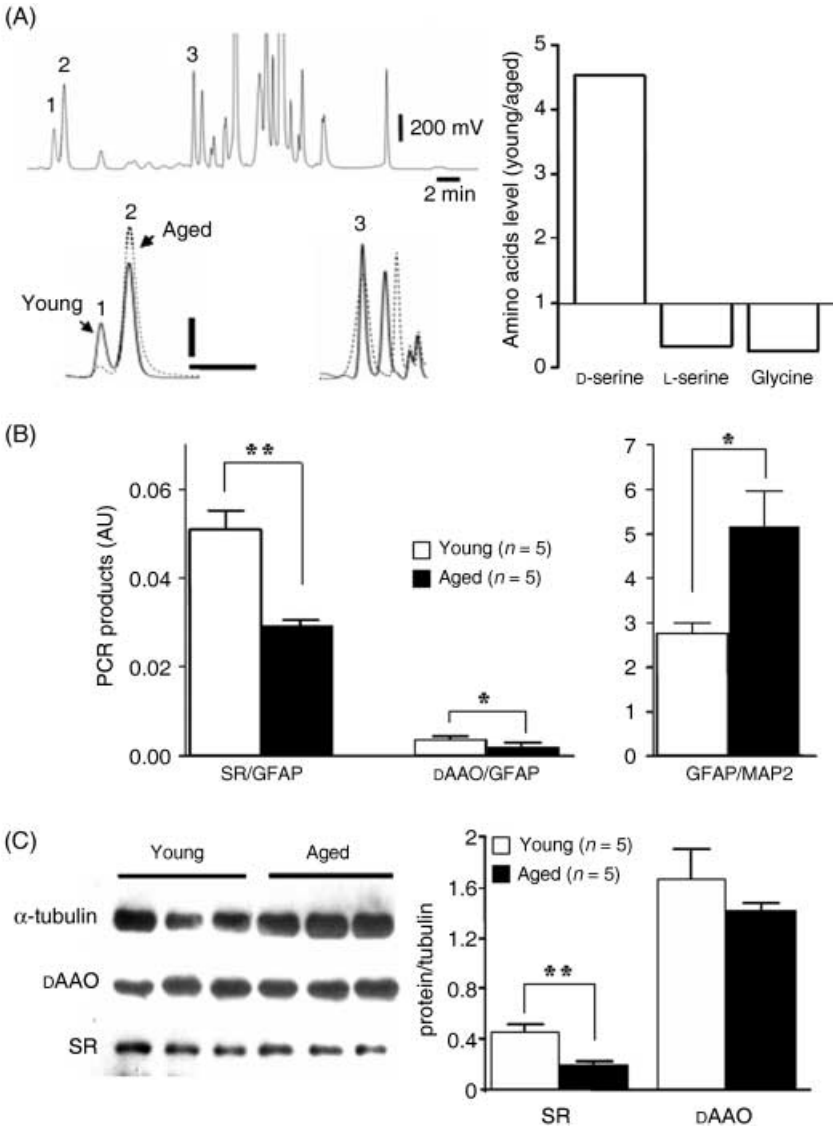

Fig. 2 D-serine biosynthesis is impaired in aged rats. (A, left) Representative amino acids chromatogram obtained from a young rat hippocampus. D-serine (1), L-serine (2) and glycine (3) were resolved with retention time 6.41, 7.04 and 15.84 min, respectively. Bar = 200 mV. Insets show the mean peaks for D-serine, L-serine and glycine in young (n = 7, solid lines) and aged (n = 8, dashed lines) rats. (A, right) Bar graphs representing the ratio young/aged for D-serine, L-serine and glycine. (B, left) mRNA levels of serine racemase (SR) and D-amino acid oxidase (DAAO) in young (n = 5) and aged (n = 5) rats. SR and DAAO levels were normalized to the housekeeping gene GFAP. (*P < 0.05 and **P < 0.01). (B, right) GFAP/MAP2 mRNA ratio determined in both groups of animals. (*P < 0.05). (C, left) Immunoblots for α-tubulin (upper bands), DAAO (middle bands) and for serine racemase (SR) (lower bands) in young and old rats. Ten micrograms of protein was loaded per lane. (C, right) Bar graphs depicted quantitative analysis of immunoreactivity for SR and DAAO in young (n = 5) and old (n = 5) rats when normalized to α-tubulin. (**P < 0.01)

Exogenous D-serine prevents age-related alterations of NMDAr-dependent LTP

Considering the lower availability of D-serine in hippocampal tissues of aged rats, we next examined whether the age-related deficit of synaptic plasticity could be rescued by providing D-serine to slices. The magnitude of TBS-induced LTP was significantly weaker in CA1 hippocampal slices of aged rats as compared to young animals ($F_{1,23}$ = 6.61, P < 0.01) (Fig. 3A, left). In the presence of D-serine (100 μM), LTP was significantly enhanced in both young (P < 0.05) and aged (P < 0.01) animals (Fig. 3B) but the increase was higher in old than in young rats (25% vs. 15%, respectively). Consequently, the age-related deficit in LTP was no longer significant after saturating glycine modulatory sites with exogenous D-serine (Fig. 3A, right). Because TBS-induced LTP is dependent on NMDAr activation, we then checked for the age-related effects of D-serine on NMDAr-mediated synaptic potentials. The magnitude of D-APV sensitive fEPSPs (Fig. 3C) was significantly reduced in aged as compared to young animals (P < 0.05) whereas the magnitude of presynaptic fibre volley was not affected. D-Serine (100 μM) prevented the age-related decrease in NMDAr-mediated synaptic potentials (Fig. 3C). The co-agonist increased the magnitude of synaptic potentials in young rats (23.6 ± 5.5%) (Fig. 3D) indicating that NMDAr glycine modulatory sites were not fully saturated in slices of young rats as already indicated by the increase in LTP magnitude. Importantly, the increase in NMDAr-mediated fEPSPs in aged animals (41.6 ± 5.9%) was significantly higher than in young rats (P < 0.05) indicating a greater efficiency of exogenous D-serine to activate NMDAr in aged rat hippocampus (Fig. 3D).

The affinity of D-serine for NMDAr binding site is not altered in aged hippocampi

We then tested whether the higher potency of D-serine in aged rats involved changes in the specific affinity of the glycine modulatory

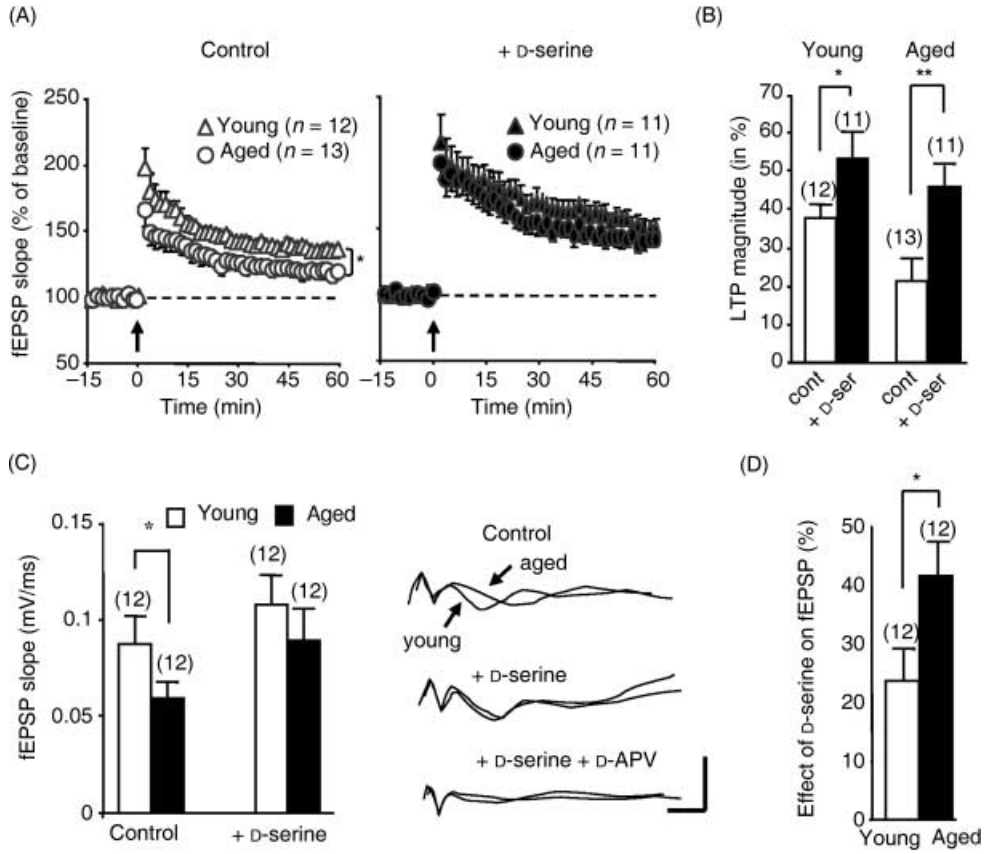

Fig. 3 D-serine rescues age-related deficit in *N*-methyl-*D*-aspartate receptor (NMDAr) activation. (A) Time course of mean Theta burst stimulation (TBS)-induced long-term potentiation (LTP) calculated from slices of young ($n = 7$) and aged ($n = 9$) rats in control medium (left panel) and in slices supplied with D-serine (100 μM; right panel) (*$P < 0.05$). (B) Bar graphs comparing LTP magnitude averaged from the last 15 min of recordings in young and aged rats in control medium (white bars) and in the presence of D-serine (black bars) (*$P < 0.05$ and **$P < 0.01$). (C) Bar graphs comparing the mean NMDAr-mediated fEPSP (field excitatory postsynaptic potential) slope averaged from young and aged rats before and 15 min after adding D-serine to slices (*$P < 0.05$). In the right are surimposed sample traces of evoked NMDAr-mediated fEPSPs recorded in a young and an aged rat in control medium, in the presence of D-serine (100 μM) and in the presence of D-serine and D-APV (30 μM) (right). Traces are averages of three consecutive responses obtained with a stimulus current intensity of 300 μA (Bar scales: 0.5 mV and 10 ms). (D) Bar graph of the increase in NMDAr-mediated fEPSP slope induced in young ($n = 8$) and aged ($n = 8$) rats by D-serine (*$P < 0.05$). In B, C and D, the number of recorded slices is indicated in parentheses.

site for the co-agonist. Using the potent radioligand [^{3}H] L689.560, high labelling was found in the strata *radiatum* and *oriens* of CA1 region and the dentate gyrus in both young and aged rats (Fig. 4A). In competitive studies with increasing concentrations of unlabeled D-serine (from 3 nM to 300 μM), IC_{50} assessed in *stratum radiatum* was not significantly affected in old animals (Fig. 4B) nor in CA1 *stratum oriens* or in the lower blade of the dentate gyrus (data not shown).

Discussion

Age-dependent deficit in spatial memory has been correlated to alterations in synaptic plasticity within the hippocampal formation (see Rosenzweig & Barnes, 2003). Changes in calcium homeostasis and alterations of NMDAr are predominantly responsible for the age-related deficit of synaptic plasticity (Magnusson, 1998; Foster, 1999). In this study, we confirm that NMDAr activation is impaired in aged animals (Barnes *et al.*, 1997; Potier *et al.*, 2000; Clayton *et al.*, 2002). However, our results indicate that changes in NMDAr expression are not the primary mechanism involved in this deficit (Magnusson, 2000; Clayton *et al.*, 2002), but rather that alterations in their functional properties occur. Indeed, no significant reduction of mRNA for the different NMDAr subunits was observed in the aged animals used in the present study (data not shown). In addition, the age-related alterations of both NMDAr-mediated synaptic potentials and TBS-induced LTP were prevented when NMDAr were extensively recruited by saturating doses of D-serine. These latter results also indicate that an impaired activation of NMDAr glycine modulatory sites by the endogenous co-agonist is involved in the age-related deficit of NMDAr activation. Initially assigned to glycine (Johnson & Ascher, 1987; Reynolds *et al.*, 1987; Kemp & Leeson, 1993), a major role in activating the strychnine-insensitive glycine modulatory site of NMDAr in the hippocampus is now attributed to the enantiomer amino acid D-serine (for review Miller, 2004). We here show that D-serine is indeed required for the induction of hippocampal LTP in adult rats, as previously reported in 16- to 18-day postnatal animals (Yang *et al.*, 2003)

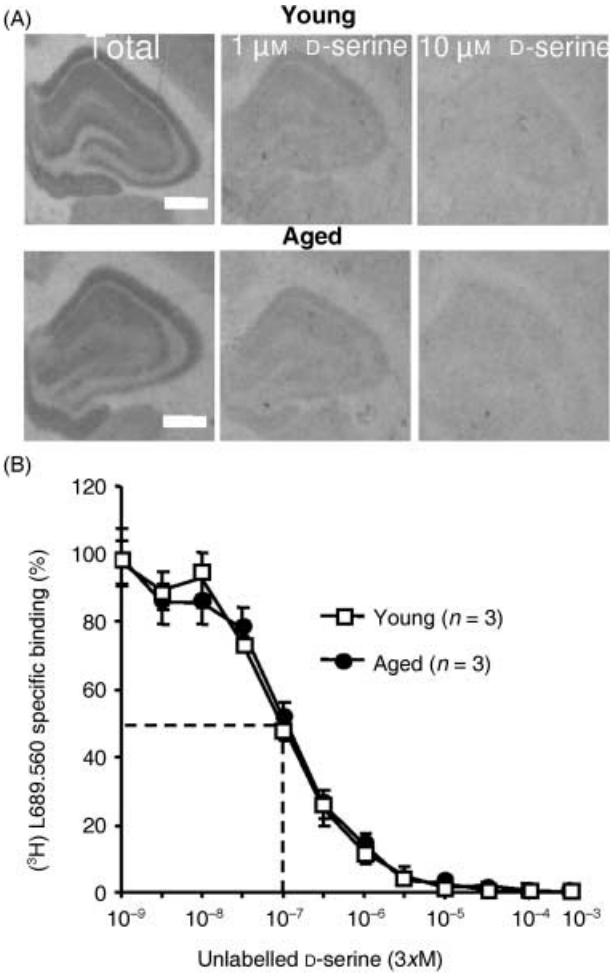

Fig. 4 The affinity of D-serine binding sites is not affected in aged rats. (A) Examples of displacements of [^{3}H] L689.560 binding in hippocampal formation by increased concentrations of unlabeled D-serine in a young and an aged rat (Bar = 1 mm). (B) Representative mean displacement curves of specific [^{3}H] L689.560 binding by D-serine in CA1 stratum radiatum of young (n = 3) and aged (n = 3) rats.

or in senescent-accelerated (SAMP8) mice (Yang *et al*., 2005). Indeed, treatment of slices with DAAO occludes LTP by neutralizing D-serine action. This effect of DAAO on LTP was comparable to that of antagonists acting at the glycine modulatory site. This latter observation extends the notion that D-serine is the main endogenous agonist that governs the activation of the glycine modulatory site of NMDAr during induction of long-term changes in synaptic plasticity (Yang *et al*., 2003), as also recently reported for NMDA-elicited neurotoxicity (Shleper *et al*., 2005). We provide evidence that exogenous D-serine facilitates both NMDAr-mediated synaptic potentials (Martina *et al*., 2003; see also Watanabe *et al*., 1992) and NMDAr-dependent LTP, indicating that glycine modulatory sites are not fully saturated in the hippocampal slice preparation of young adult rats (Krasteniakov *et al*., 2005). Interestingly, the facilitation effect of D-serine is significantly higher in aged animals. This greater efficacy of the co-agonist during aging reflects changes in the degree of receptor saturation that is determined by the specific affinity of the glycine modulatory site and/or changes in endogenous D-serine concentration. We did not observe age-related modification of receptor affinity (see also Miyoshi *et al*., 1990; Nagata *et al*., 1998). In young hippocampi, high contents of D-serine were found, at levels much higher than initially reported by the group of Hashimoto (1993). We reasoned that this apparent discrepancy may reflect either differences between rat strains and/or variations in HPLC preparation of amino acid samples and analysis. During aging, we observed a dramatic decrease in the availability of D-serine in hippocampal tissues as also recently indicated in cerebral cortex by immunohistochemistry (Williams *et al*., 2006). Interestingly, this deficit is specific to D-serine since the content of the other putative NMDAr co-agonist, glycine, is not affected. Previous studies have reported that D-serine levels do not change in aged Wistar rats (Hashimoto *et al*., 1993) and SAMP8 mice (Nagata *et al*., 1998). This discrepancy could reflect again a difference between species or strain, although an interesting possibility is that it may also be due to the fact that the experiments performed in rats used whole brain extracts instead of isolated hippocampi as in the present study. If this were the case, these results imply that cerebral D-serine may be regionally affected during aging. Although recent studies have shown that D-serine is present in both neurons and glial cells in the central nervous system (Schell *et al*., 1995, 1997; Yasuda *et al*., 2001; Williams *et al*., 2006), the metabolism of this amino acid is virtually confined to the latter cell type (Wolosker *et al*., 1999; Wu *et al*., 2004; Mothet *et al*., 2005). The decrease in D-serine in aged animals does not correlate with general astrocyte impairment. Indeed, age-related gliosis, confirmed in the present study, has been previously demonstrated (Finch, 2003). The age-related decrease in D/L-serine ratio rather suggests a significant impairment in the D-serine biosynthesis pathway during aging. Accordingly, we have shown a lower expression of serine racemase at both mRNA and protein levels in aged rats, whereas expression levels of DAAO and other glial specific markers such as glutamine synthetase were not affected. Our study therefore indicates that the age-related decrease in D-serine availability (see also Williams *et al*., 2006) is mainly due to the impairment of its biosynthesis pathway. Other mechanisms may also amplify this decrease, for example alterations in the diffusion rate (reduced tortuosity) and/or an increased reuptake by membrane transporters.

A wealth of evidence now indicates that astrocytes play a significant role in modulating synaptic transmission (Auld & Robitaille, 2003). Our results show for the first time that through the availability of the neuromodulator D-serine, glial cells are also involved in functional deficits that occur during aging. Although behavioural studies indicate that memory deficits in old rats are alleviated by related agonists such as D-cycloserine (Aura *et al*., 1998), long-term treatments of age-related memory deficits with large doses of D-serine itself seem unlikely because of nephrotoxic side-effects of the co-agonist (Silbernagl *et al*., 1999). At any rate, the role of serine racemase in age-associated deficits reported here opens new perspectives in the search of relevant therapeutic strategies for memory impairments related to brain aging.

Experimental procedures

Animal protocols

Experiments were carried out in accordance with the European Communities Council Directive (86/809/EEC) regarding the care and use of animals for experimental procedures and approved by the local ethical committee. The experiments were conducted in 3- to 6-month-old (n = 49) and 25- to 33-month-old (n = 33) male Sprague-Dawley rats purchased from IFFA-CREDO (France). Rats were maintained on a controlled light-dark cycle at a constant temperature (22 ± 2 °C) with *ad libitum* access to food and water.

Electrophysiology

Transverse hippocampal slices (400 μm) were obtained as previously described (Potier *et al*., 2000) in rats anaesthetized with halothane before decapitation. Slices were prepared in ice-cold artificial cerebrospinal fluid (aCSF) and placed in a holding chamber for at least 1 h. The composition of aCSF is as follows (in mM): NaCl 124, KCl 3.5, $MgSO_4$ 1.5, $CaCl_2$ 2.3, $NaHCO_3$ 26.2, NaH_2PO_4 1.2, and glucose 11, pH 7.4. A single slice was transferred to the recording chamber and continuously superfused with aCSF pregassed with 95% O_2/5% CO_2.

Extracellular recordings were obtained at 30–35 °C from the apical dendritic layer of CA1 area using micropipettes filled with 2 M NaCl. Presynaptic fibre volleys (PFVs) and field excitatory postsynaptic potentials (fEPSPs) were evoked by electrical stimulation of Schaffer collaterals and commissural fibres located in *stratum radiatum*. NMDAr-mediated fEPSPs were recorded in slices perfused in a low (0.1 mM) Mg^{2+} aCSF supplemented with the AMPA/kainate receptor antagonist 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulfonamide (NBQX, 10 μM) and with picrotoxin (10 μM) to block the inhibitory neurotransmission. A knife cut separating CA3 and CA1 was introduced to prevent the propagation of epileptiform discharges. The slope of three averaged PFVs and fEPSPs was measured and plotted against the stimulus intensity (from 100 to 500 μA) using the Acquis 1 software (CNRS, Paris, France). The effects of D-serine (100 μM) were determined by measuring input/ouput curves constructed 15 min after the co-agonist was added to aCSF.

In order to investigate LTP of synaptic transmission, test stimulus was applied every 15 s in a control medium and adjusted to get an fEPSP with a baseline slope of 0.1 V/s. The slope of three averaged fEPSPs was measured for 15 min before TBS was induced. TBS consisted of five trains of four pulses at 100 Hz separated by 200 ms and delivered at test intensity. This sequence was repeated three times with an interburst interval of 10 s. Testing with single pulse was then resumed for 60 min to determine the level of stable LTP. In pharmacological experiments, all drugs were applied for 10 min prior to establishment of the baseline and were maintained throughout the recording.

Determination of D-serine, L-serine and glycine content in hippocampal tissues

Free amino acids were extracted from pooled hippocampal tissues of young and aged rats with trichloroacetic acid, according to published procedures (Schell *et al*., 1997). HPLC analysis was performed by a precolumn derivatization of samples using O-phthaldialdehyde and N-acetyl cysteine and diasteroisomers were resolved in isocratic phase on a C18 novapak column. The amount of D-serine was adjusted to content of proteins determined by a Dc protein (Bio-Rad Laboratories, Hercules, CA, USA). Standards of D-serine were used to normalize results.

Quantitative real-time polymerase chain reaction

Total RNA was prepared from frozen tissues using the RNeasy Midi kit (QIAGEN, Valencia, CA, USA). Total RNA was converted to cDNA using M-MLV reverse transcriptase primed with random hexanucleotoides. Primers for serine racemase (sense: 5′-GATTCGAGGTGCCCTTAACG-3′; antisense: 5′-TTGGGCTTCCCTT CTAAAGTATCA-3′), D-amino acid oxidase (sense: 5′-CCTCAGGTCCG GCTAGAAAGA-3′; antisense: 5′-GGATGACCTCTGCACTTGAAGAT-3′), glial fibrillary acidic protein (GFAP) (sense: 5′-TGACCGCTTTGCTAGCTACATC-3′; antisense: 5′-GCGCCTTGTT TTGCTGTTC-3′) and MAP2 (sense: 5′-AGATCAGAAAGACTGGTTCATCGA-3′; antisense: 5′-CAGCTAAACCCCATTCATCCTT-3′) were designed using PrimerExpress (Applied Biosystems, Foster City, CA, USA). PCRs (20-μL total volume) were performed with Sybr Green PCR master mix using standard protocol on an ABI 7000 Sequence Detection System. Raw Ct values were obtained with SDS 2.0 and were used for relative expression calculations.

Semiquantitative immunoblotting analysis

Western blot analysis was performed as described previously (Puyal *et al*., 2002). Briefly, after lysis, protein extracts were subjected to electrophoresis (12% SDS-polyacrylamide gel) and electroblotted onto PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). Membranes were probed with polyclonal antibodies against D-amino acid oxidase (DAAO) (1: 2000, Nordic Immunological Laboratories, Tilburg, The Netherlands), and serine racemase (1 : 200, Santa Cruz Biotechnology, Wiltshire, UK) or monoclonal α-Tubulin (1 : 400, Santa Cruz Biotechnology) for 1 h at room temperature. After washing, they were incubated with peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat immunoglobulin G (IgG; 1: 2000, Vector Laboratories, Burlingame, CA, USA). Immunoblots were developed by enhanced chemiluminescence (ECL; Amersham Bioscience, Little Chalfont Buckinghamshire, UK). Molecular sizes were estimated by separating prestained molecular weight markers (6.5–175 kDa) in parallel (New England BioLabs, Hertfordshire, UK). Protein bands of interest were analysed by measuring optical density (OD) by scanning densitometry. Densitometric results for serine racemase and DAAO were normalized to α-Tubulin density. Each blot included three successive dilutions of the samples for quantification of variation of density.

Autoradiography

[^{3}H] L-689.560 (22.41 Ci/mmol or 0.83TBq/mmol) was purchased from Tocris Cookson (Bristol, UK). The brains of young and aged rats were quickly removed, frozen for 30 s in isopentane at –40 °C and conserved at –80 °C. Coronal sections (14 μm) were cut on a cryostat at –15 °C and mounted onto glass slides. Brain sections were pre-incubated in 50 mM Tris-acetate buffer (pH 7.0) at room temperature for 30 min to remove endogenous free D-serine. For competition studies, D-serine, glycine and L-689.560 were included in Tris-acetate buffer supplemented with 10 μM strychnine and 1 nM [^{3}H] L-689.560 at 4 °C for 120 min (16). After the incubation, slides were rinsed four times with cold Tris-acetate buffer (4 °C) for a total of 20 s and then in distilled water at the same temperature for 5 s. After drying, slides were placed on [^{3}H]-sensitive film (Hyperfilm-^{3}H, Amersham) for 2 months at 4 °C. The films were developed with dektol (Kodak, cf. CV) for 2 min and analysed using an X-ray film digitizer, RAG500, and Biocom software (les Ulis, France).

Drugs

All drugs were applied in aCSF and included DNQX (10 μM in DMSO), D-APV (30–50 μM), D-serine (100 μM), picrotoxin (10 μM in DMSO) and L689.560 (10 μM in DMSO). All drugs were purchased from Tocris (Illkirch, France). DAAO purified from the yeast *Rhodotorula gracilis* (RgDAAO; 120 U mg^{-1}) was used in our experiments and was a courtesy of Pr. L. Pollegioni.

Statistics

All results are expressed as mean ± SEM. In order to take into account the correlation inherent to repeated measure data, significance was calculated using multivariate analyses of variance (ANOVA) followed by post-hoc unpaired *t*-test. In all cases, differences were considered significant when $P \leq 0.05$.

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