Reduction in glutamate uptake is associated with extrasynaptic NMDA and metabotropic glutamate receptor activation at the hippocampal CA1 synapse of aged rats

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Reduction in glutamate uptake is associated with extrasynaptic NMDA and metabotropic glutamate receptor activation at the hippocampal CA1 synapse of aged rats

Brigitte Potier,[1] Jean-Marie Billard,[1] Sylvain Rivière,[1] Pierre-Marie Sinet,[1] Isabelle Denis,[2] Gaelle Champeil-Potokar,[2] Barbara Grintal,[2] Anne Jouvenceau,[1] Melanie Kollen[1] and Patrick Dutar[1]

[1]Université Paris Descartes, Faculté de Médecine, Centre de Psychiatrie et de Neurosciences, UMR 894, Paris, F-75014 France
[2]INRA, UR 909, Nutrition et Régulation Lipidique des Fonctions Cérébrales (NuRéLiCe), Jouy-en-Josas, F-78352 France

Summary

This study aims to determine whether the regulation of extracellular glutamate is altered during aging and its possible consequences on synaptic transmission and plasticity. A decrease in the expression of the glial glutamate transporters GLAST and GLT-1 and reduced glutamate uptake occur in the aged (24–27 months) Sprague–Dawley rat hippocampus. Glutamatergic excitatory postsynaptic potentials recorded extracellularly in *ex vivo* hippocampal slices from adult (3–5 months) and aged rats are depressed by DL-TBOA, an inhibitor of glutamate transporter activity, in an *N*-Methyl-D-Aspartate (NMDA)-receptor-dependent manner. In aged but not in young rats, part of the depressing effect of DL-TBOA also involves metabotropic glutamate receptor (mGluRs) activation as it is significantly reduced by the specific mGluR antagonist d-methyl-4-carboxy-phenylglycine (MCPG). The paired-pulse facilitation ratio, a functional index of glutamate release, is reduced by MCPG in aged slices to a level comparable to that in young rats both under control conditions and after being enhanced by DL-TBOA. These results suggest that the age-associated glutamate uptake deficiency favors presynaptic mGluR activation that lowers glutamate release. In parallel, 2 Hz-induced long-term depression is significantly decreased in aged animals and is fully restored by MCPG. All these data indicate a facilitated activation of extrasynaptic NMDAR and mGluRs in aged rats, possibly because of an altered distribution of glutamate in the extrasynaptic space. This in turn affects synaptic transmission and plasticity within the aged hippocampal CA1 network.

Key words: aging; extrasynaptic receptors; GLAST; GLT-1; glutamate transporters; hippocampus; mGluRs; synaptic plasticity; synaptic transmission.

Introduction

Glutamate is the most abundant excitatory neurotransmitter in the mammalian central nervous system (CNS), and glutamatergic systems are involved in a wide range of functions, such as motor behavior, cognition and memory [see Fonnum (1984) for review]. However, when present at sustained elevated concentrations, glutamate can lead to excitotoxicity and neuronal death by excessive activation of glutamate receptors (Choi, 1988; Greene & Greenamyre, 1996). Homeostasis of extracellular glutamate levels is therefore critical for normal brain function. Glutamate must be removed efficiently from the extracellular space to terminate excitatory synaptic transmission and to avoid neurotoxicity as no specific degradation enzyme has been reported so far. Consequently, a reliable system of active, potassium- and sodium-dependent high-affinity transporters, transporting glutamate against its concentration gradient, ensures the long-term maintenance of low extracellular concentrations of glutamate.

Five families of glutamate transporters have been cloned to date, EAAT1 to EAAT5 (Excitatory Amino Acid Transporters). GLAST (EAAT1) and GLT-1 (EAAT2) are expressed ubiquitously by astroglial cells, whereas EAAT3 is expressed in neurons throughout the brain. EAAT4 is present almost exclusively in Purkinje cells of the cerebellum and EAAT5 in bipolar cells of the retina [see Danbolt (2001) for review]. In the hippocampus, 90% of the extracellular glutamate uptake relies on the glial transporters GLT-1 and GLAST. Depending on their location and density at the synaptic cleft, but also on their affinity for glutamate, transporters can shape synaptic transmission by limiting glutamate spill-out to extrasynaptic receptors and glutamate spillover to neighboring synapses (Tzingounis & Wadiche, 2007).

A decline in cognition and memory occurs during normal aging i.e. in the absence of neurodegenerative diseases (Barnes, 1979; Barnes & McNaughton, 1985). This decline can be explained, at least in part, by changes in glutamatergic synaptic transmission and plasticity in central areas dedicated to learning and memory, such as the cortex and hippocampus.

Correspondence
Dr Brigitte Potier, Centre de Psychiatrie et Neurosciences UMR U894, 2 ter rue d'Alésia, 75014 Paris, France. Tel.: +33 (0)1 40 78 86 48; fax: +33 (0)1 45 80 72 93; e-mail: brigitte.potier@inserm.fr

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Searching for the role of glutamate in these alterations during the normal process of aging has led to numerous, but sometimes contradictory data [see Segovia *et al.* (2001) for a review] depending on species, experimental protocols or individual variability of the animals. *In vivo* and *in vitro* studies have failed to show any change in glutamate release, and an age-related decrease in glutamate content in the striatum, hippocampus and cortex is still a matter of debate (Price *et al.*, 1981; Banay-Schwartz *et al.*, 1989; Strolin Benedetti *et al.*, 1990; Zhang *et al.*, 2009). The expression and function of glutamate receptors are strongly subject to age-dependent variation. The expression of the AMPAR subtype of glutamate receptors is unaffected, despite weakened AMPAR-mediated transmission (Potier *et al.*, 2000; Kollen *et al.*, 2008). However, aging is associated with the alteration of *N*-Methyl-D-Aspartate receptor (NMDAR) expression (Sonntag *et al.*, 2000; Wenk & Barnes, 2000; Clayton & Browning, 2001; Shi *et al.*, 2007; Kollen *et al.*, 2010) and function (Barnes *et al.*, 1997; Billard *et al.*, 1997; Jouvenceau *et al.*, 1998; Potier *et al.*, 2000; Mothet *et al.*, 2006; Magnusson *et al.*, 2007; Boric *et al.*, 2008). These alterations contribute to the age-related decline in spatial learning [see Rosenzweig & Barnes (2003) for review].

In this study, we envisaged a role for an altered distribution of extracellular glutamate because of glutamate transporter dysfunction. As reactive gliosis is a hallmark of aging [(Schipper, 1996; Rozovsky *et al.*, 1998); see Cotrina & Nedergaard (2002) for review], changes in the astrocytic phenotype or failure of astrocyte function in the aging brain could significantly contribute to synaptic transmission defects (Perea & Araque, 2007), notably through the alteration of glutamate transporter expression and/or function. To determine whether a glutamate uptake defect occurs in memory-related structures, we first measured the expression and activity of the transporters in the hippocampus of young and aged Sprague–Dawley rats. We compared basal synaptic transmission in the CA1 hippocampal area of young and aged rats under acute glutamate uptake disruption by DL-TBOA, a nontransportable blocker of glutamate transporter activity. We then wondered whether changes in the spatiotemporal distribution of glutamate in the hippocampus of aged rats could alter the pharmacological properties of NMDAR-dependent long-term depression (LTD). Collectively, our data support the idea that a glutamate uptake deficiency contributes to a decreased regulation of extracellular glutamate and to an abnormal activation of extrasynaptic metabotropic receptors in the aged hippocampus. These age-related alterations may therefore be key contributors to cognitive aging.

Results

Protein expression of the glial glutamate transporters GLAST and GLT-1

Protein levels of GLAST [young: 2.073 ± 0.1231 (n = 11); aged: 1.351 ± 0.1518 (n = 8), $P < 0.01$] and GLT-1 [young: 1.540 ± 0.098 (n = 11); aged: 1.076 ± 0.1345 (n = 5)] glutamate transporters were significantly decreased in aged whole hippocampi compared to young ones ($P < 0.05$), whereas the level of the neuronal transporter (EAAC1) expression was not affected [young: 0.758 ± 0.033 (n = 11), aged: 0.691 ± 0.022 (n = 8), nonsignificant] (Fig. 1A,B). In contrast, the level of glial fibrillary associated protein (GFAP) expression was significantly increased in aged rats [young GFAP: 0.3573 ± 0.0013 (n = 11); aged GFAP: 0.491 ± 0.025 (n = 8), $P < 0.001$], reflecting the reactive gliosis of astrocytes that occurs in the hippocampus during aging.

The expression of another astrocyte marker, glutamine synthetase (GS), was unchanged in aged animals [young: 0.338 ± 0.022 (n = 11); aged: 0.372 ± 0.024 (n = 8), nonsignificant].

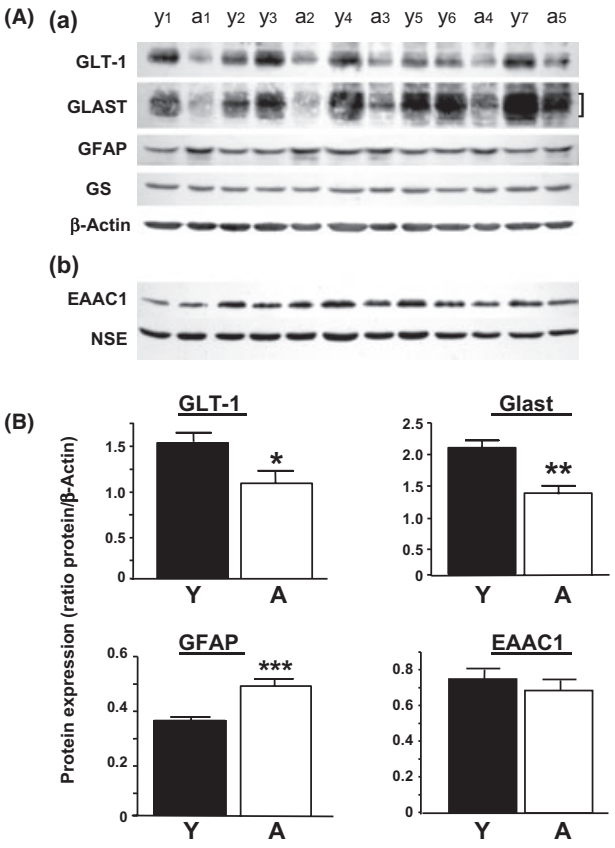

Fig. 1 The expression of astrocytic glutamate transporters is selectively reduced in aged rats. (A) Examples of immunoblots in seven young (y1 to y7) and five aged (a1 to a5) rats for the astrocytic glutamate transporters GLT1 and GLAST, for two selective astrocytic markers glial fibrillary associated protein (GFAP) and glutamine synthetase (GS), and for the neuronal glutamate transporter EAAC1. β-actin and Neuron-Specific Enolase (NSE) (Ab) are used to normalized astrocytic and neuronal markers, respectively. (a) and (b) are two different separated blots. (B) Bar graphs depicting immunoreactivity to GLT1, GLAST and GFAP in young (n = 11) and aged (n = 8) rats, normalized to β-actin levels, and to EAAC1, normalized to NSE levels. (*; **; *** $P < 0.05$; 0.01 and 0.001 respectively). (C) Saturation curves for Na^+-dependent D-[^{3}H]-aspartate uptake by cell suspensions freshly isolated from CA1 area of young (n = 7) and aged (n = 7) rats. Total and Na^+-independent uptake was measured in triplicate for each D-aspartate concentration and for each rat. (***$P < 0.0001$).

Activity of glutamate transporters

We compared the capacity of Na^+-dependent glutamate transport in young (3-month old) and aged (24-month old) rats by measuring the rate of D-[^{3}H] aspartate uptake by brain cell suspensions freshly isolated from the CA1 region of the hippocampus. D-Aspartate is taken up by astroglial glutamate transporters with the same affinity as L-Glutamate (Gundersen *et al.*, 1993; Bridges *et al.*, 1999; Danbolt, 2001). D-[^{3}H]-aspartate is a transportable analogue of L-glutamate that is metabolically inert. The saturation curves obtained by incubation of the cell suspensions with increasing concentrations of D-[^{3}H]-aspartate showed a significant decrease in the uptake rate in aged rats when compared to young ones ($P < 0.0001$, Fig. 1C). Analysis of the curves (Michaelis–Menten equation) indicated that the maximal velocity of D-[^{3}H]-aspartate uptake (i.e. maximal velocity at a saturating concentration of the substrate) was reduced by 40% ($P < 0.01$) in aged rats (V_{max} = 3.75 ± 0.56 nmoles min^{-1} mg^{-1} protein, $n = 7$) when compared to young ones (V_{max} = 6.33 ± 0.63 nmoles min^{-1} mg^{-1} protein, $n = 7$), whereas the apparent affinity of the transport for the substrate was unchanged (K_m = 8.89 ± 2.45 μM in aged rats, K_m = 12.64 ± 2.03 μM in young rats, nonsignificant). The decrease in V_{max} without a change in the K_m value is compatible with a decreased density of functional glutamate transporters in cell suspensions from aged rats.

Effect of DL-TBOA on basal synaptic transmission

In control artificial cerebrospinal fluid (aCSF), single-shock electrical stimulation of the stratum radiatum evoked a presynaptic fiber volley (PFV) followed by a field excitatory postsynaptic potential (fEPSP) both increasing with stimulus intensity. Field excitatory postsynaptic potentials were totally suppressed by CNQX (10 μM, not illustrated), reflecting the activation of AMPAR (Fig. 2A, upper traces). I/O curves of PFVs and fEPSP slopes were constructed in 15 young ($n = 22$ slices) and 14 aged rats ($n = 22$ slices). No differences in PFV slopes were found between the two groups of animals, whereas fEPSP slopes were significantly reduced (Fig. S1), as previously described [see (Potier *et al.*, 2000; Kollen *et al.*, 2010)].

When applied at a concentration of 25 μM, DL-TBOA slightly but nonsignificantly reduced fEPSPs in young rats ($n = 15$ slices in 10 animals) at all stimulus intensities (Fig. 2A2). In aged rats ($n = 13$ slices from 10 animals), the depressing effect became significant at a 300 μA stimulation intensity and above (Fig. 2B2). When the effect of DL-TBOA was expressed as a percentage of control values, the amplitude of fEPSP depression was 34 ± 9% over the 300–500 μA range ($P < 0.01$).

The magnitude of fEPSP depression increased with DL-TBOA concentration. When 50 μM DL-TBOA was applied, a 41.7 ± 9% mean reduction in fEPSPs was observed in young rats over the 300–500 μA range ($n = 12$ slices from nine rats, $P < 0.05$, Fig. 2A3), whereas the percentage of decrease reached 64.8 ± 7.3% in aged rats ($n = 14$ slices from 10 rats, Fig. 2B3, $P < 0.001$). Therefore, synaptic transmission was more prone to be blocked by DL-TBOA in aged than in young rats ($P < 0.05$). We confirmed that the PFV slope was not modified by any dose of DL-TBOA tested, indicating that the effect on synaptic transmission was not because of a reduction in presynaptic fiber activity (Fig. S2).

Pharmacological profile of the DL-TBOA depressing effect on synaptic transmission

The pharmacological properties of the depressing effect of DL-TBOA on synaptic transmission were next studied in young and aged rats. The time-course of the DL-TBOA effect on the fEPSP slope was evaluated, using 50 μM DL-TBOA as the control condition and in the presence of different glutamate receptor antagonists.

After 15 min of a baseline recording of a stable fEPSP slope, DL-TBOA was added to the bath. Within a few minutes, the drug induced a depression of the fEPSPs slope that reached a maximal value of 61 ± 6.7% in young rats ($n = 14$ in nine rats; Fig. 3A). The effect was reversible as illustrated by the complete recovery of responses following DL-TBOA wash-out. In slices from aged animals, the maximal effect of DL-TBOA on fEPSPs slope was 63 ± 4.4% ($n = 17$ in 13 rats; Fig. 3B). After the wash-out of DL-TBOA, synaptic transmission recovered but remained depressed to 20% below the level of the baseline (Fig. 3B).

Increasing the dose of DL-TBOA to 100 μM further depressed the fEPSP slope in both young and aged rats (see Fig. S4). In young rats, the fEPSP slope depressed to about 14% of baseline but still returned to baseline level after DL-TBOA removal ($n = 9$ slices in four rats). In aged rats, a 100% depression was obtained ($n = 8$ slices in four rats) and remained at a stable level of about 78% of the baseline value at the end of the recording. An application of 200 μM of DL-TBOA induced a complete depression of the fEPSP slope in young rats ($n = 4$ slices in three rats) and in this case a long-lasting depression of 20% was observed after the drug wash-out. In aged rats, this dose of DL-TBOA induced a full depression of fEPSP slope, which stabilized to a level of 80% below that of baseline ($n = 4$ slices in three rats).

A selective antagonist of NMDAR, D-APV, was applied at 50 μM for at least 15 min before DL-TBOA and remained present throughout the recording. D-APV had no effect on baseline transmission in either group of animals but fully abolished the effect of DL-TBOA (50 μM) on fEPSP slope in slices from young ($n = 5$ in three rats) and aged ($n = 6$ in five rats) rats (Fig. 3B).

Following uptake disruption, other receptor subtypes such as mGluRs could also be activated by the resulting extracellular glutamate accumulation (Scanziani *et al.*, 1997). We therefore asked whether an activation of mGluRs could be implicated in the DL-TBOA effect in young and aged rats. MCPG (500 μM) was applied in the aCSF at least 20 min before the application of DL-TBOA and remained present throughout recording. MCPG had no effect on baseline transmission in either group of

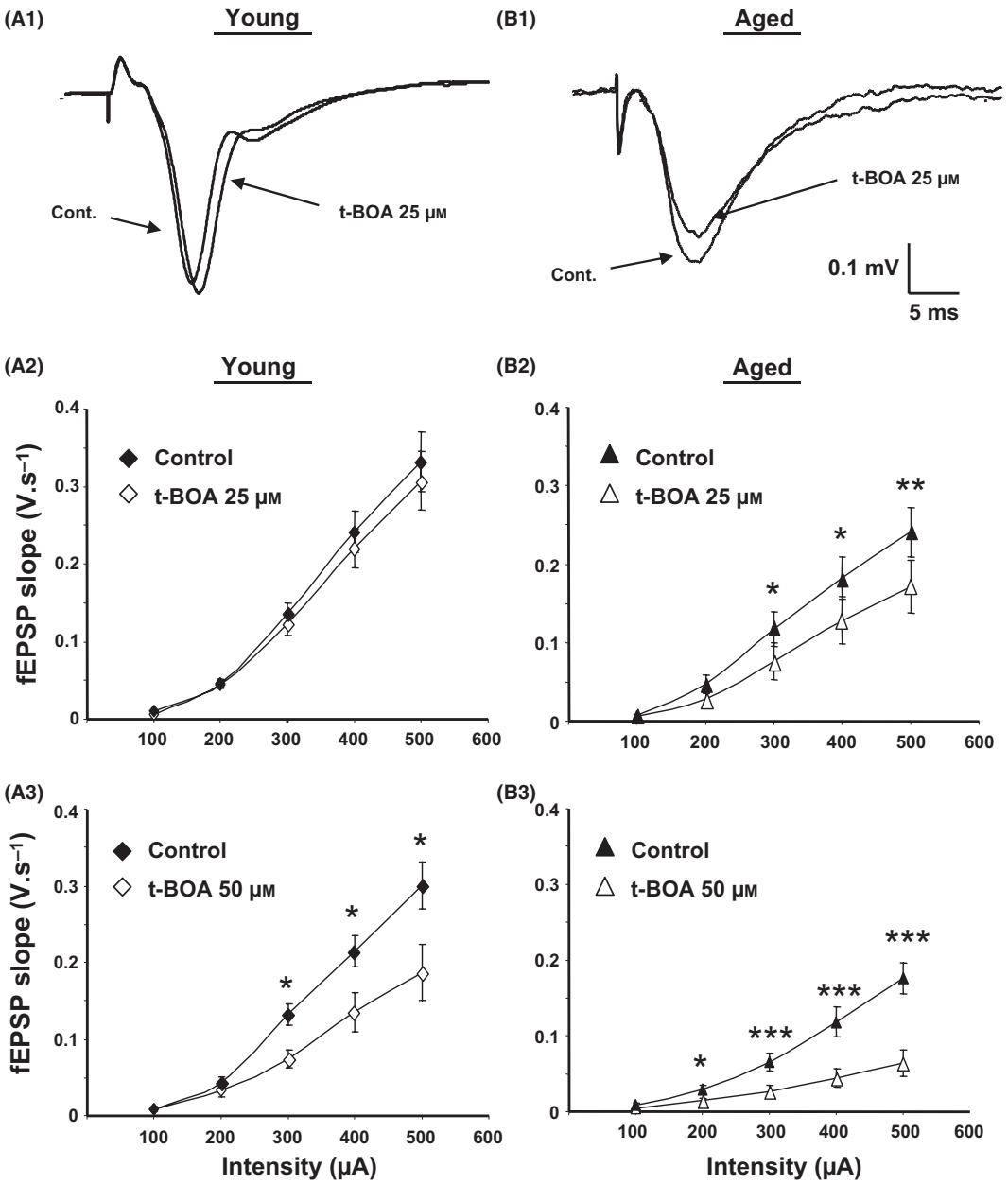

Fig. 2 Blockade of glutamate transporters by DL-TBOA depresses synaptic transmission more in aged than in young rats. Upper traces are examples of field excitatory postsynaptic potential (fEPSPs) induced by a 500 µA stimulus intensity and recorded from a young (A1) and an aged (B1) rat before and after application of DL-TBOA (25 µM). Comparison of the effects of DL-TBOA (25 µM) on the mean AMPAR-mediated fEPSP slope plotted against the stimulus intensity and averaged from 15 slices of 10 young animals (A2) and from 13 slices of 10 aged rats (B2). (*; ** $P < 0.05$ and 0.01, respectively for differences between treatment and control groups). Results obtained with a 50 µM application of DL-TBOA in 12 slices from nine young rats (A3) and in 14 slices from 10 aged animals (B3). (*; *** $P < 0.05$ and 0.001, respectively, for differences between treatment and control groups).

rats. MCPG only slightly reduced the amplitude of the DL-TBOA effect on synaptic transmission in young slices ($n = 11$ in eight rats, ns, Fig. 4A). In contrast, the mGluR antagonist significantly attenuated the DL-TBOA effect in aged rats (23.2 ± 5.1% inhibition at the peak of the DL-TBOA + MCPG effect, $n = 12$ in 10 rats, $P < 0.001$, Fig. 4B). The long-lasting depression of fEPSPs following DL-TBOA wash-out was also blocked by MCPG in aged rats.

Presynaptic effects of DL-TBOA

The hypothesis that the glutamate leakage could be sensed by presynaptic receptors was tested using a paired-pulse facilitation (PPF) protocol (Zucker, 1989). Because the probability of glutamate release can be modified during aging, PPF was not compared between young and aged rats. Only the comparison of PPF before and under drug application was made within a group

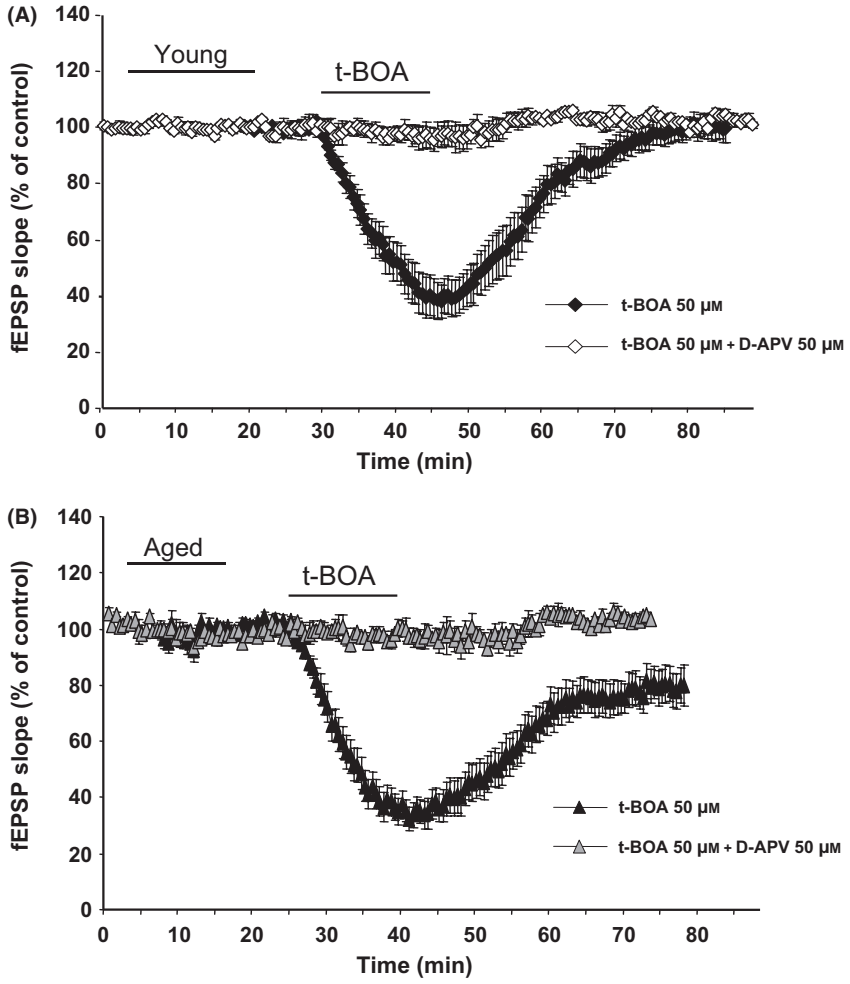

Fig. 3 DL-TBOA-induced depression of synaptic transmission is dependent on NMDAR activation. (A) Time-course of the decreased synaptic transmission induced by DL-TBOA (50 µM) in slices from young rats in control artificial cerebrospinal fluid (14 slices in nine animals) and in the presence of the NMDAR antagonist D-APV (50 µM) (five slices in three rats). APV was applied throughout the experiments. (B) The same experiments in aged rats (control medium: 17 slices in 13 animals; D-APV: six slices in five animals).

of age. DL-TBOA had no significant effect on the PPF ratio measured in young slices, either when applied at 25 µM (control PPF: 1.36 ± 0.05 vs. 1.39 ± 0.05 in the presence of DL-TBOA, $n = 9$ in six rats, nonsignificant, Fig. 5A) or at 50 µM (control PPF: 1.39 ± 0.08 vs. 1.41 ± 0.09 in the presence of DL-TBOA, $n = 5$ in three rats, nonsignificant, Fig. 5B). In contrast, the PPF ratio was dramatically increased by DL-TBOA in aged slices when applied at 25 µM (control ratio: 1.79 ± 0.11 vs. 2.57 ± 0.3 in the presence of the drug, $n = 7$ in five rats, $P < 0.01$, Fig. 5A) or at 50 µM (control PPF: 1.8 ± 0.06 vs. 3.14 ± 0.25, $n = 5$ in four rats, $P < 0.01$, Fig. 5B).

Effect of mGluR blockade on the DL-TBOA-induced enhancement of PPF

In addition to their reported postsynaptic effects in both interneurons and pyramidal cells in the hippocampus, mGluRs also influence presynaptic function, producing a reversible reduction in excitatory synaptic transmission (Forsythe & Clements, 1990; Burke & Hablitz, 1994). We therefore tested the hypothesis that presynaptic mGluRs might be tonically activated or that their activation might be facilitated in aged synapses under conditions of uptake blockade. Our data show that MCPG had no effect on PPF in young rats, in the presence of 50 µM DL-TBOA (1.41 ± 0.09 for DL-TBOA, $n = 5$ in three rats, vs. 1.4 ± 0.05 for MCPG + DL-TBOA, $n = 7$ in four rats, Fig. 6A), while it abolished the effect of DL-TBOA on the PPF ratio in aged slices (3.14 ± 0.25 for DL-TBOA, $n = 5$ in four rats vs. 1.52 ± 0.09 for MCPG + DL-TBOA, $n = 7$ in five rats, $P < 0.001$, Fig. 6B).

Effect of mGluR blockade on basal PPF

Figure 6 also shows that a facilitated activation of presynaptic mGluRs can occur physiologically in the aged hippocampal CA1 area even in the absence of DL-TBOA. Indeed, blocking mGluRs with MCPG significantly reduced the basal PPF ratio in aged rats (MCPG: 1.54 ± 0.05 vs. control: 1.85 ± 0.11; $n = 19$ slices in 14 rats; $P < 0.01$, Fig. 6D), whereas the antagonist has no

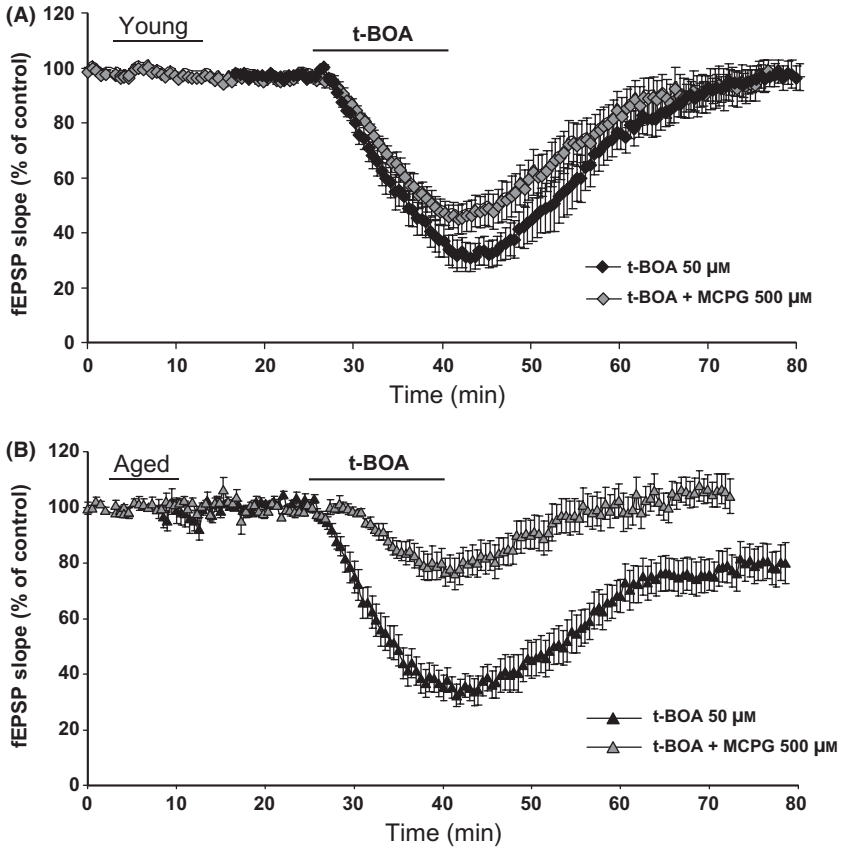

Fig. 4 DL-TBOA-induced depression of synaptic transmission is dependent on mGluR activation only in aged rats. (A) Time-course of the decreased synaptic transmission induced by DL-TBOA (50 µM) applied to slices from young rats in control artificial cerebrospinal fluid (14 slices in nine animals) and in the presence of the mGluR antagonist MCPG (500 µM) (11 slices in eight rats). MCPG is applied throughout the experiment. (B) The same experiments in aged rats (control medium: eight slices in six animals; MCPG: 12 slices in 10 animals).

significant effect on basal PPF in young rats (MCPG: 1.4 ± 0.04 vs. control: 1.38 ± 0.03; $n = 11$ in nine rats, nonsignificant, Fig. 6C).

Comparison of LTD in slices from young and aged animals

The previous data show that DL-TBOA exacerbates a preexisting alteration in the distribution of extracellular glutamate in aged rats. This situation can be readily detected in physiological conditions (in the absence of TBOA) with dual stimulations, such as those used to induce PPF. We therefore asked whether this modification could impact LTD, a mechanism of synaptic plasticity induced by low frequencies of stimulation (LFS) over a long duration.

Long-term depression was induced using two different LFS, 1 and 2 Hz, and its amplitude was compared in young and aged rats. Low frequencies of stimulation at 1 Hz induced significant LTD in slices from both young and aged rats (young rats: 89.1 ± 2.7% of baseline, $n = 8$ slices from seven animals, $F_{1,14} = 21.9$, $P < 0.001$; aged rats: 86.0 ± 2.8% of baseline, $n = 11$ slices from 11 animals, $F_{1,20} = 28.4$, $P < 0.0001$). The difference in LTD amplitude between slices from young and aged rats was not statistically significant ($F_{1,17} = 0.45$, nonsignificant) (Fig. 7A). Increasing the stimulation frequency to 2 Hz revealed differences in LTD magnitude between slices from young and aged rats. Indeed, this protocol induced significant LTD in young rats (80.2 ± 1.5% of baseline, $n = 32$ slices from 25 rats; $F_{1,62} = 188$, $P < 0.0001$) as well as in aged rats (86.9 ± 1.9% of baseline, $n = 33$ slices from 21 rats; $F_{1,64} = 51.4$, $P < 0.0001$). Nevertheless, the magnitude of LTD in this case was significantly higher in slices from young rats when compared to aged rats (young vs. aged: $F_{1,63} = 8.92$, $P < 0.01$) (Fig. 7B).

A role for mGluRs in the alteration of LTD in aged slices

The effect of mGluR antagonism was then studied on LTD induced by a 2 Hz stimulus in both groups of animals. MCPG (500 µM) had no effect on LTD in slices from young rats (79.7 ± 3.7% of baseline, $n = 10$ control slices from four rats vs. 77.5 ± 5.3% of baseline, $n = 6$ MCPG-treated slices from three rats; $F_{1,14} = 0.1$, nonsignificant) (Fig. 8A). In contrast, MCPG application significantly increased the amplitude of LTD in slices from aged rats (87.9 ± 3.8% of baseline, $n = 12$ control

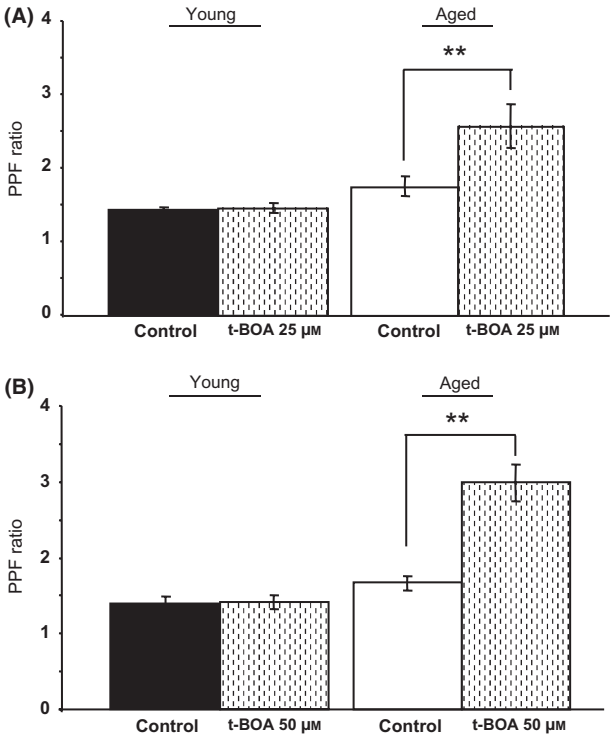

Fig. 5 Presynaptic glutamate release is affected by DL-TBOA in aged but not in young rats. (A) Bar graphs depict the effects of DL-TBOA (25 µM) on paired-pulse facilitation ratio in young (nine slices in six animals) and in aged rats (seven slices in five animals). (B) The same experiments using 50 µM DL-TBOA (five slices from three young rats and five slices from four aged rats). (**$P < 0.01$).

slices from eight rats vs. 74.6 ± 4.9% of baseline, $n = 11$ MCPG-treated slices from six rats; $F_{1,21} = 4.9$, $P < 0.05$) (Fig. 8B1). Interestingly, LFS induced a slight but not significant potentiation in the presence of D-APV in aged slices (Fig. 8C), which was blocked by MCPG (94.9 ± 3.8%, $n = 5$ slices from two rats; $F_{1,11} = 4.3$, $P = 0.06$ (Fig. 8B2). These data indicate that under LFS conditions, a sustained perisynaptic activation of mGluRs interferes with LTD expression in aged rats.

Discussion

The first observation of this study was a reduction in the expression of the glial glutamate transporters GLAST and GLT-1 in the aged rat hippocampus. This was associated with a decrease in functional glutamate uptake in the CA1 area. This alteration seemed to be restricted to the transporter system as the expression of another glial marker, glutamine synthetase, was not changed in aged animals, excluding a general astrocytic failure during aging. A few previous studies have addressed the status of glutamate uptake during aging, and most have reported a lowered uptake and a loss of glutamate transporter sites in the glutamatergic terminals of aged rodents (Wheeler & Ondo, 1986; Najlerahim *et al.*, 1990; Saransaari & Oja, 1995; Vatassery *et al.*, 1998). More recently, *in vivo* voltammetric recordings have demonstrated that the loss of plasma membrane GLAST transporters in the striatum of aged Fisher 344 rats is correlated with an increased clearance time/slower clearance of glutamate (Nickell *et al.*, 2007). The fact that the bulk of functional glutamate transport is undertaken by GLT-1 and GLAST (Rothstein *et al.*, 1996) underlines the importance of their alteration in aged animals.

Extracellular recordings of fEPSPs were performed in hippocampal slices from young and aged rats to detect the impact of weakened glutamate clearance on glutamatergic neurotransmission and its plasticity. To better understand the shaping of glutamatergic transmission by glutamate transporters in both young and aged rats, we used a nontransportable blocker of transporters, DL-TBOA (Shimamoto *et al.*, 1998), as a pharmacological tool to manipulate glutamate uptake. In the presence of high doses of DL-TBOA designed to fully disrupt the uptake cycle [i.e. 200 µM, Cavelier & Attwell (2005)], a physiologically relevant increase in extracellular glutamate occurs, unmasking the continuous release of glutamate into the extracellular space (Jabaudon *et al.*, 1999). In our study, smaller doses of DL-TBOA were used as our purpose was to lower glutamate uptake in young rats to reproduce the situation during aging. The main effect of DL-TBOA was to reversibly depress the fEPSP slope in young and aged rats, and the magnitude of depression increased with DL-TBOA concentration in both groups of animals.

We aimed to characterize the pharmacological properties of the depressing effect of DL-TBOA in both groups of animals. No effect of a 50 µM dose of DL-TBOA was observed when D-APV, a selective NMDAR antagonist, was present in the perfusion medium, indicating the activation of these subtypes of glutamatergic receptors. D-APV also abolished the long-lasting depression of fEPSP induced by DL-TBOA in slices from aged rats, indicating that this long-term effect of the uptake blocker was also dependent on NMDAR activation. Increasing concentrations of DL-TBOA in aged animals led to a critical and permanent depression. It has been shown that NMDAR activated by glutamate after uptake blockade was mainly extrasynaptically located (Le Meur *et al.*, 2007), and extrasynaptic NMDAR appeared to contribute preferentially to pathological processes linked to overexcitation of glutamatergic pathways (Hardingham *et al.*, 2003). The situation was different in slices from young rats. The DL-TBOA-induced depression of fEPSPs was transient, and a concentration as high as 200 µM was necessary to induce a long-lasting depression comparable to that induced by 50 µM DL-TBOA in slices from aged rats. This is in contrast to other forms of LTD that can be easily obtained in young rats by straightforward activation of NMDAR, such as chemical LTD (Kollen *et al.*, 2008). Chemical LTD is an APV-sensitive form of LTD that involves synaptic but also extrasynaptic NMDA receptors (Kollen *et al.*, 2010). Chemical LTD is artificially obtained by bath application of low doses of NMDA, an exogenous agonist not transported by glutamate transporters, conversely to endogenous glutamate used in this study. At doses of DL-TBOA that do not block all the transporters (i.e. 25, 50 or 100 µM) in young animals, the remaining uptake is probably still efficient enough

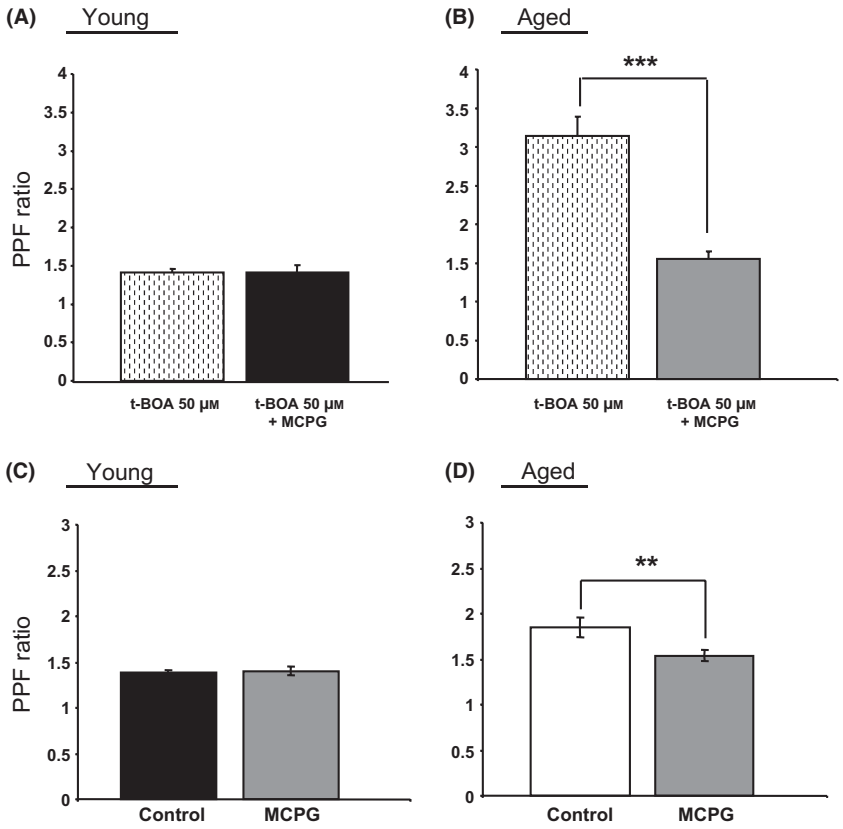

Fig. 6 Altered presynaptic glutamate release is dependent on mGluR activation in aged but not in young rats. Bar graphs depicting the effects of MCPG (500 µM) on DL-TBOA-induced changes in paired-pulse facilitation (PPF) ratio in (A) young (DL-TBOA alone: five slices in three rats vs DL-TBOA + MCPG: seven slices in four rats) and in (B) aged (DL-TBOA alone: five slices in four rats vs DL-TBOA + MCPG: seven slices in five rats) animals. Effects of MCPG (500 µM) on PPF ratio determined in control artificial cerebrospinal fluid in (C) young (control: 37 slices in 20 rats vs MCPG: 11 slices in nine rats) and in (D) aged (control: 39 slices in 24 rats vs MCPG: 19 slices in 14 rats) animals. (* and *** $P < 0.05$ and 0.001, respectively).

to reduce the extracellular glutamate concentration to avoid pathological activation of extrasynaptic NMDA receptors. This is one possible reason for the absence of DL-TBOA-induced LTD in young rats.

Because mGluRs are also expressed extrasynaptically (Baude *et al.*, 1993; Lujan *et al.*, 1996) and can be activated by a rise in extracellular glutamate (Scanziani *et al.*, 1997; Renden *et al.*, 2005; Wadiche & Jahr, 2005), we asked to what extent the manipulation of uptake by DL-TBOA could affect mGluRs in young and aged rats. We observed that a significant part of the depressing effect of DL-TBOA on fEPSPs was mGluRdependent in aged rats. Blocking mGluRs prevented the increase in the PPF ratio induced by DL-TBOA in slices from aged rats, indicating that a facilitation of presynaptic mGluR activation can be achieved by extracellular glutamate following transporter blockade.

The alteration of extracellular glutamate homeostasis can deeply affect the fine tuning of the dendritic processing of synaptic inputs, with strong repercussions on synaptic plasticity. We thus studied LTD a glutamate-dependent form of plasticity generated by LFS. Low frequencies of stimulation induced NMDAR-dependent LTD in both young and aged rats. Under our experimental conditions, the amount of LTD induced by 1 Hz stimulation was not different between young and aged rats in agreement with other studies indicating no change in LTD during aging (Kumar & Foster, 2007; Billard, 2010; Kollen *et al.* 2010). However, others demonstrate an enhanced (Norris *et al.*, 1996; Kumar & Foster, 2005) or a decreased susceptibility to LTD either in middle-aged (Kamal *et al.*, 1998) or aged animals (Lee *et al.*, 2005). These discrepancies result from differences in methodology, including age, strain of the animals and the Ca/Mg ratio of the recording solution. To observe LTD in adult slices, the Ca/Mg ratio must be > 1.5 [see Norris *et al.* (1996)]. In our conditions, LTD was maximal at 1 Hz in aged rats, and increasing the stimulation frequency induced weaker LTD in aged animals than in young ones. This age-related decrease in LTD amplitude could be the result of different modifications at the glutamatergic synapse: (i) a reduction in glutamate release, although this has been the subject of much debate [see review by Segovia *et al.* (2001)]; (ii) a change in NMDAR expression in the hippocampus of rats and mice, as illustrated by many groups (Sonntag *et al.*, 2000; Clayton & Browning, 2001); (iii) a change in NMDAR activation, as shown by the decrease in isolated NMDAR-mediated synaptic potentials recorded in slices from aged rats (Barnes *et al.*, 1997; Jouvenceau *et al.*, 1998; Potier *et al.*, 2000; Mothet *et al.*, 2006) and the alteration of modulating factors, such as the co-agonist D-serine (Junjaud *et al.*, 2006; Mothet

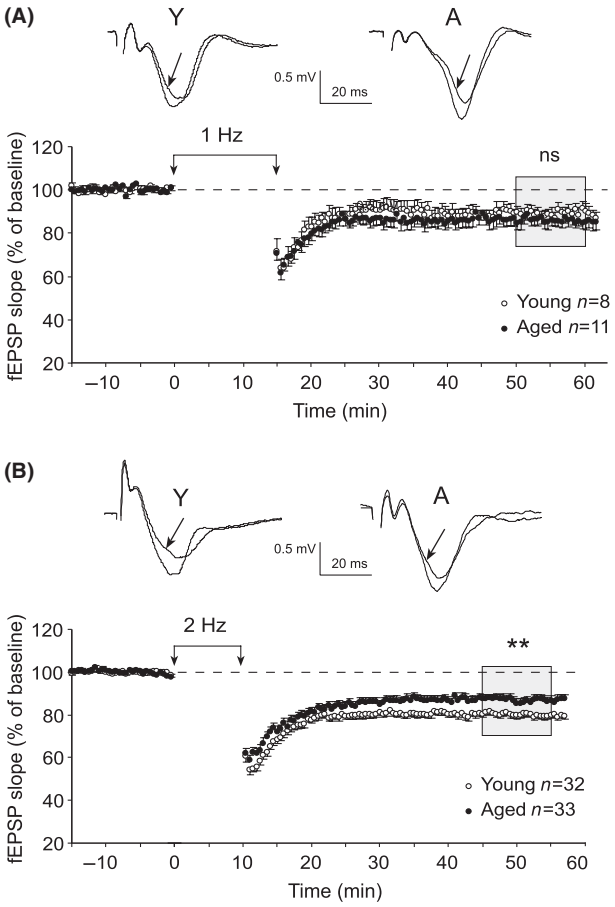

Fig. 7 Long-term depression (LTD) induced by two different frequencies in slices from young and aged rats. A 1 Hz stimulation induces slightly greater depression in slices from aged rats than from young ones, but the difference is not statistically significant (A). Increasing the stimulation frequency to 2 Hz (B) leads to a difference between young and aged rats, with weaker LTD induced in aged slices. Individual traces of EPSPs are shown for each frequency in slices from young (Y) and aged (A) rats before and 40 min after stimulation (arrow). Statistical analysis was carried out during the period indicated by the square. *P* values: **< 0.01; ns, not significant.

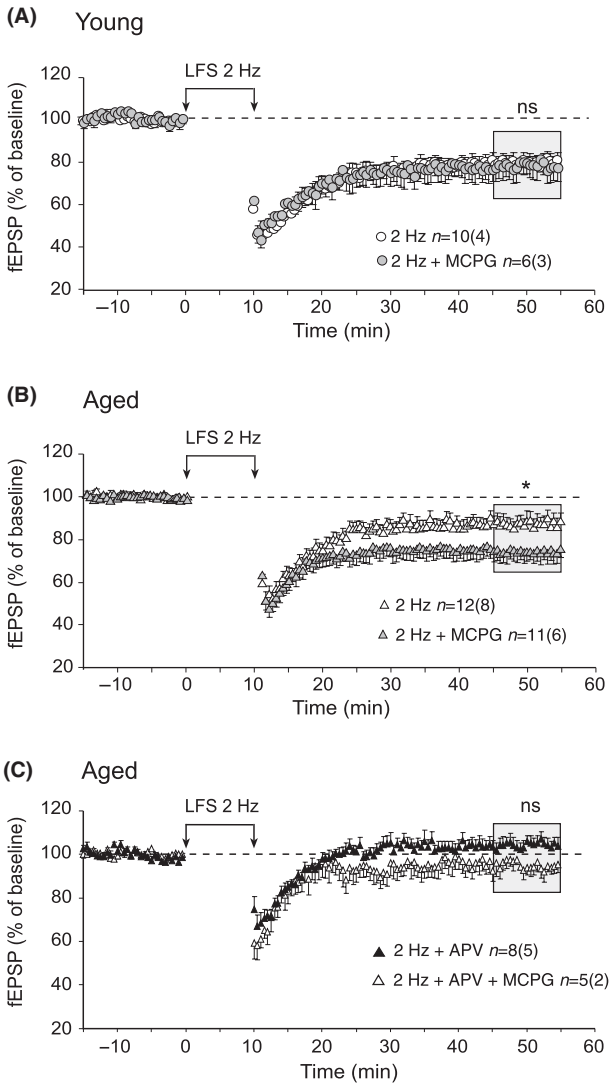

Fig. 8 Implication of mGluR in synaptic plasticity in aged rats. In slices from young rats (A), low frequencies of stimulation (LFS) (2 Hz, 10 min) induces long-term depression (LTD) to a similar extent in the presence or absence of the mGluR antagonist MCPG (500 μM). In slices from aged rats, MCPG significantly (*$P < 0.05$) reduces LTD (B), suggesting that these receptors are activated during LFS. MCPG is applied throughout the experiments. Although not significant ($F_{1,11} = 4.3$, $P = 0.006$), the small potentiation observed in aged slices in the presence of APV (50 μM) was blocked by MCPG (C).

et al., 2006; Turpin *et al.*, 2009). Age-related alterations in NMDAR expression and/or efficiency only partly explain the changes in plasticity, and our experiments provide new evidence that the deregulation of glutamate uptake is also involved in the impairment of LTD in aged rats. Indeed, the pharmacological properties of LTD in aged rats appear similar to its properties in young rats when glutamate is forced to spill out (Kollen *et al.*, 2008). We demonstrated that a significant activation of mGluRs occurred during the induction of 2 Hz-induced LTD in aged but not in young rats as the mGluR antagonist MCPG restored LTD amplitude to levels comparable to those in young rats. Our findings were reinforced by the observation that D-APV switched the response to LFS in aged rats from depression to a weak but reproducible mGluR-dependent potentiation. This observation fits with the results of (van Dam *et al.*, 2004) who showed that low concentrations of the mGluR agonist DHPG can shift plasticity in young animals, with an LTD protocol being able to induce LTP in the presence of the agonist. It is thus conceivable that an mGluR-dependent LTP was induced by the 2 Hz LFS in aged rats, partially occluding LTD. Another possible explanation for the weaker 2 Hz-induced LTD in aged rats would be the activation of presynaptic mGluRs by an excess of extracellular glutamate, decreasing glutamate release through a negative feedback mechanism (Vignes *et al.*, 1995; Scanziani *et al.*, 1997), as is strongly suggested by the present PPF data. Discrepancies in the literature regarding the induction of LTD in aged animals have already been discussed previously, but our data indicate that the functional implication of mGluRs can be another matter of debate. The role of mGluR signaling in the induction of LTD is still unclear in aged animals. Only a few studies have investigated alterations in the characteristics of mGluRs signaling

during aging, and the results are divergent. Different degree of tonic mGluRs activation can be achieved as a result of glutamate spillover in aged animals. An exhaustive study of mGluRs subtypes implicated in pre- versus postsynaptic responses remains to be performed during aging.

Our data strongly argue for an altered spatial and/or temporal distribution of glutamate at aged synapses detected by extrasynaptic NMDAR and mGluRs. Where does this glutamate come from? Glutamate can be synaptic, arising from activity-dependent release, with some proportion leaking away from its release site or of ambient origin. A tonic release of glutamate occurs in the hippocampus, (Cavelier & Attwell, 2005), and recent studies have identified astrocytes as the source of nonsynaptic glutamate (Cavelier & Attwell, 2005; Cavelier *et al.*, 2005; Le Meur *et al.*, 2007). When uptake mechanisms are fully operational, an equilibrated ambient glutamate concentration is reached, estimated to be around 25 nM in slices (Herman & Jahr, 2007). Is it conceivable that the ambient glutamate level increases in aged rats? A tonic activation of extrasynaptic NMDA receptors by ambient glutamate can be detected in the hippocampus when the voltage-dependent Mg^{2+} blockade of these receptors is relieved (Le Meur *et al.*, 2007). Under conditions closer to resting, such as ours, fEPSPs were not affected by D-APV in aged rats, suggesting that the level of ambient glutamate is still below the concentration necessary to alleviate the Mg^{2+} blockade of NMDAR. However, an elevation of local ambient glutamate, although not detected, cannot be rejected. NMDARs require the binding of 2–4 molecules of glutamate for full activation to occur (Banke & Traynelis, 2003). A rise in the extracellular glutamate concentration produced by exocytosis will have a different impact if it is superimposed on an ambient glutamate level that results in some of the receptor binding sites being tonically occupied. This could contribute to the susceptibility of aged tissues to acute hypoxic-ischemic injuries and to neuronal damage (Sheldon & Robinson, 2007).

The landscape of extracellular glutamate thus seems to be quite different between young and aged rats. The facilitated activation of extrasynaptic receptors in aged rats suggests that additional mechanisms may worsen the alteration of glutamate uptake. One of our hypotheses is that there is a morphological modification of the synaptic surroundings because of the astrocytic hypertrophy that gradually develops during aging [see Cotrina & Nedergaard (2002) for review]. Age-related astrocyte hypertrophy is characterized by the elongation of fine processes and the upregulation of GFAP expression. Modifications of the glial sheath of central synapses can have profound effects on the activation of perisynaptic receptors. In the hippocampal CA1 area, the astrocytic coverage of synapses is relatively limited, with glia occupying only $\sim$ 10% of the tissue volume and contacting synapses in only $\sim$ 25% of cases, as shown by 3D reconstruction (Ventura & Harris, 1999). This poor glial sheath may explain synaptic crosstalk by glutamate spillover (Rusakov & Kullmann, 1998), whereas enhancing glial coverage reduces the leak and increases glutamate concentration inside the sheath (Rusakov, 2001). No ultrastructural studies of the extent of neuron-astrocyte contacts during aging have been yet performed but it is conceivable that the astroglial coverage of synapses increases because of hypertrophy. Residual glutamate may then accumulate in the reduced extrasynaptic volume, facilitating the access to extrasynaptic receptors. If glutamate can easily escape from the synapse and be rapidly diluted in a larger extrasynaptic space, as is the case in young tissue, this situation is not observed. *In vivo* macroscopic studies show that the reduction in extracellular space and diffusion of neurotransmitters are correlated with learning deficits in aged rats (Sykova *et al.*, 2002; Syková & Vargová, 2008).

In conclusion, our data, though not direct, present a wealth of evidence for an increased or unusual tonic activation of extrasynaptic NMDA and mGluRs receptors in aged rats, consistent with a dysregulation of extracellular glutamate homeostasis. It would therefore be of therapeutic interest to determine whether treatments reducing astrocytic hypertrophy and/or promoting glutamate uptake can reverse synaptic plasticity and memory impairments in aged animals.

Experimental procedures

All experiments were carried out in accordance with the INSERM committee guidelines and with the European Communities Council Directive of November 24, 1986 (86/609/EEC). Thirty young (3–6 months) and 30 aged (24–27 months) Sprague–Dawley rats were used in this study.

Semi-quantitative immunoblotting analysis

Parts of the hippocampi were dissected immediately after decapitation of some of the animals used for electrophysiology, cut into small pieces and homogenized by sonication in ice-cold buffer consisting of 20 mM sodium phosphate pH 7.2, 150 mM NaCl, EDTA 10 mM, EGTA 10 mM, 150 mM sodium fluoride, 0.5% Triton ×100, also containing protease inhibitors (Protease inhibitors set III; Sigma, L'isle d'Abeau chesnes, France). Aliquots of the extracts were assayed for protein using the Bradford method, and equal amounts of protein (80 μg/lane) were loaded on sodium dodecyl sulfate–polyacrylamide gels (6.5% acrylamide), electrophoresed and transferred onto nitrocellulose membranes (Transblot; Biorad Laboratories Inc, Marnes-la-Coquette, France) by semi-dry blotting. Blots were blocked in 5% defatted milk in 10 mM Tris–HCl (pH 8.3), 150 mM NaCl containing 0.05% Tween-20 (TBST), and then incubated overnight at 4 °C with appropriate antisera dilution [GLT-1, 1/1000 (rabbit, kind gift from Pr. Rothstein); GLAST, 1/5000 (guinea-pig; Chemicon, Millipore, Molsheim, France); GFAP, 1/6000 (mouse; Chemicon); Actin, 1/10 000 (mouse; Sigma); Glutamine Synthetase, 1/1000 (rabbit; Santa Cruz, Tebu-Bio, Le Perray-en-Yvelines, France); EAAC1, 1/1000 (rabbit; Uptima, Interchim, Montluçon, France) and Neuron-Specific Enolase (NSE), 1/6000 (rabbit, TEBU)] in the TBST containing 0.5% skimmed milk. After incubation with the primary antibodies, blots were washed three times with TBST and further incubated

for one hour at room temperature in TBST supplemented with 0.5% milk containing the appropriate horse radish peroxidase–conjugated secondary antisera (1/10 000 for rabbit or goat, P.A.R.I.S). Immunoreactive bands were detected by enhanced chemiluminescence (ECL westernblotting detection kit; Amersham, GE Healthcare Europe GmbH, Orsay, France), and signals were visualized with hyperfilm ECL (Amersham) and measured by densitometry (Quantity One; Biorad). Band intensities were within the linear range of film development as checked in preliminary experiments (data not shown). Levels of actin or NSE were used as a control to correct for variations in protein loading in the gels. Extracts from young and aged rats were loaded onto the same gel and blots were successively hybridized with the different antibodies, including loading control antibodies.

Glutamate uptake

The activity of the Na+-dependent glutamate transporters was studied by measuring the uptake of D-[^{3}H]aspartate, a transportable analogue of L-Glutamate that is not metabolized, in freshly isolated brain cell suspensions, as previously described (Grintal *et al.*, 2009).

Preparation of brain cell membrane suspensions

After decapitation of the animals, the brains were quickly removed and transferred into ice-cold oxygenated Krebs' buffer (content in mM: Hepes 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ $2H_2O$ 1.8, $MgSO_4$ $7H_2O$ 0.8, Glucose 7, NaCl 120 pH 7.2). All manipulations were performed on ice. Hippocampal CA1 areas were isolated, homogenized with a Teflon homogenizer, and cell suspensions were filtered through a 40 μm filter. The suspensions were then centrifuged at 1000 *g* for 10 min, at 4 °C. The pellets were gently re-suspended in Krebs' buffer (67 mg brain tissue/mL) and kept on ice until and all during the D-[^{3}H]-Aspartate uptake assays. The uptake assays were performed no more than 1 h after the decapitation of the rat. Protein content of the final suspensions was determined on sonicated aliquots of the suspension, using a kit from BioRad (DC protein assay).

D-[^{3}H]-Aspartate uptake assay

The uptake of D-[^{3}H]-Aspartate was measured in oxygenated Na^+-containing Krebs' medium (total uptake) and in Na^+-free (choline chloride substituted for NaCl) Krebs' medium (Na^+-independent uptake), added with D-[^{3}H]Aspartate (GE Healthcare Europe; specific activity 35 Ci/mmol) and unlabeled D-Aspartate (final concentration from 0.5 to 20 μM). The reaction was started by adding an aliquot of the brain cell suspensions (containing 50 μg of protein) for 2 min at 30 °C. The reaction was terminated by adding ice-cold Na^+-free Krebs' medium containing 1 mM D-aspartate and quickly filtering the mixture through GF/B filters (Whatman International Ltd VWR, Strasbeurg, FRANCE). Filters were rinsed twice with ice-cold Na^+-free Krebs' medium containing 1 mM D-aspartate, and the level of radioactivity retained on the filter was measured in a liquid scintillation counter. Na^+-dependent uptake was calculated as the difference between total uptake and Na^+-independent uptake.

Ex vivo electrophysiology

Acute slice preparation

Conventional slices (400 μm thick) were prepared as previously described (Potier *et al.*, 2000) and placed in a holding chamber in an aCSF containing: 124 mM NaCl, 3.5 mM KCl, 1.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 11 mM glucose, continuously oxygenated (pH = 7,4; 27 °C). Slices were allowed to recover in these conditions from the slicing at least 1 h before recording.

Recordings

For electrophysiological recordings, a single slice was placed in the recording chamber, submerged and continuously superfused with gassed (95% O_2, 5% CO_2) aCSF (28–31 °C) at a constant rate (2 mL min^{-1}) for the reminder of the experiment. Extracellular fEPSPs were recorded in the CA1 *stratum radiatum* using a glass micropipette filled with 2 M NaCl. Presynaptic fiber volleys and fEPSPs were evoked by the electric stimulation of Schaffer collaterals/commissural pathway at 0.1 Hz with a bipolar tungsten stimulating electrode placed in the *stratum radiatum* (100 μs duration). Stable baseline fEPSPs were recorded by stimulating at 30% maximal field amplitude for 20 min prior to beginning experiments.

Synaptic transmission. Input/output (I/O) curves were constructed to assess the responsiveness of the AMPAR subtype of glutamate receptors to electrical stimulation in young and aged rats and to compare the effects of drugs in both groups of animals. The slope of three averaged PFVs and fEPSPs was measured and plotted against different intensities of stimulation (from 100 to 500 μA) using the WINLTP software (Anderson & Collingridge, 2007). I/O curves were constructed before and in the presence of drugs. Time-course of drug effects was also assessed by changes in fEPSP slope, expressed as the percentage of the baseline value and plotted against time.

LTD was induced by the application of LFS: 1 Hz for 15 min or 2 Hz for 10 min. Responses were recorded for at least for 45 min after LFS. For the pharmacological studies, drugs were bath applicated for at least 10 min before, and during LFS stimulation. For each pharmacological study, experiments under control conditions were systematically performed on the same day.

Paired-pulse facilitation (PPF). For paired-pulse experiments, two fEPSPs were induced at a interstimulus interval (ISI) of 40 ms for maximum facilitation of the second response in control conditions and in the presence of drugs. Paired-pulse facilitation is dependent on the probability of neurotransmitter release, and the ratio of facilitation is greater after a failure of the first response to evoke transmitter release. Because of this dependence of PPF on the size of the first response (Debanne *et al.*, 1996), stimulation intensities were adjusted to respect the

low probability of release rule for facilitation and were generally < 300 μA in young and aged rats. PPF was quantified by normalizing the slope of the second fEPSP by the slope of the first fEPSP (PPF ratio).

Drugs

Drugs used in this study include: the glutamate uptake inhibitor DL-TBOA (D,L-threo-beta-benzyloxyaspartic acid; Tocris, Illkirch, France), the NMDA receptor antagonist D-APV (D-2-amino-5-phosphonovalerate; Tocris) and the metabotropic glutamate receptor antagonist (S)-MCPG ((S)-α-methyl-4-carboxyphenylglycine; Tocris Cookson, Bristol, UK). All pharmacological agents were diluted directly in superfusion medium from stock solutions prepared in distilled water or in dimethylsulfoxide.

Analysis of data

Data are reported as mean ± SEM. Significance was calculated using multivariate analyses of variance (ANOVA) followed by *post hoc* (paired or unpaired) *t* test. The magnitude of synaptic plasticity was measured, and mean values were calculated between the 35th and 45th minute after the end of the conditioning stimulus. Differences were considered significant when $P \leq 0.05$.

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Author contributions

B.P., P.D., A.J. and J.-M.B. designed experiments. B.P. and P.D. wrote the paper. M.K., A.J., P.D., S.R., B.P. J-M.B. performed and analyzed electrophysiological recordings in slices. G.C.-P., B.G. and I.D. performed and analyzed glutamate uptake experiments. P-M.S. performed and analyzed western blotting. All authors discussed the results and implications and commented on the manuscript at all stages.

Disclosure statement

The authors declare that they have no actual or potential conflict of interest, financial or otherwise, related to the present work. The data contained in the manuscript being submitted have not been submitted elsewhere and will not be submitted elsewhere while under consideration at Aging Cell. Procedures and final version of the manuscript are approved by all authors.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 AMPAR-mediated synaptic responses in 22 slices from 15 young and 22 slices from 14 aged rats.

Fig. S2 DL-TBOA had no effect on the mean PFV slope.

Fig. S3 Effect of DL-TBOA on synaptic transmission in young and aged rats.

Fig. S4 Dose-dependence of the DL-TBOA-induced depression of synaptic transmission.

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