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Summary

Epidemiological data suggest that a poor ω3 status favoured by the low ω3/ω6 polyunsaturated fatty acids ratio in western diets contributes to cognitive decline in the elderly, but mechanistic evidence is lacking. We therefore explored the impact of ω3 deficiency on the evolution of glutamatergic transmission in the CA1 of the hippocampus during aging by comparing 4 groups of rats aged 6–22 months fed ω3-deficient or ω3/ω6-balanced diets from conception to sacrifice: Young ω3 Balanced (YB) or Deficient (YD), Old ω3 Balanced (OB) or Deficient (OD) rats. ω3 Deficiency induced a 65% decrease in the amount of docosahexaenoic acid (DHA, the main ω3 in cell membranes) in brain phospholipids, but had no impact on glutamatergic transmission and astroglial function in young rats. Aging induced a 10% decrease in brain DHA, a 35% reduction of synaptic efficacy (fEPSP/PFV) due to decreased presynaptic glutamate release and a 30% decrease in the astroglial glutamate uptake associated with a marked astrogliosis (+100% GFAP). The ω3 deficiency further decreased these hallmarks of aging (OD vs. OB rats: −35% fEPSP/PFV $P < 0.05$, −15% astroglial glutamate uptake $P < 0.001$, +30% GFAP $P < 0.01$). This cannot be attributed to aggravation of the brain DHA deficit because the brains of OD rats had more DHA than those of YD rats. Thus, ω3 deficiency worsens the age-induced degradation of glutamatergic transmission and its associated astroglial regulation in the hippocampus.

Key words: astrocytes; DHA; glutamate; hippocampus; neurotransmission; polyunsaturated fatty acids.

Introduction

The growing imbalance between ω6 and ω3 polyunsaturated fatty acids (PUFA) in western diets is a well-documented health concern. This imbalance may restrict the availability of ω3 long-chain polyunsaturated fatty acids (LC-PUFA) (mainly docosahexaenoic acid, DHA) to tissues and decrease the amount of DHA incorporated into cell membrane phospholipids (Ailhaud *et al.*, 2006). The decrease in membrane DHA may alter the physiology of tissues where DHA is abundant, like the brain. Many studies have highlighted the adverse influence of insufficient dietary ω3 PUFA (or ω3) on brain function (cognition, behaviour). In particular, ω3 intakes seem to influence cognitive function in the elderly. Correcting the ω3 imbalance of the western diet would therefore be a valuable strategy for coping with the health concerns in our aging populations. However, the underlying mechanisms of ω3 action in brain physiology are still debated, and we need to elucidate the impact of a life-long deficit in cell membrane DHA to determine how nutrition can help prevent brain aging.

Human epidemiological studies indicate an inverse correlation between ω3 intakes or fish consumption and the risk of developing Alzheimer's disease (review Cunnane *et al.*, 2009), or deteriorating cognitive performance in the elderly. A recent review concluded that ω3 helped slow cognitive decline in dementia-free old individuals (Fotuhi *et al.*, 2009). However, the dietary ω3 intakes of the subjects in most of these studies cannot be truly isolated from confounding factors that also influence cognitive function (Barberger-Gateau *et al.*, 2005).

Many experimental studies on rodents have shown that ω3 deficiency impairs memory in young animals (review Fedorova & Salem, 2006) but the relationship between ω3 supply and age-related cognitive impairment remains tentative. Some studies have shown that spatial learning capacity is altered in ω3-deficient old rodents (Yamamoto *et al.*, 1991; Umezawa *et al.*, 1995; Carrie *et al.*, 2002), while ω3 LC-PUFA supplementation (fish oil) improves learning in old rats (Gamoh *et al.*, 2001; Kelly *et al.*, 2010) and in rodent models of Alzheimer's disease (Calon *et al.*, 2004; Hashimoto *et al.*, 2011), with some contradictory results (Barcelo-Coblijn *et al.*, 2003; Arendash *et al.*, 2007; Moranis *et al.*, 2011). The beneficial effect of acute fish oil supplementation seems to be partly attributable to the anti-oxidative and anti-inflammatory actions of ω3 LC-PUFA (review Boudrault *et al.*, 2009), but little is known about the physiological mechanism linking cognitive decline and chronic ω3 deficiency.

The age-related cognitive decline is due to many physiological alterations. One is the deregulation of the glutamatergic synapses in the hippocampus, especially in the CA1 area, which is involved in spatial memory. The documented age-related alterations in its function include decreased plasticity and glutamate homoeostasis (Potier *et al.*, 2010; review Segovia *et al.*, 2001). Three components are involved in regulating the hippocampal glutamatergic synapse: the presynaptic and postsynaptic neuronal compartments and the surrounding astrocytes (review Halassa *et al.*, 2007). The astrocytes are the main regulators of the homoeostasis of the glutamatergic synapse; they maintain a safe concentration of glutamate in the neuronal environment by transporting glutamate and by controlling the extra-cellular volume (Sheldon & Robinson, 2007). The diffuse astrogliosis that occurs progressively with age alters the regulative functions of the astrocytes and exacerbates the neuronal damages, notably by disrupting glutamate homoeostasis (Cotrina & Nedergaard, 2002; Sofroniew & Vinters, 2010). Therefore, an efficient astroglial function must be maintained throughout life to prevent brain aging.

Astrocytes may be targets of ω3 in the brain. They have high concentrations of DHA in their membranes, and this depends on the amount of dietary ω3. Our studies on cultured astrocytes have shown that DHA influences major functions involved in the regulation of brain homoeostasis, such as gap junction coupling and glutamate elimination (Champeil-Potokar *et al.*, 2004, 2006; Grintal *et al.*, 2009).

We therefore postulated that a deficient brain ω3 status, as may occur in western populations eating too much ω6 and too little ω3,

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influences the homoeostasis of the glutamatergic synapse throughout life and reduces the resistance of the brain to aging.

We evaluated the influence of a ω3 deficiency on glutamatergic homoeostasis during aging by comparing young (6 months) and old (22 months) rats fed a balanced diet containing 5/1 linoleic/α-linolenic acid or an ω3-deprived diet containing only linoleic acid from conception to sacrifice. We determined the ω3 status of the rats by analysing the fatty acid composition of their brains and evaluated the efficiency and regulation of the glutamatergic synapse by electrophysiological recordings, analysis of synaptic receptors and measuring astroglial function (glutamate uptake and astrogliosis).

Results

Influence of diet and aging on brain fatty acid composition

We measured changes in the lipid composition of the frontal cortex and considered them to be representative of changes in the whole brain (Mathieu *et al.*, 2008) (Table 1). The fatty acid compositions of three classes of membrane phospholipids were determined: phosphatidylethanolamine (PE), which is 36% of the total phospholipids and has a high PUFA content (45% of total FA), phosphatidylcholine (PC), which is 40% of the total phospholipids but has a low PUFA content (13% of total FA), and phosphatidylserine (PS), which represents 14% of phospholipids and has a high PUFA content (40% of total FA).

Only the results for PE are shown; the results for PC and PS were very similar to those for PE and are given in Table S1.

Influence of diet (YD vs. YB rats)

The rats fed the ω3-deficient diet had dramatically less DHA in their brain phospholipids (−64% in the PE of YD rats than in YB rats, $P < 0.001$) and a compensatory increase in ω6 docosapentaenoic acid (DPA), a PUFA present in only small amounts in ω3-balanced-diet rats. Arachidonic acid (AA), the main ω6 PUFA in membrane phospholipids, was unaltered by ω3 deficiency. The total amounts of PUFA were similar in YD and YB rats. The amounts of saturated (SFA) and mono-unsaturated (MUFA) fatty acids in all the phospholipid classes studied were unchanged in rats on the ω3-deficient diet (data not shown).

Influence of aging (OB vs. YB rats)

Aging resulted in a slight but significant decrease in the amount of DHA (−11% in PE, $P < 0.01$), leading to the total ω3 PUFA being significantly lower in OB than in YB rats. In contrast, aging had no effect on ω6 PUFA. The age-related decrease in ω3 PUFA was offset by an increase in MUFA (mainly oleic acid): 13.8 ± 0.8% in OB rats and 11.5 ± 0.5% in YB rats, $P = 0.068$.

Combined influence of diet and aging (OD vs. OB rats)

As in young rats, the ω3 deficiency also had effects in old rats: OD rats had reduced DHA (−40% in the PE of OD vs. OB $P < 0.01$) and a compensatory increase in ω6 DPA in membrane phospholipids, which was higher than in OB rats. However, the ω3 deficiency had less impact on old rats than on young animals: OD rats had significantly more DHA (+46% in PE, $P < 0.01$) and less ω6 DPA (−43% in PE, $P < 0.01$) than did YD rats. This indicates that the brain tends to obtain the DHA even from the very small amounts of ω3 PUFA in the ω3-deficient diet or from other parts of the body throughout life. Therefore, the age-related decrease in DHA seen in rats fed a ω-3-balanced diet (OB and YB) was not observed in ω3-deficient rats (OD and YD). However, there was an age-related decrease in the 'DHA-like' PUFA compounds in ω3-deficient rats. Indeed, the sum of DHA and ω6 DPA, the fatty acid replacing DHA in ω3-deficient animals, was significantly lower (−10% in PE, $P < 0.05$) in OD than in YD rats, and in the same order of magnitude as the age-related decrease in DHA in rats fed a ω3-balanced diet (OB vs. YB: −11% in PE, $P < 0.05$). Thus, aging partly reversed the marked effect of ω3 deficiency on the brain DHA content, while ω3 deficiency did not influence the small effect of aging on the 'DHA-like' content of the brain.

ω3 deficiency aggravates the age-related decrease in glutamatergic synaptic transmission

Stimulation of the *stratum radiatum* induced a presynaptic fibre volley (PFV) followed by an NBQX-sensitive field excitatory postsynaptic potential (fEPSP) in all groups of rats, both of which increased with current intensity. The fEPSP/PFV ratio, an index of synaptic efficacy, was comparable in young rats on both balanced and deficient diets (Fig. 1A). The fEPSP/PFV ratio was lower in old rats on a balanced diet than in young rats on a balanced diet (age effect, $P < 0.05$), indicating a potent decrease in AMPA/kainate receptor-mediated synaptic transmission during aging. The PFVs and fEPSPs decreased in parallel in old rats, suggesting that the impairment had a presynaptic origin (data not shown). ω3 deficiency potentiated the effect of aging on synaptic transmission; the fEPSP/PFV ratio of OD rats was significantly lower than that of OB rats (diet effect in the aged population, $P < 0.05$) (Fig. 1A).

The paired-pulse facilitation (PPF) ratio (Fig. 1B), an index of change in the presynaptic release of glutamate, was enhanced by age and by ω3 deficiency (two-way ANOVA: effect of age $P = 0.01$; effect of diet

Table 1 Polyunsaturated fatty acid composition of phosphatidylethanolamine measured in the frontal cortex of the rats (means ± SEM)

% of total fatty acids	Young ω3-balanced rats (YB)	Young ω3-deficient rats (YD)	Old ω3-balanced rats (OB)	Old ω3-deficient rats (OD)
Total PUFA sum	45.1 ± 0.8	44 ± 0.5	42.6 ± 0.8	43.3 ± 0.8
ω6 PUFA sum	19.6 ± 1.3	34.6 ± 0.6**	19.7 ± 0.8	29.7 ± 0.9**,##
ω3 PUFA sum	25.6 ± 0.9	9.4 ± 0.2**	22.9 ± 0.5##	13.6 ± 0.6**,##
ω6/ω3 ratio	0.8 ± 0.08	3.7 ± 0.1**	0.9 ± 0.05	2.2 ± 0.1**,##
AA (20:4n-6)	13.8 ± 1.1	13.0 ± 0.3	13.1 ± 0.5	14.1 ± 0.41
DHA (22:6n-3)	25.2 ± 0.9	9.2 ± 0.2**	22.5 ± 0.5##	13.4 ± 0.6**,##
DPA (22:5n-6)	0.6 ± 0.05	16.1 ± 0.5**	0.7 ± 0.06	9.3 ± 0.4**,##
DHA + DPA	25.9 ± 0.8	25.3 ± 0.6	23.2 ± 0.5#	22.7 ± 0.7#

Statistical analysis by 2-way ANOVA (diet × age) following by post-tests (Bonferroni).

**$P < 0.01$, vs. ω3 balanced of the same age.

#$P < 0.05$, ##$P < 0.01$, vs. young with the same diet.

AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; PUFA, polyunsaturated fatty acids.

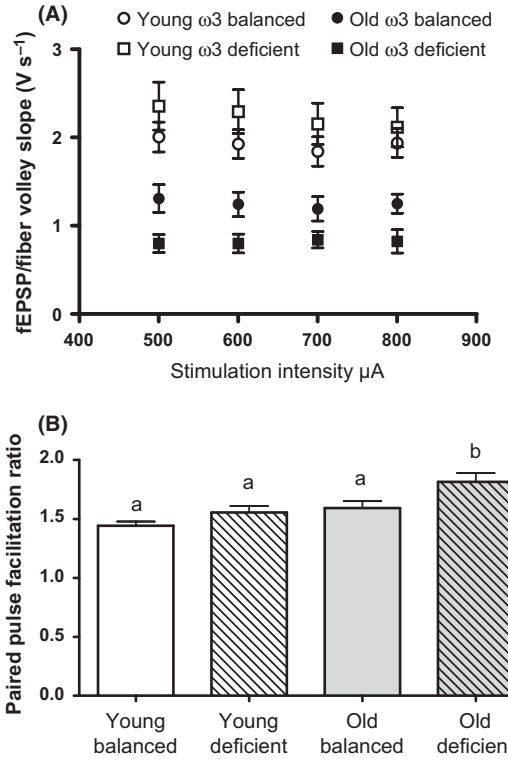

Fig. 1 ω3 deficiency aggravates the age-related decrease in glutamatergic transmission in the hippocampal CA1. The fEPSP/PFV ratio (A) was significantly lower in old rats than in young rats, whatever the diet (age effect $P < 0.001$), and was even lower in old ω3-deficient (OD) rats than in old ω3-balanced (OB) rats (age × diet interaction $P < 0.001$; Bonferroni post-test, $P = 0.016$). Values are means ± SEM; $n = 17$ slices in 6 young balanced (YB) rats, $n = 16$ slices in 6 young deficient (YD) rats, $n = 12$ slices in 6 OB rats, $n = 11$ slices in 5 OD rats. They were compared by three-way ANOVA (age × diet × stimulation intensity: no effect of diet, no effect of stimulation, effect of age $P < 0.001$; Bonferroni post-test a≠b≠c $P < 0.05$). The PPF ratio (B) was enhanced by both diet and age (two-way ANOVA, diet effect $P = 0.036$, age effect $P = 0.01$), and Bonferroni post-tests indicate that the increase in OD rats was significantly greater than in other groups (a≠b, $P < 0.05$). Values are means ± SEM; $n = 17$ slices in 6 YB rats, $n = 17$ slices in 6 YD rats, $n = 20$ slices in 11 OB rats, $n = 16$ slices in 10 OD rats.

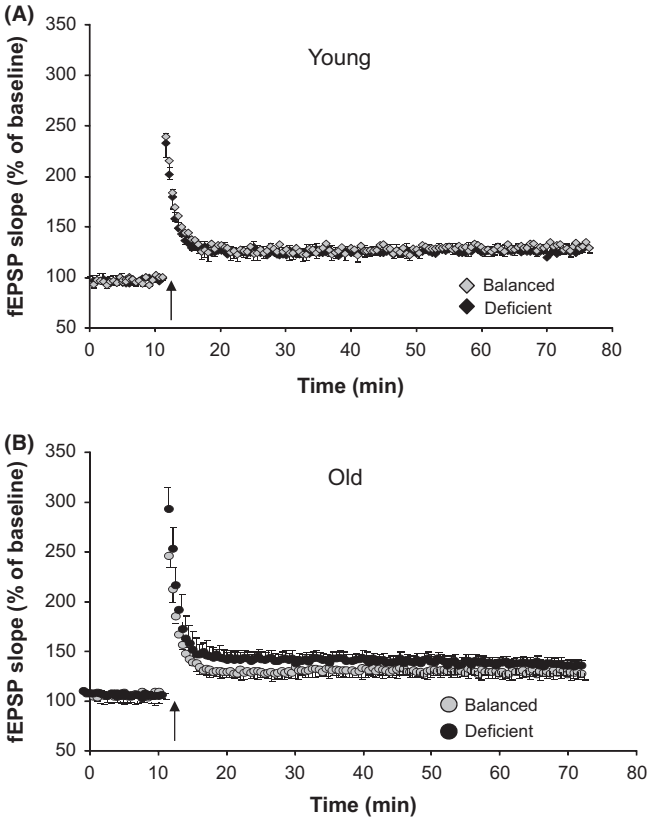

Fig. 2 ω3 deficiency does not alter long-term potentiation (LTP) in the hippocampal CA1 in young (A) and old (B) rats. Averaged LTP, expressed as per cent change in the slope of fEPSP vs. time, was induced by a 2 × 100 Hz stimulation (arrow) of glutamate afferents. There was no significant difference between groups. Values are means ± SEM; $n = 13$ slices in 10 YB rats, $n = 15$ slices in 10 YD rats, $n = 16$ slices in 10 OB rats, $n = 15$ slices in 10 OD rats.

$P < 0.05$), suggesting decreasing glutamate release with aging and with ω3 deficiency. The effect was potentiated by the combination of age and ω3 deficiency, so that the PPF was 25% higher in OD rats than in YB rats ($P < 0.05$, Bonferroni post-test).

Long-term potentiation (LTP), the major form of synaptic plasticity, was characterized in hippocampal slices from all groups of rats. The tetanus stimulation induced a significant increase in fEPSP slope over baseline levels, which persisted until the end of the recording (1 h; Fig. 2A, B). However, the amplitudes of the LTP were similar in all groups of rats (YB 33 ± 5%; YD 29 ± 6%; OB 22 ± 6%; OD 30 ± 6%, no effect of age or diet), indicating that neither age nor ω3 deficiency altered LTP in our rats.

ω3 deficiency aggravates the age-related decreases in VGlut-1 and VGlut-2

The age-related increase in PPF suggests that aging reduced the presynaptic release of glutamate. We found significant decreases in the amounts of the main vesicular glutamate transporters at the presynaptic membranes, in the CA1 with age: VGlut-1 (Fig. 3A, two-way ANOVA old vs. young rats −32%, $P < 0.01$) and VGlut-2 (Fig. 3B, two-way ANOVA, old vs. young rats −41%, $P < 0.01$). The ω3 deficiency reduced the amount of VGlut-1 (Fig. 3A, two-way ANOVA ω3 deficient vs. balanced: −27%, $P < 0.05$), but not the amount of VGlut-2 (Fig. 3B). Bonferroni post-test indicates that OD rats, only, had lower amounts of VGlut-1 (−53% vs. YB, $P = 0.017$, Fig. 3A) and VGlut-2 (−50% vs. YB, −62% vs. YD, $P < 0.05$, Fig. 3B) than did YB rats, suggesting that the ω3 deficiency reinforced the impact of aging.

We found that age and diet had no effect on the amount of synaptophysin, a nonspecific marker of synaptic vesicles (Fig. 3C). This suggests that the number of synapses and their vesicular equipment were unaltered in old and ω3-deficient rats. Therefore, the decrease in VGlut-1 and VGlut-2 found in old rats and especially in old ω3-deficient rats reflected a specific alteration in glutamate release capacity.

Neither age nor diet had any effect on the amount of GLUR1, a subunit of the AMPA receptors (Fig. 3D). This indicates that the age- and diet-related alterations found in glutamatergic transmission (Fig. 1) are not due to a lack of AMPA receptors.

ω3 deficiency aggravates the age-related decrease in astroglial glutamate uptake capacity

We compared the rates of D-[^{3}H]aspartate uptake (astroglial glutamate transport) by suspensions of brain cells freshly isolated from the CA1

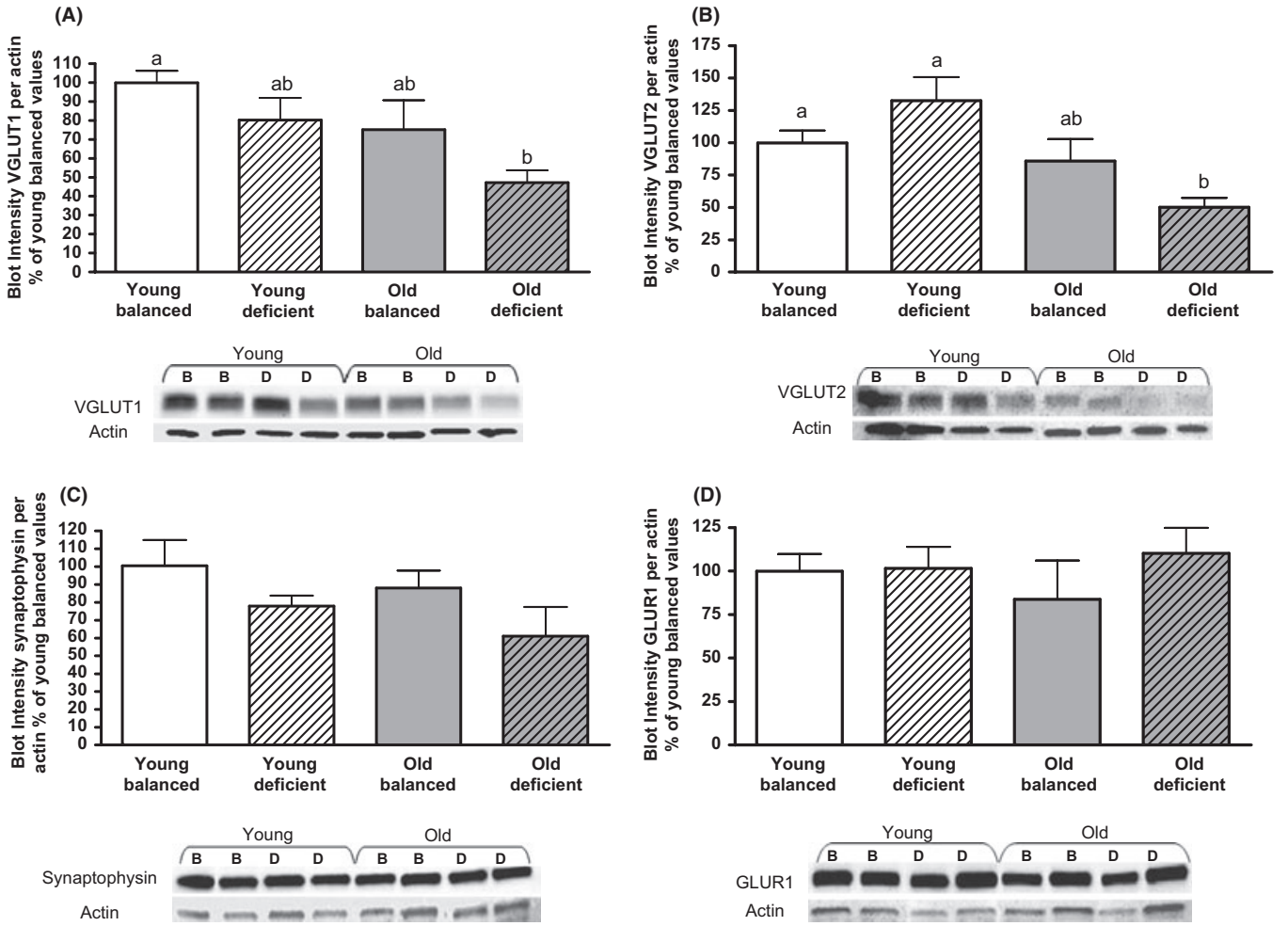

Fig. 3 Effect of ω3 deficiency on the age-related decrease in the amounts of the vesicular glutamate transporters VGlut-1 (A) and VGlut-2 (B) measured by western blotting in the hippocampal CA1. The amounts of the nonspecific synaptic marker synaptophysin (C) and the subunit of AMPA of postsynaptic receptors GLUR1 (D) in this region were not altered by age or diet. The amount of VGlut-1 was decreased by both ω3 deficiency and age (two-way ANOVA: effect of diet $P = 0.023$, effect of age $P = 0.006$); Bonferroni post-tests indicated that only old ω3-deficient (OD) rats differed significantly from the young ω3-balanced (YB) group ($a \neq b$, $P < 0.05$). The amount of VGlut-2 decreased with age, but not with diet, except in old rats (two-way ANOVA: effect of age $P = 0.006$, no effect of diet, age x diet interaction $P = 0.016$) where post-tests indicate that OD rats differ significantly from young deficient (YD) and balanced (YB) rats ($a \neq b$, $P < 0.05$). Values are means ± SEM, $n = 9$ YB rats, $n = 9$ YD rats, $n = 7$ OB rats, $n = 11$ OD rats.

region of the hippocampus obtained from all groups of rats. Most (85%) of the measured D-[^{3}H]aspartate uptake by these cell suspensions can be abolished by di-hydro-kainate (100 μM), a specific inhibitor of GLT1 transport, indicating that GLT1 activity accounts for most of the measured uptake (data not shown).

The saturation curves (Michaelis–Menten) show significant differences ($P < 0.001$) in the glutamate uptakes of cell suspensions from young and old rats, regardless of the diet, indicating that glutamate uptake capacity is reduced with age (Fig. 4A). The data for cell suspensions from young rats show similar glutamate uptakes for rats on ω3-deficient and ω3-balanced diets, indicating that ω3 status had no influence on glutamate uptake in young animals. In contrast, ω3 deficiency was associated with a further decrease in glutamate uptake in old rats ($P < 0.001$).

Age had a significant influence on the maximal velocity (Vmax) of D-[^{3}H]aspartate uptake by cell suspensions (in nmoles min^{-1} mg^{-1} protein: 4.8 ± 0.38 in young rats and 3.1 ± 0.27 in old rats $P < 0.001$), while the Km of the transporters was not significantly altered (10.7 ± 1.7 μM in young rats and 8.1 ± 2 μM in old rats). This suggests a lower density of functional glutamate transporters in the cell suspensions from aged rats.

The significant difference ($P < 0.001$) in the D-[^{3}H]aspartate uptake curves of cells from OD and OB rats was not great enough to significantly decrease the kinetic parameters Vmax (3.2 ± 0.2 in OD and 3.0 ± 0.3 nmoles min^{-1} mg^{-1} protein in OB; $P = 0.6$) or Km (7.2 ± 1.1 in OD and 8.9 ± 2.2 μM in OB, $P = 0.5$).

As suggested by the decreased Vmax in old rats, aging significantly decreased the amount of the astroglial glutamate transporter GLT-1 protein (Fig. 4B) (two-way ANOVA, effect of age $P < 0.01$). This effect of age was significant in deficient rats (−33% in OD vs. YD, $P = 0.01$) but not in balanced rats (−18% in OB vs. YB: ns). Thus, the potentiation of the age-related decrease in glutamate uptake capacity in the CA1 by ω3 deficiency is due at least partly to a decrease in GLT-1.

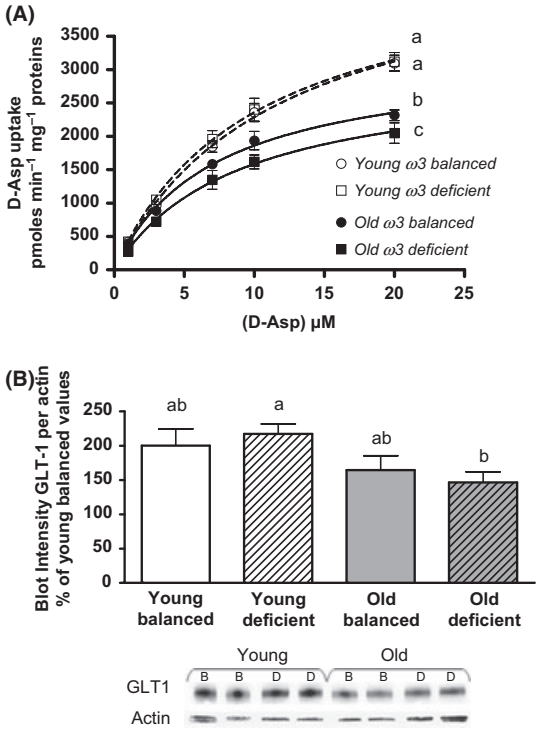

Fig. 4 ω3 deficiency aggravates the age-related decrease in astroglial glutamate transport, measured by D-[^{3}H]aspartate uptake, by suspensions of brain membranes (A) and by western blotting of the astroglial glutamate transporter GLT-1 (B), in the hippocampal CA1 of young/old ω3-balanced/deficient rats. D-[^{3}H] aspartate uptake saturation curves (A) were significantly different in young and old rats (*F*-test comparison of nonlinear curves: $P < 0.001$) and in old ω3-balanced rats and old ω3-deficient rats ($P < 0.01$) (a≠b≠c), indicating that glutamate uptake was lower in old rats than in young rats and was even lower in old ω3-deficient rats than in old ω3-balanced rats. Aging significantly reduced the amount of GLT-1 (B) (two-way ANOVA: effect of age $P = 0.009$), and Bonferroni post-tests indicate that old ω3-deficient rats differed from young ω3-deficient rats (a≠b, $P < 0.05$), while old ω3-balanced rats did not differ from young ω3-balanced rats. Values are means ± SEM; for D-[^{3}H]aspartate uptake, $n = 5$ (young) to 9 (old) rats per group; for GLT-1, $n = 9$ rats per group.

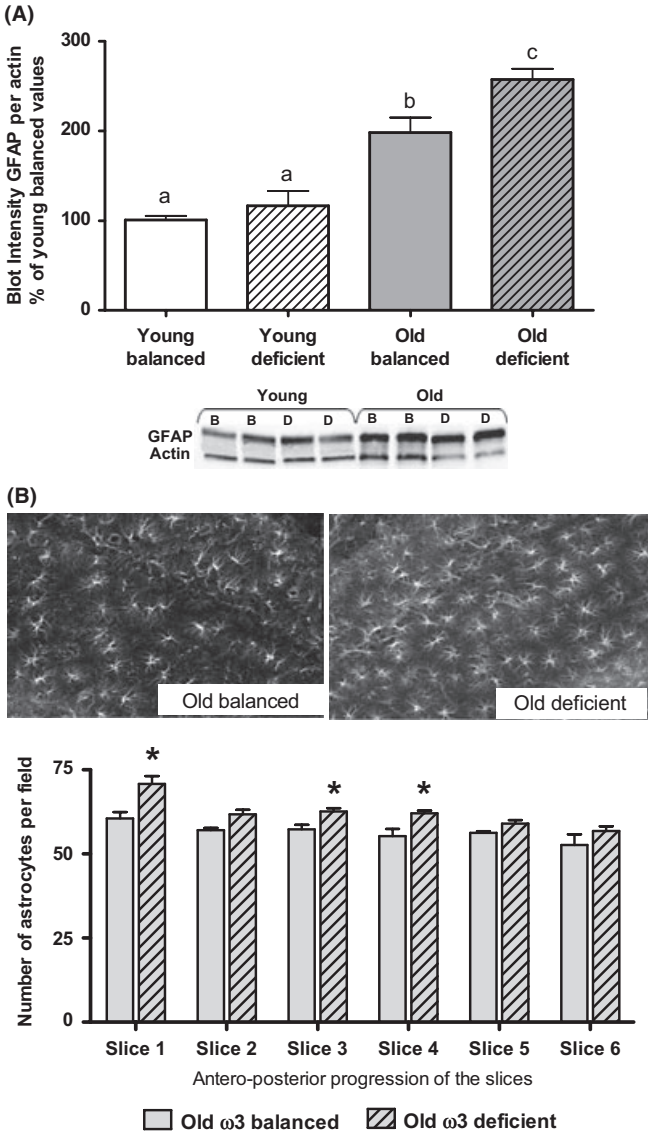

Fig. 5 ω3 deficiency aggravates the age-related astrogliosis, as indicated by western blotting of GFAP (A) and the number of GFAP-immunolabelled astrocytes (B) in the hippocampal CA1. Aging significantly increased the amount of GFAP (two-way ANOVA: effect of age $P < 0.0001$), and the increase was significantly greater in old ω3-deficient (OD) rats than in old ω3-balanced (OB) rats (effect of diet $P = 0.008$; Bonferroni post-tests: a≠b≠c, $P < 0.01$). There were significantly more GFAP+ astrocytes in the CA1 of the OD rats than in OB rats (two-way ANOVA: diet effect $P < 0.001$, effect of slice position $P < 0.001$, no interaction between factors, Bonferroni post-tests on individual slices indicated significantly more astrocytes in OD vs. OB rats in slices 1, 3 and 4, $*P < 0.05$). Values are means ± SEM. For GFAP assays: $n = 8$–10 rats per group. For astrocyte counts in hippocampal slices: 3–6 microscope fields were counted per slice for 4 OB rats and 5 OD rats.

ω3 deficiency aggravates the age-related astrogliosis

Astrogliosis, a hallmark of brain aging characterized by astroglial hypertrophy, can be identified by an increase in the amount of GFAP, the main protein of astrocyte intermediate filaments.

Our western blotting studies showed a marked increase in the amount of GFAP in the CA1 region of the hippocampus of old rats as compared to young rats (+97%, $P < 0.001$), and this increase was significantly greater in OD rats than in OB rats (+30%, $P < 0.01$) (Fig. 5A). Anteroposterior slices of the hippocampus of OD rats immunolabelled for GFAP contained slightly but significantly more astrocytes than did similar slices from OB rats (+ 10%, $P < 0.001$). Direct comparisons of sections indicated that there were significantly ($P < 0.05$) more astrocytes in slices 1, 3 and 4 from OD rats than in the corresponding sections/slices from OB rats, but not in slices 2, 5 and 6, suggesting that astrogliosis is more apparent in some areas of the CA1 than in others.

Discussion

This study on young (6 months) and old (22 months) rats fed either an ω3/ω6-balanced diet or an ω3-deprived diet from their conception shows that some major features of hippocampal aging are amplified by a lack of ω3.

Normal aging is associated with a decline in hippocampus-dependent cognitive ability, particularly a decrease in spatial memory. Alterations in glutamatergic transmission in the CA1 area of the hippocampus have been implicated in these cognitive deficits. Our data indicate that aging reduces the efficacy of glutamatergic neurotransmission between Schaffer collaterals and pyramidal neurons in CA1. This effect is

associated with a reduced release of glutamate from presynaptic terminals, as indicated by increased PPF ratio and decreased amounts of VGlut-1 and VGlut-2, the transporters involved in filling presynaptic glutamate vesicles, in the CA1 of old rats compared to young rats. There was no parallel change in the amount of synaptophysin, a generic marker of presynaptic vesicles, indicating that age probably has no effect on the number of synaptic terminals in the CA1. We also found no age-related change in GluR1, a subunit of AMPA receptors, suggesting that the AMPA postsynaptic response equipment remains intact in old rats. This agrees with our previous results showing that the synaptic efficacy in the CA1 of old Sprague-Dawley rats is poorer than that of young rats, while the amount of AMPA subunits remains unchanged (Potier *et al.*, 2000; Kollen *et al.*, 2008). Other studies have also found no change in the number of AMPA postsynaptic receptors in the CA1 of aged rats (Rosenzweig & Barnes, 2003). The decreased intensity of glutamatergic neurotransmission in the CA1 of old rats may therefore be due mainly to a reduced presynaptic release of glutamate, in line with several studies (Minkeviciene *et al.*, 2008; Canas *et al.*, 2009; Stephens *et al.*, 2011). Earlier studies found contradicting effects of age on glutamate release in the hippocampus and on the number of AMPA receptors (review Segovia *et al.*, 2001).

The other major feature that we found in older rats was hypertrophy of the astroglia, which was associated with impaired glutamate uptake. This could fundamentally alter the homoeostasis of glutamatergic synapses. The astrocytes from the CA1 of old rats was 30% less able to scavenge glutamate than the CA1 astrocytes from young rats, while there was twice as much GFAP in this area of old rats than in young rats, indicating a great expansion of astroglial processes. Astrogliosis is considered to be a hallmark of brain aging, and many studies have shown increased GFAP expression (mRNA and protein) or immunoreactivity in the brains of aged rodents and humans (review Middeldorp & Hol, 2011). Our results showing that the Vmax of glutamate uptake is 30% lower in CA1 cell suspensions from old Wistar rats than in young ones, with no change in the Km, agree well with our previous findings for the CA1 of Sprague-Dawley rats (Potier *et al.*, 2010) and with others showing a comparable 20–30% decrease in glutamate uptake in the cortex of old rats (review Segovia *et al.*, 2001) and a major reduction in Vmax in the cortex of old mice (Saransaari & Oja, 1995). Our finding of less GLT-1 in the CA1 of old rats than in young rats corroborates the decrease in the uptake Vmax, indicating that fewer glutamate transporters were active in the CA1 of the old rats than in the young ones.

The combination of increased synaptic astroglial coverage due to astrocyte hypertrophy and reduced glutamate uptake in our old rats probably acts together to enhance or prolong the elevated glutamate concentration under neuronal stimulation, or that of the ambient glutamate concentration in the synaptic area (Rusakov, 2001; Sykova, 2005). This may favour the activation of presynaptic mGluRs controlling the exocytosis of glutamate and then reduce glutamate release at the presynaptic membrane in old rats. The reduced presynaptic glutamate release in old rats suggested by the elevated PPF and decreased amounts of VGlut-1 and VGlut-2 may therefore be due, at least in part, to an increase in the synaptic glutamate concentration as a result of the hypertrophy of astroglial processes and the slower scavenging of glutamate by astrocytes. This would agree with our previous results obtained with Sprague-Dawley rats (Potier *et al.*, 2010).

Despite the observed changes in synaptic transmission and astrocyte function in old rats, we found no change in synaptic plasticity measured between the Shaffer collaterals and pyramidal neurons in CA1. The LTP values in young and aged rats were similar. The many studies that have measured the changes in LTP with age indicate that only NMDA-dependent LTP is diminished in aged animals while the voltage-dependent calcium channel-related LTP is unaltered or increased. The protocol we used to measure LTP may therefore give global results showing no change in LTP with aging (Rosenzweig & Barnes, 2003). We saw no difference in the amplitude of LTP induced by a weaker stimulation, or in the amplitude of long-term depression (LTD, data not shown), another mechanism of synaptic plasticity that is altered with aging (Billard, 2010). These data suggest that compensatory mechanisms can preserve some forms of synaptic plasticity, together with the expression of the AMPA receptor subunit and synaptophysin. We also found that ω3 deficiency had no influence on synaptic plasticity in either young or old rats. There have been reports that ω3 have a rescuing effect on LTP and postsynaptic AMPA or NMDA receptors in aged rodents, but these studies used acute treatment with long-chain ω3 (DHA and/or EPA) in models of altered plasticity with aging (Kelly *et al.*, 2010).

The age-associated alterations in the CA1 that we found were more severe in the ω3-deficient rats, whereas ω3 deficiency induced little or no change in these parameters in young rats. Indeed, glutamatergic transmission (fEPSPs) was reduced (−35%, $P < 0.05$), PPF was enhanced (+12%, $P < 0.05$), as was GFAP expression (+30%, $P < 0.01$) and the number of GFAP-positive astrocytes (+10%, $P < 0.001$), and the uptake of glutamate by astroglia was reduced (−15%, $P < 0.001$) in old ω3-deficient (OD) rats as compared to old ω3-balanced (OB) ones. In contrast, these parameters were similar in young ω3-deficient (YD) and young ω3-balanced (YB) rats. This indicates that a lack of ω3 does not alter glutamatergic transmission *per se* or its regulation by astroglia in young rats, but that it does contribute to their gradual alteration throughout life. Therefore, the aging mechanisms that lead to the reductions in synaptic glutamate release and uptake and to the astrogliosis in aged rats appear to be enhanced by ω3 deficiency. As stated above, we believe that astroglial hypertrophy is probably the major determinant of the age-associated changes in glutamatergic transmission in the CA1. This astrogliosis was particularly pronounced in old ω3-deficient rats, leading to very high concentrations of GFAP and increased number of astrocytes. The increase in GFAP (+30%) was greater than the increase in the number of astrocytes (+10%), suggesting that it also accounts for the hypertrophy of the main astrocyte processes. Age-associated astrogliosis is thought to result from gradual/repeated increases in inflammatory cytokines (IL-1β, IL-6, TNFα), which contribute to the well-characterized low-grade inflammatory status of the aging brain (Laye, 2010). There is evidence that a ω3 deficiency favours the establishment of a proinflammatory status in the brain, as in the whole body, by enhancing the production of proinflammatory eicosanoids deriving from arachidonic acid, reducing that of protective docosanoids deriving from DHA, and deregulating the release of cytokines and signalling in the brain (review Laye, 2010). Therefore, the promotion of proinflammatory pathways in our ω3-deficient rats may have enhanced the age-associated installation of astrogliosis. This will be verified in further experiments evaluating the brain inflammatory status of the rats.

The age-associated decrease in the presynaptic release of glutamate in old ω3-deficient rats may also have been worsened by a progressive alteration in the exocytosis of neurotransmitter release that is likely to be affected by the ω3 status (Mathieu *et al.*, 2010).

The ω3 deficiency had a dramatic impact on the PUFA composition of the brain phospholipids of young rats, as expected. It produced a 65% decrease in DHA and a compensatory increase in DPA, an ω6 PUFA found in only negligible amounts in ω3/ω6-balanced animals. The effect of ω3 deficiency was less pronounced in old rats; their average DHA was

50% greater than that of young ω3-deficient rats. Therefore, the changes in synaptic regulation found in old deficient rats but not in young deficient rats cannot be attributed to increased brain ω3 PUFA deficiency. This supports the hypothesis that ω3 deficiency does not directly alter synaptic transmission but rather aggravates the aging processes that alter glutamatergic transmission in old rats.

The greater DHA in old ω3-deficient rats than in young ones, previously observed in our laboratory (Delion *et al.*, 1997), probably results from an adaptive sparing of brain DHA over time, due to reduced turnover (DeMar *et al.*, 2004), and the synthesis of DHA from the trace amounts of α-linolenic acid present in ω3-deficient diet.

In conclusion, our data show that ω3 deficiency aggravates the age-associated reductions in glutamatergic synaptic efficacy and its astroglial regulation in the hippocampal CA1. ω3 deficiency does not directly target synaptic efficacy because these functions were not affected in young deficient animals. Instead, it aggravates some of the aging processes occurring in the hippocampus that lead to impaired glutamatergic transmission. The astrogliosis and the associated changes in astrocyte function that gradually take place during aging are more severe in ω3-deficient rats, indicating that astroglial aging is one of the processes influenced by ω3 deficiency. We previously used *in vitro* models to show that ω3 modulates important properties of astroglia, such as gap junction coupling (Champeil-Potokar *et al.*, 2006) and glutamate uptake (Grintal *et al.*, 2009), which also points to the preeminent influence of ω3 status on astrocyte function. We believe that ω3 deficiency exacerbates the evolution of these cells towards astrogliosis by altering some of their properties. Consequently, ω3 deficiency contributes to the many deficits of the aging brain by deregulating the neuron–astrocyte cross-talk, which is crucial for glutamatergic transmission.

While a major ω3 deficiency such as that induced in our rats never occurs nutritionally in humans, the majority of people in western countries could be suffering from a chronic slight DHA deficit due to excessive intakes of ω6 and relatively poor intakes of ω-3 (Ailhaud *et al.*, 2006). Our results indicating the deleterious impact of a long-term deficit of DHA in the brain on the processes of brain aging highlight the need for a re-equilibration of the nutritional intakes of ω3.

Experimental procedures

Products are referenced in Data S2.

Animals and diet

Animal experiments were carried out in accordance with the European Directive (86/609/EEC) and NIH guidelines (no. 85-23), under the authorization no. 78-150 from the 'DDSV Yvelines, France'.

Rats were raised under 22 ± 1°C, 50 ± 10% humidity and 7 AM–7 PM light cycles. Two weeks before mating, female Wistar rats were fed a ω3-deficient or a ω3/ω6-balanced diet containing 6% lipids (PUFA composition, provided by sunflower and rapeseed oil, in Table 2). The ω6/ω3 ratio of the balanced diet was 5, according to the dietary guidelines for humans and meeting the ratio (5 to 8) usually found in commercial standard rat chow. The experimental animals were the first generation of male rats from the mothers of each dietary group, as previously described (Ximenes da Silva *et al.*, 2002). Rats were housed two per cage from weaning, given free access to food and water, and fed the same diet as their mothers throughout their lives. Young rats were sacrificed at 6 months and old rats at 22 months. The 4 experimental groups of rats were young ω3-balanced-diet rats (YB), young ω3-deficient-diet rats (YD), old ω3-balanced-diet rats (OB) and old ω3-deficient-diet rats (OD). Body weights were similar in rats of the same age whatever the diet [at sacrifice time: YB 640.5 ± 12.2 g (n = 21), YD 625.1 ± 14.5 g (n = 20), OB 715.8 ± 25.5 g (n = 23), OD 701.3 ± 34 g (n = 20)].

Table 2 Fatty acid (FA) composition of the experimental diets

	ω3-balanced diet	ω3-deficient diet
	% weight of total fatty acids	
Sum of saturated FA	7.3	7.8
Sum of monounsaturated FA	65.6	69.8
Linoleic acid (LA 18:2n-6)	22	22.4
Sum of ω6 polyunsaturated FA	22	22.4
α-linolenic acid (LNA 18:3n-3)	5.2	0.1
Sum of ω3 polyunsaturated FA	5.2	0.1
	mg/100-g diet	
LA	1307	1370
LNA	309	6

Brain lipid analysis

The fatty acid compositions were analysed in the three main classes of membrane phospholipids, PC, PE and PS, in the frontal cortex, as previously described (Goustard-Langelier *et al.*, 1999).

Briefly, frontal cortices were dissected out from the rat brains immediately after decapitation, homogenized in aqueous NaCl (9 + 0.02 g L^{-1} butylhydroxytoluene) on ice, and the phospholipid classes were separated from total lipids by solid-phase extraction on aminopropyl-bonded silica columns. The phospholipid fractions were evaporated to dryness under nitrogen and transmethylated by incubation with 10% boron trifluoride at 90°C for 20 min. Fatty acid methyl esters were analysed by gas liquid chromatography.

Ex vivo electrophysiology

Acute slice preparation

Brain slices (400 μm thick) were prepared as previously described (Potier *et al.*, 2000) and placed in a holding chamber in continuously oxygenated artificial cerebrospinal fluid (aCSF, pH 7.4, 28°C). Slices were allowed to recover in these conditions for at least 1 h before recording.

Recordings

A single slice was placed in the recording chamber and continuously superfused with gassed (95% O_2, 5% CO_2) aCSF at 2 mL min^{-1}. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA1 *stratum radiatum* using a glass micropipette filled with 2 M NaCl. Presynaptic fibre volleys (PFVs) and fEPSPs were evoked by 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% of the maximal field amplitude for 15 min prior to beginning the experiments.

Synaptic transmission. Input/output curves were constructed to assess the responsiveness of the AMPA subtype glutamate receptors to electrical stimulation in each group of rats. The averaged slopes of three PFVs and three fEPSPs were measured using WinLTP software, and the fEPSP/PFV ratio was plotted against the stimulus intensity (100–900 μA).

Paired-pulse facilitation (PPF). Paired-pulse facilitation (PPF) of synaptic transmission was induced by electrically stimulating Schaffer collaterals/commissural fibres with paired pulses (inter-stimulus interval: 40 ms). PPF was calculated as the ratio of the slope of the second response over that of the first response.

Long-Term Potentiation (LTP). A 15-min stable baseline of the fEPSP slope was recorded; LTP was then induced by applying a high-frequency stimulation (2 stimulations at 100 Hz for 1 s, 20 s apart). Responses to single pulse were then resumed for 60 min to determine the stable LTP. The magnitude of synaptic plasticity is given by the mean of the responses over 10 last minutes at the end of the stimulus.

Ex vivo 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, by suspensions of freshly isolated brain cells (Grintal *et al.*, 2009).

Cell suspensions were obtained from hippocampal CA1 homogenized immediately after decapitation of the rats, and their protein contents were determined using a DC protein assay kit (details in Data S2).

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), containing D-[^{3}H]aspartate (specific activity 35 Ci $mmol^{-1}$) and unlabelled D-aspartate (total concentration D-Asp from 0.5 to 20 μM). The reaction was started by adding an aliquote of cell suspension to the reaction mix, with incubation for 2 min at 30°C. It was terminated by adding ice-cold Na^+-free Krebs' medium containing 1 mM D-aspartate and quickly filtering the mixture through a GF/B filter. The radioactivity 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 (which was < 10% of the Na^+-dependent D-[^{3}H]aspartate uptake).

Western blotting

The hippocampal CA1 were dissected on ice immediately after decapitation of the rats and homogenized in RIPA buffer + antiproteases (details in Data S2).

Immunoblotting was performed as previously described (Grintal *et al.*, 2009). Briefly, samples diluted in sample buffer were loaded onto 4–15% Tris-HCl polyacrylamide gels. Proteins were separated by electrophoresis and transferred to polyvinylidene fluoride membranes. These membranes were incubated overnight at 4°C with appropriate primary antibodies: guinea pig polyclonal anti-GLT1; mouse monoclonal anti-VGlut-1; mouse monoclonal anti-VGlut-2; mouse monoclonal anti-synaptophysin; rabbit polyclonal anti-GLUR1; mouse monoclonal anti-GFAP, and then for 2–3 h at room temperature with an appropriate HRP-conjugated secondary antibodies (anti-mouse HRP antibody for VGlut-1, VGlut-2, synaptophysin, GFAP; anti-rabbit HRP antibody for GLUR1; anti-HRP antibody for GLT1).

Immunoreactive bands were visualized by enhanced chemiluminescence (ECL+ detection kit) and quantified using a camera connected to image analysis software. The membranes were labelled for actin (mouse monoclonal anti-actin antibody) as a control of protein load. The intensity of the marker for each blot was normalized to that of the corresponding actin blot and expressed as a per cent of the mean value obtained on the same membrane for samples from YB rats.

Immunohistochemistry

The number of astrocytes in the hippocampal CA1 was determined on GFAP-immunolabelled brain slices prepared as previously described (Ximenes da Silva *et al.*, 2002).

Rats were anaesthetized with pentobarbital and perfused intracardially with NaCl (0.9 + 1% nitrite) followed by 4% paraformaldehyde in 0.9% NaCl. Brains were postfixed in the same fixative for 4 h and then immersed in 30% sucrose in PBS until they sank. Serial 30-μm sections containing the hippocampus were cut using a cryostat at −20°C. We selected 6 sections across the hippocampus, from a starting point for slicing at Bregma −2.8 mm (The rat brain atlas, Paxinos and Watson) and over 840 μm for each animal.

The nonspecific sites on free-floating sections were blocked by incubation with PBS + 0.3% Triton + 2% bovine serum albumin for 2 h, and the sections then immunolabelled for GFAP (1 h with a monoclonal Cy3-anti-GFAP antibody). Sections were mounted on gelatine-coated slides in fluorescence mounting medium. The images were captured using a structured illumination microscope (Apotome, MIMA2 Platform, INRA) coupled to AxioVision 4.8 software. The number of GFAP-labelled astrocytes was counted on 6 selected images in the right and left CA1 for each slice and on 6 slices for each rat, using Image J software.

Statistical analysis

SigmaStat 3.1 software was used for statistical comparisons between groups using multivariate analyses of variance (ANOVA) to determine the main effects and interaction of the two independent variables, diet and age. Then, multiple comparison procedures based on Bonferroni correction were run (post-tests). Prism software was used to compare D-[^{3}H]aspartate uptake curves by *F*-test. Differences were considered significant when $P \leq 0.05$.

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Author contributions

A.L. and G.C.P. were involved in the experiments, data and writing. B.G., M.H. and M.L. contributed to data and writing. B.P., J.M.B. and I.D. were involved in conception and design, experiments, data and writing. S.V. was involved in conception and design, data and writing.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Results: fatty acid composition of brain phosphatidylcholine and phosphatidylserine.

Data S1. Experimental procedures: 1/Products information, 2/Cell suspension preparation for glutamate uptake, 3/CA1 dissection of the hippocampal CA1.