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High tryptophan diet reduces CA1 intraneuronal β-amyloid in the triple transgenic mouse model of Alzheimer's disease

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Summary

Alzheimer's disease (AD) is a progressive neurodegenerative disease that impairs mnesic functions. The histopathology of the disease is manifested by the accumulation of intracellular β-amyloid (Aβ) and subsequent formation of neuritic plaques as well as the presence neurofibrillary tangles in specific brain regions associated with learning and memory including the hippocampus. Here, we analysed the effect of chronic (1 month) food diets containing low (LTrP), normal (NTrP) and high tryptophan (HTrP), 0.04, 0.20 and 0.40 g/100 g, respectively, on CA1 serotonin transporter (SERT) fibre density, intraneuronal Aβ deposition and total number of serotonergic (5-HT) neurons in an AD triple transgenic (3xTg-AD) mouse model. Nontransgenic (non-Tg) animals fed with HTrP displayed increased SERT fibre density in CA1 (35%) and in stratum lacunosum moleculare (S.Mol) (48%) compared to LTrP diet. Transgenic animals showed increased SERT fibre density in CA1 S.Mol compared to diet-matched non-Tg irrespective of dietary tryptophan content (104% for LTrP, 74% for NTrP and 35% for HTrP); no differences were observed in the total number of 5-HT neurons neither in the dorsal nor in the median raphe nuclei. However, and more relevant to AD, HTrP diet reduced intraneuronal Aβ density (by a 17%) in transgenic animals compared to transgenic animals fed with NTrP diet. Our results show that increased dietary TrP intake reduces intraneuronal Aβ load in the 3xTg-AD mouse model of AD, suggesting that enhanced TrP intake and in consequence a potential increase in 5-HT neurotransmission may be effective in reducing plaque pathology in AD.

Key words: Alzheimer's disease; hippocampus; L-tryptophan diet; raphe nucleus; serotonin; serotonin transporter.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that deteriorates learning, memory and cognition (Braak *et al.*, 1999). Patients with AD exhibit multiple behavioural disturbances including agitation, irritability, anxiety, delusion and depression (Lyketsos & Olin, 2002). Neuropathological hallmarks of AD include β-amyloid (Aβ) neuritic plaques, neurofibrillary tangles (NFTs), and neuronal and synaptic loss (Selkoe, 2002). Aβ is produced via amyloidogenic processing of amyloid precursor protein (APP) that involves enzymatic cleavage of the protein by β and γ-secretase (Esler & Wolfe, 2001). Aβ is first accumulated inside neurons; subsequently, it is released into the neuropil-forming neuritic plaques (Masters *et al.*, 1985). The intraneuronal accumulation of Aβ could represent a pathologically relevant part of the neurodegenerative process in AD (Rodríguez *et al.*, 2008; Umeda *et al.*, 2011).

Progression of the AD affects several neurotransmitter systems including cholinergic (Kasa *et al.*, 1997), glutamatergic (Miguel-Hidalgo *et al.*, 2002) and serotonergic (5-HT) (Mossello *et al.*, 2008). The dorsal (DR) and the median raphe (MR) nuclei encompass the majority of 5-HT neurons that project to multiple brain regions including the brainstem, the thalamus, the cortex and the hippocampus therefore playing an important role in memory and cognition (Vertes *et al.*, 1999; Schmitt *et al.*, 2006). Patients with AD show reduced 5-HT neurotransmission, which correlates with the severity of the disease (Mossello *et al.*, 2008). Treatment with selective serotonin re-uptake inhibitors (SSRI) increases 5-HT neurotransmission, improves cognition in patients with AD and reduces behavioural disturbances associated with AD (Mowla *et al.*, 2007; Mossello *et al.*, 2008).

L-tryptophan (TrP) is an essential amino acid that acts as 5-HT precursor (Cooper & Melcer, 1961); therefore, 5-HT synthesis and availability is influenced by TrP intake (van der Stelt *et al.*, 2004). Alteration in dietary TrP is frequently used as a noninvasive method to manipulate with systemic TrP levels and thus with central 5-HT neurotransmission (van der Stelt *et al.*, 2004). Changes in dietary TrP alter basal extracellular 5-HT levels in multiple brain regions including the hippocampus; therefore reducing TrP intake impairs learning and memory (van der Stelt *et al.*, 2004; Jenkins *et al.*, 2010). In patients with AD, reduced TrP intake further deteriorates cognitive function (Porter *et al.*, 2000). Chronic increase in 5-HT neurotransmission by oral administration of TrP is associated with improved memory acquisition, consolidation and storage in rodents (Haider *et al.*, 2007), whereas daily TrP injections enhanced spatial memory in aged rats (Levkovitz *et al.*, 1994).

In vitro studies have shown that exposure to 5-HT increases the non-amyloidogenic processing of APP metabolite (APP_S) (Robert *et al.*, 2001). It has also been shown that increased 5-HT neurotransmission resulted from treatment with SSRI reduced APP translation and lowered pathogenic Aβ peptide secretion hence potentially decreasing Aβ deposition in AD (Payton *et al.*, 2003; Pakaski *et al.*, 2005). These findings are supported by *in vivo* studies in which chronic treatment with SSRI reduced plaque burden in the cortex and the hippocampus in PS1APP transgenic mice, which exhibit severe amyloid pathology (Cirrito *et al.*, 2011). Similarly, chronic treatment with SSRI reduced both the presence of plaques and NFT in the triple transgenic mouse model of AD (3xTg-AD) (Nelson *et al.*, 2007). We have recently reported a bi-phasic increase in serotonin

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transporter–immunoreactive (SERT)-IR) fibres and terminals (SERT-Te) that develops concomitantly with AD-related neuropathology in 3xTg-AD animals (Noristani *et al.*, 2010, 2011). However, the relationship between altered 5-HT neurotransmission and serotonergic fibre density is not clearly understood. Furthermore, it remains to be determined how altered 5-HT neurotransmission via increased dietary TrP intake may affect

AD-related neuropathology.

In the present study, we analysed effects of acute (1 month) increasing/reducing dietary TrP intake on hippocampal SERT-IR fibre density, total number of 5-HT neurons in the DR/MR nuclei and intraneuronal Aβ accumulation in 3xTg-AD animals.

Results

Dietary TrP intake-associated changes in body weight gain, food and water intake

Animals fed with LTrP diet showed reduced body weight gain irrespective of genotype, when compared to animals on NTrP and/or high tryptophan (HTrP) diets (Fig. 1A). Both non-Tg control and 3xTg-AD animals showed significant reduction in their body weight gain from 1 week of LTrP diet compared to animals in NTrP diet (5%, $P = 0.0273$ for non-Tg and 6%, $P < 0.0001$ for 3xTg-AD, respectively, Fig. 1A). LTrP diet-induced decrease in body weight was sustained during all the experimental period in both non-Tg control and 3xTg-AD groups compared to NTrP diet group (12%, $P = 0.0115$ for non-Tg and 10.5%, $P = 0.0037$ for 3xTg-AD, respectively, Fig. 1A). Non-Tg control and 3xTg-AD animals fed with HTrP diet showed no differences in body weight gain compared to same genotype animals in NTrP diet group (Fig. 1A).

Despite the decrease in body weight gain, non-Tg control animals showed significant increase in food intake after 3 weeks of LTrP diet (103%, $P = 0.0069$, Fig 2B). LTrP-induced increase in food consumption was maintained during all the experimental period in non-Tg control animals compared to animals fed with NTrP diet (67%, $P = 0.0015$, Fig. 1B). Non-Tg animals fed with HTrP diet showed no difference in food consumption compared to non-Tg animals in NTrP diet group (Fig. 1B). No difference in food intake was observed in 3xTg-AD animals fed with either LTrP or HTrP diets compared to 3xTg-AD animals in NTrP diet.

In addition to increased food intake, non-Tg control animals also showed significant increase in water consumption after 1 week of LTrP diet (107%, $P < 0.0001$, Fig. 1C). Increased water intake was continuously evident in non-Tg control animals fed with LTrP diet during all experimental period compared to non-Tg animals in NTrP diet group (294%, $P < 0.0001$). Similarly, 3xTg-AD animals fed with LTrP diet also showed significant increase in water intake from 2 weeks of diet (55%, $P = 0.0215$), which was maintained during all experimental period compared to 3xTg-AD animals in NTrP diet (46%, $P = 0.0370$, Fig. 1C). Both non-Tg control and 3xTg-AD animals fed with HTrP diet showed no differences in water intake compared to same genotype animals in NTrP group (Fig. 1C).

When comparing water intake between the two genotypes, 3xTg-AD animals fed with NTrP and HTrP diets showed significant increase in water intake starting after 1 week of the diet (79%, $P = 0.0031$ for NTrP and 79%, $P = 0.0316$ for HTrP, respectively, Fig. 1C). This increase in water intake was sustained during all the experimental period (106%, $P = 0.0011$ for NTrP and 124%, $P = 0.0033$ for HTrP, respectively, Fig. 1C).

Overall, during the 1-month period, non-Tg animals fed with LTrP diet consumed significantly more food (25%, $P = 0.0422$, data not shown) and water (271%, $P < 0.0001$, data not shown) compared to non-Tg animals fed with NTrP diet. On the other hand, 3xTg-AD animals fed with LTrP diet showed a nonsignificant increase (6%, $P = 0.4211$, data not shown) in food consumption but a significant increase in water consumption (48%, $P = 0.0076$, data not shown) compared to 3xTg-AD animals in NTrP diet.

Dietary TrP intake-associated changes on hippocampal serotonergic fibre density

In both non-Tg control and 3xTg-AD animals, SERT-IR fibres were heterogeneously distributed throughout the hippocampal formation (Fig. 2). SERT-IR fibres appear mainly as fine and thick processes with numerous varicosities, which are characteristic of axonal profiles (Fig. 2). The highest density of SERT-IR is evident in the S.Mol with S.Rad and S.Or expressing moderate level, whilst PCL of the hippocampus display very low SERT-IR fibre density (Fig. 2). Within this SERT-IR fibres, we found axons with large circular and irregularly spaced varicosity typical of beaded fibres (BF, from the MR), which accounted for the 94% of the total SERT-IR fibres in the hippocampus, whilst thin fibres with small fusiform or granular varicosities regularly spaced classified as fine fibres (FF, originating from the DR) accounted for <5% of total SERT-IR fibres. Being the other type of MR fibres, large thick straight stem axons (SA), was rare and accounting for the remaining percentage (Noristani *et al.*, 2010). In fact, our immunohistochemical labelling showed a general increase in hippocampal SERT-IR fibres in non-Tg animals fed with HTrP compared to animals in LTrP/NTrP diets (Fig. 2A–C), as confirmed by a detailed quantitative measurement of SERT-IR fibre density by using unbiased optical density analysis.

Non-Tg control animals fed with HTrP diet displayed a significant increase in SERT-IR fibre density in the whole CA1 subfield and specifically in the S.Mol compared to non-Tg animals in LTrP diet (35%, $P = 0.0417$, for CA1 subfield and 48%, $P = 0.0253$, for S.Mol, respectively, Fig. 3A,B). This same relevant trend was also observed, when comparing non-Tg animals subjected to NTrP vs. HTrP diet (30%, $P = 0.0662$ for CA1 subfield and 31%, $P = 0.0916$ for S.Mol, respectively, Fig. 3A,B). No diet-induced changes were observed in SERT-IR fibre density in non-Tg control animals in the dentate gyrus (DG) and the CA3 subfield of the hippocampus (Fig. 3C,D). Furthermore, we found no significant differences in SERT-IR fibre density between non-Tg animals fed with LTrP and NTrP diet groups.

Comparison between the two genotypes revealed significant increase in SERT-IR fibre density in the CA1 subfield of the hippocampus in 3xTg-AD animals compared to age-matched non-Tg control animals in LTrP and NTrP but not HTrP diet group (54%, $P = 0.0234$ for LTrP, 52%, $P = 0.0188$ for NTrP and 15%, $P = 0.0998$% for HTrP diet, Fig. 3A). In addition, 3xTg-AD animals also exhibit a significant increase in SERT-IR fibre density in CA1 S.Mol compared to non-Tg control in all 3 experimental diet groups, irrespective of dietary TrP content (104%, $P = 0.0046$ for LTrP, 74%, $P = 0.0108$ for NTrP and 35%, $P = 0.0060$ for HTrP diet, Fig. 3B). However, and unlike to the non-Tg control animals, 3xTg-AD animals in all 3 experimental diet groups showed a stable SERT-IR fibre density, irrespective of dietary TrP contents, with no differences between them (Figs 2 and 3). No changes were observed in SERT-IR fibre density in the 3xTg-AD mice compared to non-Tg control animals in the DG and the CA3 subfield of the hippocampus (Fig. 3C,D).

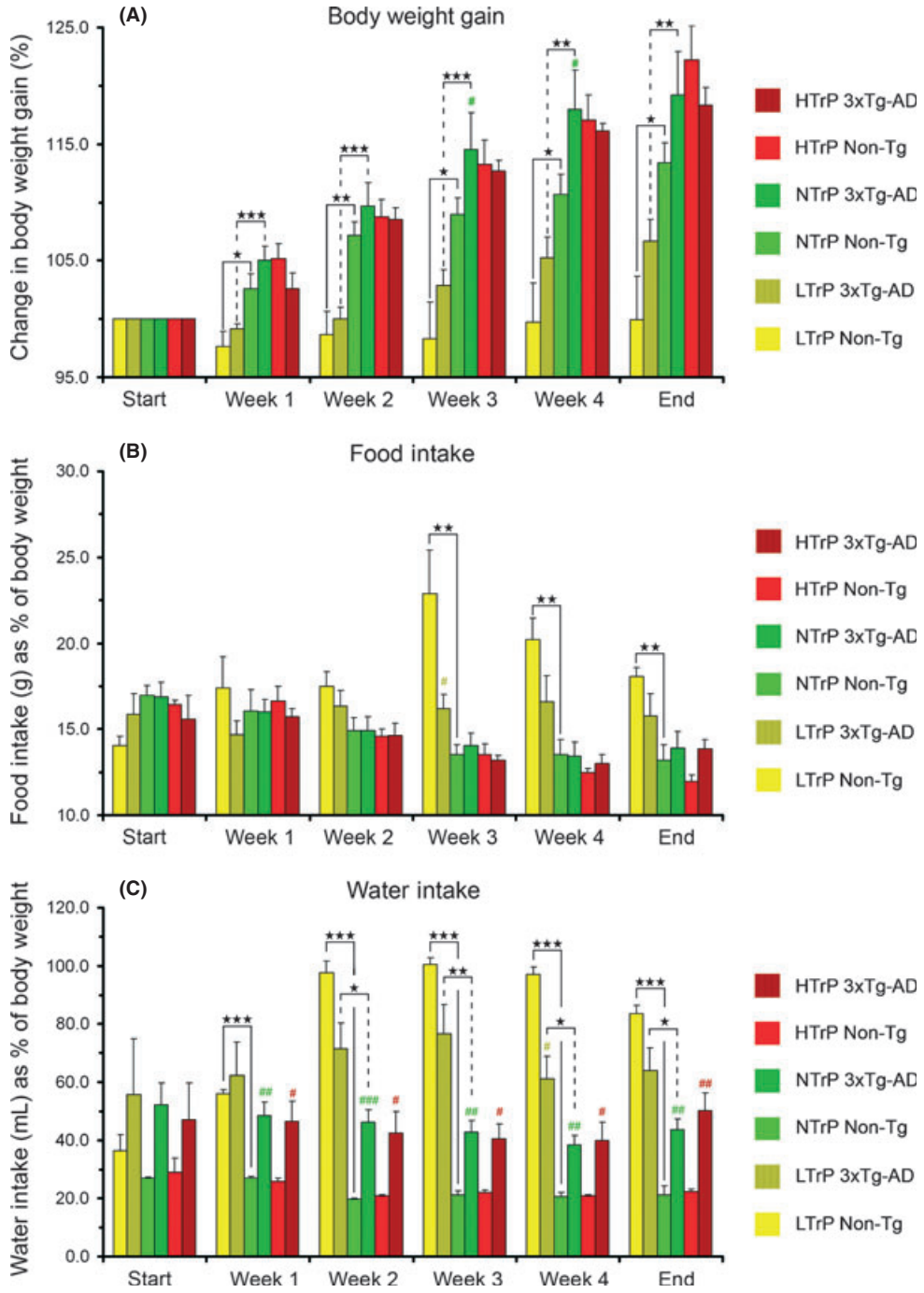

Fig. 1 Bar graphs showing the TrP diet effect on weekly body weight gain (A), food intake (B) and water intake (C). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#\#}P < 0.001$ compared to diet-matched non-Tg control group.

Dietary TrP intake does not affect the total number of 5-HT neurons in either the dorsal or the MR nuclei

5-HT immunoreactive (5-HT-IR) neurons were distributed throughout the different subdivisions of both the DR (Fig. 4A–G) and the MR nuclei (Fig. 4H). 5-HT-IR somatodendritic profiles were characterized by small rounded cell bodies with sparse dendritic arborizations (Fig. 4A–E insets). 5-HT-IR neurons were observed in different subnuclei of the DR nucleus including the dorsal raphe dorsal, the dorsal raphe ventral and the dorsal raphe interfascicular (Fig. 4A–F). The 5-HT-IR neuronal population were also detected within both the MR and the para-MR nuclei (Fig. 4H). Altered dietary TrP intake had no effect on 5-HT neuron morphology in 3xTg-AD and non-Tg control animals (Fig. 4A–F insets). The distribution and total number of 5-HT-IR neurons also showed no significant differences in either the DR or the MR nuclei between 3xTg-AD and non-Tg control mice in LTrP, NTrP and HTrP diet groups (Fig. 4G,H).

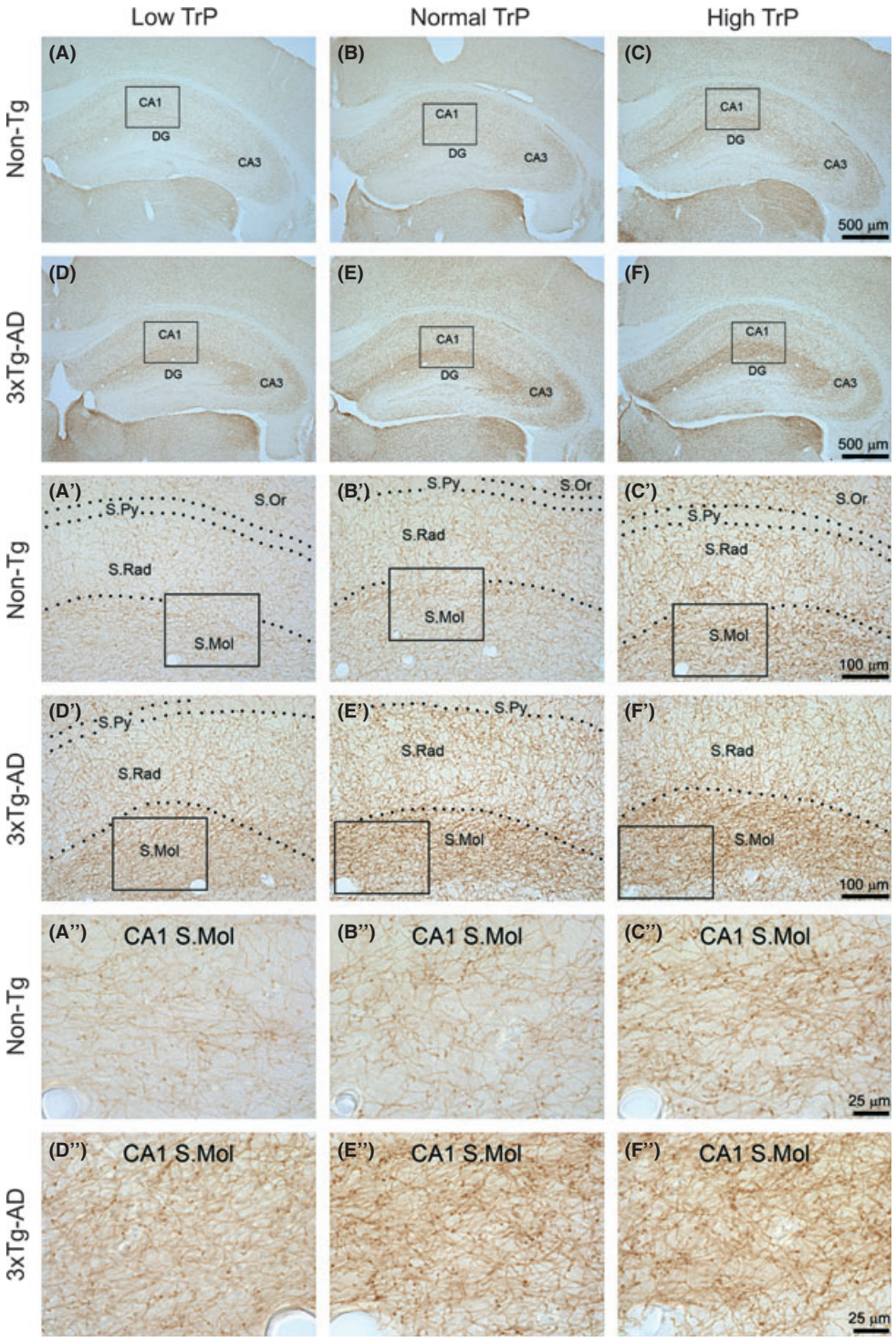

Fig. 2 Brightfield micrographs showing the distribution of SERT-IR fibres within the dorsal hippocampus of 3-months non-Tg control (A, B, C) and 3xTg-AD mice (D, E, F) fed with LTrP (A, D), NTrP (B, E) and HTrP (C, F) diets. Scale bars: A–F = 250 μm, A′–F′ = 100 μm, A″–F″ = 25 μm. DG, dentate gyrus; S.Or, stratum oriens; S.Py, stratum pyramidale, S.Rad, stratum radiatum and S.Mol, stratum lacunosum moleculare; AD, Alzheimer's disease.

Dietary TrP intake affects intraneuronal Aβ density in the hippocampus but not in either the cortex or the amygdala

Immunohistochemical analysis of brains obtained from 2- and 3-month-old 3xTg-AD animals confirmed the presence of intraneuronal Aβ in the hippocampus (Fig. 5), in the cortex and in the amygdala (Fig. 6), whereas no Aβ presence was detected in non-Tg control mice. In the hippocampus, 3xTg-AD mice showed intraneuronal Aβ immunoreactivity in the stratum pyramidale within CA1, CA2 and CA3 subfields, but with no apparent presence in the dentate gyrus (Fig. 5A–C). It was possible to

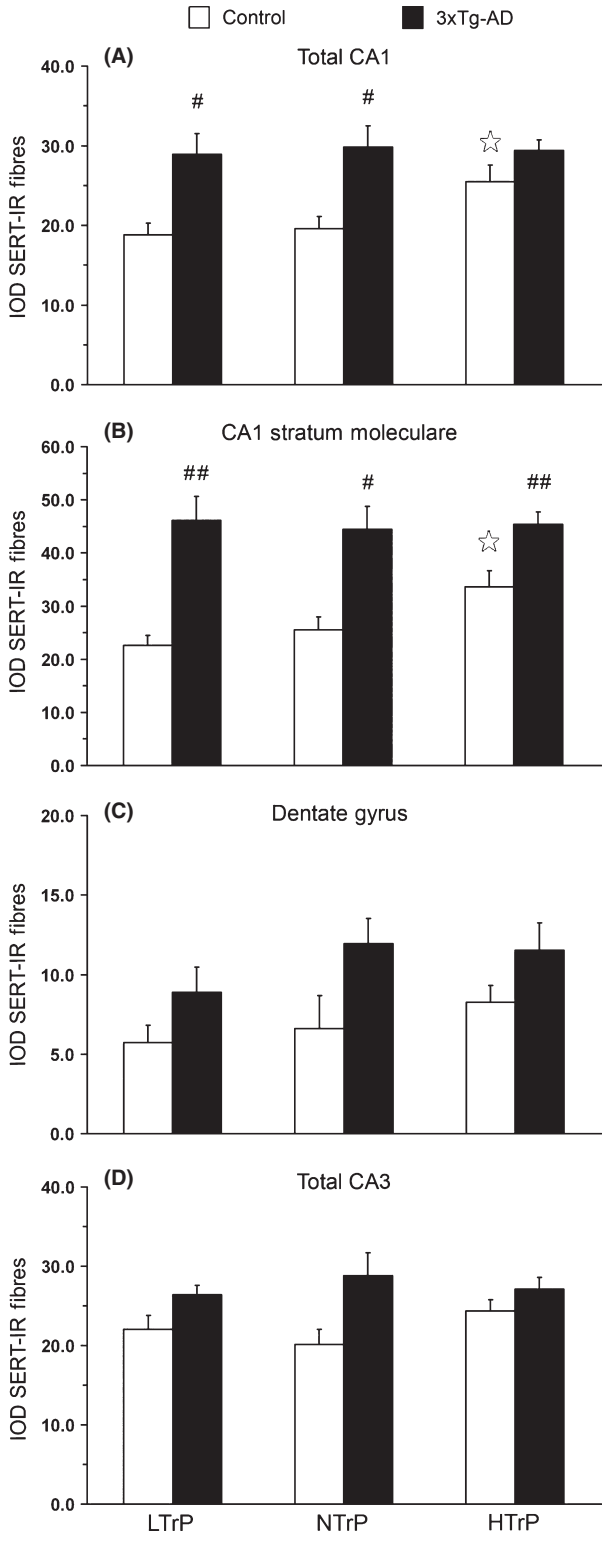

Fig. 3 Bar graphs showing the effect of altered dietary TrP intake on SERT-IR fibre density within CA1 subfield of the hippocampus (A), CA1 stratum lacunosum moleculare (B), dentate gurus (C) and the CA3 subfields of the hippocampus (D) between non-Tg control and 3xTg-AD groups. Bars represent mean ± SEM (n = 5–7). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to diet-match non-Tg; $^{\star}P < 0.05$ compared to non-Tg control mice fed with LTrP diet. LTrP, low TrP diet; NTrP, normal TrP diet; HTrP, high TrP diet; AD, Alzheimer's disease.

observe a gradient in the density of intraneuronal Aβ in different subfields of the hippocampus that was reducing from CA1 to CA2 and CA3 subfields (Fig. 5A–H). There was an age-related increase in intraneuronal Aβ density in the hippocampus, as we have described previously (Rodríguez *et al.*, 2008) (Fig. 5). In the cortex, intraneuronal Aβ aggregates were evident in the somatosensory cortex barrel field (S1BF) and other adjacent cortical areas (Fig. 6A–C). In the amygdala, intense intraneuronal Aβ deposits were observed in both the posterior and the anterior parts of the basolateral amygdala nucleus (BLA and MLP), whilst only scattered Aβ neurons were seen in the basomedial amygdala nucleus posterior part (BMP, Fig. 6D–F).

Quantitative analysis revealed that 3-months-old 3xTg-AD animals fed with LTrP and NTrP diets for 1 month showed a significant increase in intraneuronal Aβ accumulation and optical density compared to 3xTg-AD animals before the start of the diet (2 months of age; 18%, $P = 0.0371$ for LTrP and 27%, $P = 0.0006$ for NTrP diets, respectively, Fig. 5G,H). However, and more relevant, 3xTg-AD animals fed with HTrP diet showed a significant decrease in intraneuronal Aβ density in CA1 stratum pyramidale compared to age-matched 3xTg-AD animals in NTrP diet (17% decrease, $P = 0.0460$, Fig. 5G), being equivalent to 3xTg-AD animals before the start of the diet at 2 months of age, when its presence is minimal ($p = 0.6197$, Fig. 5C,F,G). However, no significant differences were observed in intraneuronal Aβ density in either the CA3 subfield of the hippocampus, the cortex or the amygdala between 3xTg-AD animals fed with HTrP and NTrP diets (Fig. 6G,H). On the other hand, 3xTg-AD animals fed with LTrP diet showed no significant difference in intraneuronal Aβ density compared to 3xTg-AD animals in NTrP diet in any of the studied brain regions (Figs 5 and 6).

Discussion

The present study demonstrates that increase in dietary intake of L-tryptophan (TrP) reduces intraneuronal Aβ accumulation in the hippocampal CA1 subfield in the 3xTg-AD mouse model of AD; surprizingly, this increased TrP intake does not affect 5-HT neurons and projections.

LTrP diet reduces body weight gain whilst increasing food and water consumptions

Young 3xTg-AD animals have significantly larger body mass compared to age-matched non-Tg control animals. This observation is consistent with previous reports, which also showed higher body weight in 3xTg-AD animals at 2 months (Knight *et al.*, 2012) and 4 months of age compared to non-Tg control animals (Arsenault *et al.*, 2011). The underlying mechanism responsible for increased body weight is not clear; possible factors may include accelerated growth and an increase in deposition of fat tissue (adiposity), although it remains to be determined (Knight *et al.*, 2012).

LTrP diet reduced body weight gain in both non-Tg control and 3xTg-AD groups compared to animals fed with NTrP diet (Fig. 1A). These findings are in agreement with previous studies in normal rats (Jenkins *et al.*, 2010), mice (De Marte & Enesco, 1986) and chickens (Carew *et al.*, 1983). LTrP diet decreases plasma level of TrP that may in turn reduce the level of protein synthesis necessary for normal growth, as suggested previously (De Marte & Enesco, 1986). In addition, deficiency in dietary TrP intake also alters thyroid and growth hormone levels, which is associated with reduced bone growth and body weight gain in chickens (Carew *et al.*, 1983).

Chronic LTrP diet induced increase in food and water consumption (Fig. 1B,C). Repeated intraperitoneal injection of TrP administration sup-

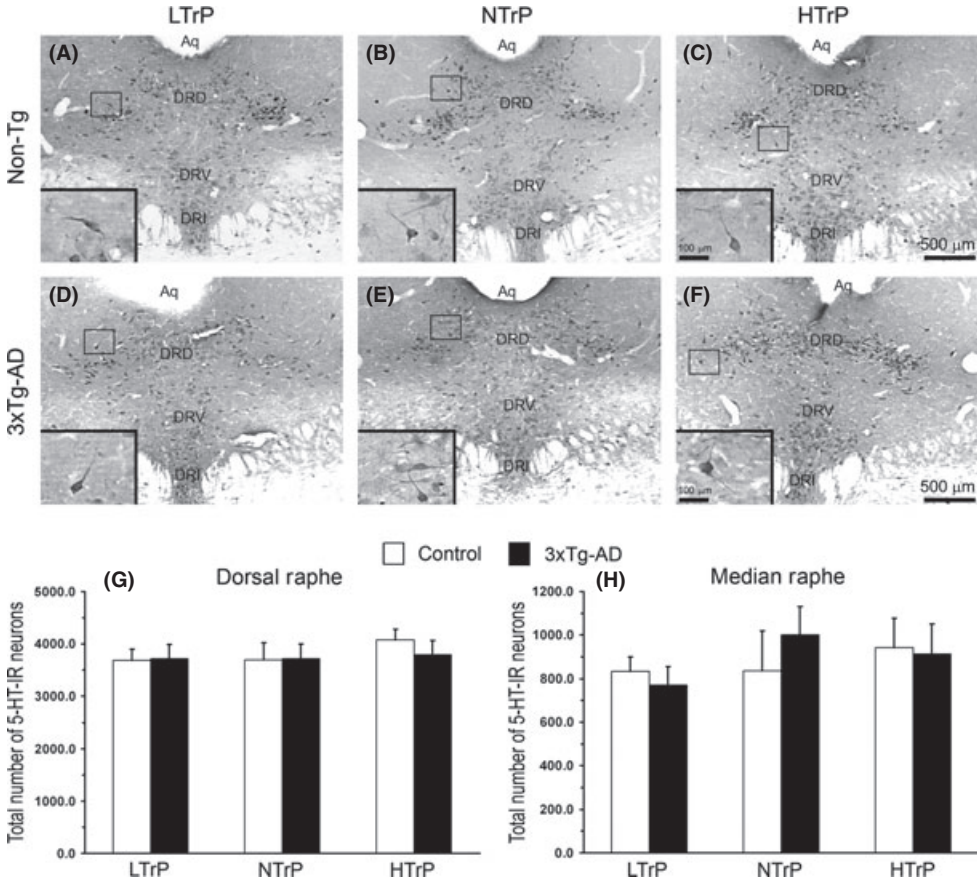

Fig. 4 Brightfield micrographs showing the distribution of 5-HT-IR neurons within raphe nuclei of non-Tg control (A, B, C) and 3xTg-AD mice (D, E, F) fed with LTrP (A, D), NTrP (B, E) and HTrP (C, F) diets. (G–H) Bar graphs showing the effect of altered dietary TrP intake on total number of 5-HT-IR neurons in the dorsal (G) and the median (H) raphe nuclei between non-Tg control and 3xTg-AD group. Scale bars: A–F = 500 μm, insets = 100 μm. Aq, aqueduct; DRD, dorsal raphe dorsal; DRV, dorsal raphe ventral; DRI, dorsal raphe interfascicular part; LTrP, low TrP diet; NTrP, normal TrP diet; HTrP, high TrP diet; AD, Alzheimer's disease.

presses food intake in nonfasted mice (Coskun *et al.*, 2006) and rats (Ju & Tsai, 1995). As a possible mechanism, increased TrP level in plasma may inhibit food consumption. Increasing evidence also suggests that 5-HT exerts a negative effect on drinking behaviour, through central 5-HT availability and activation of 5-HT receptors that mediate a specific inhibitory effect on water intake (de Arruda Camargo *et al.*, 2010).

Enhanced dietary TrP intake increases serotonergic fibre density in non-Tg control but has no effect on 3xTg-AD animals

Chronic increase in dietary TrP intake induced a significant increase in hippocampal SERT-IR fibre density in non-Tg control mice compared to animals fed with LTrP diet (Figs 2 and 3). However, the effect of reducing dietary TrP content on hippocampal SERT-IR fibre density was less evident between LTrP and NTrP diets. Several mechanisms may be responsible for the lack of change in SERT-IR fibre density between LTrP and NTrP diet groups including (i) differences in TrP contents between LTrP and NTrP diets and (ii) relatively later start of dietary experiment in relation to brain development.

The minimum TrP level required for serotonergic fibre development is 0.01% (0.01 g in 100 g of diet) (Bell & John, 1981). Our LTrP diet contained 0.02%, which is double the minimum required amount for normal 5-HT fibre development (see Table 1). To induce an effect on SERT-IR fibre density, greater differences in TrP content might be required between the two diets. In support of this phenomenon, SERT-IR fibre density is significantly increased in non-Tg control animals fed with HTrP diet compared to LTrP diet groups, which has 10 times more TrP content (Figs 2 and 3). In addition, we started the TrP diet experiment at 2 months of age, when the developmental process for 5-HT projections has already taken place (Lauder, 1990).

We observed increased hippocampal SERT-IR fibre density in the CA1 subfield of the hippocampus in 3-months-old 3xTg-AD animals compared to age-matched non-Tg control groups, irrespective of dietary TrP content (Figs 2 and 3). This observation is consistent with our previous studies where we reported increase in SERT-IR fibre and SERT-Te density at same age in 3xTg-AD compared to control animals fed with standard rodent chow (Noristani *et al.*, 2010, 2011). Increased SERT-IR fibre density is associated with an increase in the area density (#/mm^2) of SERT-IR fibres (Noristani *et al.*, 2010) and an increase in the numerical density (#/mm^3) of SERT-IR terminals (Noristani *et al.*, 2011), suggesting heterotrophic sprouting of hippocampal serotonergic fibres in 3xTg-AD mice as an intrinsic defensive mechanism against intraneuronal accumulation of Aβ (Noristani *et al.*, 2010, 2011). Furthermore, we have previously shown that increased SERT-IR fibre density is also associated with an increase in 5-HT terminal size in the 3xTg-AD mouse model of AD (Noristani *et al.*, 2011), therefore directly affecting 5-HT neurotransmission.

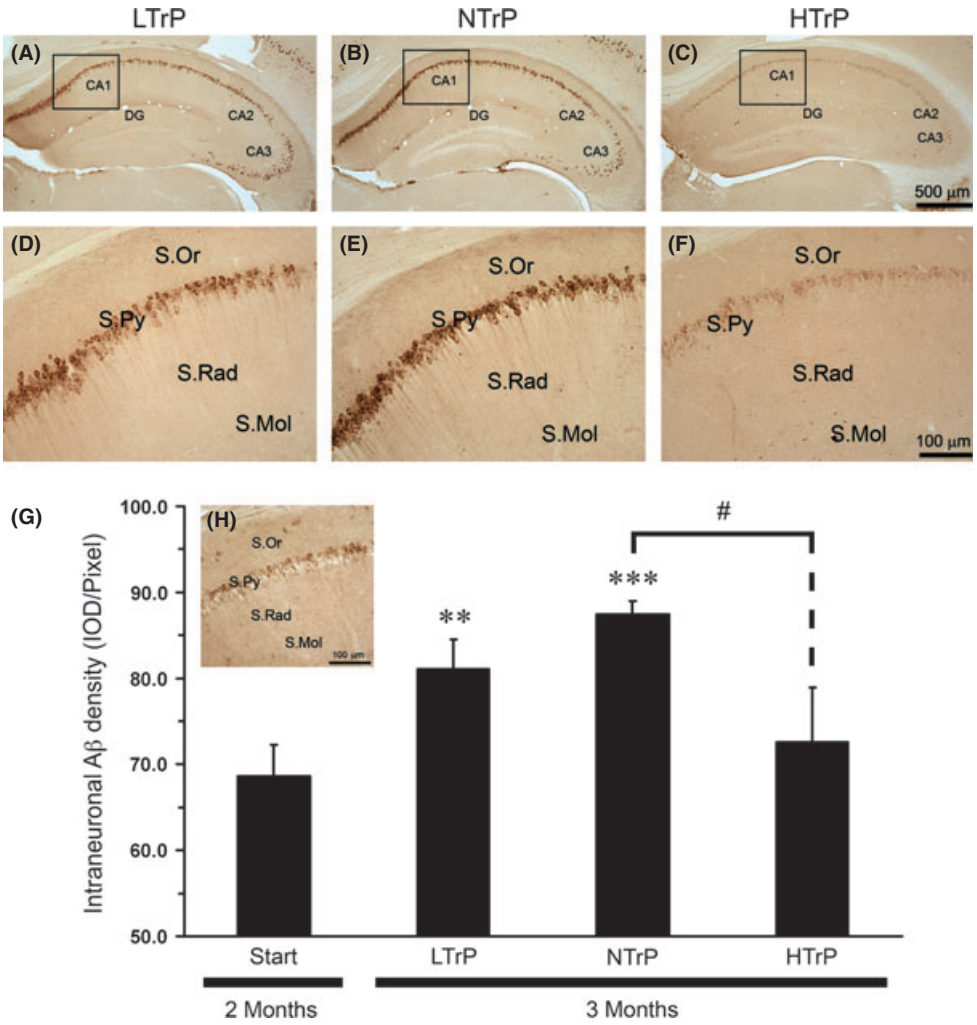

Fig. 5 Brightfield micrographs showing the distribution of intraneuronal Aβ in the stratum pyramidale of the hippocampus in 3xTg-AD animals fed with LTrP (A, D), NTrP (B, E) and HTrP (C, F) diets. (G) Bar graph showing the effect of altered dietary TrP intake on intraneuronal Aβ density in the hippocampal CA1 stratum pyramidale in 3xTg-AD animals fed with LTrP, NTrP and HTrP diets. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to 2 months 3xTg-AD. (H) Brightfield micrographs showing the distribution of intraneuronal Aβ in the stratum pyramidale of the hippocampus in 3xTg-AD animals at the start of the experiment. Scale bars: A–C = 500 μm, D–F = 100 μm. LTrP, low TrP diet; NTrP, normal TrP diet; HTrP, high TrP diet; AD, Alzheimer's disease.

Although previous studies have shown that altered TrP intake modifies 5-HT neurotransmission in multiple brain regions including the cortex and the hippocampus (Stancampiano *et al.*, 1997; Fadda, 2000), we just observed a clear and specific increase in SERT-IR fibres within the CA1 subfield, whilst no changes were observed in either the CA3 or the dentate gyrus of the hippocampus (Fig. 3). This fact amongst other potential reasons is attributable to higher constitutional density of SERT-IR fibres in the CA1 region, arising from the raphe nuclei, compared to other subfields of the hippocampus (Fig. 2). Supporting this phenomenon, no changes in SERT-IR fibre density was observed in the CA3 subfield and the dentate gyrus of the hippocampus, which encompass lower densities of serotonergic fibres compared to the CA1 subfield of the hippocampus (Fig. 3C,D). In addition, the CA1 subfield of the hippocampus is one of the earliest brain regions affected during AD progression and shows the most extensive AD-related neuronal loss compared to other brain regions (West *et al.*, 1994, 2000) that may also trigger the observed sprouting of SERT-IR fibres.

Aβ-induced neurotoxicity involves glutamatergic excitotoxicity that is mediated by intracellular calcium (Ca^{2+}) load (Miguel-Hidalgo *et al.*, 2002; Supnet & Bezprozvanny, 2010). Increased 5-HT input counteracts excitotoxic effect of glutamate by preventing postsynaptic membrane depolarization and by blocking Ca^{2+} channels acting mainly through
5-HT_{1A} receptors (Bayliss *et al.*, 1997; Williams *et al.*, 1998). Previously, we have reported decreased in the number of asymmetric perforated synapses that further support altered hippocampal glutamatergic neurotransmission in 3xTg-AD animals at 3 months of age (Noristani *et al.*, 2011). Increased hippocampal serotonergic input in 3xTg-AD mice may indicate an intrinsic neuroprotective response to intraneuronal Aβ-induced damage by maintaining hippocampal functionality and connectivity.

Altered dietary TrP content had no effect on SERT-IR fibre density in 3xTg-AD animals. Lack of dietary TrP content on SERT-IR fibre density in 3xTg-AD animals may be due to fact that these animals may already express maximum SERT-IR fibre density compared to non-Tg control ani-

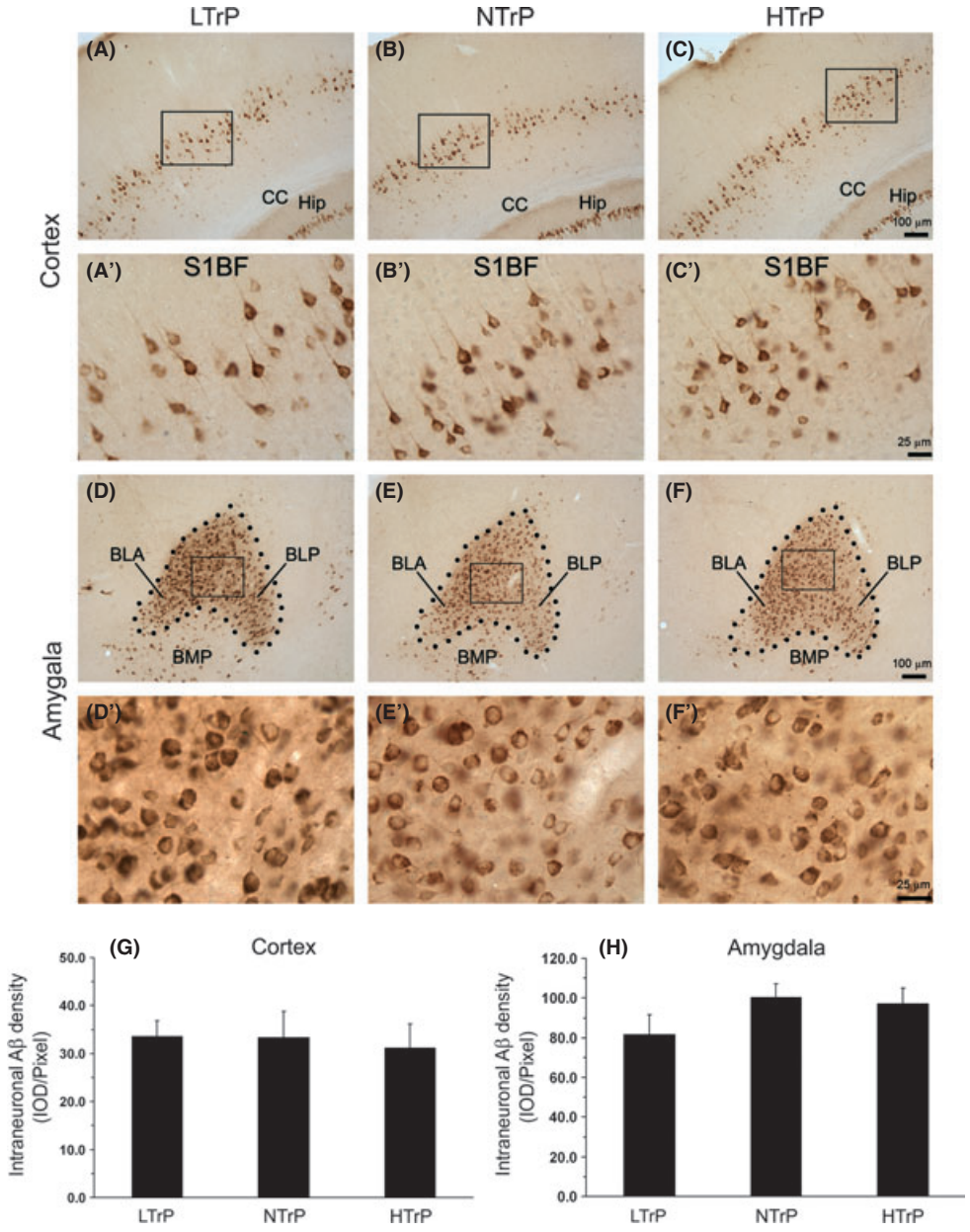

Fig. 6 Brightfield micrographs showing the distribution of intraneuronal Aβ in the cortex (A–C) and the amygdala (D–F) in 3xTg-AD animals fed with LTrP (A, D), NTrP (B, E) and HTrP (C, F) diets.(G and H) Bar graphs showing the effect of altered dietary TrP intake on intraneuronal Aβ density in the cortex (G) and the amygdala (H) in 3xTg-AD animals fed with LTrP, NTrP and HTrP diets. Scale bars: A–F = 100 μm, A'–F' = 25 μm, S1B1, somatosensory cortex barrel field; BLA, basolateral amygdala nucleus anterior part; BLP, basolateral amygdala nucleus posterior part; BMP, basomedial amygdala nucleus posterior part; LTrP, low TrP diet; NTrP, normal TrP diet; HTrP, high TrP diet; AD, Alzheimer's disease.

mals, in response to intraneuronal Aβ accumulation and impaired hippocampal functionality and connectivity (Noristani *et al.*, 2010, 2011).

Increased dietary TrP intake reduces intraneuronal Aβ in the CA1 subfield of the hippocampus

Previous studies, including ours, demonstrated the presence of intraneuronal Aβ in the hippocampus of 3xTg-AD animals from 2 months of age (Rodríguez *et al.*, 2008); the Aβ intraneuronal load increases with advanced age (Oddo *et al.*, 2003; Rodríguez *et al.*, 2008). Intraneuronal accumulation of Aβ in the hippocampus and the amygdala correlates with early cognitive impairment (deficit in long-term memory retention (Billings *et al.*, 2005) as well as neurogenic impairment (Rodríguez *et al.*, 2008) in 3xTg-AD mice.

Increased TrP intake enhances 5-HT neurotransmission in multiple brain regions including the hippocampus, which is associated with improved cognition in rodents (Haider *et al.*, 2007). Clinical studies also have shown beneficial effects of selective serotonin re-uptake inhibitor (SSRI) not only in improving memory and cognitive functions but also in reducing behavioural abnormalities in patients with AD (Mowla *et al.*, 2007; Mos-

Table 1 Amino acid composition (g/100 g) of Low, Normal and High TrP diets

Amino Acid	LTrP diet	NTrP diet	High tryptophan diet
Arginine	1.19	1.19	1.19
Lysine	1.09	1.09	1.09
Methionine	0.59	0.59	0.59
Cystine	0.39	0.39	0.39
Tryptophan	**0.04**	**0.20**	**0.40**
Histidine	0.59	0.59	0.59
Threonine	0.78	0.78	0.78
Isoleucine	0.80	0.80	0.80
Leucine	1.18	1.18	1.18
Phenylalanine	0.79	0.79	0.79
Valine	0.79	0.79	0.79
Tyrosine	0.40	0.40	0.40
Taurine	0.00	0.00	0.00
Glycine	1.98	1.98	1.98
Aspartic acid	0.00	0.00	0.00
Glutamic acid	2.97	2.97	2.97
Energy (kcal kg^{-1})	**3840.28**	**3839.51**	**3838.54**

sello *et al.*, 2008). Our data demonstrate that chronic increase in dietary TrP intake reduced (by 17%) intraneuronal Aβ density in 3xTg-AD animals compared to age-matched transgenic animals fed with NTrP diet (Fig. 5G). These findings are in general agreement with a previous study in 3xTg-AD animals, where increasing 5-HT neurotransmission via chronic treatment with SSRI (paroxetine) reduced Aβ and tau neuropathology (Nelson *et al.*, 2007). In addition, chronic treatment with another commonly prescribed SSRI (citalopram) reduced plaque burden in the cortex and the hippocampus in PS1APP transgenic mice (Cirrito *et al.*, 2011). Furthermore, a recent positron emission tomography (PET) clinical study had reported that exposure to SSRI antidepressants (for at least 5 years) reduced Aβ plaque load in cognitively normal individuals (Cirrito *et al.*, 2011). Interestingly, increased dietary TrP intake enhanced extracellular 5-HT and induced antidepressant effect similar to SSRI administration in rats (van der Stelt *et al.*, 2004).

However, and although significant, currently it is unclear whether the observed 17% reduction in intraneuronal Aβ density would have an effect on 3xTg-AD mice behaviour. Given that the earliest sign of cognitive deficits does not appear before 6 months of age (Oddo *et al.*, 2003; Billings *et al.*, 2005), whereas in the present work, we studied animals at 3 months of age owing to the marked 5-HT sprouting observed at this age (Noristani *et al.*, 2010, 2011). Thus, further studies at older ages are required to determine the effect of HTrP diet on cognitive status in 3xTg-AD mouse model of AD.

The HTrP-induced decrease in intraneuronal Aβ deposition was mainly localized to the CA1 subfield of the hippocampus but not in the cortex or the amygdala (Fig. 6), the latter two brain regions that are deeply affected in AD. This specific and restricted effect may be due to the facts that (i) CA1 subfield of the hippocampus is one of the first region affected during AD progression within the hippocampus that shows the most extensive AD-related neuronal loss compared to other brain regions (West *et al.*, 1994, 2000) and (ii) to its critical importance for the cognitive and associative functions, as the CA1 subfield of the hippocampus is the final hippocampal integration area and output to high cortical areas. Increased neuronal activity in the CA1 subfield of the hippocampus is closely associated with improved memory formation, an aspect of cognitive function that is classically impaired in patients with AD (Walsh & Selkoe, 2004).

Although the underlying mechanisms involved in 5-HT-mediated decrease in intraneuronal Aβ accumulation has not been addressed *in vivo*, experiments *in vitro* suggested that 5-HT stimulates the nonamyloidogenic processing of APP metabolite (APP_S) by stimulating sAPPα cellular release (Robert *et al.*, 2001) and (Fig. 7). In addition, paroxetine reduces APP translation and lowers pathogenic Aβ peptide secretion *in vitro* (Payton *et al.*, 2003). Administration of citalopram (another commonly prescribed SSRI) and imipramine (tricyclic anti-depressant) facilitates the soluble form of APP (APP_S) secretion *in vitro* and may potentially prevent the accumulation of insoluble Aβ in AD (Pakaski *et al.*, 2005). Enhanced 5-HT neurotransmission following increased dietary TrP intake may alleviate AD-related neuropathology by stimulating the nonamyloidogenic processing of APP, where the secreted protein is no longer available for the amyloidogenic accumulation mediated by β- and γ-secretase cleavage (Fig. 7), as previously suggested for treatment with SSRI (Pakaski *et al.*, 2005). Another possible mechanism responsible for HTrP diet-induced decrease in intraneuronal Aβ density could be due to inhibition of β-secretase activity as a result of increased central 5-HT neurotransmission (Fig. 7). In supported of this phenomenon, a recent study by Takahashi & Miyazawa (2011) has reported that 5-HT derivatives may inhibit β-secretase hence possibly reducing amyloidogenic accumulation of Aβ in AD (Takahashi & Miyazawa, 2011).

In conclusion, our results support a possible neuroprotective role of 5-HT neurotransmission in AD pathology. Increasing 5-HT level by TrP supplement not only improves behavioural abnormalities, but it may also reduce the underlying neuropathology associated with AD, as shown by a clear diminution of intraneuronal Aβ accumulation in the hippocampus. Direct increase in 5-HT neurotransmission may provide a promising therapeutic approach to the halt or better treatment of AD.

Experimental procedures

All animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 under the licence from the Home Office. All efforts were made to reduce the number of animals by following the 3Rs.

Animals

The procedure for generating 3xTg-AD mice has been described previously elsewhere (Oddo *et al.*, 2003; Rodríguez *et al.*, 2008). All 3xTg-AD and non-Tg control mice were obtained by crossing homozygous breeders. The animals were housed in the same-sex cage, kept in 12 h light-dark cycles with free access to food and water independent of the diet.

Diets

Following birth and weaning period (P21), the animals were housed in same-sex groups of at least 4 animals in standard laboratory housing environment until 2 months of age. At 2 months of age, male non-Tg control (n = 15) and 3xTg-AD (n = 21) were weighted and randomly assigned to three dietary conditions consisting of low, normal and high TrP contents (LTrP, NTrP and HTrP, respectively; n = 5 for non-Tg control and n = 7 for 3xTg-AD in each diet condition, Fig. 8). All TrP diets were manufactured and purchased from Special Diets Services Ltd. (SDS, Horley, Surrey, UK). The diets were prepared in pellet forms and were isocaloric (Table 1). NTrP diet contained 0.20 g TrP/100 g of the food, whilst TrP contents in LTrP and HTrP diets were 0.04 and 0.40 g TrP/100 g, respectively (20 and 200% of the NTrP diet). Mice had free access to their respective diet and water during the 30 days of the experiment allowing them to acclimate to their respective diets, thus minimizing metabolic disequilibrium

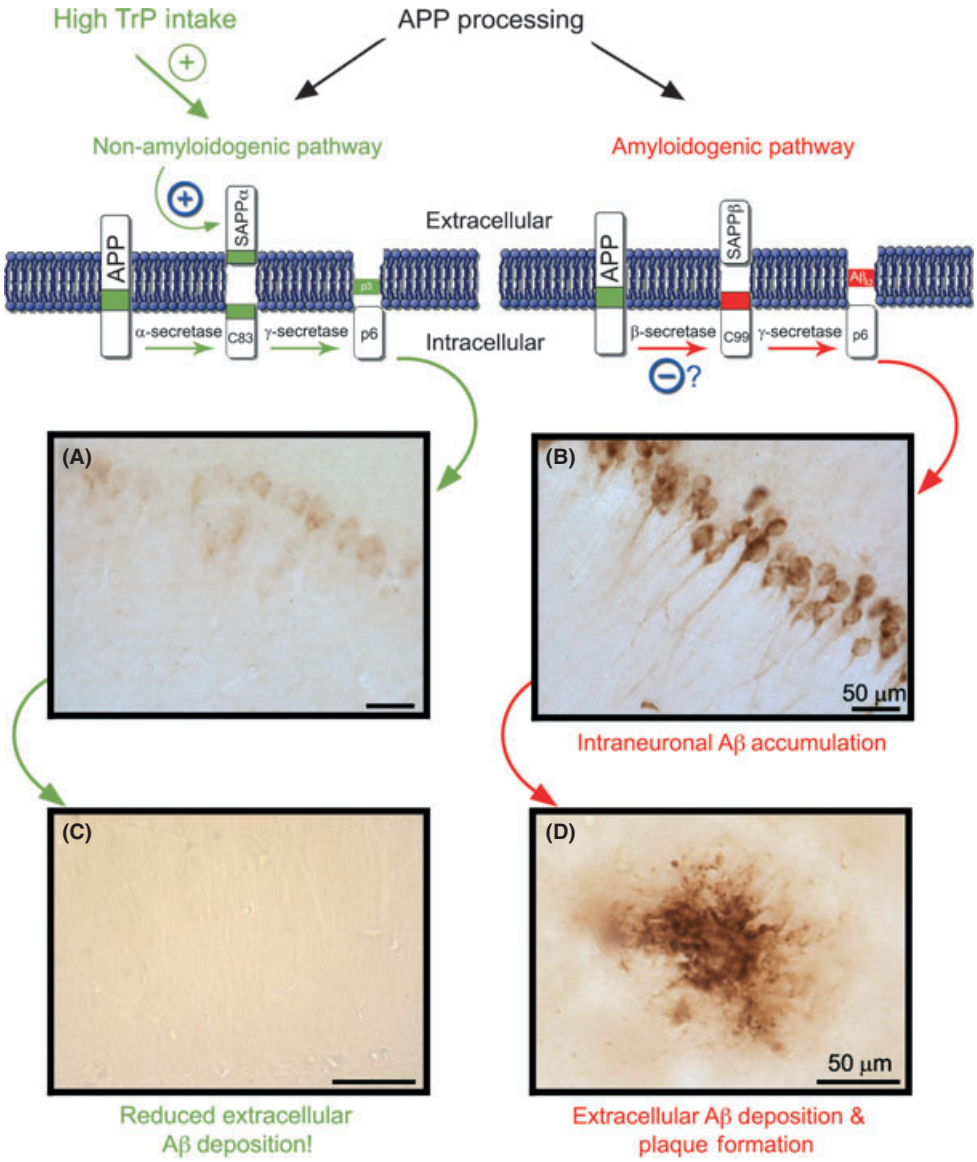

Fig. 7 Amyloid precursor protein (APP) processing in Alzheimer's disease. Nonamyloidogenic processing of APP via α-secretase (left) and the amyloidogenic processing of APP (right). Increase in serotonin levels hypothetically favours the nonamyloidogenic route.

because of diet alterations. Body weight, food intake and water intake were measured daily throughout the experiment. All animals were perfused at 3 months of age (Fig. 8). We chose 1 month of dietary TrP intake because previous studies have shown that 1 month of TrP intake is sufficient to induce significant changes in 5-HT neurotransmission in multiple brain regions including the hippocampus (Haider *et al.*, 2007; Jenkins *et al.*, 2010). In addition, we had chosen 3 months of age because previous studies including our own have shown that in 3xTg-AD animals, intraneuronal accumulation of Aβ initiates at this age (Oddo *et al.*, 2003; Rodríguez *et al.*, 2008). Furthermore, our earlier studies have shown increased hippocampal SERT-IR fibre and terminal (SERT-Te) density in 3xTg-AD animals at 3 months of age (Noristani *et al.*, 2010, 2011).

Fixation and tissue processing

Male 3xTg-AD and non-Tg control mice were anaesthetized with intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}) at 3 months of age. To investigate the level of intraneuronal Aβ in 3xTg-AD before TrP diet experiment, another group of male 3xTg-AD were perfused at 2 months of age (n = 7). All mice were perfused through the aortic arch with 3.75% acrolein (TAAB, Berkshire, UK) in a solution of 2% paraformaldehyde (Sigma, Cambridge, UK) and 0.1 M phosphate buffer (PB) pH 7.4, followed by 2% paraformaldehyde. Brains were then removed and cut into 4- to 5-mm coronal slabs of tissue containing the entire rostrocaudal extent of the hippocampus. The brain sections were postfixed in 2% paraformaldehyde for 24 h and kept in 0.1 M PB, pH 7.4. Coronal sections of the brain were cut into 40- to 50-µm thickness using a vibrating microtome (VT1000S; Leica, Milton Keynes, UK). Free floating brain sections in 0.1 M PB, pH 7.4, were collected and stored in cryoprotectant solution containing 25% sucrose and 3.5% glycerol in 0.05 M PB at pH 7.4. Coronal sections at levels −1.58/−2.46 mm (hippocampus) and −4.36/−4.96 mm (raphe nuclei) posterior to bregma were selected for immunohistochemistry according to the mouse brain atlas of Paxinos & Franklin (2004).

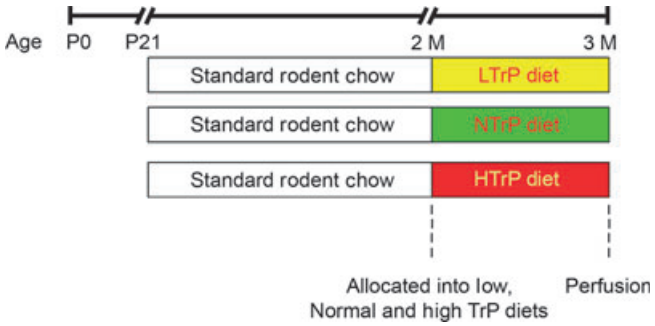

Fig. 8 Experimental design. Following the weaning period (P21), male 3xTg-AD and nontransgenic (non-Tg) control animals were housed in standard laboratory housing environment until 2 months of age. At 2 months of age, animals were randomly assigned into three dietary conditions consisting of LTrP, NTrP and HTrP contents (0.04, 0.20 and 0.40 g/100 g) for 1-month period. All animals were sacrificed by perfusion at 3 months of age. LTrP, low TrP diet; NTrP, normal TrP diet; HTrP, high TrP diet; AD, Alzheimer's disease.

Antibodies

A polyclonal rabbit antibody raised against a synthetic peptide sequence corresponding to amino acids 602–622 of rat 5HT transporter (Immunostar, Hudson, WI, USA) was used for the determination of SERT-IR fibre density in the hippocampus. 5-HT neurons in the raphe nuclei were studied using a polyclonal rabbit antibody antiserum generated against 5-HT (Immunostar). Monoclonal mouse antibody against amino acid residues 1–16 of beta amyloid (Covance, Emeryville, CA, USA) was used to detect intraneuronal Aβ accumulation in the hippocampus, the cortex and the amygdala. The specificity of the antibodies has been reported previously using immunohistochemistry (Rodríguez *et al.*, 2008; Noristani *et al.*, 2010) and western blots (Albright *et al.*, 2007). To determine the specificity of the antibodies, adsorption controls were done using SERT and 5-HT specific peptides, respectively, which resulted in total absence of target labelling. Furthermore, omission of primary and/or secondary antibodies also showed no immunoreactivity (data not shown).

Immunohistochemistry

The sections were incubated for 30 min in 30% methanol in 0.1 M PB and 3% hydrogen peroxide (H_2O_2) (Sigma, Gillingham, UK). Sections were then rinsed with 0.1 M PB for 5 min and placed in 1% sodium borohydride (Aldrich, Gilligham, UK) for 30 min. The sections were then washed with PB profusely before rinsing in 0.1 M Trizma base saline (TS) for 10 min. Brain sections were then incubated in 0.5% bovine serum albumin (BSA) (Sigma) in 0.1 M TS and 0.25% Triton (Sigma; ×100) for 30 min. Sections were incubated for 48 h at room temperature in primary antibody (rabbit anti-SERT, 1:2500, rabbit anti-5-HT, 1:5000; Immunostar and mouse anti-Aβ, 1:2000; Covance). The sections were rinsed in 0.1 M TS for 30 min and incubated in 1:400 dilutions of biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch; Stratech Scientific Ltd, Soham; UK) for 1 h at room temperature, washed and then incubated for 30 min in avidin–biotin peroxidase complex (Vector Laboratories Ltd, Peterborough, UK). The peroxidase reaction product was visualized by incubating in a solution containing 0.022% of 3,3′-diaminobenzidine (DAB; Aldrich) and 0.003% H_2O_2 for 6 min (Rodríguez *et al.*, 2008). The reaction was stopped by rinsing the sections in 0.1 M TS for 6 min followed by 0.1 M PB for 15 min. Brain sections were permanently mounted onto gelatinized slides and allowed to dry overnight. Sections were then dehydrated in ascending concentration of ethanol (50, 70, 80, 90, 95 and 100%) and finally xylene. Cover slips were applied using Entellan (Merck KGaA, Darmstadt, Germany), and slides were left to dry overnight.

Optical density (OD) measurement

Using computer-assisted imaging analysis (IMAGEJ 1.32j; NIH, Bethesda, MD, USA), we analysed the expression and density of intraneuronal Aβ and SERT-IR fibres by measuring their optical density (OD), as we have described previously (Noristani *et al.*, 2010). In brief and to exclude any experimental errors and/or bias, all images were taken at a constant light intensity. Optical filters were used to ensure the specificity of the signal recorded by the camera. The staining was observed throughout the thickness of the section (40 μm) using confocal scanning microscopy (Leica Microsystems, Nussloch, Germany TCS sp2 upright AOBS) recording optical sections at every 0.2 μm. No differences were observed in Aβ and SERT antibody penetration (Pickel *et al.*, 1992) and immunoreactivity throughout the thickness of the section between 3xTg-AD and non-Tg control animals; hence, the changes in OD were used as measure of altered intraneuronal Aβ and SERT-IR fibres density. The OD was calculated from a relative scale of intensity ranging from 0 to 255, with a measurement of 255 corresponding to the area with very low intraneuronal Aβ accumulation and 0 corresponding to the densest area of labelling (Noristani *et al.*, 2010). The calibration density was kept constant for measuring all section to avoid experimental variances. Nonspecific OD in sections was measured from the corpus callosum. The density of intraneuronal Aβ was measured in stratum pyramidale (PCL) of CA1, CA2 and CA3 subfields of the hippocampus. Within the same brain sections, intraneuronal Aβ deposition was also quantified in the somatosensory cortex barrel field (S1BF) and the amygdala in the 3xTg-AD mouse model of AD at 3 months of age following chronic exposure to LTrP, NTrP and HTrP diets. SERT-IR fibre density of the complete CA1 subfield of the hippocampus and its different layers [PCL, stratum oriens (S.Or), stratum radiatum (S.Rad) and stratum lacunosum moleculare (S.Mol)], except CA3 where we also studied stratum lucidum, were measured independently. To analyse the changes in SERT-IR fibre density and intraneuronal Aβ against constant control, 255 was divided by the control region (corpus callosum) and the obtained factor was multiplied by the region of interest in every given section (Noristani *et al.*, 2010). Inverse optical density was obtained by subtracting from the obtained background level (255). Measurement of mean density was taken and averaged, after background subtraction, from each hippocampal layers in both the left and the right hemisphere of each slice. The results are shown as inverse optical density (IOD/pixel).

Cell count of 5-HT neurons in the raphe nuclei

To determine whether alteration in dietary TrP intake has an effect on 5-HT neuron density, we estimated total number of 5-HT immunoreactive (5-HT-IR) neurons in the DR and in the MR nuclei of 3xTg-AD and non-Tg control mice. The areas analysed for 5-HT cell count included the dorsal raphe dorsal, the dorsal raphe ventral, the dorsal raphe interfascicular part, the MR and the para-MR nuclei. The boundaries of areas in which cells were to be counted were clearly delineated; thus, counts were reproducible and counting 5-HT stained cell profiles in every third section constituted a true random sample (Noristani *et al.*, 2010). All 5-HT-IR neurons were intensely labelled against light background, which made them easy to identify with equal chance of being counted (Noristani *et al.*, 2010). The main source of error in using this calculation is the potential multiple counting of the same profile in more than one section (Noristani *et al.*, 2010). However, in this case, one has to consider

that the maximum cell diameter of the neurons counted was approximately 25–30 μm, and every third 40-μm section was 120 μm distant from the adjacent one, making multiple counting of the same cell profile in adjacent sections unlikely as described previously (Noristani *et al.*, 2010). To obtain a systemic random sampling of 5-HT-IR neurons, a sampling grid, consisting of counting frame, was positioned over the DR and the MR nuclei on each section. A single observer using 10 × 10 mm graticule determined the number of 5-HT-IR neurons blindly. All visible 5-HT-IR neurons were counted in every third 40-μm thickness coronal section throughout rostrocaudal extent of the different subdivisions of the DR nucleus corresponding to bregma –4.36/–4.96 mm (Paxinos & Franklin, 2004). To define the DR and the MR nuclei as well as their subnuclei boundaries, adjacent sections were counterstained with toluidine blue. The estimated total number of 5-HT-IR neurons within different raphe nuclei were calculated according to Konigsmark equation (Konigsmark, 1970) as described previously (Noristani *et al.*, 2010).

$$N_t/n_s = V_t/v_s$$

where N_t is total count, n_s is sample count, V_t is total volume (range from 0.55 to 0. 65 mm^3) and v_s is sample volume. Composite figures, adjusted for brightness, contrast and sharpness, were generated using Adobe Photoshop CS2 (Adobe Systems Inc., San Jose, CA, USA) and Microsoft Excel 2002 (Microsoft Corporation, Reading, UK).

Statistical analysis

Results are expressed as mean ± standard errors of the mean. Individual body weight was measured daily; average food and water intake was measured by dividing the total amount consumed per number of mice per group. At 2 months of age, 3xTg-AD showed significant increase in body weight (by 11%, $P = 0.0288$) and food intake (by 10%, $P = 0.0097$) compared to age-matched non-Tg control animals. To avoid the possible effect of differences in body weight on food and water intake, we analysed food and water consumption as percentage of body weight between 3xTg-AD and non-Tg control animals (Fig. 1). Unpaired *t*-test was used to determine changes in body weight, food intake and water intake as well as for the differences in SERT-IR fibre density, 5-HT neurons and intraneuronal Aβ density. Significance was accepted at $P \leq 0.05$. The data were analysed using GRAPHPAD PRISM 4.0 (GraphPad Software, Inc. La Jolla, CA, USA).

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Author contribution

H.N.N and J.J.R designed the studies, analysed and interpreted the data. A.V contributed to the writing of the manuscript. All authors critically edited each draft.

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