Research

Effect of anti-inflammatory agents on transforming growth factor beta over-expressing mouse brains: a model revised

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Abstract

Background: The over-expression of transforming growth factor β-1(TGF-β1) has been reported to cause hydrocephalus, glia activation, and vascular amyloidβ (Aβ) deposition in mouse brains. Since these phenomena partially mimic the cerebral amyloid angiopathy (CAA) concomitant to Alzheimer's disease, the findings in TGF-β1 over-expressing mice prompted the hypothesis that CAA could be caused or enhanced by the abnormal production of TGF-β1. This idea was in accordance with the view that chronic inflammation contributes to Alzheimer's disease, and drew attention to the therapeutic potential of anti-inflammatory drugs for the treatment of Aβ-elicited CAA. We thus studied the effect of anti-inflammatory drug administration in TGF-β1-induced pathology.

Methods: Two-month-old TGF-β1 mice and littermate controls were orally administered pioglitazone, a peroxisome proliferator-activated receptor-γ agonist, or ibuprofen, a non steroidal anti-inflammatory agent, for two months. Glia activation was assessed by immunohistochemistry and western blot analysis; Aβ precursor protein (APP) by western blot analysis; Aβ deposition by immunohistochemistry, thioflavin-S staining and ELISA; and hydrocephalus by measurements of ventricle size on autoradiographies of brain sections. Results are expressed as means ± SD. Data comparisons were carried with the Student's T test when two groups were compared, or ANOVA analysis when more than three groups were analyzed.

Results: Animals displayed glia activation, hydrocephalus and a robust thioflavin-S-positive vascular deposition. Unexpectedly, these deposits contained no Aβ or serum amyloid P component, a common constituent of amyloid deposits. The thioflavin-S-positive material thus remains to be identified. Pioglitazone decreased glia activation and *basal* levels of Aβ42- with no change in APP contents – while it increased hydrocephalus, and had no effect on the thioflavin-S deposits. Ibuprofen mimicked the reduction of glia activation caused by pioglitazone and the lack of effect on the thioflavin-S-labeled deposits.

Conclusions: i) TGF-β1 over-expressing mice may not be an appropriate model of Aβ-elicited CAA; and ii) pioglitazone has paradoxical effects on TGF-β1-induced pathology suggesting that anti-inflammatory therapy may reduce the damage resulting from active glia, but not from vascular alterations or hydrocephalus. Identification of the thioflavin-S-positive material will facilitate the full appraisal of the clinical implication of the effects of anti-inflammatory drugs, and provide a more thorough understanding of TGF-β1 actions in brain.

Background

Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine implicated in developmental processes, immune host defense, and injury repair [for review, [1]]. TGF-β1 contributes to the resolution of injuries by inhibiting local inflammation, and by stimulating the synthesis and deposition of matrix components leading to the reconstitution of the basal membrane in the final stages of angiogenesis. A paradoxical feature of TGF-β1 is that, when produced in excess or in the absence of counter-regulatory elements, it can become pro-inflammatory, induce abnormal vascular growth, and thus be detrimental [2]. This occurs in the mouse brain, where the transgenic over-expression of TGF-β1 in astrocytes causes chronic astrocytosis and microglia activation, as well as fibrosis – i.e. increased production of matrix proteins and abnormal thickening of vascular basal membranes – and endothelial cell atrophy [3,4]. The mice develop hydrocephalus [3], revealing an alteration of cerebrospinal fluid (CSF) dynamics – probably due to clearance obstruction – and present, at one year of age, a reduction in brain blood flow and metabolism, signs of vascular and neuronal dysfunction [5].

Another abnormality reported in TGF-β1 over-expressing brains was the vascular and meningeal deposition of endogenous mouse amyloidβ (Aβ), as well as of human Aβ when TGF-β1 was over-expressed together with a mutated form of the human Aβ precursor protein (APP) [6,7]. The vascular deposition was a likely consequence of the fibrosis, since components of the extracellular matrix have been shown to trigger the fibrillation of Aβ proteins [8]. Vascular Aβ deposition is often seen *postmortem* in individuals with Alzheimer's disease as cerebral amyloid angiopathy (CAA), which appears to be an important pathogenic factor in the disease [9-12]. The detection of TGF-β1 and fibrosis in vessels bearing Aβ deposits in human brains [6], together with the β amyloidogenic role of TGF-β1 in mouse models, led to the hypothesis that Aβ-related CAA could be caused or enhanced by the aberrant production of TGF-β1 [6]. This idea was in accordance with the view that chronic inflammation contributes to the pathogenesis of Alzheimer's disease [for review, [13]], as supported by: i) epidemiological studies showing that the use of non steroidal anti-inflammatory drugs (NSAIDs) like ibuprofen delays the onset of Alzheimer's disease [14]; and ii) studies in APP mice showing that treatment with ibuprofen reduces gliosis, microglia activation, interleukin-1β production, and amyloid plaque burden, and improves cognition [15,16]. The TGF-β1 over-expressing mice thus emerged as a model to gain insight into the relationship between chronic inflammation and vascular Aβ deposition, and to evaluate the therapeutic potential of drugs.

We sought to determine whether pioglitazone, a novel anti-inflammatory drug, reduces glia activation, APP expression, and Aβ production and deposition in TGF-β1 over-expressing mice. For comparison, we tested the effect of ibuprofen on some of these parameters since this drug is beneficial in patients with Alzheimer's disease and APP mouse models. Pioglitazone is a thiazolidinedione (TZD) drug and an agonist of the peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear receptor that plays a key role in the regulation of glucose and lipid metabolism in non cerebral tissues. The rationale for the use of pioglitazone stems from: i) the anti-inflammatory effect of PPARγ *in vivo* and *in vitro* [17-19]; ii) the commercial availability and safety record of pioglitazone and other TZDs, which are currently in use for the treatment of type 2 diabetes; and iii) the fact that pioglitazone, unlike ibuprofen, does not cause gastric problems after prolonged treatment and thus it would be better suited for chronic use. TZD-PPARγ agonists can block in mice the development of experimental allergic encephalomyelitis [20], and reduce the degeneration of dopaminergic neurons caused by methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [ref [21]. In both cases, the protective actions of TZDs have been attributed to their anti-inflammatory capacities. A review of the therapeutic potential and mechanisms of action of TZDs in Alzheimer's disease has been presented elsewhere [22].

The mice under study showed astrocyte and microglia activation, hydrocephalus and thioflavin-S-positive deposits. A two-month treatment with pioglitazone reduced glia activation, but it increased hydrocephalus unexpectedly, while it had no effect on the thioflavin-S-positive deposits. Immunohistochemical analyses and ELISAs failed to confirm the presence of Aβ in such deposits, despite an increase in Aβ40 levels detected at 9 months. These findings contradict the view that TGF-β1 over-expressing mice are a model of Aβ-elicited CAA, and reveal paradoxical effects of pioglitazone on TGF-β1-induced chronic inflammation whose clinical relevance is discussed.

Material and methods

Generation of mice and anti-inflammatory treatment

The study was carried out in heterozygous C57BL/6 mice genetically modified to produce a constitutively active form of TGF-β1 under the control of the GFAP promoter. The mice were derived from the heterozygous line T65, generated similarly to the line T64 on a BALB/c background [3], and changed to C57BL/6 by successive crossings. APP mice over-expressing the human V711F mutation [23] were used as positive controls for immunohistochemistry. Animal care was carried out according to the European Community's regulations, the principles of

which agree with the National Institutes of Health guide for the care and use of laboratory animals.

TGF-β1 over-expressing mice and littermate controls were sacrificed at 2, 4 and 9 months of age to assess the evolution over time of inflammation and vascular Aβ deposition. Treatment with the anti-inflammatory drugs started at 2 months of age, soon after weaning, and was carried out for 2 months.

Ibuprofen was purchased from Sigma, and pioglitazone, manufactured by Takeda Pharmaceuticals, was obtained from a local pharmacy. The drugs were pulverized, and mixed with Purina chow to give concentrations of 120 ppm and 375 ppm of pioglitazone and ibuprofen, respectively. Mice were allowed free access to the chow. Neither pioglitazone nor ibuprofen caused detectable weight changes, or affected the amount of food consumed by the mice. The animal weights, monitored in 42 animals, were 23.4 ± 4.2 g at the start of the treatments, and 25.2 ± 4.6 g at the end. The amount of food consumed, expressed in g/mouse/week, was: in the control group, 30.8 ± 3.8 (n = 17); in the ibuprofen group, 31.0 ± 6.4 (n = 11); and in the pioglitazone group, 25.9 ± 4.2 (n = 14). Values are the means ± SD. These values translate to a daily dosing of approximately 18 mg/kg of pioglitazone, and 60 mg/kg of ibuprofen.

Immunohistochemistry

The mice were sacrificed under anesthesia. The brains were taken out of the skulls, and fixed by submersion in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 hours at 4°C. The brains were transferred to 10% sucrose in 0.1 M phosphate buffer for 24 hours at 4°C, snap frozen in isopentane at -40°C, and kept at -80°C until further use. Brain sections (35 μm) were cut in a cryostat and collected in phosphate-buffered saline (PBS). The immunohistochemistry was carried out on free-floating sections. The sections were incubated 15 min in 3% H_2O_2/20% methanol to inactivate endogenous peroxidase activity. The sections were blocked with 0.5% bovine serum albumin (BSA) in PBS, and incubated with primary antibodies in PBS containing 0.2% Triton X-100 and 0.1% BSA. The incubations were performed overnight at 4°C with gentle shaking. After several washes in PBS, the sections were incubated with biotinylated secondary antibodies (1:200, Vector Laboratories, Burlingame, CA) for 30 min at room temperature. The staining was visualized by the biotin-avidin-peroxidase method (Elite Kit, Vector Laboratories, Burlingame, CA) using diaminobenzidine as the chromogen. To reveal Aβ deposits, the sections were incubated in 80% formic acid for 5 min before blocking with BSA. The primary antibodies used were: rabbit anti-mouse GFAP (1: 1000, Sigma), rat anti-mouse Mac-1 (1: 250, Serotec, Oxford, United Kingdom), rabbit anti-

human Aβ40 FCA40 (1: 500, courtesy of Frederic Checler); mouse anti Aβ clone 4G8 (1: 500, Signet, Dedham, MA); and sheep anti-mouse serum amyloid P component (SAP, 1: 500, Calbiochem, San Diego, CA).

Analysis of microglia activation

Microglia activation was assessed in the hippocampus by measuring the soma surfaces of Mac-1 positive cells with the image analysis system VisioScan (BIOCOM, Les Ulis, France), following a variation of the method described by Breidert et col. [21]. Brain sections were analyzed at a 50× magnification under high contrast. A minimum of 100 microglia cells were examined per brain. Each cell was scanned to find the plane containing the largest soma surface. Figure 1G,1H,1I shows the contours of microglia soma in different states of activation.

Thioflavin-S labeling

The number of thioflavin-S-containing vessels was counted in three levels of the hippocampus (the distance from Bregma is indicated in parentheses): i) rostral (-1.75- to -2.5 mm), ii) intermediate (-2.5 to -3.25 mm), and iii) dorsal (-3.25 to -4.5 mm). Coronal brain sections were mounted on slides, air-dried, and incubated with 1% thioflavin-S (Sigma) for 10 min, followed by short rinses in 80–90% alcohol, and a final rinse in H_2O. The sections were mounted in Vectastain fluorescent mounting media, and the staining visualized with UV light or FITC filters. In the case of branched vessels each branch was counted as "one". The thioflavin-S-positive vascular density per hippocampus level was the average of 4 sections.

Western blots

The hippocampi were snap frozen in isopentane cooled on dry ice, and stored at -80°C. Brain tissue was homogenized by passage through 18-, 21-, and 26-G syringes, in a buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP40, and protease inhibitors. The homogenates were left 20 min on ice, and centrifuged at 15,000 × g for 15 min. The supernatants were processed for PAGE-SDS electrophoresis. Proteins were then transferred to PVDF membranes (BioRad) by semi-dry electrophoresis. The membranes were blocked in 10% milk in 10 mM Tris/150 mM NaCl containing 0.1% Tween-20 (TBS) and incubated overnight in TBS containing the primary antibodies. The membranes were incubated with HRP-conjugated IgGs for 1 hour. Washes between steps were carried out with TBS. The bands were visualized with enhanced chemiluminescence reagents (New England Nuclear, MA, USA), and exposure to X-ray films. Three antibodies were used: rat anti-mouse GFAP (1: 5000, clone MAB 2.2B10, provided by Virginia Lee); mouse anti-APP C-terminus (1: 1000, clone C1/6.1, ref 24), and rabbit anti-human actin (1: 500, Sigma, Santa Cruz, CA) to assure equal loading of protein. HRP-conjugated
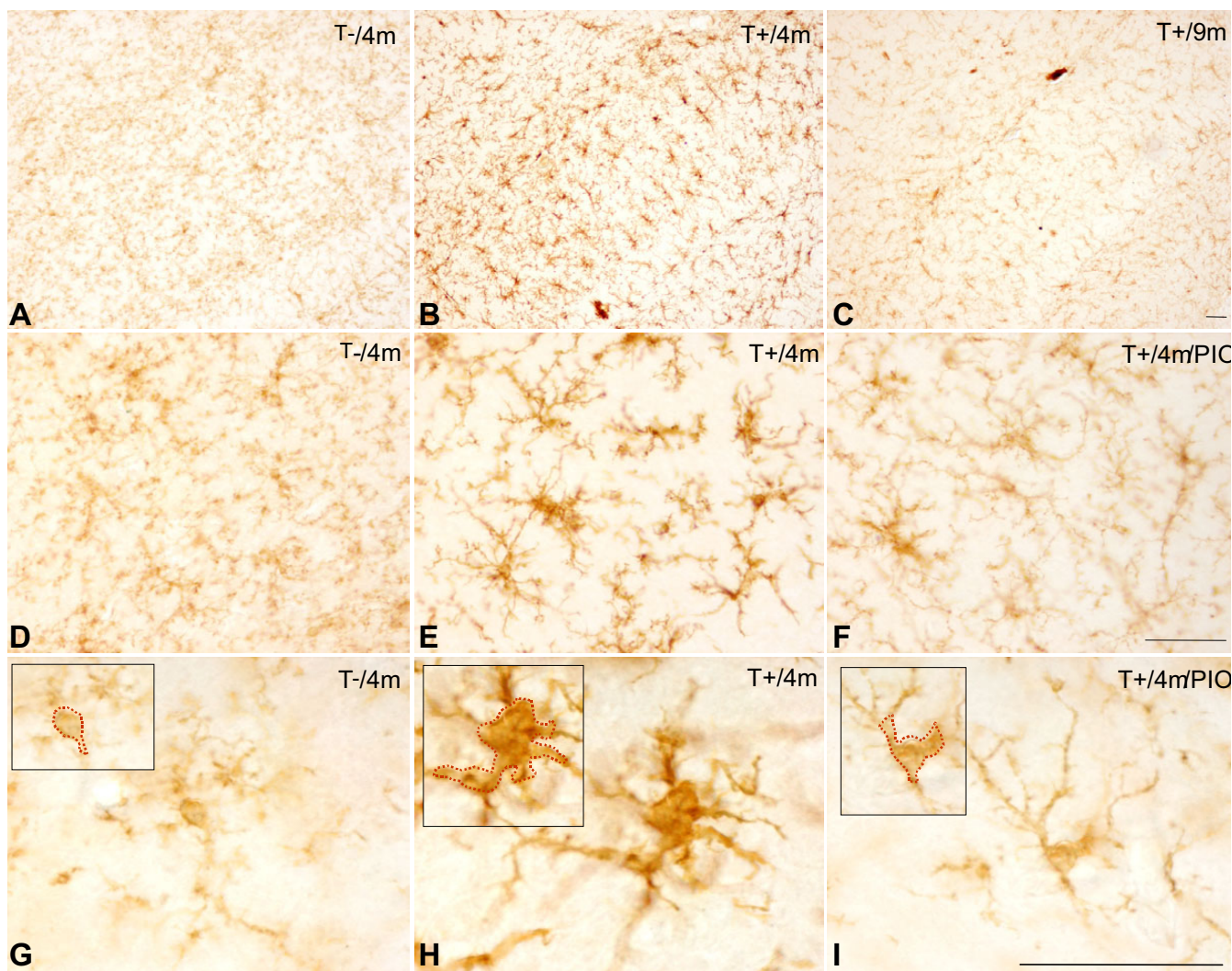
Figure 1

Mac-1 immunoreactivity in the hippocampus. The age, phenotype and treatment of the animals are indicated in the upper right corners. T-: control and T+: TGF-β1 mice; PIO: treated with pioglitazone for 2 months; 4 m and 9 m: 4 and 9-month-old mice. Each row shows images taken at increasing magnifications (bars are 50 μM). Microglia cells were "activated" in TGF-β1 mice at 4 months, revealed by increased Mac-1 immunoreactivity, retraction of processes, and increased soma size (compare B to A and E to D). Microglia activation decreased with age (compare C to B) and with PIO (compare F to E). G-I are high magnification images of single microglia cells. The insets illustrate the soma surfaces as determined for the quantification of microglia activation. DG: dentate gyrus; HiF: hippocampal fissure.

secondary antibodies were purchased from Vector Labs. For quantitative assessment of bands, the autoradiographs were scanned and analyzed with Image J from the National Institutes of Health.

ELISA

Hippocampi were homogenized individually by passage through 18-26-G syringes in 0.25 mL of buffer (250 mM sucrose, 20 mM Tris base, 1 mM EDTA, and 1 mM EGTA) in the presence of protease inhibitors. The homogenates were extracted with a diethylamine (DEA)/NaCl solution as previously described [25], and processed for sandwich ELISA measurements using monoclonal antibodies JRF/cAβ40/10 and JRF/cAβ42/26, which specifically recognize the carboxyl-terminals of mouse Aβ40 and Aβ42, respectively and JRF/rAβ1-15/2, which binds to the N-terminus of murine Aβ. Applications of this ELISA have been previously described elsewhere [24-26].

Evaluation of ventricle size

The degree of hydrocephalus was assessed in coronal sections of 20 μm thickness obtained from fresh-frozen brains processed for ^{14}C-deoxyglucose autoradiography for a parallel study. The areas of the lateral ventricles were measured at three levels using VisioLab software (BIO-COM): i) rostral, at bregma +1.1 (the anatomical reference was the genu of the corpus callosum); ii) intermediate, at bregma -0.7 mm (references were the anterior fimbria hippocampus, the dorsal 3rd ventricle and the subfornical organ); and iii) caudal, at bregma -2.5 mm (posterior ventral end of the 3rd ventricle and the dorsoventral hippocampal horn). An average ventricle surface per animal was calculated from the three values.

Statistical analysis

Results are expressed as means ± SD. Data comparisons were carried with the Student's T test when two groups were compared, or one or two-way ("treatment" versus "genotype") ANOVA analysis followed by the Bonferroni test when more than three groups were analyzed. Differences were considered significant in the 95% confidence interval when $p < 0.05$. Statistical analyses were performed with Prism Graphpad version 3.0 software.

Results

Age-dependence of glia activation and thioflavin-S-labeled deposits

Microglia cells were activated in TGF-β1 mice 2–4 months old, mostly in the hippocampus. The cells showed increased Mac-1 immunoreactivity, shortening and thickening of processes, and enlargement of somas (Fig 1B,1E and Fig 2). In control animals nearly all of the somas were smaller than 150 μm², whereas in TGF-β1 animals the somas ranged between 100–400 μm² (Fig 2A). We hence defined "activated microglia" as cells with somas larger than 150 μm² irregardless of the intensity of Mac-1 expression.

The microglia were clearly activated (60% of all cells) at 2 months of age, the earliest time analyzed (Fig 2B). The extent of activation was comparable at 4 months, but decreased to 20% at 9 months ($p < 0.01$) (Fig 1C and Fig 2B).

TGF-β1 animals also had astrocytosis at all ages analyzed, defined as increased GFAP immunoreactivity (Fig 3), and increased GFAP protein content assessed by Western blot analysis (Fig 4). The astrocytosis was detected throughout the brain, but it characteristically affected the hippocampus in particular the dentate gyrus.

In TGF-β1 animals, but not in control mice (Fig 5A,5C), thioflavin-S labeled meninges and vessels of 10–20 μm diameter concentrated primarily around the hippocampal

fissure (Fig 5B) and, on occasion, in larger penetrating vessels directly originating from the pial vasculature (Fig 5D). The density of thioflavin-positive vessels was higher in rostral hippocampus and decreased caudally by 30% (Fig 6). Both meningeal and vascular deposits were evident at 2 months (Fig 6A). The density of thioflavin-S positive vessels increased by 40% at 4 months, and did not change thereafter (Fig 6A), indicating that the thioflavin-S-labeled material accumulated early, and achieved a steady-state by 4 months.

Comparison of the thioflavin-labeled material in vessels from TGF-β1 and APP mice revealed two differences (Fig 5E,5F,5G). First, in APP mice the deposition affected long vessels in the cortical parenchyma, while in TGF-β1 mice the labeled vessels were shorter, frequently branched, and localized to the hippocampal fissure. Secondly, the thioflavin-S deposits appeared to have spread over the vessels in patches in APP brains, while deposits appeared smoother and more uniformly covered entire vascular stretches in TGF-β1 mice. This strongly suggests a different composition of the vascular deposits in APP and TGF-β1 transgenic mice.

Effect of anti-inflammatory drugs on glia activation and vascular amyloid deposition

Ibuprofen or pioglitazone treatments were started at 2 months of age and continued for 2 months. Two reasons justified the length of the treatments: i) we had established that the largest change detected in vascular deposits, as assessed with thioflavin-S, took place before 4 months of age; and ii) since Mac-1 expression dramatically decreased with age, a longer treatment would have potentially complicated the assessment of whether the drugs had efficiently inhibited inflammation.

Both ibuprofen and pioglitazone effectively reversed the TGF-β1-induced microglia activation. Pioglitazone reduced the number of activated microglia by 40% ($p < 0.01$), and ibuprofen by 70% ($p < 0.001$) (Fig 2C). Accordingly, Mac-1 expression and soma sizes were reduced, and the cellular processes recovered some of the diffuse pattern that characterizes resting microglia (Fig 1D,1E,1F). The anti-inflammatory treatment also reduced the astrogliosis (Fig 3C,3D and Fig 4A). By contrast, neither pioglitazone nor ibuprofen changed the density of thioflavin-S-positive vessels (Fig 6B). This suggests that none of the drugs interfered with the vascular deposition that occurred between 2 and 4 months of age.
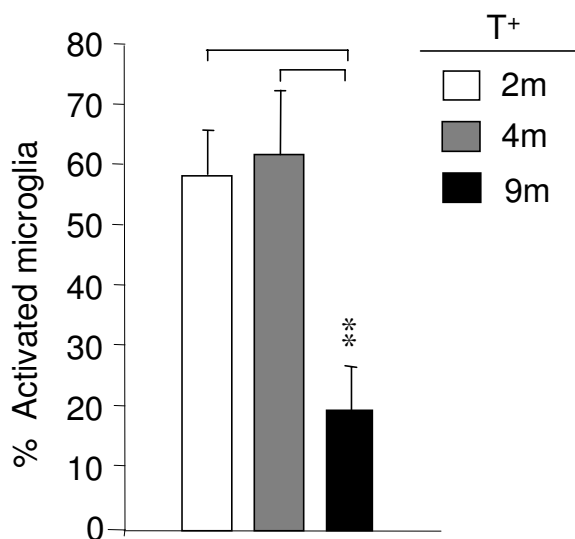
Measurement of Aβ and APP

Aβ production was assessed by ELISA and immunohistochemistry. It should be stressed that the antibodies used in both procedures, FCA40 [27,28], 4G8 [29], JRF/cAβ40/10, JRF/cAβ42/26, JRF/rAβ1-15/2 [24-26] recognize
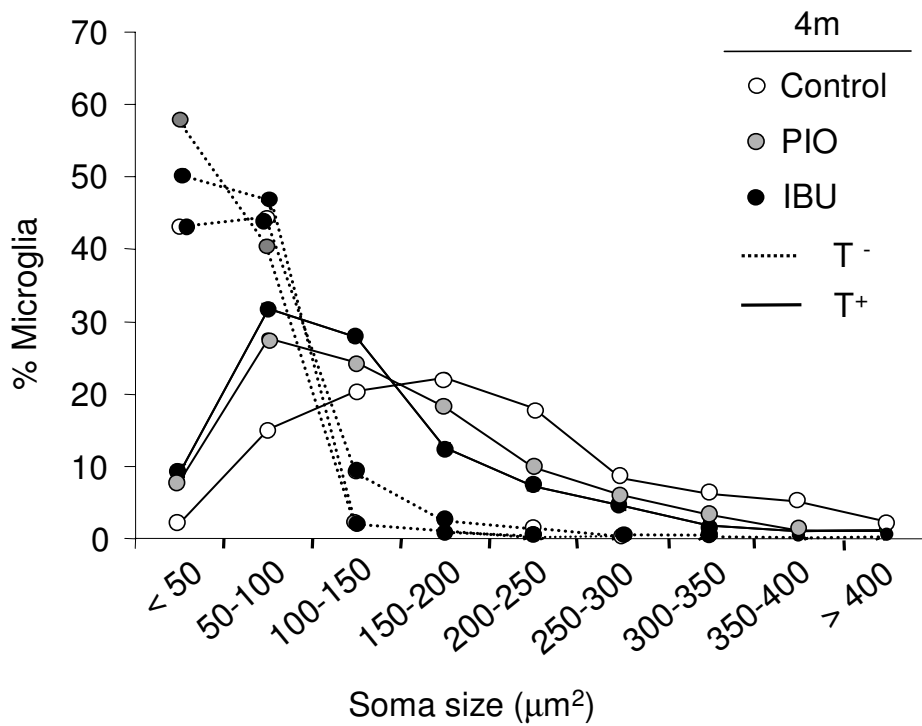
Figure 2
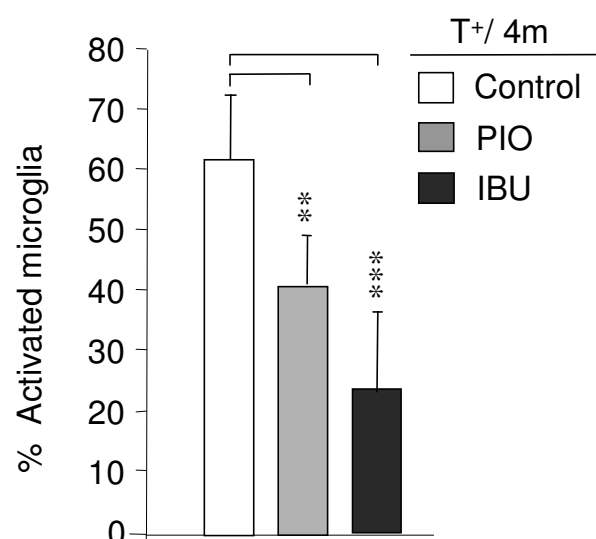Quantification of microglia activation. A) Distribution of microglia according to soma sizes in 4-month-old control and TGF-β1 mice fed for 2 months with control chow, or chow containing pioglitazone or ibuprofen; B) Evolution of microglia activation with age; C) Effect of anti-inflammatory drugs. "Activated microglia" were cells with somas larger than 150 μm^2. The values are the means ± SD; (**) $p < 0.01$; (***) $p < 0.001$, one-way ANOVA analysis, Bonferroni *post hoc* test. N = 2 for 2-month-old mice, and N = 4–6 for 4 or 9-month-old mice.
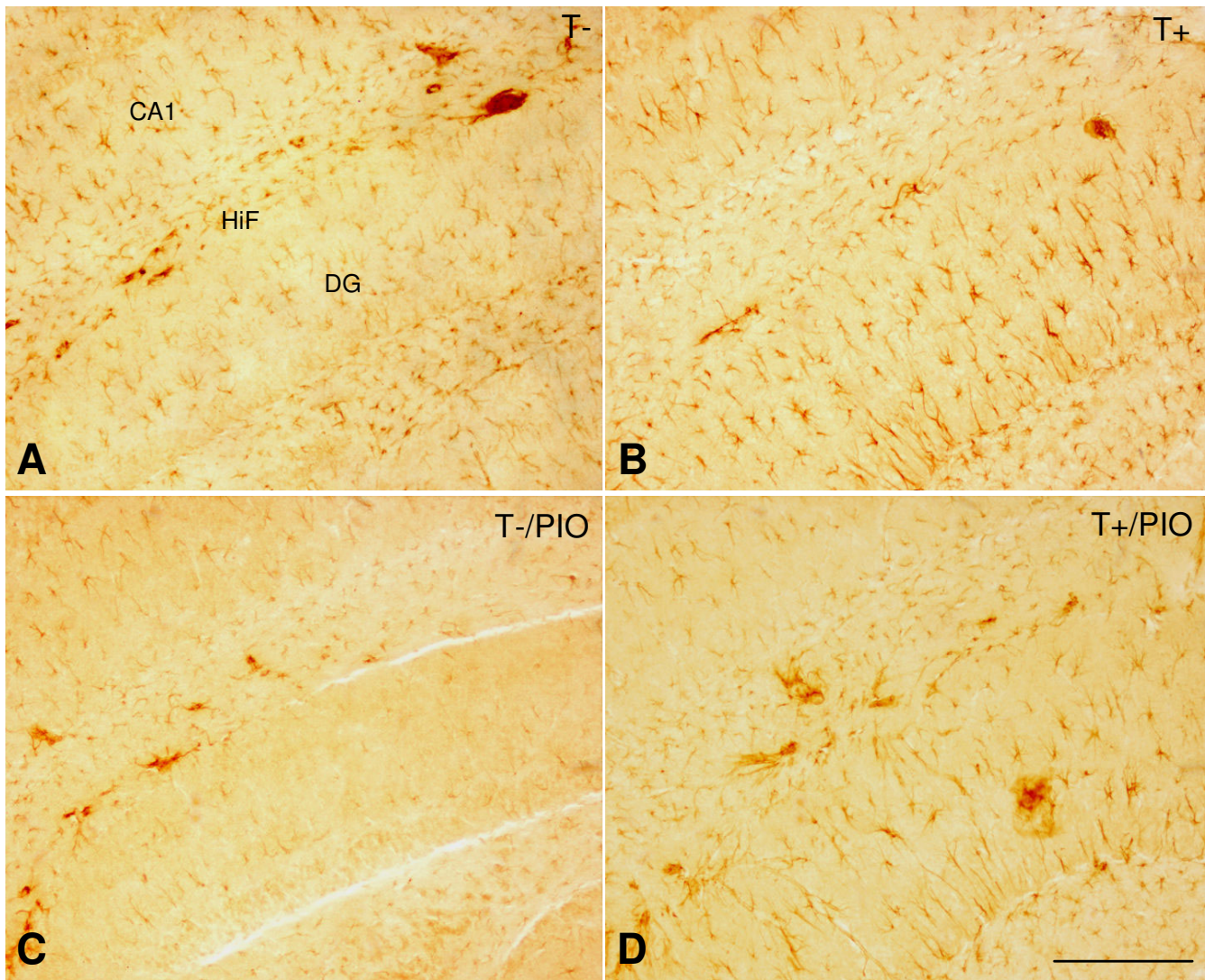
Figure 3

GFAP immunohistochemistry in hippocampus. T-: control mice, T+: TGF-β1 mice. Animals had been fed for 2 months with control (A-B) or pioglitazone-containing (C, D) chow. GFAP expression was increased in TGF-β1 animals mostly in astrocytes in the dentate gyrus (DG) and in perivascular astrocytes at the hippocampal fissure (HiF). The astrocytosis was decreased by pioglitazone. Bars are 100 μM.

mouse Aβ and hence should detect endogenous mouse Aβ in TGF-β1 mice.

ELISA measurements of Aβ40 and Aβ42 at 2 and 4 months revealed no significant differences between control and TGF-β1 mice (Table 1, p = 0.78 in the 4-month-old group), although at 9 months there was an increase (102%) in Aβ40 levels (Table 1). FCA40 and 4G8 revealed extensive plaque deposition in the parenchyma of the hippocampus and cortex of over one-year-old APP mice (Fig 7A,7C), but produced no specific staining in TGF-β1 mice at 4 or 9 months of age (Fig 7B,7D). The ELISA and the

immunohistochemical analysis combined suggest that the thioflavin-S-labeled vascular deposits, which were abundant at 4 months of age, contained no Aβ. Immuno-histochemical analysis for SAP, which is considered a common component of amyloid deposits [47], gave negative results (Fig 7E,7F). The SAP antibody has been shown to detect mouse SAP by immunocytochemistry [49].

The ELISA also showed that pioglitazone decreased by 23–32% the basal Aβ42 production in control and TGF-β1 mice (Table 1, p = 0.013; 2-way ANOVA). Pioglitazone
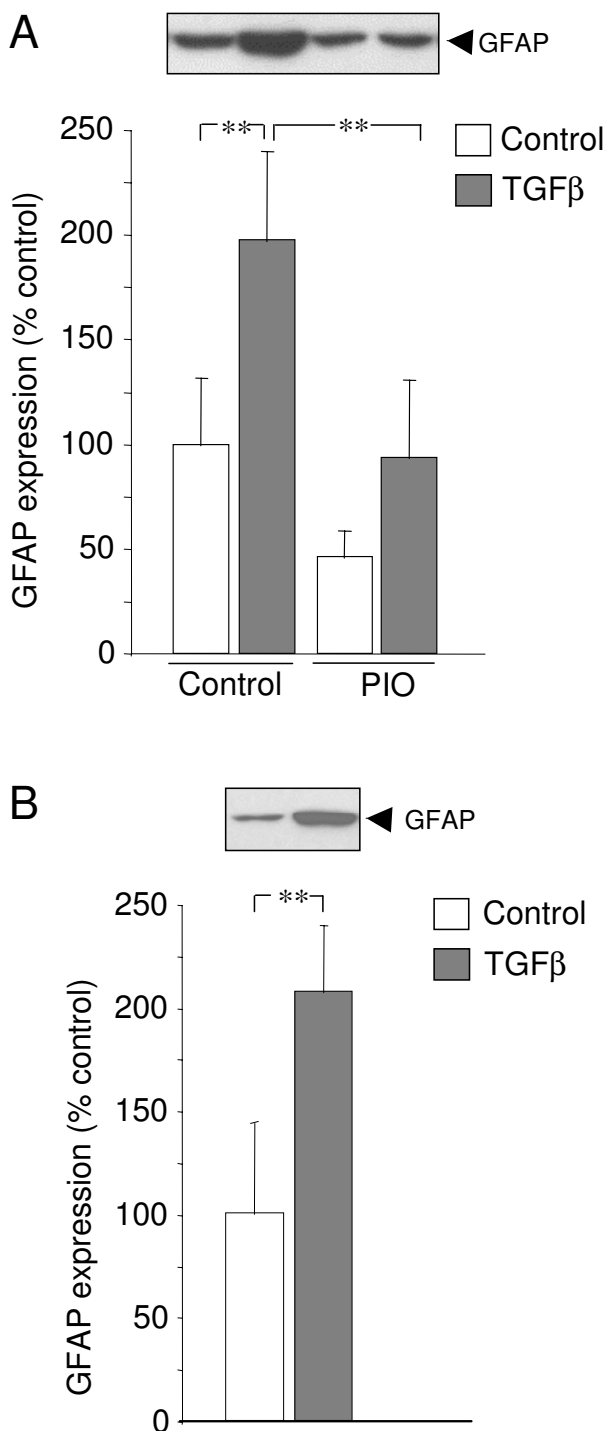
Figure 4
Western blot analysis of GFAP expression in hippocampus.
A) 4-month-old animals, treated for 2 months with control
food or pioglitazone. B) 9-month-old mice. Values are the
means ± SD; In A: (**) p < 0.01, (*) p < 0.05, two-way
ANOVA analysis followed by Bonferroni analysis; n = 4–5
per group. In B: (**) p < 0.01, Student's T-test; n = 5 per
group.

had a negligible effect on Aβ40 – the ratio Aβ42/40 was
accordingly reduced – while ibuprofen had no effect on
the levels of Aβ40 or Aβ42.

Analysis of APP expression by Western blot showed sev-
eral bands around 100 kDa (Fig 8) that are probably post-
transcriptional modifications of the 695 amino acid-long
APP isoform predominant in brain. Neither the combined
nor the individual expression of APP bands was altered by
TGF-β1 over-expression at 4 or 9 months, or by treatment
with pioglitazone. Thus, the increase in Aβ40 detected at
9 months did not correlate with an increase in APP
expression.

Effect of pioglitazone on the hydrocephalus
TGF-β1 animals were hydrocephalic, reflected by an over
2-fold increase in ventricle surface assessed in coronal sec-
tions (p < 0.001) (Figs 9 and 10). Surprisingly,
pioglitazone increased the ventricle surface in non trans-
genic animals by 22% (p > 0.05), and exacerbated the
TGF-β1-induced increase by 55% (p < 0.001) (Figs 9 and
10). Although the effect of pioglitazone in control ani-
mals was not statistically significant when values at the
three brain levels were averaged, the drug caused a signif-
icant (p < 0.05) 40% increase at level 3, an area largely
comprising the ventrocaudal hippocampal horn (Fig
10G,10H). Conversely, pioglitazone acted rostrally in
TGF-β1 mice (110% increase at Bregma level 1) (Fig
10A,10B).

Discussion
The TGFβ1-mice used in this study showed between 2–4
months of age several of the pathological signs associated
with the anomalous expression of this cytokine in brain:
glia activation, vascular and meningeal deposition of a
material positive to thioflavin-S, and hydrocephalus.
Microglia activation was severely reduced at 9 months,
indicating that the cells became refractory to TGF-β1 stim-
ulation over time, whereas the thioflavin-S labeling, astro-
cytosis, and hydrocephalus persisted. Pioglitazone exerted
paradoxical actions on TGF-β1-elicited pathology: it
inhibited both astrocyte and microglia activation, but it
did not interfere with the vascular and meningeal deposi-
tions, and exacerbated the hydrocephalus. In addition,
pioglitazone decreased Aβ42 basal levels, an effect unre-
lated to the anti-inflammatory actions of the TZD since it
was equally observed in transgenic and controls, and the
latter do not display inflammation.

PPAR agonists are currently being considered as a treat-
ment of neurological diseases where chronic inflamma-
tion is suspected to be a pathogenic factor. There are NIH-
sponsored pilot clinical trials of TZDs ongoing for
Alzheimer's disease (Gary Landreth, personal communi-
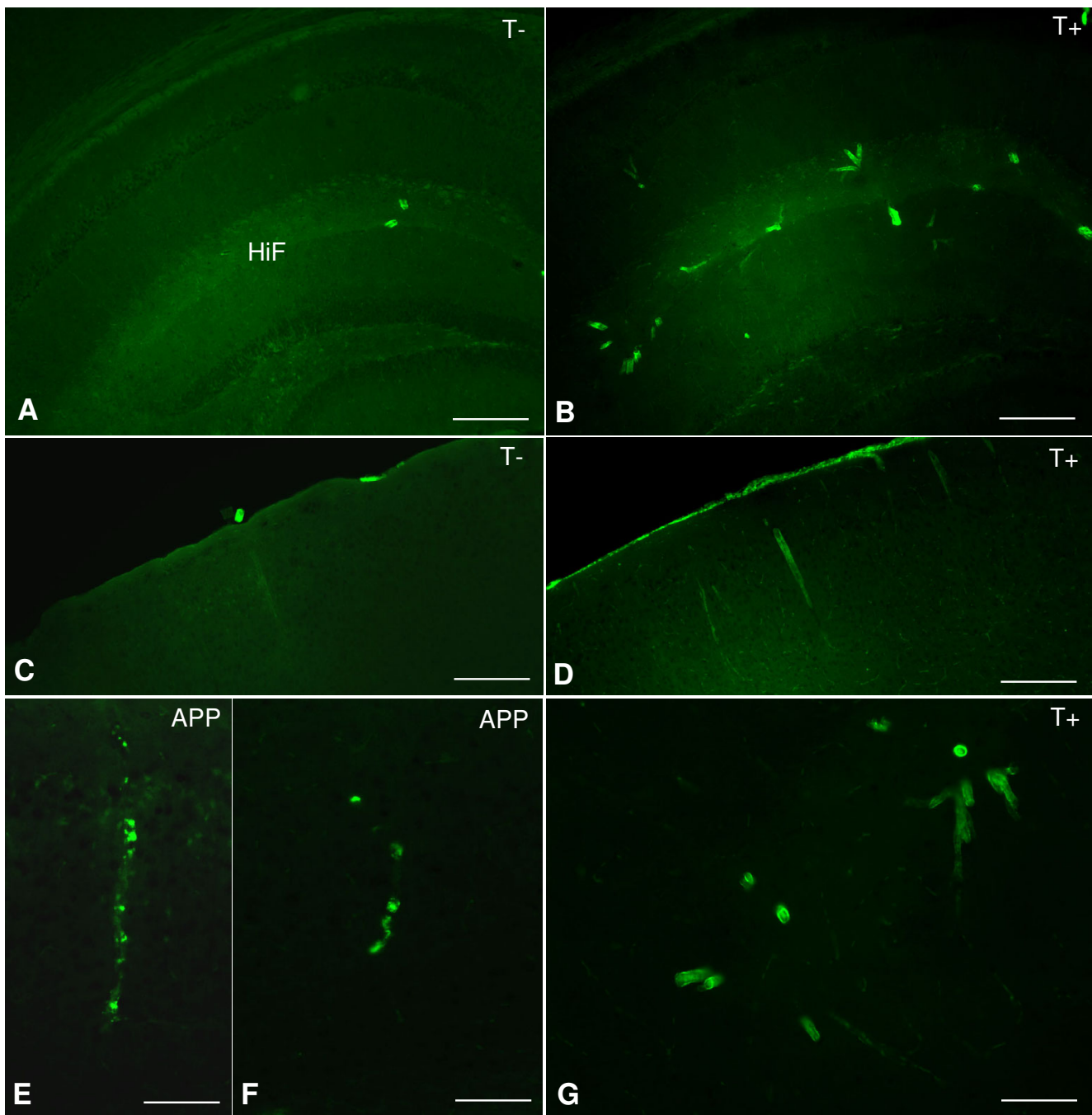cation) and multiple sclerosis (D.L. Feinstein, personal

Figure 5
Thioflavin-S labeling in coronal brain sections. A, B show hippocampus and C, D show cortex of 4-month-old mice; A, C are control mice, B, D, G are TGF-β1 mice, and E, F are a one-year-old APP mouse. Bars are 200 μm (A-D) or 100 μm (E-G). Thioflavin-positive material accumulated in the meninges and in vessels around the hippocampal fissure (HiF) in TGF-β1 mice. Vascular deposits in TGF-β1 mice were smoother in appearance and uniformly covered entire vascular segments, while in APP brains the deposits spread over the vessels in patches (compare E and F to G).
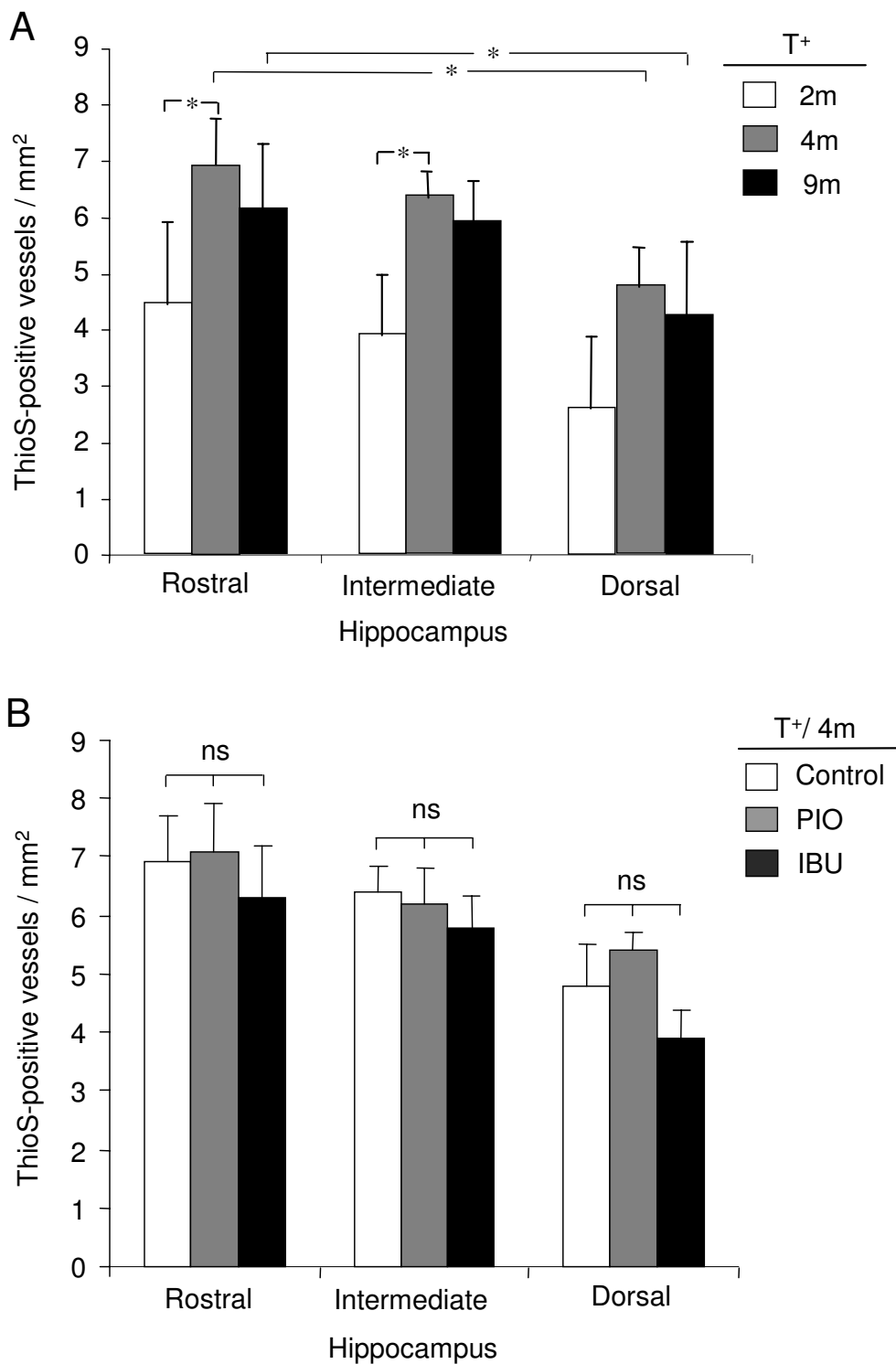
Figure 6
Density of thioflavin-S positive vessels along the hippocampus. A) Evolution with age. B) Effect of anti-inflammatory drugs. Values are the means ± SD; (*) $p < 0.05$, ns=non significant, ANOVA and Bonferroni *posthoc* analyses. N = 4–6 mice per group. The vascular accumulation of thioflavin-S-positive material increases between 2–4 months, and it is not altered by pioglitazone or ibuprofen.

Table 1: ELISA measurement of Aβ proteins in hippocampus

	2 months		4 months						9 months	
	control		control		PIO		IBU		control	
	T-	T+	T-	T+	T-	T+	T-	T+	T-	T+
Aβ40	9.8 ± 1.7	10.1 ± 1.1	9.5 ± 2.5	9.6 ± 2.1	8.5 ± 2.2	8.3 ± 1.5	9.9 ± 0.9	10.5 ± 2.0	9.2	18.6
Aβ42	7.2 ± 1.5	7.2 ± 1.0	6.8 ± 2.7	6.9 ± 1.7	5.2 ± 1.5*	4.7 ± 0.7*	7.2 ± 1.3	7.2 ± 1.8	6.5	7.8
ratio 42/40	0.74	0.71	0.71	0.73	0.61	0.57	0.73	0.68	0.71	0.42
n	6	4	8	8	6	5	6	7	4	5

Values are means in fmol / mg protein ± SD, except at 9 months where measurements were performed in pooled hippocampi. Results: i) no difference between T+ and T- groups at 2 or 4 months; ii) increase in Aβ40 in T+ at 9 months; iii) decrease of basal Aβ42 but not Aβ40 by pioglitazone in T- and T+ groups (*) $p = 0.013$, PIO *versus* control, two-way ANOVA analysis; iv) no effect of Ibuprofen. n, number of animals, T-, non transgenic mice; T+, TGF-β1 mice.

communication), and GlaxoSmithKline has initiated a clinical trial of pioglitazone in Europe for both Alzheimer's disease and multiple sclerosis. The findings in the present study are relevant to the clinical trials because TGF-β1 animals display CAA-related vascular alterations and dysfunction [3-6], and can develop a more severe experimental allergic encephalomyelitis than normal mice [31].

The observation that pioglitazone reduces glia activation indicates that orally administered pioglitazone, like ibuprofen, can traverse the blood-brain barrier and exert anti-inflammatory actions in brain. This finding confirms the pioglitazone-mediated decrease in glia activation reported in the MPTP mouse model of Parkinson's disease [21]. Since activated microglia produce cytotoxic substances [32], and astrogliosis leads to the dysregulation of astrocyte-neuron networks [33], administration of pioglitazone may help to prevent the damage caused by chronic glia activation.

Despite the decreased inflammation, pioglitazone exacerbated the hydrocephalus in TGF-β1 mice. It also increased the ventricle size in normal animals. Hydrocephalus is a salient feature of TGF-β1 homozygous transgenic mice and, though to a lesser extent, heterozygous mice like the ones generated for this study. That our mice were less affected than the homozygous ones was indicated by the lack of behavioral alterations clinically associated to hydrocephalus such as aggression, or deficits in posture and balance. TGF-β1 overproduction has been reported to severely alter CSF dynamics, and studies indicate that the underlying mechanism is obstruction of CSF clearance by meningeal fibrosis [34,35]. Other examples exist of hydrocephalus caused by growth factor-induced fibrosis [36]. The site and mechanism of action of pioglitazone – whether it stimulates CSF production or inhibits its reab-

sorption – remain to be investigated. Nevertheless TZDs would not appear indicated for the treatment of diseases presenting CSF flow alterations.

The anti-inflammatory drugs did not reverse the thioflavin-S-positive vascular deposition. These deposits were identified as Aβ by immunostaining with an array of antibodies raised against human and rat Aβ proteins that apparently stained vessels in a pattern identical to that obtained with thioflavin-S [6]. At variance with this claim, FCA40 and 4G8, antibodies that can recognize mouse Aβ [27-29] produced no vascular labeling in the TGF-β1 mice used in the present study. Also, the ELISA did not reveal any change in the production of endogenous Aβ40 or Aβ42 within 2–4 months, despite the unequivocal presence of vascular and meningeal thioflavin-S reactivity. It is unlikely that these results were due to insufficient sensitivity of the techniques used, since the antibodies detected Aβ deposition in APP transgenic mice, and these ELISAs have been successfully used to measure very low levels of endogenous murine Aβ [25,26]. Rather, these results, together with the different appearance of vascular deposits in APP and TGFβ-1 mice, strongly suggest that the thioflavin-S-positive material is not Aβ. This is in agreement with the view that spontaneous deposition of *endogenous* mouse Aβ does not readily occur in the mouse brain [37].

An increase in Aβ40 was however detected in TGF-β1 mice 9 months old, suggesting changes in APP metabolism later in the life of the animals. This finding reproduces in part a study reporting up-regulation of APP mRNA and protein, and increased production of Aβ40 and Aβ42 in 6-month-old TGF-β1 heterozygous mice [38]. This and one other study [39] also showed that TGF-β1 induces the transcription of the APP gene, and the production of APP and Aβ species in astrocyte cultures. Although these studies demonstrate that TGF-β1 canstimulate the

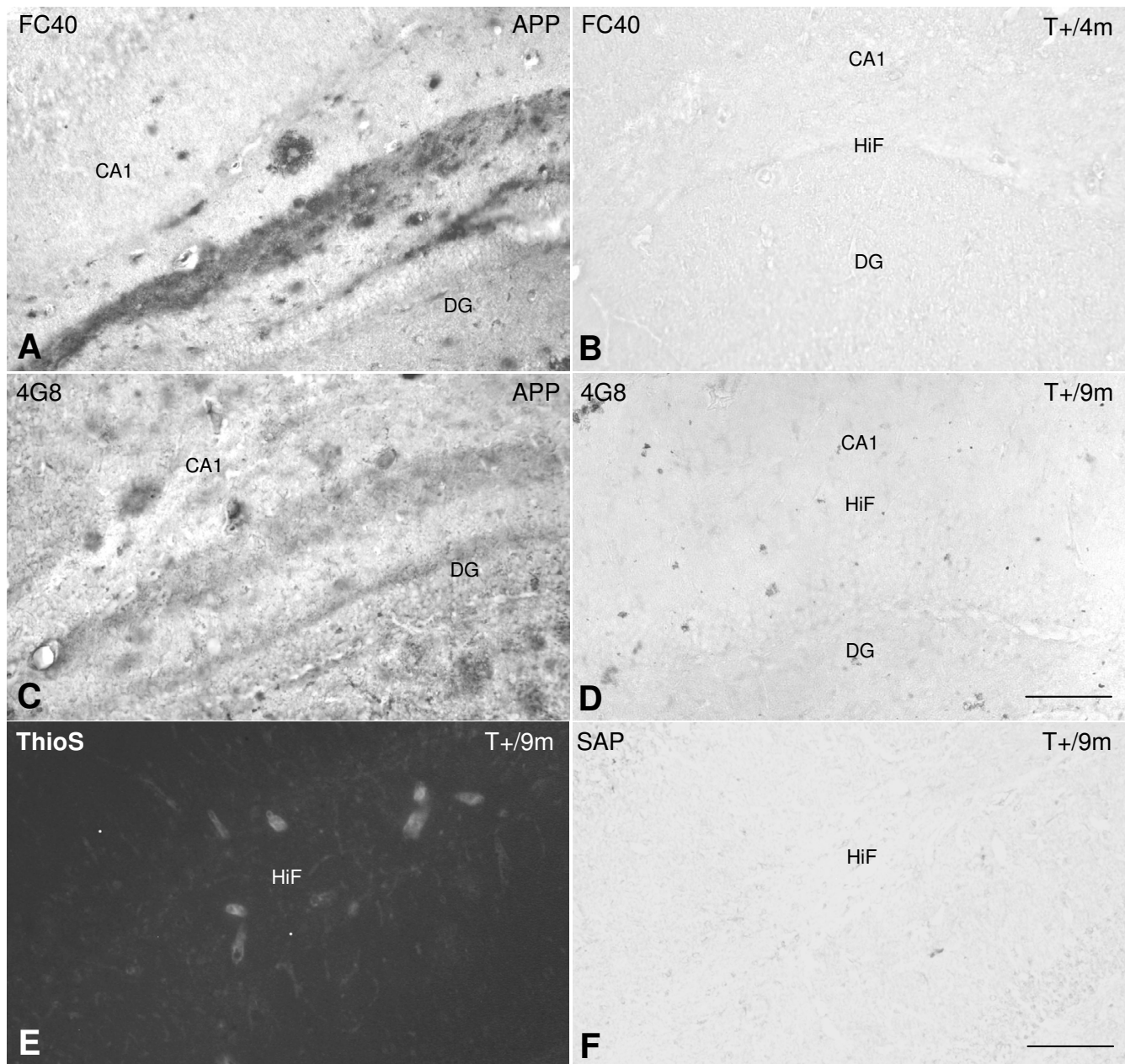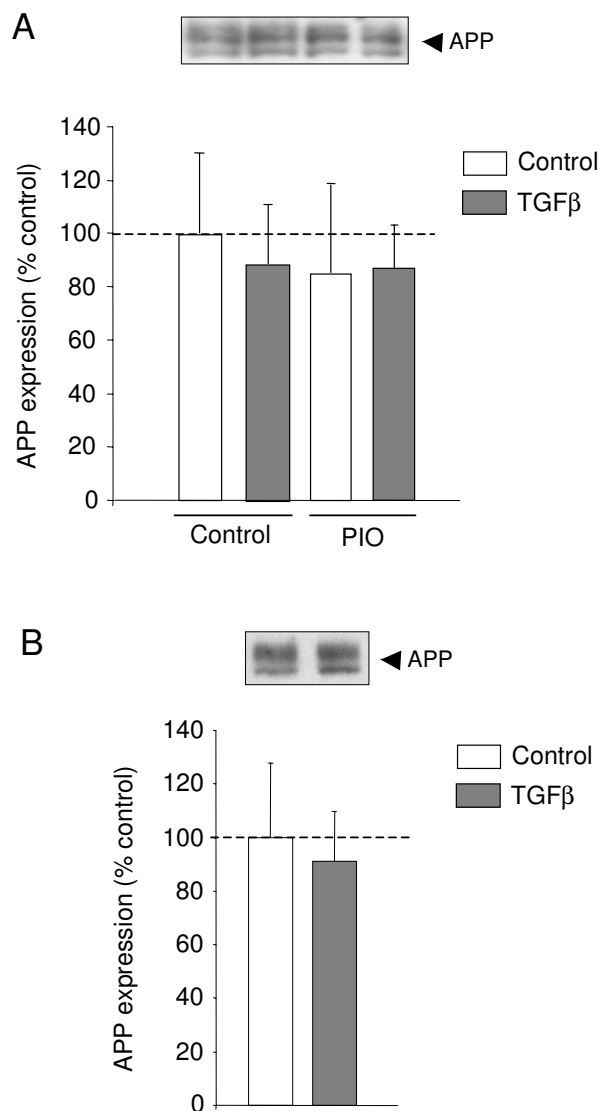**Figure 7**
Aβ and SAP immunoreactivity. The images show coronal sections of hippocampi from over one-year old APP mice (A, C), a 4-month-old TGF-β1 mouse (B) and a 9-month-old (D), stained with FC40 (A, B), 4G8 (C, D), thioflavin (E) or SAP (F). E, F are images of the same field in consecutive sections. FCA40 and 4G8 labeled Aβ plaques in APP mice and showed no specific reactivity in TGF-β1 mice. No SAP was detected in the thioflavin-reactive vascular deposits. Bar equals 100 μm. CC: corpus callosum. HiF: Hippocampal fissure; DG: dentate gyrus.

synthesis and cleavage of glial APP, caution has to be exerted when extrapolating this conclusion to an *in vivo* context: the mice used in the present study produ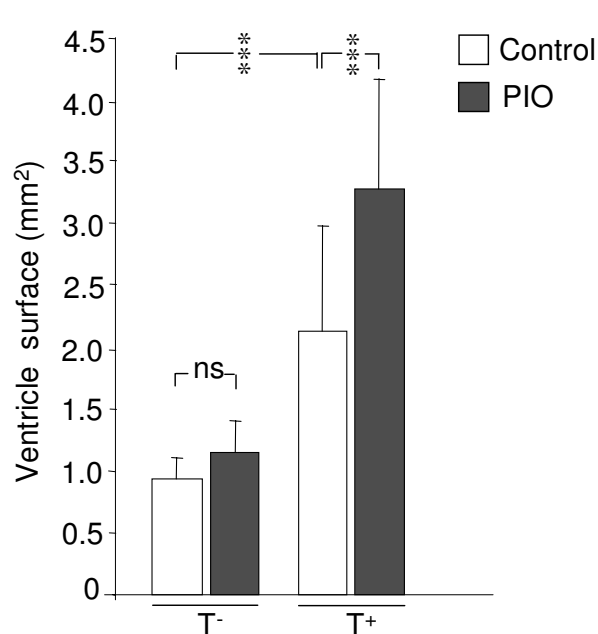ce TGF-β1 constitutively since birth, while Aβ40 – and not Aβ42 – was detected only at midlife, in the absence of increased APP expression. This indicates that factors other than TGF-β1, or the amount of TGF-β1 produced, are of critical importance in the regulation of APP metabolism *in vivo*.

Figure 8
Western blot analysis of APP expression. A) 4-month-old mice, after treatment with pioglitazone; B) 9-month-old mice. Values are the means ± SD. N = 5–6 animals per group in A, and 5 in B. No change with genotype or treatment was observed.

Figure 9
Effect of pioglitazone on ventricle size. Values are the means ± SD of ventricle surfaces at three bregma levels. (ns) non significant, (***) p < 0.001, two-way ANOVA analysis, Bonferroni *post hoc*. N = 10–12 animals per group.

Altogether, the present study supports a β amyloidogenic effect of TGF-β1 in brain cells, but it provides no evidence of vascular accumulation of Aβ peptides.

What is then the material labeled by thioflavin-S in TGF-β1 mice Thioflavin is a recognized marker of "amyloid", a term that applies to any protein that upon adopting a β-pleated sheet configuration instead of the α-helical one becomes fibrillar, insoluble, and precipitates [40]. Over twenty proteins qualify as amyloids by the criterion of thioflavin-S staining. Other than Aβ, amyloids to consider are transthyretin, gelsolin, BRI, prion proteins or cystatin C, which are prone to misfold and deposit in human brain vessels and meninges, leading to several forms of CAA [10,41]. Among possible mechanisms causing amyloid deposition in vessels, the one supported by *postmortem* evidence in brains with Alzheimer's disease is altered drainage of Aβ along periarterial interstitial fluid (ISF) channels in the parenchyma and meninges [42,43]. By analogy with Aβ, the precise location of thioflavin reactivity in meninges, penetrating arteries, and vessels in the hippocampal fissure – also a "brain surface" – serves to argue that TGFβ1 may impair the clearance of a given protein and cause its deposition as an amyloid in brain. As discussed for Aβ [43], local factors affecting the production or solubility of the thioflavin-positive material may contribute to its precipitation.

But arguments exist as well against the amyloid nature of the thioflavin-reactive molecule. First, electron microscopy analysis revealed the presence in TGF-β over-express-
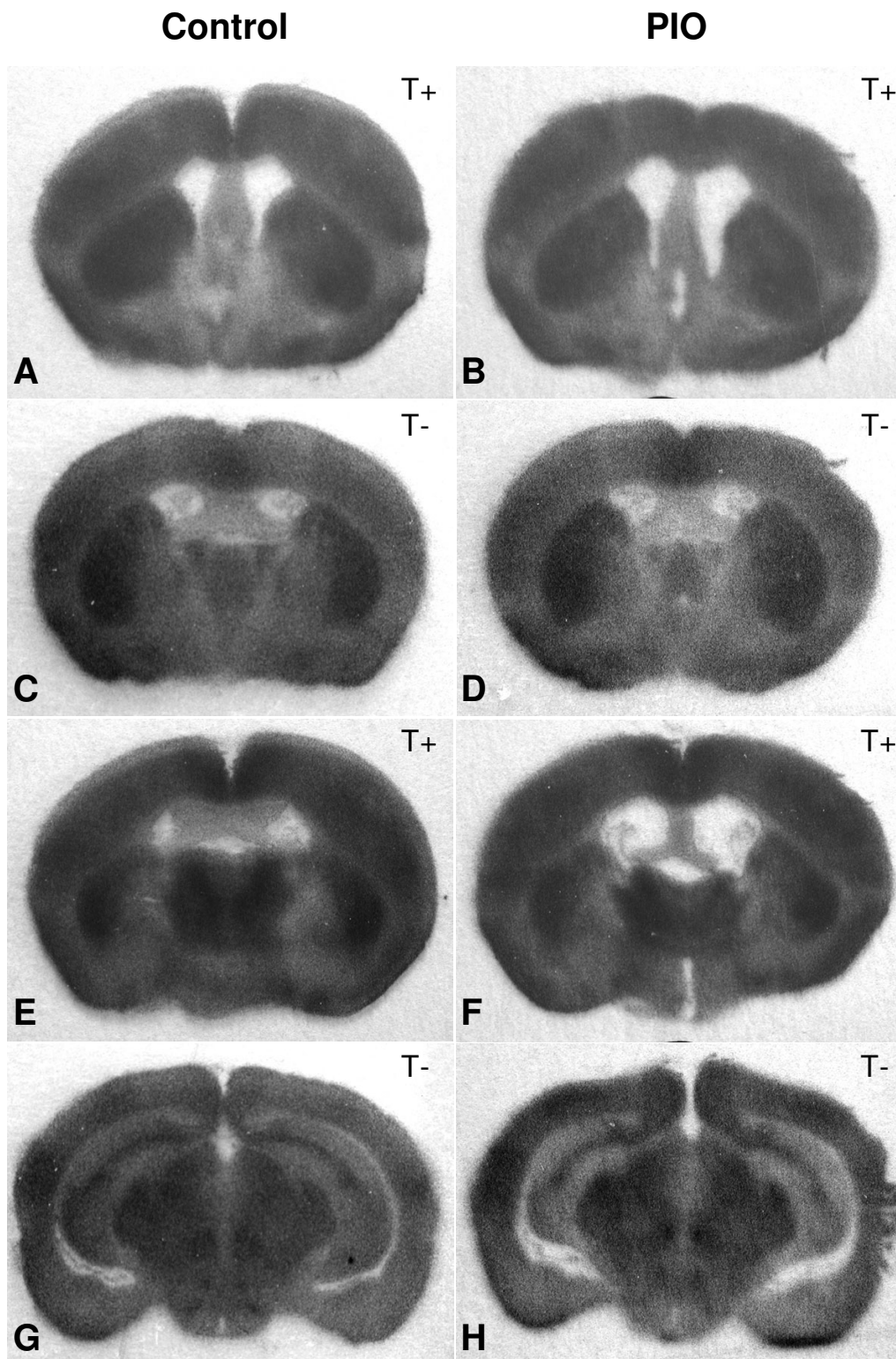
Figure 10
Zone-dependence of the effect of pioglitazone on ventricle size. A, B show rostral, C-F, mid-; and G, H dorsal brain sections. Pioglitazone increased ventricle volumes dorsally in non transgenic mice (T-) (compare H to G), while the effect on TGF-β1 mice (T+) was more apparent rostrally (compare B to A).

ing brains of vascular deposits that do not display [44] the fibrillar structure quintessential to amyloids [45]. Second, SAP was not detected in the vascular deposits by immunocytochemistry. SAP is a non-fibrillar pentraxin plasma protein that contributes to amyloidosis by stabilizing amyloid fibrils, and is widely considered as a common component of amyloid deposits [46]. The latter tenet may, however, not apply to brain. SAP is not produced by brain cells, and the association to Aβ plaques in Alzheimer's disease is probably the result of blood-brain barrier damage [47], which may not occur in animal models. Accordingly, SAP has not been detected in plaques from APP-overexpressing brains [48], a result confirmed by our laboratory (data not shown). Thus, the absence of SAP in TGF-β1 brains does not unequivocally prove that the vascular deposit is not amyloid.

We thus conclude that the thioflavin-positive material has the tinctorial properties and clearance routes of an amyloid, but there is no direct evidence that it is such.

Whatever the nature of the vascular material, we speculate that the lack of effect of pioglitazone on its deposition is related to the increase in hydrocephalus caused by the TZD. Since a continuity exists between ISF and CSF, alterations of flow in either compartment would be transmitted to the other [49]. Hence, in worsening the TGF-β1-induced alteration of CSF dynamics, pioglitazone would impair the ISF drainage of the yet unidentified substance thus promoting – or not preventing – its deposition in meninges and vessels around the hippocampal fissure. If so, pioglitazone may not be beneficial for the treatment of diseases that are caused to some extent by altered vascular drainage of amyloid-like proteins.

Finally, pioglitazone, but not ibuprofen, selectively decreased the content of the allegedly more toxic Aβ42, while not altering that of Aβ40, thus causing a change in the Aβ42/40 ratio. This evidence supports a novel effect of PPARγ activation on Aβ metabolism independent of inflammation. The possible therapeutic relevance of this phenomenon lies in the nature of the Aβ measured that is: i) endogenous, ii) wild type, and iii) soluble, for the animals under study have no Aβ deposits. An effect on wild type Aβ production may render PPARγ agonists appropriate for the treatment of the more common non familial forms of Alzheimer's disease. An effect on soluble Aβ gains importance in light of recent studies suggesting that cognitive dysfunction in Alzheimer's disease may correlate better with soluble than insoluble levels of Aβ [50]. The parallel finding that APP expression was not changed by pioglitazone rules out that the Aβ42 reduction was caused by a decrease in APP production. Rather, the TZD may act on the production or degradation of Aβ42. Also ibuprofen has been shown to reduce plaque-associated

and soluble Aβ42 in mice over-expressing the Swedish mutation of human APP [15,16]. The lack of effect of ibuprofen in our model could be due to a preferential action of NSAIDs on the metabolism of mutated Aβ. Interestingly, a selective action on Aβ42 forms appears to be the rule amongst anti-inflammatory drugs. This calls into question the use of NSAIDs or TZDs for the treatment of Aβ-related CAA caused by Aβ40 deposition [51].

Conclusions
In summary, the present study: i) supports β amyloidogenic actions of TGF-β1 in brain (i.e. increased production of Aβ40), but provides no evidence of vascular accumulation of Aβ peptides, ii) shows that TGF-β1 over-expression results in the early deposition in vessels and meninges of a thioflavin-S-positive molecule yet to identify; and iii) reveals a mix of potentially beneficial (i.e. decrease of chronic glia activation and reduction of basal Aβ42 levels) and detrimental actions (i.e. increased hydrocephalus, and lack of effect on thioflavin-S-positive deposits) of pioglitazone that will have to be weighed when considering the therapeutic applications of this TZD.

Abbreviations
Aβ, amyloid β; APP, amyloid β precursor protein; CAA, cerebral amyloid angiopathy; CSF, cerebrospinal fluid; ISF, interstitial fluid, NSAIDs, non steroidal anti-inflammatory drugs, PPARγ, peroxisome proliferator-activated receptor γ ; TGF-β1, transforming growth factor β1, TZD, thiazolidinedione.

Competing interests
Gary Landreth received a honorarium for a talk given to Takeda North America in 2003, and received funding from GlaxoSmithKline for research in his laboratory in 2002–2003.

Authors' contributions
PL, co-director with EG, and person responsible for the generation and physiological characterization of the TGFβ animals used in this study; PM and SS, ELISA determination of amyloid β proteins. Theirs is one of the few laboratories that can measure *endogenous* murine amyloid beta, an essential requirement for the study; TB, western blots and development of the morphometric method to quantitate microglia activation; GL and MH organized and paid for the synthesis and delivery of the chow containing pioglitazone and ibuprofen. DLF, constant provider of key insights and discussion; EG was the project director and carried out the immunohistochemistry.

Acknowledgements

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