

# Regulation of oligodendrocyte progenitor cell maturation by PPAR $\delta$ : effects on bone morphogenetic proteins

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## ABSTRACT

In EAE (experimental autoimmune encephalomyelitis), agonists of PPARs (peroxisome proliferator-activated receptors) provide clinical benefit and reduce damage. In contrast with PPAR $\gamma$ , agonists of PPAR $\delta$  are more effective when given at later stages of EAE and increase myelin gene expression, suggesting effects on OL (oligodendrocyte) maturation. In the present study we examined effects of the PPAR $\delta$  agonist GW0742 on OPCs (OL progenitor cells), and tested whether the effects involve modulation of BMPs (bone morphogenetic proteins). We show that effects of GW0742 are mediated through PPAR $\delta$  since no amelioration of EAE clinical scores was observed in PPAR $\delta$ -null mice. In OPCs derived from E13 mice (where E is embryonic day), GW0742, but not the PPAR $\gamma$  agonist pioglitazone, increased the number of myelin-producing OLs. This was due to activation of PPAR $\delta$  since process formation was reduced in PPAR $\delta$ -null compared with wild-type OPCs. In both OPCs and enriched astrocyte cultures, GW0742 increased noggin protein expression; however, noggin mRNA was only increased in astrocytes. In contrast, GW0742 reduced BMP2 and BMP4 mRNA levels in OPCs, with lesser effects in astrocytes. These findings demonstrate that PPAR $\delta$  plays a role in OPC maturation, mediated, in part, by regulation of BMP and BMP antagonists.

**Key words:** astrocyte, bone morphogenetic protein (BMP), experimental autoimmune encephalomyelitis (EAE), multiple sclerosis, myelin, noggin.

## INTRODUCTION

Studies from our laboratory (Feinstein et al., 2002) and others (Diab et al., 2002; Natarajan and Bright, 2002) have shown that agonists of PPAR $\gamma$  (peroxisome proliferator-activated receptor  $\gamma$ ) reduce clinical and histological symptoms in EAE (experimental autoimmune encephalomyelitis), an animal model of MS (multiple sclerosis). These effects are due, in part, to suppression of inflammatory gene expression (Drew et al., 2006; Xu et al., 2007), inhibition of activated T-cell proliferation and production of inflammatory mediators (Feinstein, 2003; Kielian and Drew, 2003), and reduction of leucocyte infiltration into the CNS (central nervous system) (Klotz et al., 2007). These findings have led to the design of small clinical trials in relapsing remitting MS patients (Miller et al., 2005; Kaiser et al., 2009) with encouraging results. Similarly, agonists of PPAR $\alpha$  show anti-inflammatory actions on glial cells (Deplanque et al., 2003; Xu et al., 2005, 2006) and benefit in EAE (Lovett-Racke et al., 2004; Dasgupta et al., 2007; Xu et al., 2007).

In contrast with PPAR $\alpha$  and PPAR $\gamma$ , studies of the PPAR $\delta$  (also referred to as PPAR $\beta$  or PPAR $\beta/\delta$ ) isoform in EAE are

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**Abbreviations:** ANGPTL-4, angiopoietin-like 4; bFGF, basic fibroblast growth factor; bHLH, basic helix-loop-helix; BMP, bone morphogenetic protein; CGT, galactose ceramide galactosyl transferase; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; E13 etc., embryonic day 13 etc; EAE, experimental autoimmune encephalomyelitis; GalC, galactosyl ceramidase; GDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; Id, inhibitors of differentiation; IFN $\gamma$ , interferon  $\gamma$ ; MBP, myelin basic protein; MOG<sub>35-55</sub>, myelin oligodendrocyte glycoprotein peptide 35–55; MS, multiple sclerosis; NPM, neural proliferation medium; ODM, oligodendrocyte differentiation medium; OL, oligodendrocyte; Olig, OL transcription factor; OPC, OL progenitor cell; P1 etc., post-natal day 1 etc; PDGF $\alpha$ , platelet-derived growth factor  $\alpha$ ; PDL, poly-D-lysine; PLP, proteolipid protein; PPAR, peroxisome proliferator-activated receptor; PT, pertussis toxin; qPCR, quantitative PCR; TNF $\alpha$ , tumour necrosis factor  $\alpha$ ; TRITC, tetramethylrhodamine  $\beta$ -isothiocyanate; UCP, uncoupling protein; WT, wild-type.

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limited. PPAR $\delta$  is expressed throughout the body in most tissues and is the most abundant PPAR in brain (Michalik et al., 2003). The knowledge that activation of PPAR $\delta$  regulates lipid metabolism (Basu-Modak et al., 1999; Kliewer et al., 2001; Rosenberger et al., 2002), raised the possibility that PPARs might regulate lipid metabolism in OLs (oligodendrocytes). Studies from the Skoff laboratory (Granneman et al., 1998) have shown that PPAR $\delta$  is the major PPAR isotype expressed in optic and sciatic nerve, and is mainly expressed in the OL population. A subsequent study (Saluja et al., 2001) confirmed that PPAR $\delta$ , but not PPAR $\gamma$ , selective agonists increased OL differentiation, including increasing MBP (myelin basic protein) and PLP (proteolipid protein) protein and mRNA levels.

Based on the above findings, we previously tested whether PPAR $\delta$  agonists could provide protection in EAE (Polak et al., 2005). We found that, in contrast with PPAR $\alpha$  and PPAR $\gamma$  agonists, treatment with a PPAR $\delta$  agonist did not significantly reduce disease severity during the early stages of EAE, but instead showed benefit when given at the peak of disease. This was accompanied by reductions in the appearance of cortical lesions, neuronal damage and glial inflammation. Moreover, in contrast with PPAR $\gamma$  agonists, the selective PPAR $\delta$  agonist GW0742 did not suppress pro-inflammatory cytokine production from T-cells, which may account for its reduced efficacy to influence early stages of EAE. GW0742 also caused an increase in myelin gene expression in EAE brains, suggesting a distinct mechanism of action possibly involving effects on OL maturation or survival.

Among the many factors implicated in OPC (OL progenitor cell) maturation are members of the BMP (bone morphogenetic protein) family. BMPs were originally identified as extracellular factors able to induce bone formation, but were later shown to be expressed in other tissues and play a role in development of other organs, including the nervous system (Goumans and Mummery, 2000; ten Dijke et al., 2003). BMPs belong to the TGF (transforming growth factor) superfamily which, upon binding to their cognate receptors, activate phosphorylation of Smad proteins, which in turn bind to specific promoter elements and regulate gene transcription. A key gene target of BMP signalling are Id (inhibitors of differentiation) proteins similar in structure to bHLH (basic helix-loop-helix) transcription factors, but lacking the DNA-binding domain (Miyazono and Miyazawa, 2002). Id proteins can therefore form heterodimers with other bHLH proteins, but the resulting complex is inactive. Both BMPs and their cognate receptors have been shown to be expressed by OLs during normal development (Cheng et al., 2007; See and Grinspan, 2009).

BMP signalling is regulated by interactions with a class of molecules referred to collectively as BMP antagonists (Yanagita, 2005) which function primarily by direct association with BMPs, thereby preventing binding to BMP receptors. BMP signalling has been shown to play a role in OL maturation and survival, since treatment with BMP

antagonists promotes OL maturation (Mehler et al., 1997; Mabie et al., 1999; Mehler et al., 2000; Mekki-Dauriac et al., 2002), and the BMP antagonist noggin induced oligodendrogenesis in human embryonic stem cells (Izrael et al., 2007). The ability of BMPs to reduce OL maturation is due in large part to formation of complexes between Id proteins and the bHLH proteins Olig1 (OL transcription factor 1) and Olig2 which have been well characterized for their involvement in OL maturation (Cheng et al., 2007; Bilican et al., 2008). Given the importance of this signalling system in OL maturation, and in view of the fact that GW0742 increased myelin expression, we hypothesized that the effects of PPAR $\delta$  agonists could involve modulation of the BMP signalling system.

In the present study we have used PPAR $\delta$ -null mice to show that the beneficial effects of GW0742 in EAE are dependent upon the presence of functional PPAR $\delta$ , and that PPAR $\delta$  plays a role in regulating the normal processes of OL maturation. We also demonstrate that the effects of PPAR $\delta$  involve regulation of BMP and BMP antagonist expression in OPCs and astrocytes. Taken together these findings support the concept that PPAR $\delta$  plays an important role in the normal maturation of OPCs, and suggest that PPAR $\delta$  agonists provide benefit in EAE by accelerating OPC maturation.

## MATERIAL AND METHODS

### Animals

Female C57BL/6 mice, aged 6–8 weeks, were from Charles River Breeding. PPAR $\delta$ -null mice were generated as previously described (Peters et al., 2000). Pregnant Sprague-Dawley rats were purchased from Charles River Breeding, and used to provide P1 (where P is post-natal day) pups. Mice were maintained in a controlled 12 h:12 h light/dark environment and provided food *ad libitum*. All experiments were approved by the local IACUC (Institutional Animal Care and Use Committee).

### Cell culture

Enriched cultures of primary mouse or rat astrocytes were prepared from P1 pups using procedures described previously, including complete change of media [DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal calf serum and antibiotics] every 3 days (Dello Russo et al., 2003). The cells reached confluency after 7–8 days. At that time the cells were shaken for 2 h to remove adhering microglia, and overnight at 225 rev./min at 37°C to dislodge OPCs. The remaining cells are approximately 95% astrocytes by staining for the astrocyte-specific protein GFAP (glial fibrillary acidic protein), and 5% adherent microglia.

OPCs were prepared from E13 (where E is embryonic day) mouse pups using a recently described method (Pedraza et al., 2008). In brief, E13 embryos were removed, and then washed in cold PBS; the cerebellum was removed and meninges dissected away. The tissue from six to eight brains was triturated by 40 passages through a 1 ml pipette tip in DMEM/F12 and B27 neuronal supplement, and then filtered through a 70  $\mu$ m-pore-size cell strainer. The cells were plated at the equivalent of two brains per T25 flask in 8 ml of NPM (neural proliferation medium) containing DMEM/F12/B27 neuronal supplement and 10 ng/ml EGF (epidermal growth factor; Sigma). The cells were passaged every 3 days by trituration and plated at a 1:5 ratio in NPM. After two passages, the cells were mechanically dissociated with a 1 ml pipette, then plated on to PDL (poly-D-lysine)-coated coverslips in NPM supplemented with 10 ng/ml bFGF (basic fibroblast growth factor) and 10 ng/ml PDGF $\alpha$  (platelet-derived growth factor  $\alpha$ ) [this is ODM (OL differentiation medium)].

For the data shown in Figure 2, cells were grown for 48 h on PDL-coated coverslips in ODM, the medium was then changed, and cells grown for a further 5 days in Sato-Bottenstein media in the absence of bFGF/PDGF, but containing 30 nM T3 and 10  $\mu$ M AraC to reduce astrocyte proliferation, and either 10  $\mu$ M of the PPAR $\gamma$  agonist pioglitazone, 10  $\mu$ M of the PPAR $\delta$  agonist GW0742, or the equivalent amount of DMSO vehicle to determine whether these drugs would induce further OPC maturation.

### Induction of EAE

EAE was actively induced in 6–8-week-old mice using synthetic MOG<sub>35–55</sub> (myelin OL glycoprotein peptide 35–55) as described previously (Feinstein et al., 2002). The MOG<sub>35–55</sub> peptide (MEVGWYRSPFSRWVHLYRNGK) was purchased from Anaspec. Mice were injected subcutaneously (two 100  $\mu$ l injections into adjacent areas in one hind limb) with an emulsion of 300  $\mu$ g of MOG<sub>35–55</sub> dissolved in 100  $\mu$ l of PBS, mixed with 100  $\mu$ l of complete Freund's adjuvant containing 500  $\mu$ g of *Mycobacterium tuberculosis* (Difco). Immediately after MOG<sub>35–55</sub> injection, the animals received an i.p. (intraperitoneal) injection of 200 ng of PT (pertussis toxin; List Biochemicals) in 200  $\mu$ l of PBS. At 2 days later, the mice received a second PT injection, and 1 week later they received a booster injection of MOG<sub>35–55</sub>.

### Clinical assessment of EAE

Clinical signs were scored on a 5 point scale: grade 0, no clinical signs; 1, limp tail; 2, impaired righting; 3, paresis of one hind limb; 4, paresis of two hind limbs; 5, death. Scoring was performed at the same time each day by a blinded investigator.

### Treatment of mice with PPAR $\delta$ agonist

The selective PPAR $\delta$  agonist GW0742 {4-[2-(3-fluoro-4-trifluoromethylphenyl)-4-methylthiazol-5-ylmethylsulfanyl]-2-methylphenoxy}-acetic acid} was synthesized at GlaxoSmithKline as described previously (Sznajdman et al., 2003) and was provided by Dr Tim Willson (GlaxoSmithKline,

Raleigh, NC, U.S.A.). Chow containing 100 p.p.m. GW0742 was prepared by Research Diets by mixing 100 mg of drug with 1 kg of Purina mouse chow 5001. Mice were provided free access to chow, and on average consumed 2 g per mouse per day, giving an average daily dose of 10 mg of GW0742/kg. This dose is similar to that previously used by our group and others for studies of other PPAR agonists in EAE models (Feinstein et al., 2002).

### mRNA analysis

Total RNA from cells and tissues was isolated using TRIzol<sup>®</sup> reagent (Invitrogen/Gibco); aliquots were converted into cDNA using random hexamer primers, and mRNA levels estimated by qPCR (quantitative PCR). PCR conditions were 35 cycles of denaturation at 94°C for 10 s, annealing at 58–64°C for 15 s and extension at 72°C for 20 s on a Corbett Rotorgene real-time PCR unit. PCR was performed using Taq DNA polymerase (Invitrogen), and contained SYBR Green (SybrGreen1 10000 $\times$  concentrate, diluted 1:10000; Molecular Probes). Relative mRNA concentrations were calculated from takeoff point of reactions using the software provided by the manufacturer, and normalized to  $\alpha$ -tubulin and GDH (glyceraldehyde-3-phosphate dehydrogenase) measured in the same samples. Melting curve analysis and agarose gel electrophoresis ensured production of single and correct sized products. Primers were derived from published sequence information using Perl Primer software (perlprimer.sourceforge.net) to generate PCR products ranging from 100 to 250 bp. The primers used were: *GDH* forward, 5'-GCCAAGTATGATGACATCAAGAAG-3'; *GDH* reverse, 5'-TCCAGGGTTTCTTACTCCTTGGGA-3'; *Olig1* forward, 5'-ATGCCATCGGTGTTCCGGACTTACT-3'; *Olig1* reverse, 5'-TGTGGTTAAGGACCAGCTGTGAA-3'; *GalC* (galactosyl ceramidase) forward, 5'-AGGACATGCGGACGTTACAGCTAA-3'; *GalC* reverse, 5'-TCCATAGGATCGTGCCGTTCAACA-3'; *CGT* (galactose ceramide galactosyl transferase) forward, 5'-ACATTTGCAGTTCTCC-TTGCTGCC-3'; *CGT* reverse, 5'-AAGGCTACTGAGTTGGGCTGATGT-3'; *PDGFR- $\alpha$*  forward, 5'-ACCTTGACACAATAACGGGAG-3'; *PDGFR- $\alpha$*  reverse, 5'-GAAGCCTTCTCGTGGACAG-3'; *PPAR $\delta$*  forward, 5'-GCCCAAGTTCGAGTTTGCTGTCAA-3'; *PPAR $\delta$*  reverse, 5'-TTAGCCACTGCATCATCTGGG-3'; *UCP3* (uncoupling protein 3) forward, 5'-GAGAGGAAATACAGAGGGAC-3'; *UCP3* reverse, 5'-GGGAGGTTGTGAGTAAACAG-3'; *ANGPTL-4* (angiopoietin-like 4) forward, 5'-GCCACCCACTTACACAGGCCG-3'; *ANGPTL-4* reverse, 5'-CCAGGCCAGCCAGAACTCG-3'; *Noggin* forward, 5'-TGAGCAAGAAGCTGAGGAGGAAGT-3'; *Noggin* reverse, 5'-AGGTGCACAGACTGGATGGCTTA-3'; *Gremlin* forward, 5'-ACAGAGCGCAAGTATCTGAAGCGA-3'; *Gremlin* reverse, 5'-AGGAGTTGCACTGGCCATAACAGA-3'; *Follistatin* forward, 5'-TGGA-TCTTGCAACTCCATCTCGGA-3'; *Follistatin* reverse, 5'-TGC-CCAAAGGCTATGTCAACACTG-3'; *Bambi* forward, 5'-TTCT-GTGTGGCTGGCCTGTTTC-3'; *Bambi* reverse, 5'-AGCAAGCTG-TGGAGAGGTCAAGAR-3'; *BMP2* forward, 5'-TGATCACCTG-AACTCCACCAACCA-3'; *BMP2* reverse, 5'-AACCTCC-ACAACCATGCTCTGAT-3'; *BMP4* forward, 5'-AGAAGTCCG-TGCCATTACTAT-3'; and *BMP4* reverse, 5'-AGTTGAGGTGATCAGCCAGTGGAA-3'.

## Immunocytochemical staining

E13 OPCs were grown on PDL-coated coverslips, treated and prepared for staining. Coverslips were then rinsed in PBS, fixed with 4% PFA (paraformaldehyde) for 10 min, rinsed in PBS, incubated with mouse anti-MBP (1:200; Chemicon), goat anti-noggin (1:40; R&D Systems), rabbit anti-PLP (1:300; Santa Cruz Biotechnology), rabbit anti-GFAP (1:500; Dako) or rabbit anti-PDGFR $\alpha$  (1:300; Santa Cruz Biotechnology) diluted in 1% donkey serum/PBS, overnight at 4°C. Cells were rinsed in PBS, incubated with anti-rabbit-TRITC (tetramethylrhodamine  $\beta$ -isothiocyanate, 1:200; Southern Biotechnology) and anti-mouse-FITC (1:200; Sigma) for 2 h at 37°C, rinsed in PBS, incubated with DAPI (at 1:500) in PBS for 10 min, rinsed in PBS, and then mounted on coverslips.

## Data analysis

Quantification of the cell numbers in Figure 2 was performed manually, and in Figures 4 and 6 performed using Zeiss Axiovision version 4.5. Comparisons between groups were made using a Student's unpaired *t* test. Comparisons of the number of stained cells in Figure 2 was done using  $\chi^2$  analysis. Comparison of clinical signs in WT (wild-type) compared with PPAR $\delta$ -null mice was performed by two-way repeated measures ANOVA using data from day 25 (the start of treatment) to the end of the study (day 49). Values are means  $\pm$  S.E.M., and for all comparisons significance was taken at  $P < 0.05$ .

## RESULTS

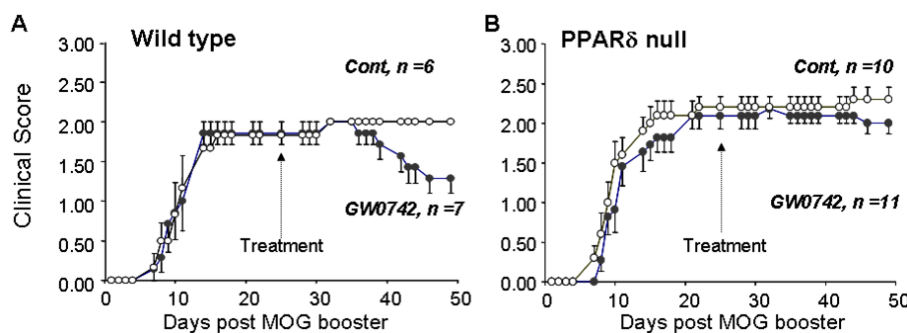
### PPAR $\delta$ mediates protective effects of GW0742

We have previously shown that treatment of C57BL/6 mice with the PPAR $\delta$  agonist GW0742 ameliorated clinical and

histological signs of EAE when administered to mice with moderate disease severity (Polak et al., 2005). To confirm that these effects were mediated via activation of PPAR $\delta$ , and not by off-target actions of the agonist, we tested whether GW0742 influenced the course of disease in PPAR $\delta$ -null mice in which endogenous PPAR $\delta$  is inactivated in all cells and tissues by insertion of the neomycin gene into the DNA-binding domain. As previously observed, providing GW0742 to WT mice at 25 days after immunization (at which time they show moderate clinical signs) significantly reduced clinical signs beginning approx. 15 days later (Figure 1A). Immunization of PPAR $\delta$ -null mice with MOG peptide resulted in a similar disease incidence and severity as WT mice, suggesting that PPAR $\delta$  does not play a significant role in the early stages of EAE. However, in contrast with the WT mice, treatment with GW0742 did not effect disease progression for up to 25 days of treatment (Figure 1B). This provides strong evidence that the effects of GW0742 are mediated through this receptor and are not due to off-target actions. Since the receptor is inactivated in all cells throughout the body, these results do not allow us to conclude whether the loss of GW0742 benefit is due to lack of PPAR $\delta$  from brain, or from some other tissue; however, our previous studies did not reveal any effect of GW0742 on splenic T-cells, suggesting that lack of PPAR $\delta$  from brain may account for the current findings.

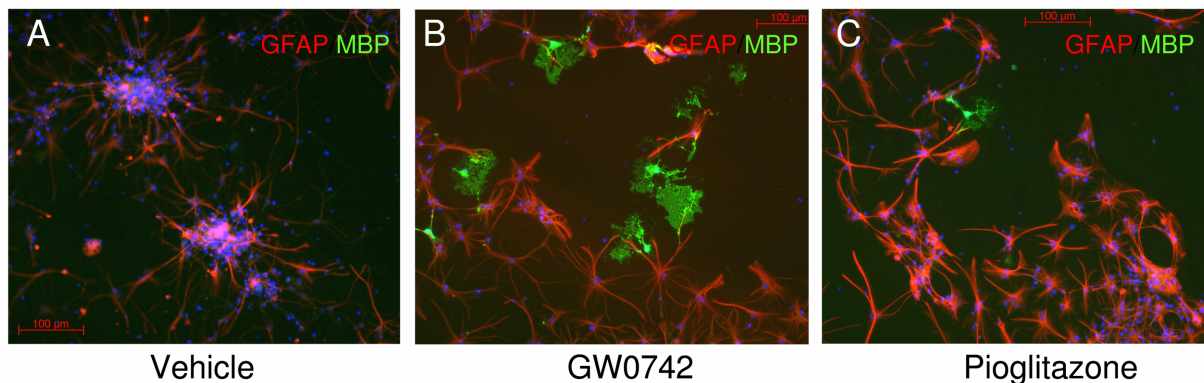
### Effects of GW0742 on OPC maturation

We hypothesized that GW0742 could provide benefit in EAE involving effects on OL maturation or survival. To address this, we first tested whether GW0742 influenced maturation of OPCs generated from E13 mice. These preparations are grown as neurospheres, then grown on PDL-coated plates in medium containing bFGF and PDGF which leads to differentiation (Pedraza et al., 2008). After 7 days growth on PDL-coated slides, OPCs show little staining for MBP (Figure 2A)



**Figure 1** GW0742 does not reduce EAE symptoms in PPAR $\delta$ -null mice  
 C57BL/6 control mice (A) and PPAR $\delta$ -null (B) mice were immunized with MOG<sub>35-55</sub> peptide and clinical scores monitored to 50 days. On day 25 the mice were split into two subgroups having comparable disease progression (the two subgroups are indicated by the open and closed circles) and were provided free access to chow containing 0, control (○) or 100 p.p.m. GW0742 (●). Values are means  $\pm$  S.E.M. of clinical scores. The incidence of disease reached 100% in all groups by day 14; there was no difference in the average day of onset ( $9.3 \pm 0.4$  compared with  $10.8 \pm 0.9$ , null compared with WT; means  $\pm$  S.E.M.). In the WT mice, there was a statistically significant effect of GW0742 on clinical scores over time (two-way repeated measures ANOVA of day 25–49 scores,  $F[14,1] = 2.75$ ,  $P = 0.0012$ ). Cont, control.





**Figure 2** Effect of GW0742 on OPC maturation

Mouse E13 OPCs were plated on to PDL-coated coverslips, grown for 48 h in differentiation medium, medium replaced with Sato-Bottenstein medium, and cells grown for a further 5 days in the absence of growth factors and in the presence of (A) vehicle, (B) 10  $\mu$ M GW0742 or (C) 10  $\mu$ M pioglitazone. Cells were fixed and stained for GFAP (TRITC, red), MBP (FITC, green) and nuclei [DAPI (4',6'-diamidino-2-phenylindole), blue]. Images shown are representative fields of view from three or four slides examined per condition. There was a significant increase in the number of GFAP<sup>+</sup> cells from 130/993 (13.0%) in vehicle-treated, to 140/643 (25.5%) in GW0742-treated cultures ( $P < 0.0001$ , as measured using a  $\chi^2$  test), but not in the pioglitazone-treated cultures (119/742; 16.1%). The number of MBP<sup>+</sup> cells was significantly increased from none in the vehicle-treated to 20/643 (4.0%) in the GW0742-treated cells ( $P < 0.0001$ ), but not in the pioglitazone-treated cultures (4/742; 0.5%).

and the presence of numerous GFAP<sup>+</sup>-stained cells. After 5 days growth in GW0742, the number of GFAP-stained cells was significantly increased from 13% (in vehicle-treated cultures) to 26% ( $P < 0.0001$ , as measured using a  $\chi^2$  test); and their morphology more closely resembled that of mature astrocytes. In these cultures we also observed an increased number of cells stained for MBP, which increased from none in vehicle-treated cultures to 4% in the treated cultures ( $P < 0.0001$ , as measured using a  $\chi^2$  test), as well as the appearance of myelin sheaths and longer processes (Figure 2B). Treatment with the PPAR $\gamma$  agonist pioglitazone did not significantly increase the number of GFAP<sup>+</sup>-stained cells (16% of all cells,  $P > 0.05$  compared with vehicle), although they again showed a more mature phenotype; very few cells (0.5% of all cells,  $P = 0.07$ ) showed positive staining for MBP (Figure 2C).

To look at the initial events involved in the effects of GW0742 on OPC maturation, we measured mRNA levels of known markers of OPC maturation (Figure 3). After treatment of E13 OPCs with 3  $\mu$ M GW0742 for 24 h, there was a significant increase in expression of *Olig1*, *CGT*, *PDGFR $\alpha$*  and an increase in *GaIC* compared with vehicle-treated cells (Figures 3A–3D). However, at this timepoint there was no change in *PLP* mRNA levels (Figure 3E). Levels of PPAR $\delta$  were also significantly increased by GW0742 (Figure 3F), as were levels of the well-characterized PPAR $\delta$  target gene *ANGPTL-4*, although interestingly, not of a second target gene *UCP3* (Figures 3G and 3H).

Staining for the early OPC marker PDGFR $\alpha$  confirmed that treatment with GW0742 for 24 h increased OPC maturation as indicated by an increase in cell migration (Figure 4). GW0742 did not modify the total number of spheres present; however, the average size of the spheres was significantly reduced (diameter of  $40.7 \pm 2.1$   $\mu$ m compared with  $30.4 \pm 1.6$

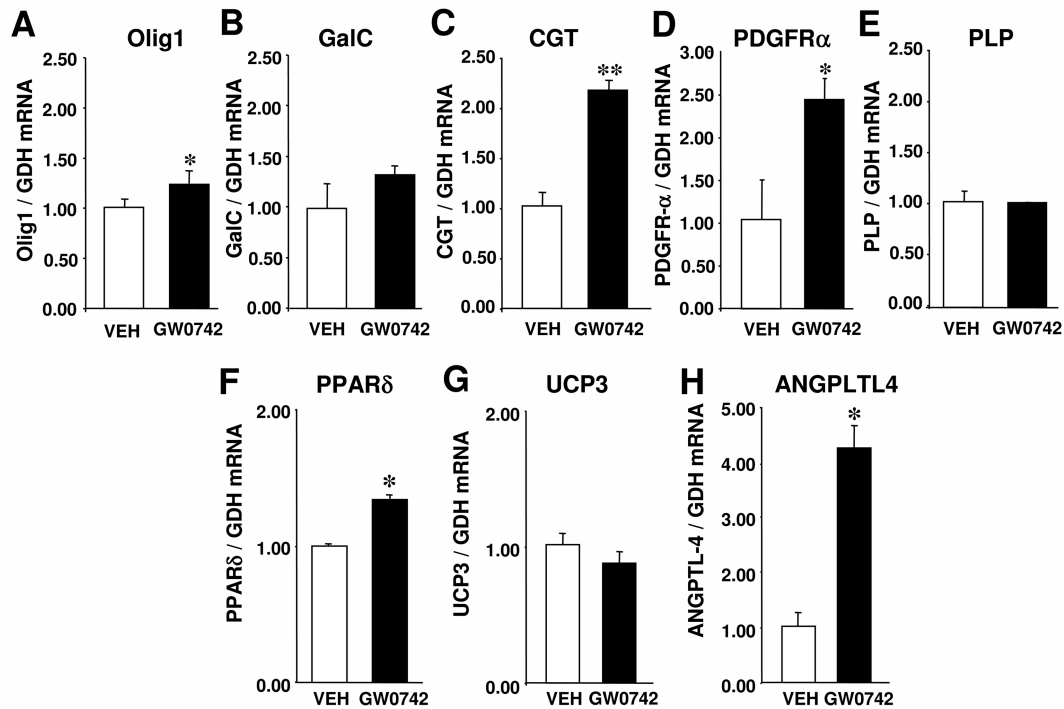
$\mu$ m,  $P < 0.0005$ ) (Figures 4A and 4B). At the same time, the total number of cells that migrated out from the spheres was significantly increased by GW0742 ( $814 \pm 38$  compared with  $1135 \pm 105$  cells per field; DMSO compared with GW0742,  $P < 0.05$ ) (Figures 4C and 4D), and those cells showed a greater number of processes (Figures 4E and 4F).

### PPAR $\delta$ mediates OPC maturation

To determine whether endogenous PPAR $\delta$  plays a role in normal OL maturation, we prepared primary OPCs from E13 WT and PPAR $\delta$ -null mice and examined process formation under full differentiation conditions (e.g. medium containing PDGF $\alpha$ , bFGF and B27 supplement). After 7 days, WT cells had more primary processes than did PPAR $\delta$ -null cells (Figure 5A), and quantification of process number (Figure 5B) revealed that PPAR $\delta$ -null cells had significantly fewer processes per cell ( $2.3 \pm 0.04$ ) than WT cells ( $3.1 \pm 0.1$ ). Analysis of process distribution (Figure 5C) showed a left shift in the average number of processes per cell in PPAR $\delta$ -null cells, suggesting that process maturation was not completely inhibited, but either temporally delayed or limited to fewer processes in the null cells.

### GW0742 increases noggin expression

In view of reports that BMPs inhibit OL maturation (Gross et al., 1996; Hardy and Friedrich, 1996; Zhu et al., 1999; Mehler et al., 2000; Mekki-Dauriac et al., 2002; Gomes et al., 2003), we hypothesized that GW0742 might act by increasing BMP antagonist expression. Immunostaining of E13 OPCs treated for 24 h with GW0742 showed staining for the BMP antagonist noggin, primarily in the non-migrating cells which remained within spheres (Figures 6A and 6B). Quantification of cell numbers revealed no significant effect of treatment on



**Figure 3** Effects of GW0742 on myelin gene and PPAR $\delta$  target gene expression  
 Mouse E13 OPCs were plated on to PDL-coated coverslips, grown for 5 days in differentiation medium, medium replaced with Sato-Bottenstein medium, and cells grown in the absence of growth factors and in the presence of vehicle or 3  $\mu$ M GW0742. After 24 h, relative levels of (A) *Olig1*, (B) *GaIC*, (C) *CGT*, (D) *PDGFR $\alpha$* , (E) *PLP*, and the PPAR $\delta$  target genes (F) *PPAR $\delta$* , (G) *UCP3*; and (H) *ANGPTL4* mRNAs were determined by qPCR. Values are shown as means  $\pm$  S.E.M. of  $n=3$  samples in each group, normalized to values measured for *GDH* in the same samples; and then normalized values in vehicle treated samples are set to 1.0; \* $P<0.05$  and \*\* $P<0.01$ , compared with vehicle (VEH).

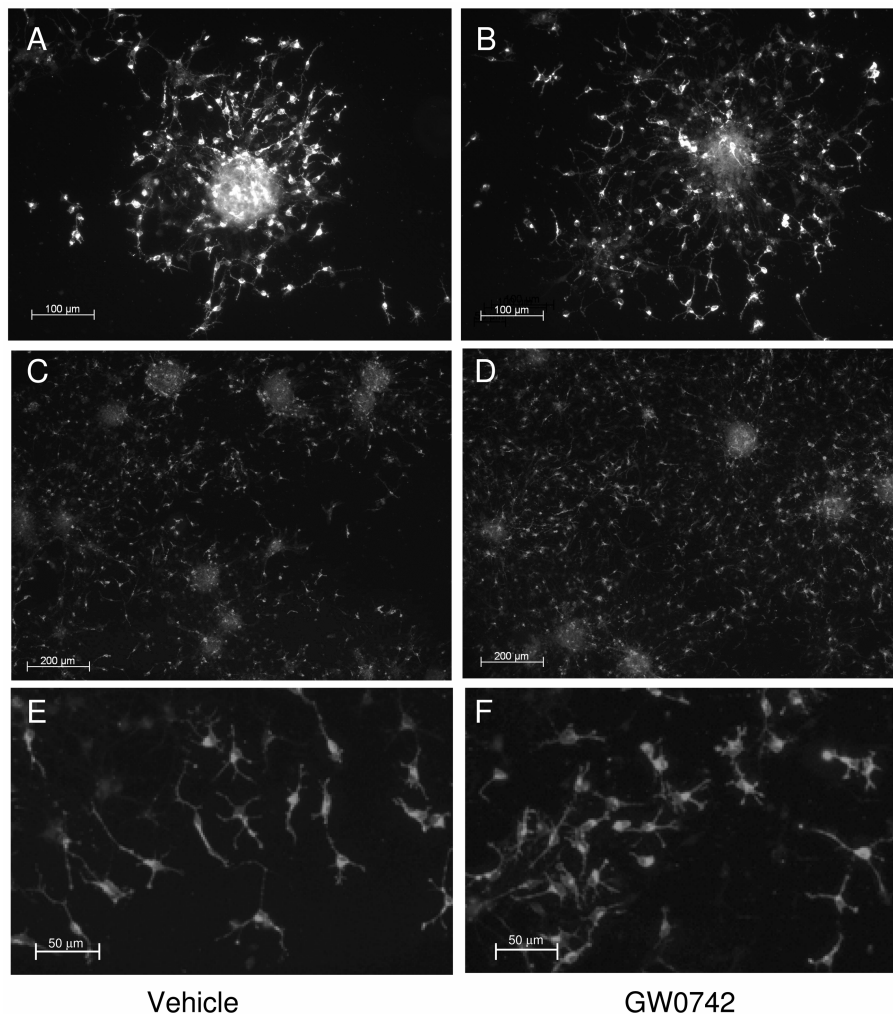
the total number of spheres ( $20.4 \pm 3$  compared with  $28.0 \pm 5$ , average number of spheres per field, DMSO compared with GW0742,  $P>0.05$ ). However, the number of noggin-stained spheres was significantly increased (from  $7.4 \pm 2$  to  $21.4 \pm 4$  per field,  $P<0.01$ ). This increased number of noggin-stained spheres reflects a significant increase from  $40 \pm 10\%$  to  $79 \pm 6\%$  ( $P<0.01$ ) of all spheres. We did not observe any significant co-localization of noggin with either PDGFR $\alpha$  (Figures 7A and 7B), PLP (Figures 7C and 7D) or GFAP (Figures 7E and 7F), suggesting that expression was restricted to immature progenitor cells. Analysis by qPCR showed that, after 24 h of treatment, GW0742 did not increase *noggin* mRNA levels, although we did observe a significant decrease in both *BMP2* and *BMP4* mRNA levels (Figure 8A).

Since there are some astrocytes present in the OPC cultures, we tested whether GW0742 influenced BMP or BMP antagonist expression in enriched astrocyte cultures. After 24 h we observed a significant increase of *noggin* mRNA levels in astrocytes (Figure 8B); interestingly, this increase appeared to be selective for *noggin* since mRNA levels of other BMP antagonists (*gremlin*, *folistatin* and *bambi*) were not increased (in fact *gremlin* mRNA levels were significantly reduced). In contrast with OPCs, GW0742 did not increase *PPAR $\delta$*  mRNA levels (Figure 9A). Interestingly, GW0742 caused a significant increase in the PPAR $\delta$  target gene UCP3, but not in ANGPTL-4.

Consistent with the increase in *noggin* mRNA, we observed a large increase in *noggin* staining, present in vesicular structures around the nucleus of primary astrocytes treated with 3  $\mu$ M GW0742 for 24 h (Figure 10).

## DISCUSSION

In the present study we show that PPAR $\delta$  is involved in the regulation of OPC maturation and is associated with changes in the expression of BMPs and BMP antagonists. We previously have shown that treatment of EAE-immunized mice with GW0742 did not offer significant protection when administered early during disease evolution, but instead reduced clinical signs when given to mice showing moderate clinical signs (Polak et al., 2005). In those studies, GW0742 did not suppress the ability of T-cells to produce IFN $\gamma$  (interferon  $\gamma$ ), providing a possible explanation for its inability to reduce early-stage EAE. In contrast, GW0742 significantly increased *PLP* and *MBP* mRNA levels in EAE mice, suggesting possible effects on OL maturation, survival or proliferation. The findings of the present study support this possibility since, in E13 OPCs, GW0742 induced maturation as determined by



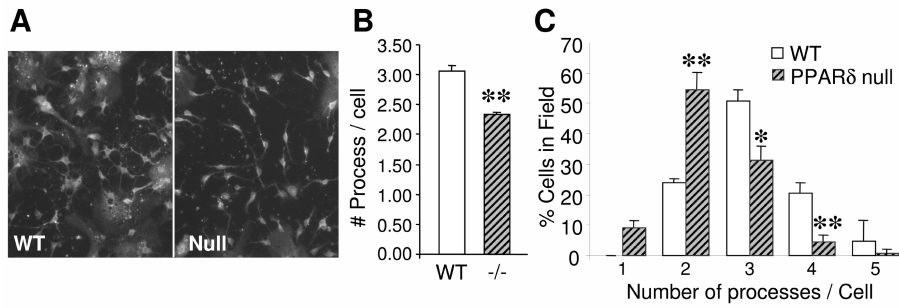
**Figure 4** Effect of GW0742 on PDGFR $\alpha$  expression

Mouse E13 OPCs were plated on to PDL-coated coverslips, grown for 5 days in differentiation medium, the medium replaced with Sato–Bottenstein medium, and the cells grown for 24 h in the absence of growth factors and in the presence of vehicle (A, C and E) or 3  $\mu$ M GW0742 (B, D and F). Cells were fixed, stained for PDGFR $\alpha$ , and cell numbers and size quantified in six fields of view taken at 20 $\times$  magnification. The total number of spheres ( $16.8 \pm 2.5$  compared with  $15 \pm 1.3$  spheres per field, DMSO compared with GW0742,  $P > 0.05$ ) was not modified by treatment. (A and B) are representative images showing the smaller average size of spheres in GW0742-treated cultures ( $40.7 \pm 2.1$  compared with  $30.4 \pm 1.6$   $\mu$ m diameter,  $P < 0.0005$ ). (C and D) are representative images showing increased numbers of PDGFR $\alpha$ -stained migrating cells ( $814 \pm 38$  compared with  $1135 \pm 105$  cells per field, DMSO compared with GW0742,  $P < 0.05$ ). (E and F) are representative images showing increased number of processes on the migrating cells in the GW0742-treated cultures.

increased myelin gene expression, increased myelin sheets and increased numbers of pre-myelinating OPCs. The finding that mRNA levels for PLP were not increased suggests that GW0742 affects earlier stages of OPC maturation, although whether PLP is increased at later times is not yet known. Overall, these findings are in agreement with earlier results showing that the weaker PPAR $\delta$  agonist bromopalmitate (Granneman et al., 1998) and the more selective agonist L-796449 (Saluja et al., 2001) induced myelin expression in post-natal mouse OPC cultures. Furthermore, the results that GW0742 was ineffective at reducing clinical signs in PPAR $\delta$ -null mice confirms that the actions of this drug

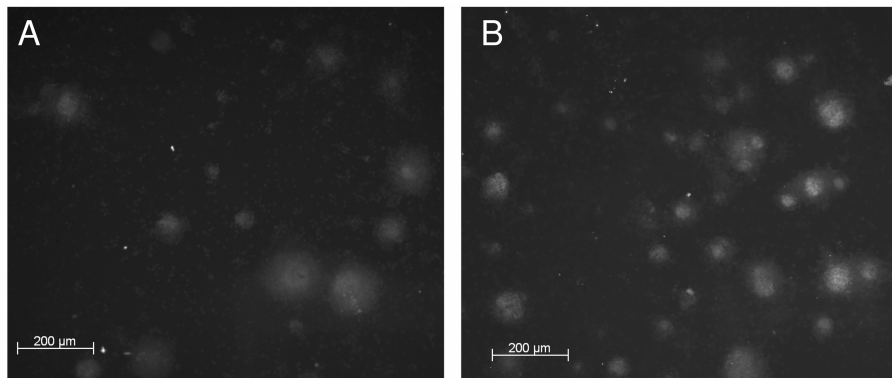
depend upon PPAR $\delta$  and are not due to off-target effects as has been reported for other PPAR agonists (Dello Russo et al., 2003).

We examined the specificity of PPAR agonist effects by comparing the actions of GW0742 with those of the selective PPAR $\gamma$  agonist pioglitazone on OPC maturation (Figure 2). Increased MBP staining was seen primarily in the GW0742-treated OPCs, suggesting a more important role for PPAR $\delta$  as compared with PPAR $\gamma$  in the OPC maturation process. Interestingly, treatment with either GW0742 or pioglitazone led to more mature astrocyte morphology in the GFAP-positive-stained cells, suggesting that both PPARs may be



**Figure 5 PPAR $\delta$  deficiency reduces OPC maturation**

(A) OPCs from WT and PPAR $\delta$ -null mice were plated on to PDL coverslides and kept in full differentiation medium for 7 days, after which the number of primary processes per cell were counted in three different slides of each, with approx. 100 cells counted per slide. (B) Average number of processes per cell (a total of 300–325 cells were counted in each group). (C) The percentage of cells having the indicated number of processes. Values are means  $\pm$  S.D.; \* $P$ <0.05 and \*\* $P$ <0.005, compared with WT.



**Figure 6 GW0742 increases noggin expression in OPC cultures**

Mouse E13 OPCs were grown for 5 days in differentiation medium, then treated with (A) vehicle or (B) 3  $\mu$ M GW0742 for 24 h. Cells were then fixed and immunostained for noggin. Cell counting was carried out to quantify the total number of spheres and total number of spheres stained for noggin in seven fields of view for each condition taken at 20 $\times$  magnification. There was a similar number of spheres in the two groups (20.4  $\pm$  3 compared with 28.0  $\pm$  5, average number of spheres per field, DMSO compared with GW0742,  $P$ >0.05). GW0742 significantly increased the number of noggin-stained spheres (from 7.4  $\pm$  2 to 21.4  $\pm$  4 per field,  $P$ <0.01) and the percentage of noggin-stained spheres increased from 40  $\pm$  10% to 79  $\pm$  6% ( $P$ <0.01). Representative images are shown for each condition.

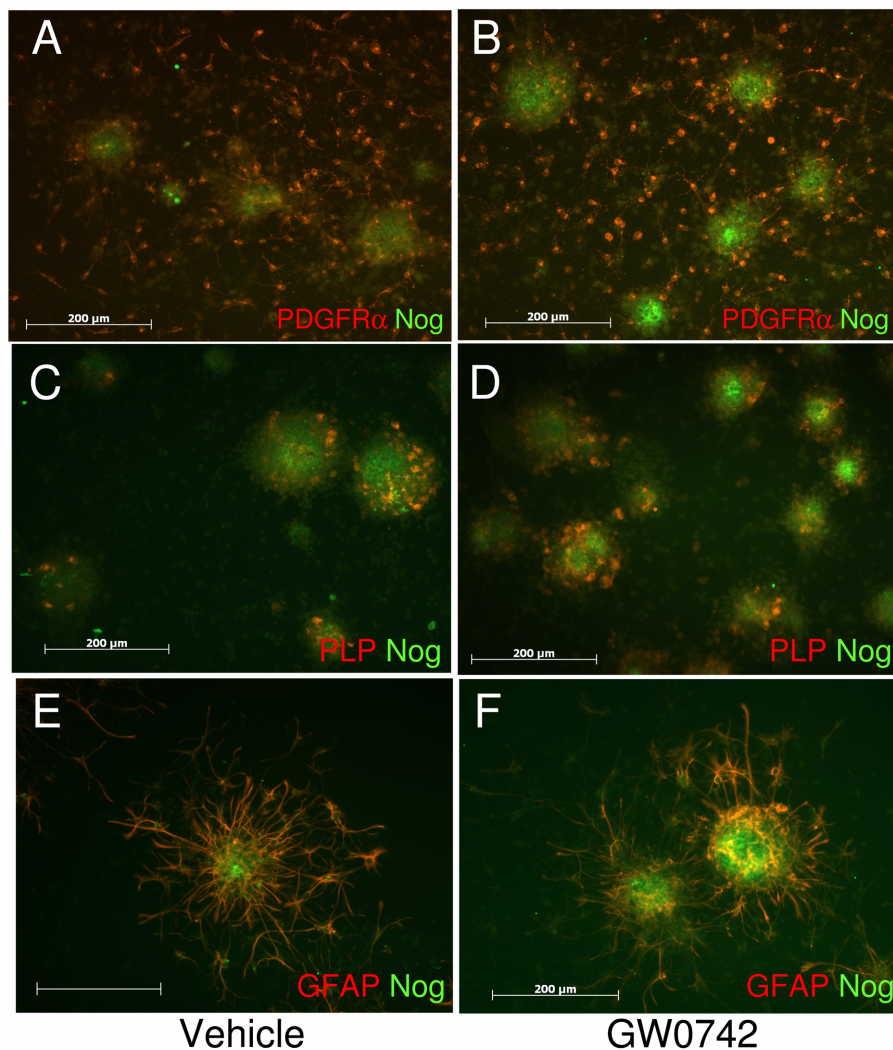
involved in astrocyte maturation. Our findings also show that endogenous PPAR $\delta$  plays a role in OPC maturation, since in PPAR $\delta$ -deficient OPCs the distribution and average number of processes was significantly reduced compared with WT OPCs. It should be noted that, despite the absence of PPAR $\delta$ , these OPCs still developed processes and sheaths, but OPC maturation was reduced, pointing to PPAR $\delta$  as a modulator of the maturation process. PPAR $\delta$  may be considered as a potential feed-forward factor along OPC maturation, even though it is not yet clear at which step through the maturation PPAR $\delta$  exerts its role.

We focused attention on the class of BMPs and BMP antagonists for several reasons. Numerous studies have shown a role for BMPs in regulating neural stem cell commitment (Kondo and Raff, 2004; Gaughwin et al., 2006; Hampton et al., 2007a; Talbott et al., 2006; Cheng et al., 2007); and in restricting OPC maturation during normal

development (Gross et al., 1996; Hardy and Friedrich, 1996; Zhu et al., 1999; Mehler et al., 2000; Mekki-Dauriac et al., 2002; Gomes et al., 2003). This has been thought to be a BMP-dependent induction of Id proteins that can bind to and inactivate the bHLH proteins Olig1 and Olig2 (Samanta and Kessler, 2004) which promote OPC maturation.

Recently, it was shown that in EAE, *BMP4*, -6 and -7 are up-regulated in lumbar spinal cord; with *BMP4* being the most abundant mRNA detected, and being detected in astrocytes as well as in oligodendrocytes and macrophages (Ara et al., 2008). In a second related study, it was found that *BMP4* and *BMP7* were increased following lysolecithin-induced demyelination, and interestingly that phosphorylated Smad 1/5/8 was detected in astrocytes (Fuller et al., 2007), suggesting that BMPs can induce astrogliosis and inhibit remyelination. Taken together, these findings raised the possibility that treatments or interventions that increase BMP





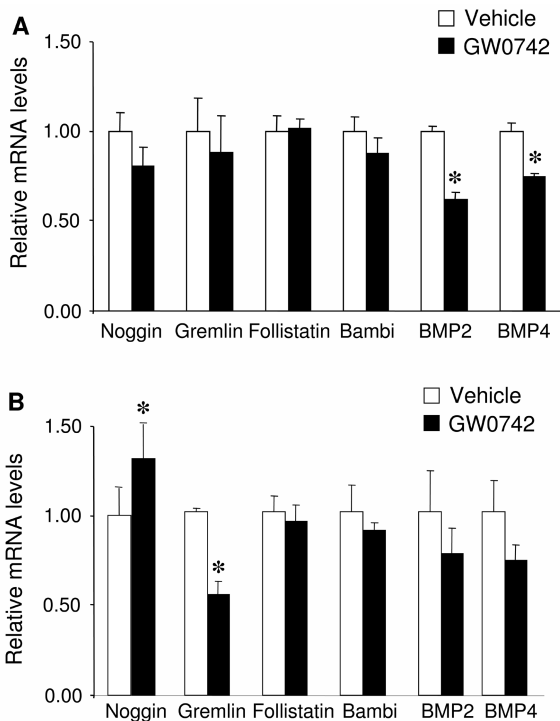
**Figure 7** **Noggin does not co-localize with OPC markers**

Mouse E13 OPCs were grown for 5 days in differentiation medium, then treated for 24 h with 3  $\mu$ M GW0742 or vehicle. Cells were then fixed and immunostained for noggin (FITC, green) together with PDGFR $\alpha$  (A and B), PLP (C and D) or GFAP (E and F). Representative images are shown for each condition. Nog, noggin.

antagonist expression, or reduce BMP expression, would facilitate OPC maturation. Several previous studies have shown that PPAR agonists can influence BMP signalling. In human umbilical vein endothelial cells, pioglitazone suppressed *BMP2* expression (Zhang et al., 2008), several PPAR $\gamma$  agonists decreased *BMP2* expression in human osteoblasts (Lin et al., 2007), and in mouse gonadotropinoma cells, pioglitazone reduced BMP signalling including activation of Id1 expression and DNA synthesis (Takeda et al., 2007). In the present study we extend this list by demonstrating that a PPAR $\delta$  agonist can decrease *BMP2* and *BMP4* expression in OPCs, and to our knowledge this is the first demonstration of any PPAR agonist increasing a BMP antagonist.

Our results point to distinct effects of GW0742 on BMPs and their antagonists' in astrocytes and OPCs. In OPCs

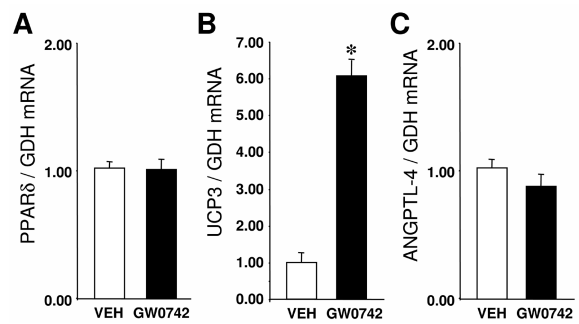
GW0742 primarily affected expression of *BMP2* and *BMP4* mRNAs with lesser effects on the BMP antagonists; in astrocytes the PPAR $\delta$  agonist had greater effects on expression of the BMP antagonists noggin and gremlin, with smaller effects on BMPs. The lack of significant changes in *BMP2* and *BMP4* expression in GW0742-treated astrocytes, together with down-regulation of the same BMPs in GW0742-treated OPCs, suggests the possibility of an autocrine short-range effect of BMPs in astrocytes and OPCs. This may be similar to the balance of interactions between BMPs and their antagonists that occur in the optic nerve which result in enhanced OPC maturation (Kondo and Raff, 2004). This may guarantee cellular identity for astrocytes and precursor cell identity for OPCs. On the other hand, increase in noggin expression in astrocytes following GW0742



**Figure 8 Regulation of BMP and BMP antagonist expression by GW0742**  
 Mouse E13 OPCs (A) grown for 5 days in full medium and primary rat astrocytes (B) were treated with 3  $\mu$ M GW0742 or vehicle for 24 h, and then RNA was isolated and qPCR used to measure relative mRNA levels of indicated genes. Values are means  $\pm$  S.E.M. of the relative mRNA level normalized to values for *GDH* measured in the same samples,  $n=3$ . The mRNA values for the vehicle groups were set to 1.0; \* $P<0.05$ , compared with the corresponding vehicle treated sample (as measured using an unpaired Student's *t* test).

treatment could represent a modulatory mechanism of the intercellular communication between astrocyte and OPCs, and may suggest a PPAR $\delta$ -mediated role of astrocytes in OPC maturation.

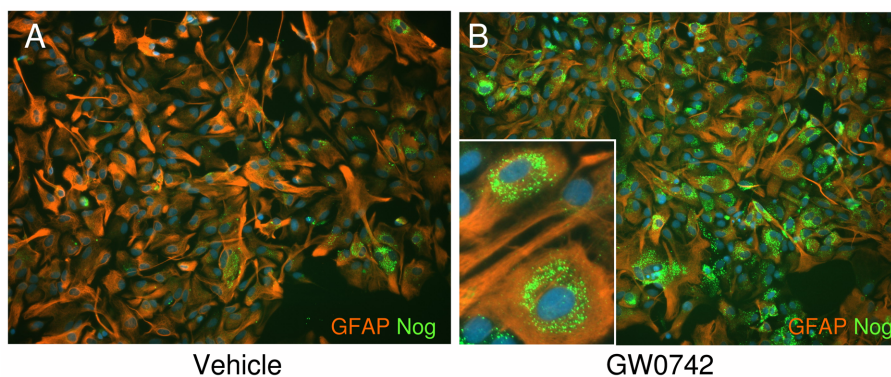
At the protein level we observed in both OPCs and astrocytes that GW0742 treatment increased staining for noggin protein, although the increase was more robust in the



**Figure 9 Regulation of PPAR $\delta$  target gene expression in astrocytes**  
 Primary rat astrocytes were treated with 3  $\mu$ M GW0742 or vehicle (VEH) for 24 h then RNA isolated and qPCR used to measure relative mRNA levels of (A) PPAR $\delta$  and its target genes, (B) *UCP3* and (C) *ANGPTL-4*. Values are means  $\pm$  S.E.M. of the relative mRNA level normalized to values for *GDH* measured in the same samples,  $n=3$ . The mRNA values for the vehicle groups were set to 1.0; \* $P<0.05$ , compared with the corresponding vehicle-treated sample (as measured using an unpaired Student's *t* test).

primary astrocyte cultures. Whereas increased staining could be due to an overall increase in noggin expression, the fact that noggin is normally released raises the possibility that GW0742 reduced release from astrocytes. If so, the absence of strong intracellular noggin staining in the OPCs could be due to increased release owing to GW0742, suggesting cell-specific means of regulating release. The greater increase in primary astrocyte cultures compared with the astrocytes present in the OPC cultures could also be due to differences in the maturation state of astrocytes, suggesting that only more mature cells can highly express noggin.

Previous characterization of BMPs and BMP antagonists in astrocytes is limited. In optic nerve astrocytes, the mRNAs for *gremlin*, *follistatin*, *chordin* and *bambi*, but not *noggin*, were detected (Wordinger et al., 2002); and *noggin* mRNA was expressed in type 1 astrocytes in P6 rat optic nerve (Kondo and Raff, 2004). Immunohistochemical staining demonstrated that noggin was primarily expressed in astrocytes in the dorsal spinal cord following rhizotomy, but was absent from non-injured spinal cords (Hampton et al., 2007b).



**Figure 10 GW0742 increases noggin expression in astrocytes**  
 Primary rat astrocytes were treated for 24 h with 3  $\mu$ M GW0742 or vehicle, after which cells were fixed and immunostained for noggin (Nog) and GFAP.

Astrocytes have been shown to express *BMP4* in the adult rat CNS (Mikawa et al., 2006), *BMP4* and 7 primarily in olfactory bulb astrocytes throughout development (Peretto et al., 2002), and the mRNAs for *BMP2*, 4, 5 and 7 in cultured adult optic head astrocytes (Wordinger et al., 2002). It has also been shown that *BMP2/4* is increased in ischaemic astrocytes (Xin et al., 2006), and that the mRNA levels of *BMP4*, but not *BMP2*, are increased in astrocytes after spinal cord injury (Chen et al., 2005), suggesting regulation following injury. These indications that BMP levels are increased under pathological conditions are consistent with our findings that a treatment to reduce pathology leads to a reduction in astroglial BMP levels.

The findings in the present study that agonists of PPAR $\delta$  can down-regulate the BMP signalling system may be of particular relevance during diseases such as EAE since inflammatory conditions have been shown to increase BMP signalling. For example, in prostate cancer cells, NF- $\kappa$ B (nuclear factor  $\kappa$ B) binds to the *BMP2* promoter and induces *BMP2* expression, and in chondrocytes, TNF $\alpha$  (tumour necrosis factor  $\alpha$ ) induces *BMP2* (Fukui et al., 2006), most probably by also binding to the *BMP2* promoter (Feng et al., 2003). It is known that certain cytokines including TNF $\alpha$  and IFN $\gamma$ , which are present in the EAE brain, cause reversible inhibition of OPC proliferation and maturation (Agresti et al., 1996), and that neurogenesis is sensitive to the inflammatory milieu and that chronic inflammation can reduce neurogenesis (Monje et al., 2003; Pluchino et al., 2008; Ekdahl et al., 2009; Whitney et al., 2009). Therefore, in addition to direct effects on BMPs and BMP antagonists, it is likely that PPAR $\delta$  down-regulates BMP signalling by attenuating inflammatory activation, as it has been shown to do in different cells and tissues (Delerive et al., 1999; Planavila et al., 2005; Kilgore and Billin, 2008; Smeets et al., 2008).

In summary, the results of the present study confirm that PPAR $\delta$  mediates the effects of the synthetic agonist GW0742 in EAE and also plays a role in normal OPC maturation. Treatment with GW0742 regulates both BMP, as well as BMP antagonist, expression in astrocytes and OPCs, although with distinct effects. The molecular mechanisms underlying the ability of PPAR $\delta$  agonists to modulate BMP and BMP antagonist regulation could involve both direct effects on transcription via binding to PPAR-responsive elements, and indirect effects due to anti-inflammatory actions which could reduce inflammatory up-regulation of BMPs. The recent demonstration that PPAR $\gamma$  agonists can be protective in relapsing/remitting MS patients (Kaiser et al., 2009), together with the observations that PPAR $\delta$  agonists promote OPC maturation, suggests that clinical trials of pure or mixed PPAR agonists may be of therapeutic value in the treatment of MS.

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