PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ LIGANDS REGULATE NEURAL STEM CELL PROLIFERATION AND DIFFERENTIATION IN VITRO AND IN VIVO

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ABSTRACT

Peroxisome proliferator-activated receptor gamma (PPARγ) belongs to a family of ligand-activated nuclear receptors and its ligands are known to control many physiological and pathological situations. Its role in the central nervous system has been under intense analysis during the last years. Here we show a novel function for PPARγ in controlling stem cell expansion in the adult mammalian brain. Adult rats treated with pioglitazone, a specific ligand of PPARγ, had elevated numbers of proliferating progenitor cells in the subventricular zone and the rostral migratory stream. Electron microscopy analysis also showed important changes in the subventricular zone ultrastructure of pioglitazone-treated animals including an increased number of migratory cell chains. These results were further confirmed *in vitro*. Neurosphere assays revealed significant increases in the number of neurosphere forming cells from pioglitazone- and rosiglitazone (two specific ligands of PPARγ receptor)-treated cultures that exhibited enhanced capacity for cell migration and differentiation. The effects of pioglitazone were blocked by the PPARγ receptor antagonists GW9662 and T0070907, suggesting that its effects are mediated by a mechanism dependent on PPARγ activation. These results indicate for the first time that activation of PPARγ receptor directly regulates proliferation, differentiation, and migration of neural stem cells *in vivo*.

INTRODUCTION

The ability of the adult central nervous system to produce new neurons is limited, rendering the brain particularly vulnerable to injury and disease. In mammals, the majority of neurons are born by the prenatal period, but it is well established that neurons continue to arise in two niches of the adult brain, the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (Gage et al. 1998; Luskin et al. 1997). Progenitor cells in the SVZ migrate to the olfactory bulb (OB) through the rostral migratory stream (RMS) where they differentiate into granule and periglomerular neurons. In the hippocampus, new neurons arise in the subgranular zone of the dentate gyrus. Both cell-extrinsic and cell-intrinsic factors have been shown to influence the maintenance and regulation of the neurogenic system *in vivo* (Ostenfeld and Svendsen 2003). Among the extrinsic factors a number of growth factors have been shown to affect the proliferation and differentiation of precursor cell populations, including insulin-like growth factor-1 (Anderson et al. 2002), epidermal growth factor (EGF) (Doetsch et al. 2002), basic fibroblast growth factor (bFGF) (Bartlett et al. 1995; Johe et al. 1996), and other factors like the brain-derived neurotrophic factor (Larsen et al. 2007) and Noggin (Lim et al. 2000). Additionally, adult neurogenesis has also been shown to be influenced by the activation of several intrinsic transcription factors among which are the transcription factors Pax 6 (Hack et al. 2005; Kohwi et al. 2005), Notch 1 (Lutolf et al. 2002), and Mash 1 (Parras et al. 2004).

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear receptor family of transcription factors, which includes different proteins mediating ligand-dependent transcriptional activation (Debril et al. 2001; Dreyer et al. 1992; Kliewer et al. 1994; Michalik et al. 2006). PPARγ participates in many biological processes including adipocyte differentiation, lipid and glucose homeostasis (Desvergne and Wahli 1999; Rosen and Spiegelman 2001; Tontonoz et al. 1994), and regulation of inflammatory responses (Ricote et al. 1999; Szeles et al. 2007). Several natural and synthetic ligands for PPARγ have been described. Among the natural ligands, polyunsaturated fatty acids and some prostaglandins and leukotrienes have been shown to be potent activators of this receptor (Forman et al. 1995; Jiang et al. 1998; Paruchuri et al. 2008). Synthetic ligands include the anti-diabetic thiazolidinediones (TZDs) such

as troglitazone, pioglitazone, and rosiglitazone (Blaschke et al. 2006; Desvergne and Wahli 1999; Rosen and Spiegelman 2001) as well as some new compounds, the aryl-tyorosine derivatives, described as novel PPARγ ligands (Brown et al. 1999).

Beyond these functions, recent data have shown that PPARγ acts as a regulator of central nervous system inflammation (Heneka et al. 2005) and is a powerful pharmacological target for counteracting neurodegeneration, as shown in animal models (e.g. of Parkinson's and Alzheimer's diseases) review in (Heneka and Landreth 2007). In this context, data from animal experiments strongly indicate that ligands of PPARγ confer neuroprotection and neurological improvement following brain injury (Abdelrahman et al. 2005; Bernardo et al. 2000; Landreth and Heneka 2001; Townsend and Pratico 2005) and PPARγ ligands, including the antidiabetic thiazolidinediones (TZDs), have been implicated in anti-inflammatory process in diverse tissues, including the brain (Kielian and Drew 2003; Ricote et al. 1999). Consistent with this idea, we have recently demonstrated that different members of the thiadiazolidinone (TDZD) family (chemical structure-related derivatives to TZDs) inhibited the activation of astrocytes and microglial cells *in vitro* and are potent anti-inflammatory and neuroprotective agents against kainic acid-induced *in vivo* excitotoxicity, through a mechanism apparently involving PPARγ activation (Luna-Medina et al. 2005; Luna-Medina et al. 2007b). We found a significant preservation of hippocampal cells in TDZD-injected rats compared with abundant neuronal loss in CA1, CA3, and hilus after injection with kainic acid (Luna-Medina et al. 2007b), suggesting a possible neurogenic action of the TDZD compound. Also, we have preliminary data showing that the TDZD compound NP031112, a potent anti-inflammatory and neuroprotective agent, which is currently under Phase II clinical trial for the treatment of Alzheimer´s disease, is a powerful inducer of neurogenesis in the two neurogenic niches, subventricular zone and hippocampus, of adult rats (Luna-Medina et al. 2007a).

On the basis of the previous evidence, in this study we were interested in determining whether activation of PPARγ might have a role in the proliferation, differentiation, and migration of neural progenitor cells. Our studies showed that pioglitazone, a specific ligand of PPARγ, is a potent inducer of neuroblasts formation and migration in the SVZ of adult rats. *In vitro*, PPARγ ligands increased the number, differentiation and

migration capacity of adult rat neurospheres. Altogether, these findings suggest that ligands of PPARγ regulate neural progenitor cell proliferation and migration and may influence their differentiation in adult forebrain.

MATERIALS AND METHODS

Animals. All animal related procedures were approved by the Laboratory Animal Care and Use Committee of the Consejo Superior de Investigaciones Científicas and were conducted in accordance with the guidelines of the European Communities Council, directive 86/609/EEC. All efforts were made to minimize animal suffering and to reduce the number of animals used. Adult (8-12 weeks old) Wistar rats were used throughout the study.

Pioglitazone and bromodeoxyuridine administration. Adult male Wistar rats (*n*=5 per group) housed in a 12-hour light-dark cycle animal facility received daily intragastrical administration of pioglitazone (20mg/kg body weight) for 3 consecutive days. This dose of pioglitazone was chosen because it has been shown to be effective *in vivo* in different previously published works (Breidert et al. 2002; Ji et al. 2009; Peiris et al. 2007; Saitoh et al. 2007; Schmerbach et al. 2008). In order to label the entire population of fast proliferating SVZ and RMS cells, rats were intraperitoneally injected with 5-bromo-2-deoxyuridine (BrdU, 50 mg/kg) 24 h or 21 days before sacrifice.

Tissue preparation,histology and immunohistochemistry. After treatment, the animals were anaesthetized and perfused transcardially with 4% paraformaldehyde solution. The brains were removed, postfixed in the same solution at 4ºC overnight, cryoprotected in the paraformaldehyde solution containing 30% sucrose, frozen, and 30 µm coronal and sagital sections were obtained in a cryostat. Free floating sections were processed for cresyl violet (Nissl stain) and immunohistochemistry using immunofluorescence analysis or diaminobenzidine method as previously described (Luna-Medina et al. 2007b). For BrdU detection, samples were first incubated with 2M HCl for 30 min at 37º before blocking 1 hour in PBS containing 5% normal serum, 0.1M lysine and 0.1% Triton X-100. Sections were then incubated with anti-BrdU mouse monoclonal (DAKO, Denmark) and anti-neuN rabbit polyclonal (Chemicon) antibodies at 4ºC overnight, washed three times and incubated with AlexaFluor 488 and Alexa 647 secondary antibodies for 1h at room temperature. After rinses, sections were mounted with Vectashield. Images were obtained using a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA) with a 488 laser line to excite Alexa-488 and a 647 laser line to excite Alexa-647. Confocal microscope settings were adjusted to

produce the optimum signal-to-noise ratio. BrdU-positive cells were scored throughout the entire SVZ and RMS. One section every 60μm was analyzed at high magnification (x 40). For the quantification of immunoreactive BrdU/NeuN cells in the olfactory bulb, the number of positive cells was counted in 5 independent random fields (x400 magnification), in 5 different sagital sections per animal (n=5 animals). For doublecortin (DCX) and β-III-tubulin detection floating sections were immersed in 3% H_2O_2 to inactivate endogenous peroxidase, blocked for 2 h at room temperature in 5% normal horse serum in PBS, containing 4% bovine serum albumin, 0.1 M lysine and 0.1% Triton X-100. Afterwards, the sections were incubated overnight with anti-doublecortin goat (Santa Cruz, CA), anti-PSA-NCAM mouse (Chemicon), and anti- β-III-tubulin mouse (Sigma) antibodies. After several rinses, sections were incubated for 1 h with the corresponding biotinylated secondary antibody and then processed following the avidin-biotin protocol (ABC, Vectastain kit. Vector Labs). Tissues were then mounted onto gelatin-coated slides, dried, dehydrated in xylene, and mounted with DePeX (Serva, Heidelberg, Germany). The slides were examined with a Zeiss Axiophot microscope, equipped with an Olympus DP-50 digital camera, and a Leica MZ6 modular stereomicroscope.

Electron microscopy. Rats were deeply anesthetized and perfused transcardially with 0.9% saline, followed by Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde). Brains were postfixed overnight in the same fixative and washed in 0.1 M phosphate buffer (PB). Coronal 200 μm sections were cut on a vibratome. Sections were then post-fixed in 2% osmium for 2 hours, rinsed, dehydrated and finally embedded in Araldite (Durcupan ACM, Fluka, Switzerland). Serial 1.5 μm semithin sections were cut with a diamond knife and stained with 1% toluidine blue to study the organization of the SVZ and RMS. In order to identify the different individual cell types in the SVZ, ultrathin (70 nm) sections were cut with a diamond knife. Photomicrographs were obtained under a Fei microscope (Tecnai-Spirit) using a digital camera (Morada, Soft-imaging System).

Neurosphere cultures. Neurosphere (NS) cultures were derived from adult rats and induced to proliferate using established passaging methods to achieve optimal cellular expansion according to published protocols (Ferron et al. 2007). Briefly, rats were

decapitated, brains removed and the subventricular zone was dissected, minced and dissociated with DMEM (Invitrogen) containing glutamine, gentamicin and fungizone. After treatment with trypsin-EDTA, hialuronidase and DNAse, myelin was removed by using DPBS (Invitrogen). Cells were seeded into 6-well dishes and cultured in Dubecco's Modified Eagle's Medium (DMEM)/F12 (1:1, Invitrogen) containing 10 ng/ml epidermal growth factor (EGF, Peprotech, London, UK), 10 ng/ml fibroblast growth factor (FGF, Peprotech) and N2 medium (Gibco). After 3 days in culture, some primary NS cultures were treated with pioglitazone (5, 10 and 30 μM, Takeda Europe R&D Centre Ltd., London, UK), rosiglitazone (5, 10 and 30 μM, Cayman, Ann Arbor, MI), or vehicle for another 7 days. Some cultures were pretreated for 30 min with GW9662 (10 and 30μM, Cayman) and T0070907 (25 and 50μM, Cayman). These primary neurospheres were then dissociated and replated in normal proliferative conditions for another 7-9 days to score the number of secondary neurospheres generated. Six to eight wells per condition tested were counted.

Protein extraction and Western blot analysis. After sacrifice, brains were immediately dissected and cortex, hippocampus and cerebellum isolated and frozen on dry ice and stored at −80°C until protein extraction. For protein extraction, samples were homogenized in ice-cold RIPA buffer and equal quantities of total protein were separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes (Protran, Whatman, Dassel, Germany) and blots were probed with anti-PPARγ (Santa Cruz, Santa Cruz, CA) antibody. For each sample, the α-tubulin level expression (Sigma) was determined as a loading control.

Growth and proliferation measurements. Primary and secondary NS cells were counted and their size was analyzed using the Nikon Digital Sight, SD-L1 (Nikon, Japan). Proliferation assays were carried out by culturing whole primary NS, in the presence or absence of the indicated stimuli, during 10 days. Afterwards they were plated onto poly-l-lysine coated coverslips for 24 h. Cells were then fixed in cold methanol, stained with anti-Ki67 antibody (Novo Castra Laboratories, Newcastle, UK) and processed for immunocytochemistry as previously described (Luna-Medina et al. 2005).

Differentiation of NS cultures. In order to determine the ability of rosiglitazone and pioglitazone to produce neurons, astrocytes or oligodendrocytes, whole neurospheres from 10-day old cultures were plated onto poly-l-lysine coated coverslips in the absence of exogenous growth factors. Cells were allowed to adhere for 24 h and fixed using cold methanol. Cells were then processed for immunocytochemistry.

Assay of cell migration. Single spheres were picked up with a pipette and plated onto poly-L-lysine coated 35 mm dishes. At 48 h post-plating, the outgrowth of the neurosphere cells was examined under the phase contrast microscope, and the images were acquired with a Nikon Digital Sight, SD-L1 software. The farthest distance of cell migration was calculated from the edge of the sphere. At least 10 plated neurospheres per situation were analyzed.

Immunocytochemistry. Cells were processed for immunocytochemistry as previously described (Luna-Medina et al. 2005). Briefly, at the end of the treatment period, NS cultures were grown on glass cover-slips in 24-well cell culture plates. Cultures were then washed with phosphate-buffered-saline (PBS) and fixed for 30 minutes with 4% paraformaldehyde at 25°C, and permeabilized with 0.1% Triton X-100 for 30 minutes at 37ºC. After 1 hour incubation with the corresponding primary antibody, cells were washed with phosphate-buffered-saline and incubated with an Alexa-labeled secondary antibody (Molecular Probes; Leiden, The Netherlands) for 45 minutes at 37°C. Later on, images were obtained using a Radiance 2100 confocal microscope (Bio-Rad, Hercules, CA) as described for immunohistochemistry. Quantification was undertaken using the image analySIS software (Soft Imaging System Corp., Münster, Germany) and normalized to total nuclei. Primary antibodies were directed against the following: MAP-2 (mouse; Sigma), GFAP (mouse; Sigma), active caspase-3 (rabbit, R&D) and CNPase (mouse, Chemicon, Temecula, CA). Dapi staining was used as a nuclear marker. For quantification of the number of cells producing a given marker, in any given experiment the number of positive cells leaving the neurosphere body were then counted. Cell numbers were estimated from a total of six to eight neurospheres per condition over three independent experiments.

Statistical determinations. Data are given as the mean ± SD. of at least five different animals per group. Comparisons of different groups were performed using the Student's *t* test with $p \leq 0.05$ being considered significant.

RESULTS

Since pioglitazone is a specific ligand of the nuclear receptor PPARγ, we first analyzed whether PPARγ was expressed in the adult brain, particularly in neurogenic areas. Western blot and immunohistochemistry (Supplementary Figure 1) analysis demonstrated that PPARγ was expressed throughout the central nervous system of the adult rat. Interestingly, significant levels of PPARγ protein were found in the two neurogenic niches, the SVZ and the hippocampus. PPARgamma expression was found in the SVZ as well as in the RMS. PPARγ is expressed at high levels in the RMS and remains detectable in these cells as they migrate into the OB.

Effect of pioglitazone on proliferation of adult progenitor cells in vivo in the subventricular zone and rostral migratory stream.

We first analyzed whether pioglitazone affected the proliferation kinetics of mitotically active progenitor cells in the SVZ and RMS. For this *in vivo* study we decided to use this PPARγ ligand because of its well known ability to cross the blood brain barrier. To this end, rats were treated during 3 days with pioglitazone or vehicle followed by one BrdU injection 24 h before sacrifice. Coronal sections were stained with cressyl violet (Nissl staining) or with specific anti-BrdU and anti-NeuN antibodies (Fig 1). Nissl staining showed an increase in the size of the SVZ in animals treated with pioglitazone (Fig. 1A, right panel), in comparison with controls. No morphological alterations were observed on the rest of the neuropil or in other cortical, septal or striatal structures. Also a widespread distribution of the RMS was observed in pioglitazone-treated animals (Fig. 1C, right panel). Twenty-four hours after the BrdU injection there was a considerable increase in the number of labeled cells both in the SVZ (Fig. 1B, right panel) and RMS (Fig. 1D, right panel) of pioglitazone-treated rats relative to the vehicle-treated control group SVZ (Fig 1B, left panel) and RMS (Fig 1D, left panel). Pioglitazone treatment increased SVZ BrdU-labeled cell numbers by about 35 % above control values (Fig. 1E). Similar results were obtained in the RMS where a significant increase in the number of BrdU cells was found in pioglitazone-treated animals in comparison with control ones (Fig. F). Besides a higher number of proliferating cells in the RMS, we also found that pioglitazone-treated animals showed a widespread RMS

(Fig 1D, right panel), compared to control rats, in which the RMS usually appeared as a dense cluster of cells (Fig 1D, left panel).

In order to determine the cell types that were increased in the RMS and the SVZ, rats were treated during 3 days with pioglitazone or vehicle and sagittal or coronal brain sections were stained for DCX^+. DCX is a microtubule-associated protein, which is a valuable endogenous marker for dividing neuroblasts and immature neurons (Brown et al. 2003; Couillard-Despres et al. 2005). As shown in Figure 2A-B, pioglitazone treatment clearly increases the number of DCX^+ cells in the RMS (Fig. 2A), specially in some areas, such as the anterior arm or the elbow (Fig. 2A, right panel). Similar results were observed in the SVZ of pioglitazone-treated animals (Fig. 2B, right panel) with an increase in the migrating chain of cells. The pioglitazone-induced increase in the number of neuroblast was further substantiated by staining with PSA-NCAM, a marker of migrating neuroblasts (Fig. 2C). As happened with the number of DCX^+ cells, animals treated with pioglitazone presented a higher number of PSA-NCAM-stained cells in the RMS. To characterize the cells marked in the SVZ, coronal sections were stained with an specific anti-β tubulin antibody, a molecule present in the migrating neuronal precursors (Doetsch and Alvarez-Buylla 1996). Our results clearly showed an increase in β-tubulin staining in the pioglitazone-treated animals (Fig. 2D, right panel).

Effect of pioglitazone on migrating cells and the subventricular zone.

We then analyzed the morphological features of the SVZ in the different groups of animals. To this end, we performed light and electron microscopy (EM) analysis on coronal sections of the ventricular lateral wall of the SVZ. EM allows characterization and identification of SZV cell types through their specific ultrastructural features, which have been well established in rodents (Danilov et al. 2009; Doetsch et al. 1997).

Analizing toluidine blue-stained semithin sections of the SVZ of control animals, next to the ependymal lining we found chains of neuroblasts (Fig 3A). However, pioglitazone-treated animals (Fig 3B) displayed an expanded SVZ, with an increased number of migrating neuroblasts. These cell clusters were located deeply in the neuropil.

EM analysis showed a typical SVZ structure in control animals (Fig 3C). Close to the ependymal cell lining, we found chains (Fig. 3C, pointed line) of migrating cells (type

A). These cells were identified by their small size and dark scanty cytoplasm. Astrocytes or type B cells were identified as large, less electrodense cells, rich of intermediate filaments. After pioglitazone treatment, the SVZ showed several ultrastructural changes. First, an increased number of neuroblasts were observed. These neuroblasts, in comparison with control animals, showed an irregular shape (Fig 3D). Moreover, migrating cell chains were located deeper in the SVZ compared to control rats. Additionally, intercellular spaces characteristic of migrating neuroblasts were more prominent in the pioglitazone-treated animals (Fig.3F), compared with controls (Fig 3 E). Signs of pathological events or tumoral cells were also absent. Regarding the RMS, migrating type A cells and astrocytes were observed in control animals; these cells accumulated forming a typical dense cell mass (Fig 3 G). The RMS of pioglitazone-treated animals (Fig 3 H) showed dispersed chains of migrating neuroblasts, which tended to spread invading a larger surface.

Effect of pioglitazone on newly generated cells in the olfactory bulb.

The neuronal differentiation of newborn cells in the olfactory bulb was assessed using double immunofluorescence labelling for BrdU and NeuN (postmitotic and postmigrational neurons), 21 days after treatment with pioglitazone and BrdU injection (Fig. 4). At this time most of BrdU-positive cells have reached the granule cell layer via the RMS, and express a neuronal phenotype (Winner et al. 2002). The number of BrdU/NeuN-positive cells in the granule cell layer was quantified as described in Material and Methods. Our results showed that after 21 days, there is a significant increase in the amount of newly generated neurons (BrdU/NeuN immunoreactive cells) in this area, in the animals treated with pioglitazone (Fig. 4A-B). That means that pioglitazone increases the number of new neurons into the olfactory bulb.

The PPARγ ligands pioglitazone and rosiglitazone control the growth and survival of neurospheres.

As a step toward understanding the role of PPARγ ligands on neurogenesis, we used primary and secondary derived neural stem cells as a model system. These studies allow us to better understand the mechanism by which pioglitazone acts as a neurogenic agent and the possible involvement of the nuclear receptor PPARγ. Primary neurospheres were obtained from adult rats and maintained and expanded as described in Materials

and Methods. We first analyzed by Western blotting whether PPARγ was expressed in the neurosphere cultures. As shown in Fig 5A, PPARγ was notably expressed in NS, regardless of their differentiation state. Next, we investigated whether addition of pioglitazone and rosiglitazone, another well-known and potent PPARγ ligand, to the medium of growing NS would increase their rate of formation and/or their size. To that point, different concentrations of pioglitazone and rosiglitazone on primary NS were used (Fig. 5C).

After 10 days of growth in suspension, there was a significant increase in both the number and diameter of primary NS growing in the presence of both ligands (Fig. 5B, 5C). Consistent with a role in stem cell regulation suggested by the *in vivo* experiments, pioglitazone- and rosiglitazone-treated NS exhibited an enhanced self-renewal capacity. Primary spheres derived from pioglitazone- and rosiglitazone-treated progenitors formed "secondary" NS when dissociated and replated (Fig. 5C), indicating self-renewal capacity. Specifically, neonatal pioglitazone- and rosiglitazone-treated primary NS produced respectively 341 ± 15.7 and 336 ± 12.2 secondary NS, while non-treated neurospheres only produced 210 ± 10.5 secondary neurospheres. Treatment of neurosphere cultures with pioglitazone or rosiglitazone did not alter cell viability, as measured by active caspase-3 staining (Supplementary Figure 2).

Next, we tested the possible implication of the PPARγ receptor in the observed effect of pioglitazone and rosiglitazone. To this end we analyzed whether the specific PPARγ antagonists GW9662 and T0070907 impaired the action of the drugs. As shown in Fig. 5B and 5C, treatment of primary NS with these antagonists significantly inhibited the effects of the drugs on primary NS formation and size. Similar results were obtained on secondary NS pre-treated with GW (Fig. 5C, lower panel). These results suggest that both rosiglitazone and pioglitazone are acting through a PPARγ-dependent mechanism.

These studies thus far demonstrate that PPARγ is expressed by NS and that their specific ligands pioglitazone and rosiglitazone enhance the number as well as the size of the NS generated from neonatal SVZ, suggesting that PPARγ activation by these factors regulates the proliferation of sphere forming progenitors. Therefore, proliferation of pioglitazone- and rosiglitazone-treated cells was assessed 7 days after treatment by

staining for Ki67, a marker of dividing cells. As expected, treatment of adherent primary NSC significantly increased Ki67-staining, indicating a direct effect of pioglitazone on proliferation (Fig. 5D).

The PPARγ ligands pioglitazone and rosiglitazone alter migration patterns from neurospheres.

In order to test whether pioglitazone and rosiglitazone modulate migration of NS, we exposed NS to both agents during 48 h. The results summarized in Fig. 6 indicate that migration in the presence of both, pioglitazone and rosiglitazone for 48 h resulted in an increased migration (1.6- and 1.5-fold, respectively of the controls). These cells moved long distances from the centre of the NS to often create overlapping zones of migration between adjacent NS. The cells from control NS remained close to the neurosphere origin and were observed in closely associated groups.

Next, we investigated whether PPARγ activation is involved in the effects upon migration of both pioglitazone and rosiglitazone. To this end, NS cultures were pretreated with the selective PPARγ antagonist GW9662 before exposure to these agents. As shown in Fig. 6, GW9662 suppressed the enhanced migration detected in pioglitazone- and rosiglitazone-treated NS cultures. These results suggest again an involvement of the nuclear receptor PPARγ in the migration effects of both compounds.

Altogether these data implicate the PPARγ receptor as a regulator of neuroblast formation and migration in the adult rat brain.

The PPARγ ligands pioglitazone and rosiglitazone enhance differentiation of neurospheres after adhesion.

To investigate whether PPARγ ligands influence cell differentiation after adhesion of NS, we analyzed by immunocytochemistry the different central nervous system cell types in the inside and the outgrowth of control, pioglitazone- and rosiglitazone-treated NS. Twenty-four hours after plating in the absence of EGF and FGF, NS extended multiple processes, which stained for the uncommitted neural precursor marker nestin. To identify the cell types generated by the effect of pioglitazone and rosiglitazone, cells were stained for immunofluorescence using antibodies against MAP-2 for neurons, GFAP for astrocytes, and CNPase for immature oligodendrocytes. As shown in Fig 7A

and 7B, in control cultures only scattered cells stained with GFAP or MAP-2 were observed. After treatment with PPARγ ligands the number of MAP-2- and GFAP-positive cells was significantly increased specially in the outgrowth of the NS (Fig. 7). An increase of the length of GFAP immunoreactive fibers was observed, with long processes extended from the NS (high magnification in figure 7A). Figure 7B also shows in detail an increase of the number of migrating MAP-2 positive cells outside the NS. On the other hand, the number of CNPase positive oligodendrocytes precursors was not significantly altered by either rosiglitazone or pioglitazone (data not shown). Addition of GW9662 completely abolished the effects of both compounds on differentiation.

DISCUSSION

Although its functional effect on brain damage has been recently investigated, there is almost no information about the effect that PPARγ activation has on neurogenesis in the adult brain. In the present study we used the TZD compound pioglitazone to demonstrate that PPARγ activation promotes proliferation, differentiation and migration of neuroblasts in the SVZ and RMS of adult rats. The results of this study demonstrate that PPARγ plays a role in regulating the expansion and differentiation of the stem cell population. This is evident *in vitro* by enhanced numbers of primary neurospheres and by rosiglitazone- and pioglitazone-mediated induction of $GFAP^+$ and $MAP2^+$ cells, and *in vivo* by an increased proliferation and a larger population of neuroblasts that finally integrate into the olfactory bulb as newly generated neurons. Enhancement of neural stem cells proliferation and differentiation by pioglitazone and rosiglitazone is possibly provoked by an activation of PPARγ, as demonstrated by the inhibition of their effects by the PPARγ antagonists GW9662 and T0070907. These studies reveal a novel role for PPARγ and may represent a potential therapeutic strategy for stem cell activation.

Neurogenesis can be regulated at multiple points, such as proliferation, differentiation, and migration, processes that are controlled by a number of extracellular signalling cues (Alvarez-Buylla and Lim 2004; Lie et al. 2004). The SVZ harbors the largest pool of proliferating cells in adult mammals (Lois and Alvarez-Buylla 1993; Morshead et al. 1994). Pioglitazone-treatment increased cell proliferation in the SVZ and the RMS, as indicated by the changes in the number of BrdU-labeled cells after 3 days of pioglitazone treatment. We also present evidence that PPARγ activation induces expansion of highly migratory SVZ progenitors in the adult rat. The increased proliferation, neuroblast migration in the SVZ and RMS, and subsequent increase of newborn neurons incorporated within the OB can be relevant in olfactory-associated behavior. There is evidence that adult mice short term memory is dependent of neurogenesis. Mice housed in an odor-enriched environment showed an increased survival of progenitor cells in the OB as well as an enhanced olfactory short memory (Rochefort et al. 2002). On the other hand, chemical inhibition of neurogenesis with the antimitotic drug araC, drastically reduced short-term olfactory memory (Breton-Provencher et al. 2009). More recently, also long-term olfactory memory has been

shown to be dependent of neurogenesis. Both neurogenesis and long term memory are impaired after treatment with anti-mitotic drugs and protein synthesis inhibitors (Kermen et al. 2010 ; Sultan et al. 2010). Irradiation-induced decrease in constitutive neurogenesis in the OB has been shown to decrease olfactory long-term memory but not other olfactory functions (Lazarini et al. 2009). Also, Mak and Weiss (Mak and Weiss. 2010) have shown that paternal-adult offspring recognition depends of OB neurogenesis and implicates prolactin signaling pathway (Mak and Weiss. 2010). Finally, it has been shown that chronic systemic inhibition of nitrite production increases proliferation in the subventricular zone and neurogenesis in the OB associated with a better olfactory learning performance (Romero-Grimaldi et al. 2006). Based on these data, together with our results, we could infer that pioglitazone -induced OB neurogenesis may improve olfactory-associated memory.

We observed large chains of neuroblasts far away from the SVZ, deep in the striatum. This effect reminds to that observed when mice where administered EGF with an intraventricular osmotic pump (Doetsch et al. 2002). Speaking of stimulating neurogenesis, the advantage of pioglitazone is that it can be administered intragastrically for a systemic effect. To our knowledge, this paper is the first direct demonstration of an involvement of PPARγ in the proliferation, migration, and differentiation of neural progenitor cells. Regarding the possible mechanism of action through which PPARγ regulates neural stem cell proliferation and differentiation, since this receptor is a transcription factor we favor the idea that this regulation takes place through direct activation of some genes involved in these processes (Mu et al. 2010).

The data obtained here also show that activation of PPARγ also stimulates neural progenitor cell proliferation in neurospheres *in vitro*. Our results suggest that PPARγ may regulate neural stem cell number by regulating the type of cell division. The formation of secondary neurospheres from primary neurospheres is indicative of self-renewing stem cell division (Reynolds and Weiss 1996). The higher number of secondary neurospheres in pioglitazone- and rosiglitazone-treated NS cultures, indicate that the neural stem cells generated in presence of these factors underwent a higher proportion of self-renewing or symmetric cell divisions, leading to an expansion of the

stem cell pool. This lends support to the view that PPARγ activation might stimulate the capability to maintain stemness.

Neural stem cells differentiate into neurons, astrocytes and oligodendrocytes (Gage 2000; Temple 2001). Here, we show that PPARγ is involved in the control of neural stem cell differentiation. Our results indicate that activation of PPARγ causes neural stem cells to differentiate into astrocytes and neurons. The finding that rosiglitazone and pioglitazone are able to induce differentiation of NS cells differs from a previous report indicating that rosiglitazone induced proliferation of embryonic mouse NS cells and concurrently inhibited their differentiation into neurons (Wada et al. 2006b). This discrepancy may simply reflect methodological differences in isolating neural stem cells or different experimental conditions. Additionally, our work was performed with neurospheres isolated from the SVZ of adult rats, whereas the neurospheres by Wada et al. came from whole brain of mice embryos on days 13-14 of gestation, where other factors might be involved.

Our work shows that PPARγ activation has a dual function in neural stem cells proliferation and differentiation, and suggests that ligands of PPARγ are not only mitogens for neural stem cells, but are also inducers of neuronal differentiation. It seems interesting that activation of PPARγ can promote both proliferation and differentiation. However, precedents for this phenomenon are seen in the case of insulin-like growth factor 1 (Arsenijevic and Weiss 1998) and leukotriene B4 (Wada et al. 2006a). In this respect, we suggest that PPARγ can present a new strategy for restoring neurogenesis.

Cell division in the SVZ is responsible for new neurons being added to the granular and the periglomerular layers of the olfactory bulb. These cells migrate through the RMS, where the majority disperses throughout the granule layer and a small percentage develops into interneurons in the periglomerular layer (Altman 1969; Doetsch et al. 1997; Lois and Alvarez-Buylla 1994). The identification of factors that promote neural stem cell division and regulate proliferation, differentiation, and migration of their progeny would facilitate attempts to manipulate the production of new neurons and glia and help in understanding the ongoing maintenance of neural circuitry in the mature central nervous system. Here, we demonstrate an increased capacity of migration of

neurosphere-derived cells in response to both pioglitazone and rosiglitazone. This is especially important in a clinical setting since the identification of factors that not only promote neural stem cells proliferation and differentiation but also have a significant effect on their migration capacity might have an important regulatory role in SVZ migratory events in a brain injury context. One important finding in this study is that activation of PPARγ is required for the observed effects of rosiglitazone and pioglitazone. These results lend support to the view that activation of this receptor can have an important regulatory role in neurogenesis in the normal brain as well as after a brain injury or a neurodegenerative disease. In contrast with this, very recently Lee et al have shown that oral administration of rosiglitazone decreases neurogenesis in the mouse hippocampus (Lee et al.). The reasons for this discrepancy are uncertain, although it could be due to an indirect effect of the long treatment with rosiglitazone since it is well established that this drug, in contrast with pioglitazone, does not cross the blood brain barrier (Brodbeck et al. 2008; Landreth et al. 2008; Pedersen et al. 2006; Risner et al. 2006; Watson et al. 2005).

In summary these studies clearly demonstrate that ligands of PPARγ play a versatile role, both in maintaining the progenitor pools and in inducing differentiation and migration of various cell types in the central nervous system through activation of this receptor.

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FIGURE LEGENDS

Fig. 1. Pioglitazone action on proliferating cells in the subventricular zone (SVZ) and the rostral migratory stream (RMS). Representative coronal sections showing Nissl staining and micrographs of BrdU-labeled cells (green) and NeuN-stained cells (red) in the SVZ (**A, B**) and in the RMS (**C, D**). Insets show higher magnifications of representative areas in the SVZ. (**E, F**) Quantification of BrdU-positive cells from the SVZ and RMS. Values are the mean ± sd from five different animals. *, $P \leq 0.05$. Scale bar in **B**, **D**: 200 μm; insets: 100 μm.

Fig. 2. Effect of PPARγ ligands on neural progenitor cells and their migration in the SVZ and RMS. Representative immunohistochemical images of DCX expression on sagital sections of RMS (**A**) and coronal sections of SVZ (**B**) of control and pioglitazone-treated animals. Insets show higher magnifications of the elbow area of RMS (**A**) and the SVZ (**B**). (**C**) Representative immunohistochemical images of PSA-NCAM expression on sagital sections of control and pioglitazone-treated animals. Insets show higher magnifications of the elbow area of the RMS (**D**) Representative Coronal sections showing staining of the SVZ with an specific anti-β tubulin antibody. Scale bar in **A, B, C, D**: 250 μm, Insets: 25μm.

Fig. 3. Effect of PPARγ ligands on migrating cells in the subventricular zone and the rostral migratory stream. (**A**) Toluidine blue-stained semithin section of the SVZ of a control rat, with dark round cells forming groups. (**B**) The SVZ of pioglitazone-treated rats displayed an increased number of migrating neuroblasts clusters. (**C**) Ultrastructure of the SVZ of an untreated rat. Close to the ependymal cell lining, chains of migrating type A cells (pointed line) showed a dark scanty cytoplasm. Astrocytes (type B cells) were large and less electron-dense and contained abundant intermediate filaments. (**D**) After pioglitazone treatment, the number of neuroblasts increased and they showed an irregular shape. Migrating cell chains (pointed line) were deeper located in the SVZ compared to control rats. (**E**) Migrating neuroblasts typically displayed intercellular spaces (arrows), indicative of cell movement, in untreated rats. (**F**) In pioglitazone-treated mice, intercellular spaces were more prominent than in control animals (arrows). (**G**) Rostral migratory stream of a control animal. Migrating cells and

astrocytes accumulate forming a dense cell mass. (**H)** The rostral migratory stream of pioglitazone-treated mice showed a larger size, with an increased cell number. Scale bar in (**A-B**) 20 μm; (**C-D**) 10 μm; (**E-F**) 2 μm; (**G-H**) 50 μm.

Fig.4. Effect of PPARγ ligands on newly generated neurons in the olfactory bulb. **(A)** Sagital sections showing the granule cell layer (GCL) of the olfactory bulb stained with BrdU (green) and NeuN (red). (**B)** Quantification of the number of BrdU/NeuN double stained cells into the GCL. Values are the mean ± sd from five different animals. ***, $P \leq 0.001$. Scale bar in (**A**) 100 μm; insets: 20 μm.

Fig. 5. Effect of PPARγ ligands on neurosphere formation and growth. (**A**) Western blot analysis showing expression of PPARγ in neurospheres grown for 7 days (undifferentiated) or after an additional 2 days of adhesion (differentiated cultures). (**B)** Representative phase-contrast micrographs showing the size of neurospheres cultures for 7 days in the presence or absence of pioglitazone (10 μM) or rosiglitazone (30 μM). Some cultures were pre-incubated 1 hour with 30 μM GW9662 (GW) or 50 μM T0070907 prior to the addition of the compounds. Scale bars, 50 μm. (**C)** Effect of different doses of pioglitazone and rosiglitazone (5, 10 and 30 μM) on the growth of primary neurospheres. The number of neurospheres was counted and its diameter measured. The diameter of 50 primary and secondary neurospheres and the total number of neurospheres was determined in control and in pioglitazone- or rosiglitazone- treated cells. Some cultures were preincubated with GW9662 (GW, 30 μM) or with T0070907 (T, 50 μM). The graphs in (**C**) demonstrate a significant increase in the number and diameter of the neurospheres in those cultures treated with pioglitazone or rosiglitazone. (**D)** Representative confocal images of Ki67 immunoreactivity (green) in primary neurospheres. Dapi staining (blue) was used as a nuclear marker. Quantification of Ki67+ cells reveals a significant induction of the Ki67+ cells in pioglitazone- and rosiglitazone-treated neurospheres. Results are mean values ± SD from three independent experiments performed in triplicate. **, $P \leq 0.01$.

Fig. 6. Effect of PPARγ ligands on cell migration out of the neurosphere. Single neurospheres were plated on a poly-lysine-coated culture Petri dish in the presence or absence of pioglitazone (10 μM) or rosiglitazone (30 μM), and the cell migration out of

the sphere was monitored 48 h later. Some cultures were pre-incubated 1 hour with 30 μM GW9662 (GW) prior to the addition of the compounds. (**A)** Shown are representative photomicrographs of three independent experiments. Scale bars, 25 μm. Insets show higher magnifications of the migrating area. Scale bars, 10 μM (**B)** Quantitative data of farthest distance of neural progenitor cell migration. **P≤ 0.01, versus control non-treated cultures.

Fig. 7. Effect of PPARγ ligands on neurosphere differentiation. The neurospheres were grown for 7 days in the presence or absence of pioglitazone (10 μM) or rosiglitazone (30 μM) and then adhered for 2 days to allow differentiation. Cultures were labeled with MAP-2 (red, for neuronal cells), or GFAP (red, for astrocytes) antibodies and counterstained with DAPI (blue). Some cultures were pre-incubated 1 hour with 30 μM GW9662 (GW) prior to the addition of pioglitazone. Addition of pioglitazone enhanced the number of GFAP (**A**) and MAP2 (**B**) positive cells inside the neurosphere. High magnification pictures in A and B show in detail some of the GFAP- or MAP-2-immunoreactive cells migrated from the neurosphere in culture. Representative confocal images of at least five independent experiments are shown. Scale bars, 10 μm. *, $P \leq 0.05$; ***, $P \leq 0.001$.

Figure 1

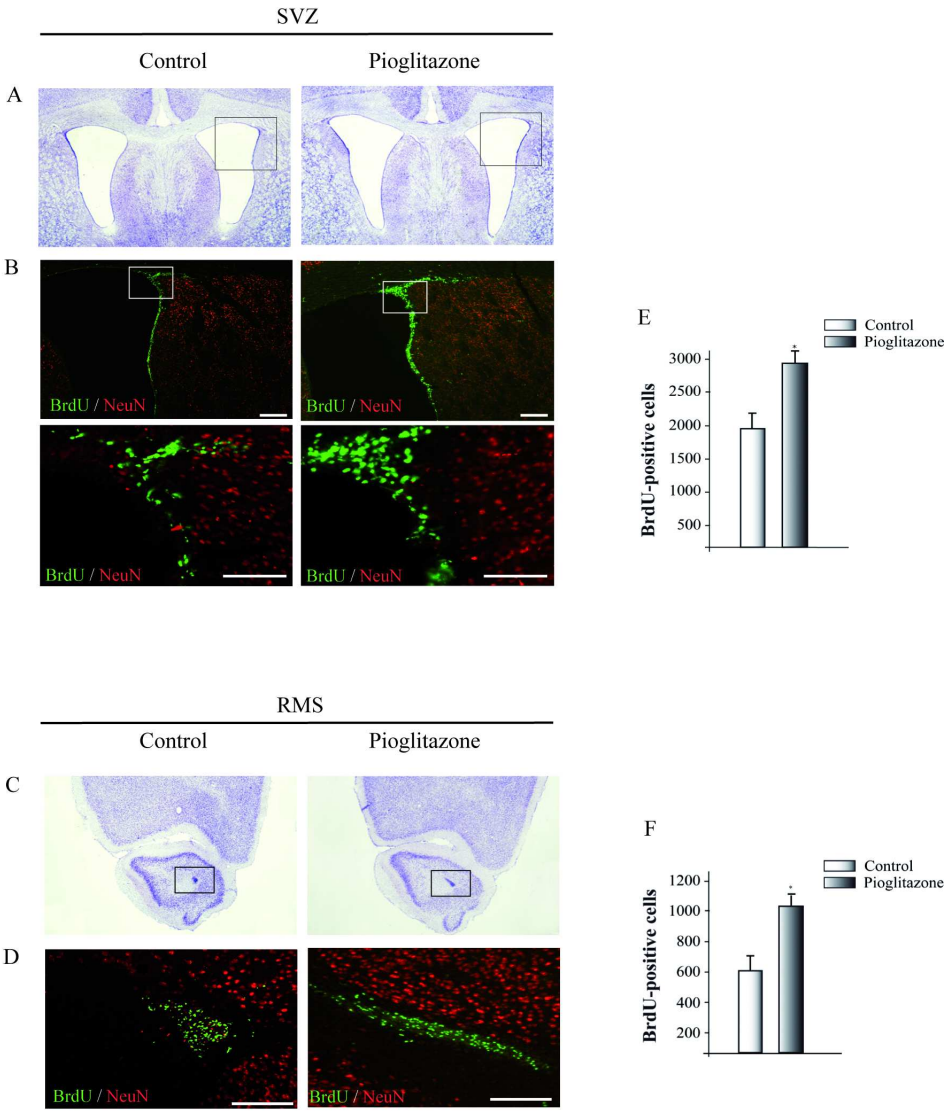

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Figure 2

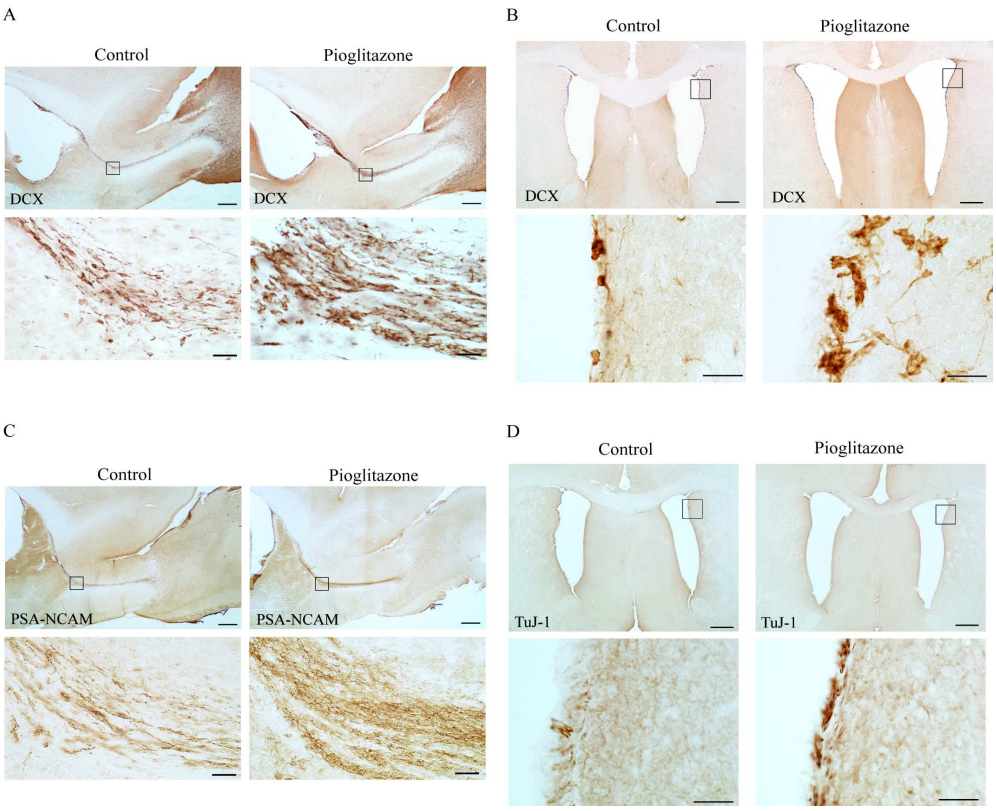

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Figure 3

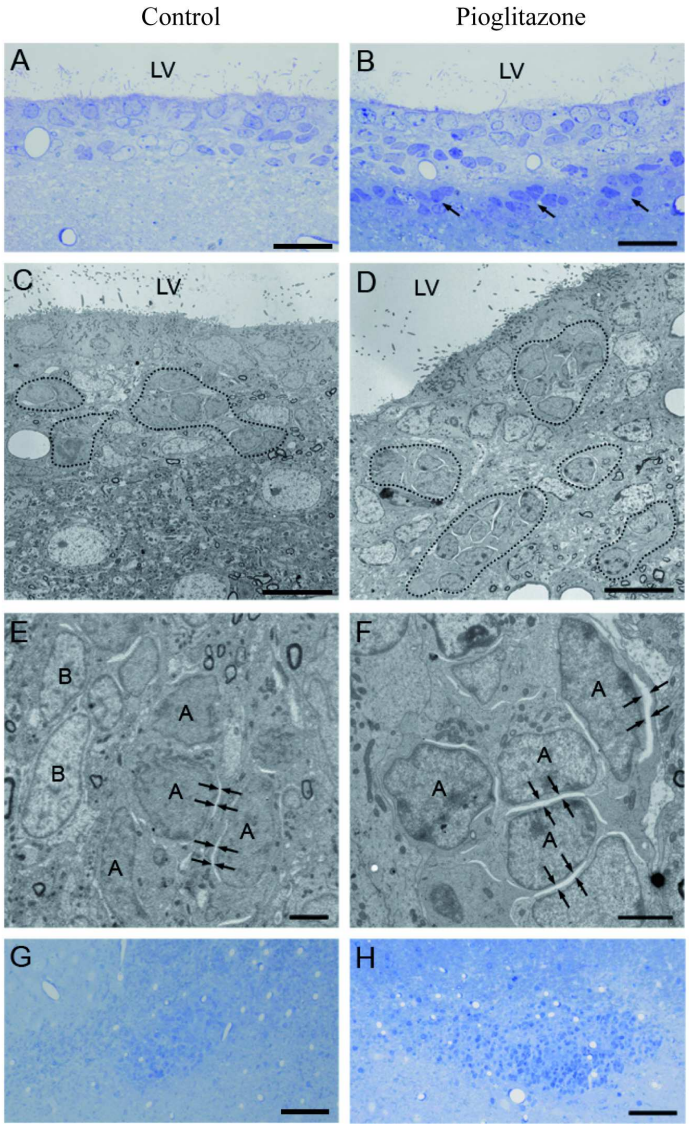

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Figure 4

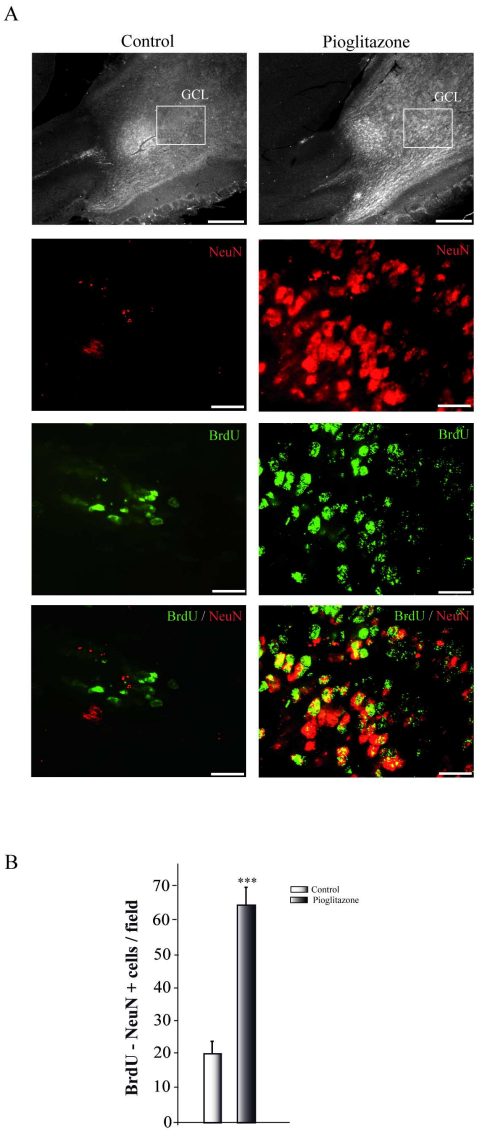

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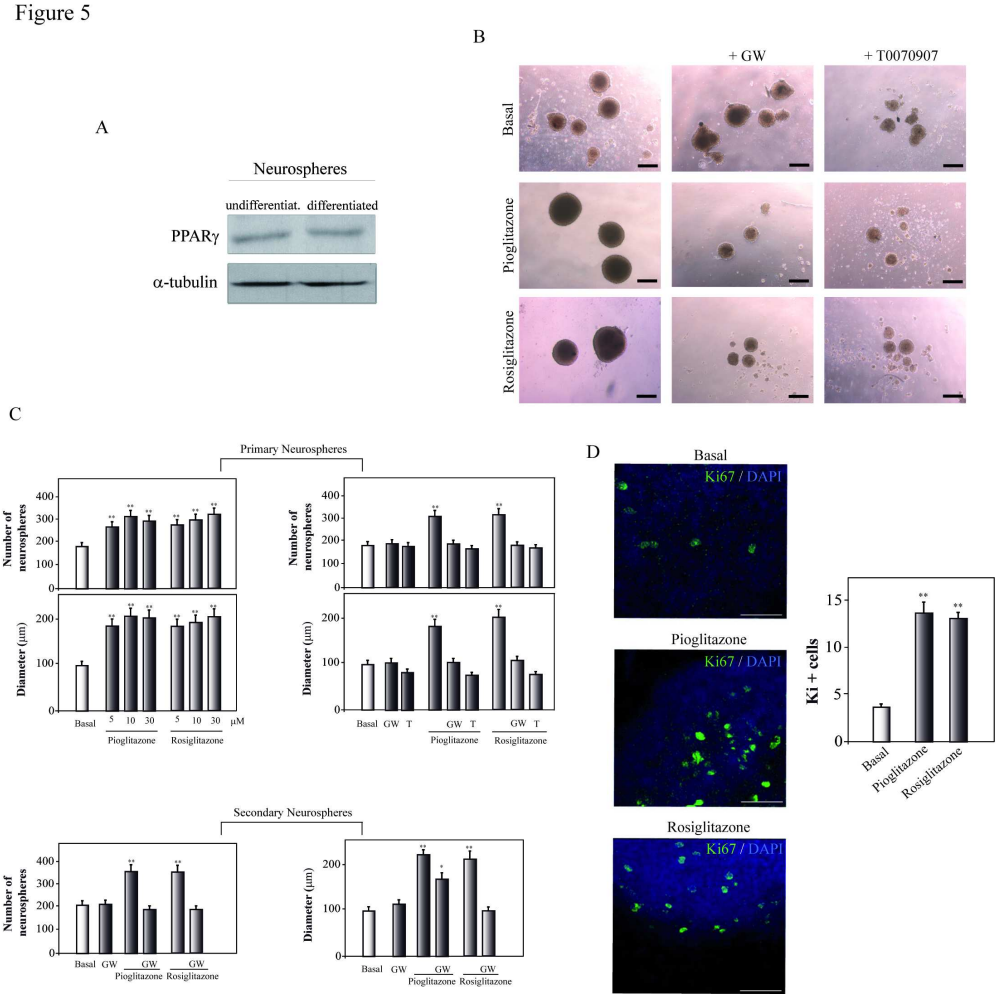
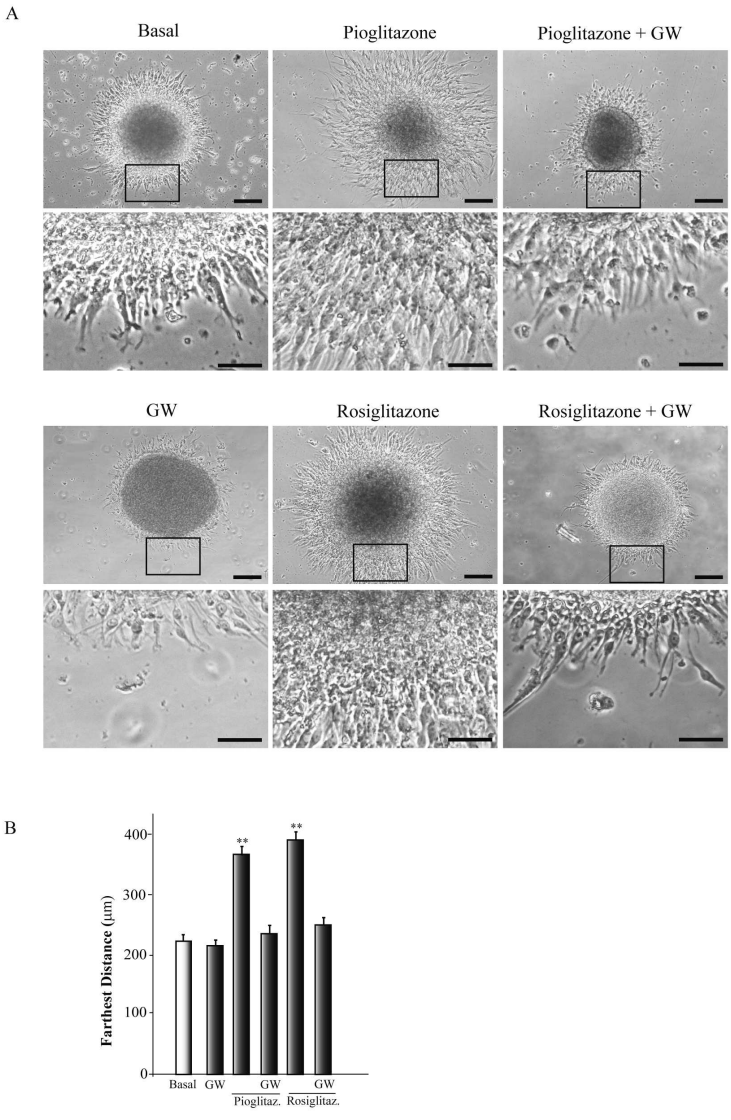
Figure 5



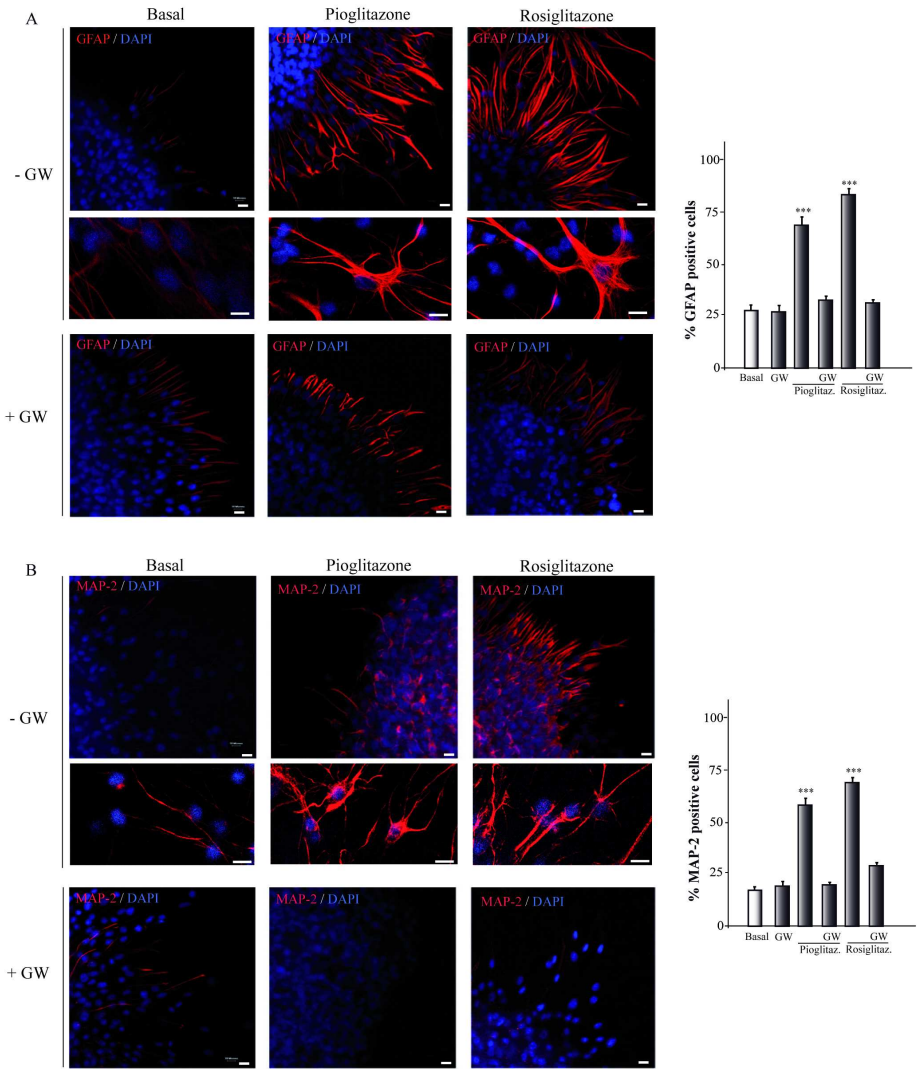
186x186mm (300 x 300 DPI)

Figure 6



139x226mm (300 x 300 DPI)

Figure 7

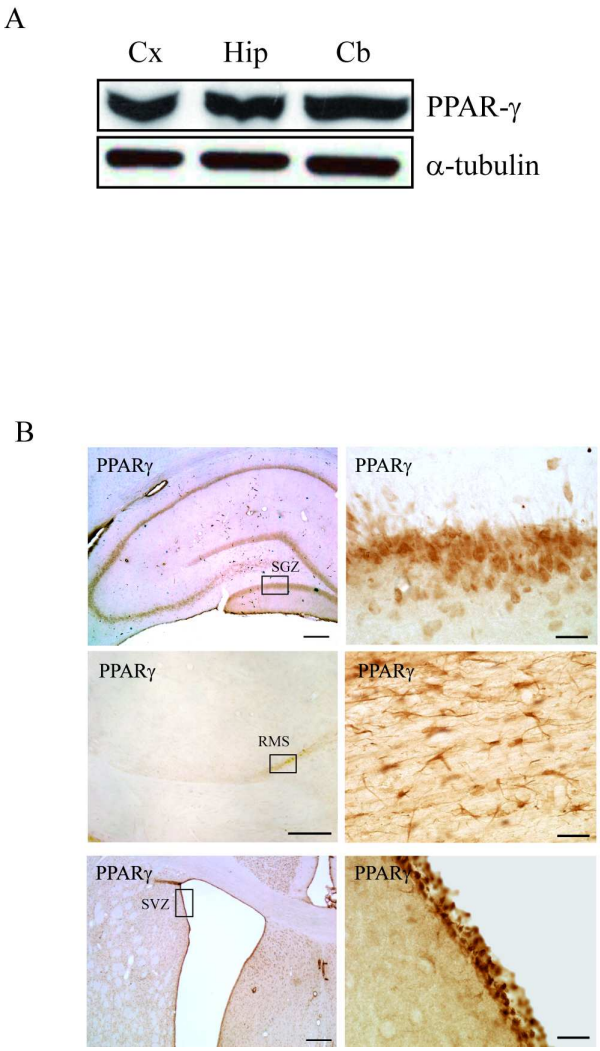


191x223mm (300 x 300 DPI)

SUPPLEMENTARY MATERIAL

Figure S1. Expression of PPARγ in adult rat brain. (**A**): Western blot analysis showing expression of PPARγ in different regions of the brain. (**B**): Immunohistochemical analysis of brain sections showing expression of PPARγ in different cerebral areas. Among other areas, expression of PPARγ was detected in the subgranular zone (SVG) of the hippocampus, the rostral migratory stream (RMS) and in the subventricular zone (SVZ). Cb, cerebellum; Cx, cortex; Hip, hippocampus. Scale bars, 250 μm. Insets 25 μm.

Figure S1

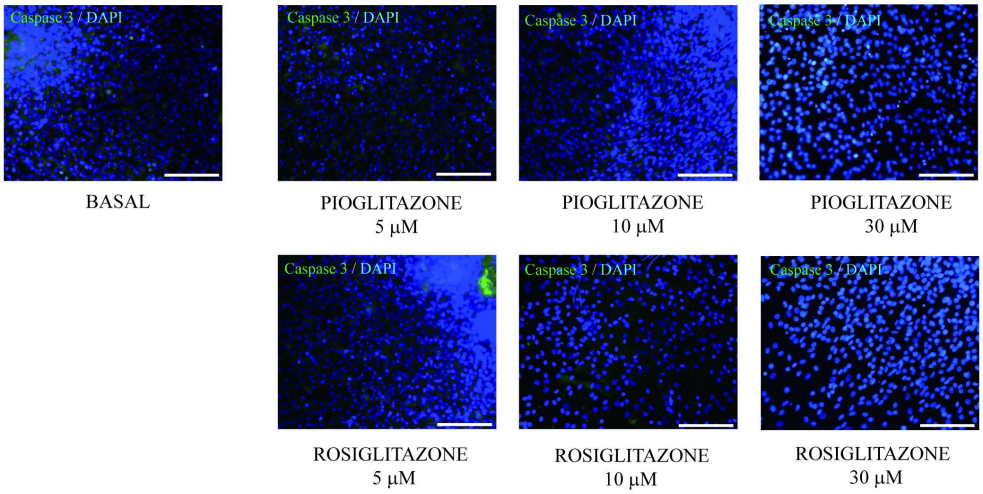


86x168mm (300 x 300 DPI)

SUPPLEMENTARY MATERIAL

Figure S2. Effect of pioglitazone or rosiglitazone on neurosphere´s viability. Treatment of neurosphere cultures with pioglitazone or rosiglitazone at different concentrations did not alter cell viability, as measured by active caspase-3 staining (green staining). DAPI staining is shown in blue. Scale bar, 100 μm.

Figure S2



179x105mm (300 x 300 DPI)