PPARs Expression in Adult Mouse Neural Stem Cells: Modulation of PPARs during Astroglial Differentiation of NSC

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Received 1 March 2007; Accepted 1 April 2007

Recommended by Jeffrey M. Gimble

PPAR isoforms are involved in the regulation of cell proliferation, death, and differentiation, with different roles and mechanisms depending on the specific isotype and ligand and on the differentiated, undifferentiated, or transformed status of the cell. Differentiation stimuli are integrated by key transcription factors which regulate specific sets of specialized genes to allow proliferative cells to exit the cell cycle and acquire specialized functions. The main differentiation programs known to be controlled by PPARs both during development and in the adult are placental differentiation, adipogenesis, osteoblast differentiation, skin differentiation, and gut differentiation. PPARs may also be involved in the differentiation of macrophages, brain, and breast. However, their functions in this cell type and organs still await further elucidation. PPARs may be involved in cell proliferation and differentiation processes of neural stem cells (NSC). To this aim, in this work the expression of the three PPAR isoforms and RXRs in NSC has been investigated.

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1. INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily [1]. After the isolation of PPARα (NR1C1) as the receptor mediating peroxisome proliferation in rodent hepatocytes in 1990 [2], two related isoforms, PPARβ/δ (NR1C2; referred to as PPARβ herein) and PPARγ (NR1C3), have been characterized [3]. PPARs exhibit a broad but isotype-specific tissue expression pattern which can account for the variety of cellular functions they regulate. PPARα is expressed in tissues with high fatty acid catabolism such as the liver, the heart, the brown adipose tissue, the kidney, and the intestine. The two PPARγ isoforms γ1 and γ2 act in the white and brown adipose tissues to promote adipocyte differentiation and lipid storage [4] while only the expression of PPARγ1 extends to other tissues such as the gut or immune cells. PPARβ has a broad expression being detected in all tested tissues but important functions have been assigned to this isotype in the skeletal muscle, the adipose tissue, the skin, the gut, and the brain.

PPARs are sensors capable of adapting gene expression to integrate various lipid signals. The diversity of functions in which they are implicated is also reflected by the diversity of ligands that can be accommodated within their ligand binding pocket. Indeed, PPARs are activated by a wide range of naturally occurring or metabolized lipids derived from the diet or from intracellular signaling pathways, which include saturated and unsaturated fatty acids and fatty acid derivatives such as prostaglandins and leukotriens [5, 6].

In contrast to steroid hormone receptors which act as homodimers, PPARs activate the transcription of their target genes as heterodimers with retinoid X receptors (RXR, NR2B) [7, 8]. The three RXR isoforms (α, β, and γ) can dimerize with PPARs, and specific association with each isotype seems to influence the recognition of target gene promoters [9]. However, very little is known on the specificity of RXR isotype utilized by the different PPARs in vivo. The observation that 9-cis retinoic acid and synthetic RXR agonists can promote the transcription of PPAR target genes leads to a model of permissive transcriptional activation where PPAR/RXR heterodimers can induce transcription in response to PPAR or RXR activation [10, 11]. Moreover, concomitant treatment with both PPAR and RXR agonists potentiates the effects observed with each ligand alone. However, the molecular mechanisms underlying transcriptional permissivity and synergy are not well understood in terms of cofactor recruitment by each partner of the heterodimer.
Table 1: Primers and PCR cycling. The adopted sequences of specific primers and relative cycling conditions of each RT-PCR are indicated.

| Gene   | Gene bank number | Size (bp) | Sequence | Annealing (°C) | Cycles |
|--------|------------------|-----------|----------|----------------|--------|
| PPAR α | Gazouli et al., 2002 | 741 | F 5′ggtcaaggcccagctcactgcagc3′
R 5′tcagctagttctggatgatctc3′ | 69 | 40 |
| PPAR β | Gazouli et al., 2002 | 130 | F 5′gtcatgggaagccccagcagagtcctc3′
R 5′gggaggaattctggagaggtctgcagc3′ | 69 | 40 |
| PPAR δ | Gazouli et al., 2002 | 421 | F 5′gagatgccattctggcccaccacttcgg3′
R 5′tatcataataaaagctcctccttc3′ | 69 | 40 |
| β -Actin | NM_031144 | 661 | F 5′tgacggggtcacccacactgtgcccatcta3′
R 5′ctagaagcattgcgggtggacgatggaggg3′ | 65 | 28 |

Finally, the interplay between PPAR and RXR pathways is further illustrated by PPAR target gene activation in response to RXR homodimers [12].

Cellular proliferation allows the renewal of tissues by providing a pool of undifferentiated cells or progenitors from stem cells. All three PPAR isotypes are involved in the regulation of cell proliferation, death, and differentiation, with different roles and mechanisms depending on the specific isotype and ligand and on the differentiated, undifferentiated, or transformed status of the cell. Thus, proliferative and antiapoptotic or antiproliferative, prodifferentiating and proapoptotic effects, and even procarcinogenic effects have been reported for PPARs [13].

Differentiation stimuli are integrated by key transcription factors which regulate specific sets of specialized genes to allow proliferative cells to exit the cell cycle and acquire specialized functions. The main differentiation programs known to be controlled by PPARs both during development and in the adult are placental differentiation, adipogenesis, osteoblast differentiation, skin differentiation, and gut differentiation. PPARs may also be involved in the differentiation of macrophages, brain, and breast [14]. However, their functions in this cell type and organs still await further elucidation.

In astroglial cells, we have demonstrated the involvement of PPARα in astrocytic differentiation [14]. The expression of PPARβ in the brain peaks between days 13.5 and 15.5 of rat embryonic development [15]. The role of PPARβ in the development of the central nervous system is further illustrated by the myelination defects of the corpus callosum observed in PPARβ null mice [16]. However, the outputs in terms of brain development and the mechanisms regulating the potential implication of PPARβ in the differentiation of cerebral cells are unknown. Recently we have demonstrated that PPARβ expression and activation are increased during neuronal in vitro maturation, thus suggesting a role for this transcription factor in this process [17]. Moreover, we have demonstrated that PPARβ agonists trigger neuronal differentiation in a human neuroblastoma cell line [18]. Very recently we found that PPARβ activation by the synthetic agonist GW0742 leads to early neuronal maturation and BDNF increase, thus suggesting a role for PPARβ in neuronal plasticity (Benedetti et al., manuscript in preparation).

On the basis of the previous evidences, we hypothesize that PPARs may be involved in cell proliferation and differentiation processes of neural stem cells (NSC). To this aim, the expression of the three PPAR isotypes and RXRs in NSC has been investigated.

2. MATERIALS AND METHODS

2.1. Materials

CD1 mice were from Charles River (Harlan, Lecco, Italy); fetal bovine serum (FBS) and Earl’s balanced salt solution (EBSS) were obtained from Invitrogen SRL (Milan, Italy); papain was from Worthington Biochemical (Lakewood, NJ, USA); the culture media was a kind gift of Dr Rosella Galli SCRI-DIBIT (Milan, Italy); EGF and bFGF were from Pepro- tech (Rocky Hill, NJ, USA); matrigel basement membrane matrix-GFR was from Becton Dickinson (Lincoln Park, NJ, USA); BCA protein detection kit from Pierce (Rockford,
2.2. Primary culture and culture propagation
differentiation

Adult CD1 Swiss-Albino mice were killed by cervical dislocation and their brains removed and placed into PBS with penicillin and streptomycin (0.1 mg/mL). The tissues containing the forebrain periventricular region SVZ were dissected and incubated in Earl’s balanced salt solution (EBSS) containing papain (1 mg/mL), EDTA (0.2 mg/mL), and cysteine (0.2 mg/mL) at 37°C for 1 hour. The pieces of tissue were collected by centrifugation at 200 g for 5 minutes and resuspended in 1 mL of the DMEM/F12 containing 0.7 mg of ovomucoid inhibitor. The cells were dissociated using a fire-polished Pasteur pipette and were collected by centrifugation at 300 g for 5 minutes. The cellular pellets were resuspended in DMEM/F12 containing HEPES buffer (5 mM), glucose (0.6%), sodium bicarbonate (3 mM), L-glutamine (2 mM), insulin (25 mg/mL), putrescine (60 μM), apo transferrin (100 μM), progesterone (6.3 ng/mL), sodium selenite (5.2 ng/mL), heparin (2 μg/mL), EGF (20 ng/mL), and bFGF (10 ng/mL), counted and plated in uncoated 25 cm² flask at 8 × 10⁵ cells/cm².

Neurospheres were passaged by harvesting them by centrifugation (200 g for 5 minutes) and triturating them in 200 μL of medium with an automatic pipetter (P200 Gilson).

2.3. Differentiation of stem cell progeny and immunofluorescence

For differentiation, neurospheres were plated onto Matrigel basement membrane matrix-coated (100 μg/mL) well in the medium described above with addition of FBS (10%) without EGF and bFGF for 5 days (S10).

Indifferentiated (S0) and differentiated (S10) neurospheres grown on Matrigel GFR glass coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature (RT) and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT. Nonspecific binding sites were blocked with 10% bovine serum albumin (BSA); in PBS, for 10 minutes at RT. This procedure was performed prior to incubation with primary antibodies, except when the A2B5 or the O4 mouse monoclonal antibodies were used. In this case, fixation followed incubation.

For single immunofluorescent staining, cells were incubated with either of the following primary antibodies: 1:5 mouse monoclonal antinestin, 1:200 mouse monoclonal antigial fibrillary acidic protein (GFAP), 1:300 mouse monoclonal anti-β-tubulin III, 1:30 mouse monoclonal PLP, 1:100 rabbit polyclonal anti-PPARα, β/δ, γ, and with 1:200 antimouse monoclonal A2B5 and O4 overnight at 4°C. All the slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG, antimouse IgG, or antimouse IgM antibodies (1:100), for 30 minutes at RT.
Both primary and secondary antibodies were diluted with PBS containing 10% BSA. Controls were performed by substituting the primary antibody with PBS-BSA, containing or not rabbit nonimmune serum.

Double immunofluorescence with anti-A2B5 and anti-GFAP antibodies was performed as described. Briefly, cells were first incubated with 1:100 anti-A2B5, then fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), and incubated with 1:100 secondary FITC-conjugated goat anti-IgM antibodies. Subsequently, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT. Nonspecific binding sites were blocked with PBS containing 10% BSA for 30 minutes at RT. Immunocytochemistry staining was performed with mouse antinestin (1:5) and anti-GFAP 1:2000 in PBS containing 10% BSA for 1 hour at RT and then with peroxidase-conjugated antirabbit IgG secondary antibodies (1:200 in PBS containing 10% BSA) for 30 minutes at RT; the immunoreactivity was detected with the 3,3′-diaminobenzidine (DAB) reaction. Subsequently, the oil red O staining was performed by the method of Diascro et al. (1998), with minor modifications. Briefly, the cells were stained with 0.35% oil red O, for 1 hour at RT. The working solution of oil red O was prepared as described by Ramirez-Zacarias et al. [19].

After washing with distilled water, cells were counterstained with Mayer’s hematoxylin and allowed to air dry. Coverslips were mounted with Kaiser’s glycerol gelatin and observed with a Leitz Wetzlar Ortholux light microscope.

2.4. Immunocytochemistry oil red O staining

Indifferentiated (S0) and differentiated (S10) neurospheres grown on Matrigel GFR glass coverslips were fixed with 10% formalin in PBS for 10 minutes at room temperature (RT) and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT. Nonspecific binding sites were blocked with PBS containing 10% BSA for 30 minutes at RT. Immunocytochemistry staining was performed with mouse antinestin (1:5) and anti-GFAP 1:2000 in PBS containing 10% BSA for 1 hour at RT and then with peroxidase-conjugated antirabbit IgG secondary antibodies (1:200 in PBS containing 10% BSA) for 30 minutes at RT; the immunoreactivity was detected with the 3,3′-diaminobenzidine (DAB) reaction. Subsequently, the oil red O staining was performed by the method of Diascro et al. (1998), with minor modifications. Briefly, the cells were stained with 0.35% oil red O, for 1 hour at RT. The working solution of oil red O was prepared as described by Ramirez-Zacarias et al. [19].

After washing with distilled water, cells were counterstained with Mayer’s hematoxylin and allowed to air dry. Coverslips were mounted with Kaiser’s glycerol gelatin and observed with a Leitz Wetzlar Ortholux light microscope.

2.5. Protein detection

For cell lysis, 10^7 cells were suspended in 150 μL of RIPA lysis buffer containing NaF [100 mM], Na_4P_2O_7 [2 mM], Na_3VO_4 [2 mM], NP-40 [0.1%], SDS [0.1%], EDTA [5 mM], DOC [0.5%], protease inhibitor cocktail, in PBS 1x solution. The lysates were cleared by centrifugation at 12000 rpm for 20 minutes.

Protein concentration was determined by BCA protein assay kit, using bovine serum albumin as a standard. Samples (20/500 μg protein) were run on 10%–15% polyacrylamide denaturing gels according to Laemmli [20]. Protein bands were transferred on polyvinylidene difluoride (PVDF) sheets by wet electrophoretic transfer according to Towbin et al. [21]. Nonspecific binding sites were blocked for 1 hour at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.25% Tween 20 (TBS-T). Membranes were incubated with the primary antibody at the appropriate dilutions [1:50 for mouse antinestin, 1:1000 mouse anti-GFAP, 1:2000 rabbit antiactin, rabbit anti-PPARα, β, γ] overnight at +4°C in blocking solution, followed by incubation with HP-conjugated secondary antibody (antirabbit; antimouse), at the appropriate dilution (1:2000 in blocking solution), for 1 hour at 4°C. After rinsing, the specific immune complexes were detected by ECL method. Band relative densities were determined and normalized using a semiquantitative densitometric analysis and values are given as relative units.

2.6. RT-PCR

Total cellular RNA was extracted by trizol reagent (Invitrogen) according to the manufacturer’s instructions. The total RNA concentration was determined spectrophotometrically in RNAase-free water and 1 μg aliquots of total RNA were reverse transcribed into cDNA using Kit Gene Specific Relative RT-PCR. After RT 2 μL of the cDNA was used as template in 20 μL of PCR mixture and Taq platinum. The number of cycles was obtained empirically by sampling the PCR amplification of positive control between 22 and 40 cycles and selecting the approximate midpoint of a linear
amplification. Table 1 reports primers sequences and amplification conditions for each gene studied. β-Actin was used as internal control and used for normalization. PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide (0.5 μg/mL) in Tris-borate EDTA buffer. A molecular weight marker was run in parallel and bands of the expected molecular size were detected under UV light. The relative densities of the PCR fragments were determined and normalized using a semiquantitative densitometric analysis and values are given as relative units.

2.7. Statistics

Statistical analysis for multiple comparisons was performed by one-way ANOVA followed by Scheffé’s post hoc test. All statistical calculations were performed using SPSS software. P values <.05 were considered statistically significant.

3. RESULTS

In Figure 1, contrast phase microscopy of neural stem cells growing in neurospheres (Figure 1(a)) and after BrdU incorporation (Figure 1(c)) are shown. Nuclear staining with Hoechst 33258 (Figure 1(b)) clearly shows that almost all cells appear positive for BrdU indicating that they are mitotic in our experimental conditions. Since the proliferation ability is not only exclusive of stem cells, but is shared with progenitors of different lineages, markers of undifferentiated status have also been investigated.

The immunolocalization of nestin (Figure 2(b)) as compared with Hoechst nuclear staining (Figure 2(a)) shows that almost all cells are immunopositive for nestin, which is asymmetrically concentrated in the perinuclear region. Proteolipid protein (PLP) immunolocalization Figure 2(e), membrane protein of differentiated status, shows that almost all cells appear immunopositive for PLP (compare with Figure 2(d)). Only few cells are immunopositive for A2B5, marker of astroglial restricted precursors (Figure 2(c)). GFAP, β tubulin III, and O4, markers of astrocytes, neurons, and oligodendrocytes, respectively, are not expressed (not shown).

Figure 3 shows the immunolocalization of the three PPAR isotypes in neurospheres. Nuclear staining of the same fields is shown in Figures 3(a), 3(b), and 3(c). All the three PPARs are present, almost exclusively localized in the nuclei. See Figures (3(b), 3(d), 3(f)).

Western blotting analysis for nestin, GFAP, PPARα, β, and γ, and RXRs in neurosphere cell lysates confirms the presence of the three PPARs and shows that the only RXR isotype detectable in these cells is the RXRβ (Figure 4).

To assess the possible quantitative/qualitative variations of the receptors during differentiation, neurospheres were cultured in absence of growth factors and in the presence of 10% FBS for 5 days (S10). Figure 5 shows the
immunolocalization of the above-mentioned differentiation markers in S10 neurospheres. Nestin is still expressed, but with lower fluorescence intensity (Figures 5(a) and 5(b)). Moreover, the protein is no more concentrated in the perinuclear region, but uniformly localized throughout the cytoplasm, including the cellular processes; the number of A2B5 immunopositive cells appears slightly increased (Figures 5(b) and 5(e)), while a clear immunofluorescence for GFAP (Figures 5(c) and 5(f)) is observed in many S10 cells. β-Tubulin III and O4 are absent (not shown).

These results demonstrated that, in our differentiating conditions, S10 neurospheres are mainly composed by differentiated astrocytes and their A2B5 precursors.

In Figure 6, double immunofluorescence staining for GFAP and PPARs in S10 neurospheres is shown. In these cells the PPARs are still present but with different fluorescence intensity. In particular, PPARα immunostaining (Figure 6(a)) is stronger, while PPARβ appears weaker than in S0 neurospheres (Figure 6(b)); PPARγ appears unchanged (Figure 6(c)).

Figure 7 shows the western blotting analysis for nestin, GFAP, PPARs, and RXRs in S0 and S10 neurosphere cell lysates. In S10 cells, nestin is significantly decreased, while GFAP is strongly expressed. Interestingly, RXRα, not present in S0 neurospheres, is now detected while RXRβ is unchanged. In agreement with the immunofluorescence data, PPARβ is strongly decreased and PPARγ appears unchanged; concerning PPARα, no significant quantitative differences are observed.

The RT-PCR analysis of PPAR mRNAs in S0 and S10 neurospheres (Figure 8) shows that, during astroglial differentiation, PPARα is significantly increased while PPARβ expression is significantly decreased. PPARγ appears unchanged.

Figure 9 shows the double staining of oil red positive lipid droplets and nestin in S0 (Figure 9(a)) and oil red/GFAP in S10 (Figure 9(b)) neurospheres. Nuclei were counterstained with Mayer haemalum. In S0 neurospheres, almost all immunoreactive nestin cells show several lipid droplets in their cytoplasm, some of which being very large. In S10 GFAP-positive cells, lipid droplets are no more observed.

4. DISCUSSION

In this paper, the presence of all three isotypes of PPARs in mouse adult neural stem cells has been established for the first time. Moreover, we demonstrated that PPARs are subjected to both quantitative and qualitative variations during astroglial differentiation.

The proliferative and undifferentiated status has been demonstrated by immunofluorescence and western blotting.
BrdU incorporation demonstrates that almost all cells of the neurospheres are proliferative and the presence of nestin and PLP, in the absence of markers of differentiation such as GFAP, β-tubulin III, and O4, is consistent with the undifferentiated status and allows to conclude that the cellular population of our neurospheres is constituted by undifferentiated cells [22].

The strongly polarized immunolocalization of nestin suggests that the cells are dividing by asymmetric divisions. In fact, recent studies have demonstrated that, in stem cells, some proteins exhibit different distribution according to their division modality [23, 24].

The result that neural stem cells possess all three PPAR isotypes is new and unexpected. In fact, one would have hypothesized that PPARβ could be the most abundant owing to its relevant presence and early expression during brain development [15] and owing to its involvement in cell proliferation and in the first stages of cellular differentiation [25–27]. Our results demonstrate that all three PPARs are expressed and that they have a nuclear localization in agreement with their function as transcription factors.

It is known that PPARs act in heterodimeric form with RXRs. The immunoblotting data reveal that in neural stem cells only RXRβ is present. This finding is in agreement with previous results demonstrating this isotype as the mainly present in rodent brain [28, 29] and suggests that one or more PPAR isotypes may heterodimerize with RXRβ.

**Figure 7:** Western blotting and relative densitometric analysis in S10 neurosphere cell lysates. An example of western blotting is shown. Densitometric data are means ± SD of 5 different experiments. *, *P < .05; **, *P < .001.
The simultaneous presence of the three PPARs in the nucleus does not indicate that they are all transcriptionally active; in fact it has been proposed that unliganded PPARβ may act as potent inhibitor of the transcriptional activity of the α and γ isotypes [30]. It is possible to hypothesize that in neural stem cells PPARβ contributes to the maintenance of the undifferentiated, proliferative status, by regulating both genes involved in cell cycle control, as observed in other cell types [18, 31, 32], and inhibiting the activity of the other PPARs, which may be, in turn, involved in cellular differentiation [13, 14].

The finding of large lipid droplets in the cytoplasm of NSC is new and suggests a role for PPARγ in this phenomenon. In fact, the importance of this transcription factor is well known in adipocyte differentiation as well as in cellular types where lipidogenesis occurs, such as oligodendrocytes and macrophages [33, 34]. In agreement with this hypothesis, the PPARγ appears to be strongly expressed both at mRNA level and at protein level in undifferentiated NSC.
When NSC were subjected to astroglial differentiation, as expected, GFAP was highly expressed and the nestin was significantly decreased. Moreover, its intracellular distribution is completely different from S0 neurospheres, with the asymmetrical concentration of the protein in the juxtanuclear region being no more observed. The persistence of nestin in these differentiated cells is consistent with data from other authors that have reported a coexpression of GFAP and nestin in astrocytes in culture from postnatal animals; this coexpression, which is not observed in vivo, is induced by in vitro conditions and in vivo during astrogliosis [14, 35].

In the S10 cells, PPARs undergo quantitative modifications. A modulation of PPARs both at protein and mRNA levels is observed. The observed strong decrease of PPARβ is particularly interesting, since it could indicate the removal or reduction of its inhibitory effect on the other PPARs [30]. In this respect, PPARβ might be considered as inhibitor of astroglial differentiation [30, 36]. PPARγ does not vary, both at mRNA and protein levels, while PPARα is significantly increased only at mRNA level. This might be due to the fact that the RT-PCR and western blotting analyses were performed after 5 days of differentiation in vitro. Probably, to observe a significant increase of the protein, a longer time should be tested. However, the increase of PPARα suggests a role for this transcription factor in astroglial differentiation, supported by our previous findings on astrocyte in vitro differentiation [14]. Moreover, the appearance of RXRα, its heterodimeric pattern [29], is in agreement with this suggestion. As regards RXRs, during NSC astroglial differentiation, the data obtained demonstrate that RXRγ is never expressed, in agreement with its restricted localization in adult brain [29, 37], RXRβ remains unchanged, while RXRα is expressed de novo by differentiated cells. Thus, a downregulation of PPARβ, accompanied by PPARα and RXRα increase may be a condition for the differentiation toward astroglial lineage.

As regards PPARγ, the fact that this receptor is not modified may indicate that it is not crucial for astrocyte differentiation, at least concerning the differentiation of type I astrocytes. However, the presence of some A2B5/GFAP immunopositive cells may indicate that, in our experimental conditions, differentiation toward type II astrocytes may also occur. Since type II astrocytes share a common progenitor with oligodendrocytes, the O2A cells, the persistence of PPARγ in differentiating neurospheres could indicate that it may be involved in the oligodendrocyte differentiation pathway.

Regarding the presence of lipid droplets in undifferentiated cells, their disappearance during differentiation may be in agreement with the hypothesis that in our experimental conditions, the differentiation toward type I astrocytes is preferred. In fact, differentiated astrocytes are able to utilize lipids as energy fuel [38] through catabolic lipid pathways requiring PPARα and not PPARγ activity, involved instead in lipidogenesis.

Overall, the data presented in this work indicate that the decrease of PPARβ and the concomitant increase and/or activation of PPARα together with RXRα are involved in astroglial differentiation of NSC.

In our opinion, however, it should be underlined that the regulation of different differentiation pathways and/or the maintenance of undifferentiated status are more affected by the quantitative ratios existing among the receptors isotypes (both PPARs and RXRs) rather than by the absolute amounts of each one of them.

ACKNOWLEDGMENT

This work has been supported by MIUR PRIA 2007 (Professor A. Cimini).

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