Sequential RARβ and α signalling in vivo can induce adult forebrain neural progenitor cells to differentiate into neurons through Shh and FGF signalling pathways

Maria Beatriz C.V. Goncalves a,1, Marta Agudo a,1, Siobhán Connor a,2, Stephen McMahon a, Stephen L Minger a,2, Malcolm Maden b, Jonathan P.T. Corcoran a,⁎

a The Wolfson Centre For Age-Related Diseases, King’s College London, Guy’s Campus, London SE1 1UL, UK
b MRC Centre for Developmental Neurobiology, King’s College London, Guy’s Campus, London SE1 1UL, UK

Abstract

We show here the role of retinoic acid receptor (RAR) β and α signalling in proliferation and differentiation of endogenous adult forebrain neural progenitor cells (NPCs). RARβ activation stimulates Sonic hedgehog signalling (Shh), and induces the proliferation of the NPCs. They can be induced to become Doublecortin (DCX) expressing migrating neuroblasts by RARβ signalling, some of which differentiate into cholinergic neurons. The same signalling pathways cause the proliferation of embryonic forebrain NPCs. These cells express glial fibrillary acidic protein (GFAP) and are predominantly uni/bipolar, two characteristics of neuronal progenitor cells. We further show that fibroblast growth factor (FGF) signalling, induces the expression of the retinoic acid degrading enzyme cytochrome P450 (cyp) 26a1, and that one of its products, 4-oxo-RA, mimics the action of the RARα agonist in the differentiation of the NPCs into cholinergic neurons.

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Introduction

In mammals, active neurogenesis occurs throughout life in the subventricular zone (SVZ) of the lateral ventricle and in the subgranular zone of the dentate gyrus in the hippocampus (Ming and Song, 2005). In the SVZ, glial fibrillary acidic protein (GFAP)-expressing cells include a neurogenic cell population (Doetsch et al., 1999; García et al., 2004) that gives rise to migrating neuroblasts (DCX) before the neurons mature (Englund et al., 2002; Gleeson et al., 1999).

Retinoids represent a family of compounds derived from vitamin A, which have been shown to play a role in both proliferation and differentiation in adult neurogenesis (Haskell and LaMantia, 2005; Wang et al., 2005). Cellular effects of retinoids are mediated by the binding to specific nuclear receptors, retinoic acid receptors (RARs), and retinoid X receptors (RXRs). (Mangelsdorf and Evans, 1995). There are three subtypes of each receptor: α, β and γ and multiple isoforms of each subtype due to alternative splicing and differential promoter usage (Leid et al., 1992). RARs heterodimerize with RXRs by binding to retinoic acid response elements (RAREs) in the upstream regions of target genes (Bastien and Rochette-Egly, 2004) and thus mediate gene expression. The biosynthesis and degradation of retinoids involve retinal dehydrogenases (Raldh), 1–3 which synthesise all trans retinoic acid (atRA) (Duester et al., 2003) and cytochrome P450 (cyp) enzymes a1, b1 c1 and d1, which hydrolyses atRA to the polar metabolites 4-hydroxyl RA and 4-oxo-RA (Gu et al., 2006; Ray et al., 1997; White et al., 1996).

In vitro, the sequential activation of RARβ and α, induces neuronal differentiation of cultured spinal cord progenitor cells (Goncalves et al., 2005) and although both receptors have been shown to be expressed in the adult SVZ (Haskell and LaMantia, 2005) their role in neurogenesis in vivo is unknown. The RA signalling pathway interacts with other pathways in neurogenesis, in Raldh-2 null mice proliferation and differentiation of forebrain NPCs is impaired, which correlates with defects in fibroblast growth factor (FGF) and Sonic hedgehog (Shh) signalling (Ribes et al., 2006). The FGF signalling pathway promotes neurogenesis in the SVZ (Jin et al., 2005, 2003; Kosaka et al., 2006) and drives neuronal differentiation along particular lineages (Kosaka et al., 2006). Shh signalling has also been linked to neurogenesis in the adult brain (Ahn and Joyner, 2005; Machold et al., 2003; Palma et al., 2005), in Shh null mice dorsalventral patterning, the specification of ventral cell populations, and general brain proliferation are all affected (Chiang et al., 1996; Rallu et al., 2002).

Using RAR specific agonists in vivo, we have defined the roles of RARβ and α signalling in adult neurogenesis and their interaction with the FGF and Shh signalling pathways. We show that RARβ agonists cause an increase in proliferation of the NPCs via stimulation of the Shh signalling and RARα signalling induces the differentiation...
of NPCs into migrating neuroblasts followed by maturation into neurons via FGF signalling.

Results

At the time of embryonic neurogenesis retinoids including atRA can be detected in all regions of the brain (Horton and Maden, 1995). Therefore, we asked if the limited neurogenesis in the adult subventricular zone (SVZ) correlates with a lack of retinoids. These were measured by HPLC in adult mouse brain (3 month old). In the cerebrum (Fig. 1A) polar retinoids were detected eluting at 8 min (Fig. 1A, peak 1); a very high level of a retinoid eluting at 16 min with a two peak absorption spectrum, maxima at 309 nm and 323 nm (Fig. 1A, peak 2 and inset). There was little or no atRA (Fig. 1A, peak 3), a retinoid with maxima of 322 nm which is probably 4-OH retinol (Fig. 1A, peak 4 and Fig. 1B, inset) and very low levels of at-Retinol (Fig. 1A, peak 5). The brain stem had a similar retinoid profile except for the absence of polar retinoids (Fig. 1B). In contrast in the cerebellum there were high levels of atRA (Fig. 1C, peak 3), a retinoid with maxima of 322 nm and a UV maxima of 319 nm, 333 nm and 349 nm. Peak 2 is a known retinoid with 2 UV maxima at 309 nm and 323 nm, full spectrum is shown in the inset in panel A. Peak 3 is atRA, full spectrum is shown in the inset in panel C. Peak 4 is possibly 4-OH retinol with a UV maxima at 322 nm, full spectrum is shown in the inset in panel B. Peak 5 is at-Retinol.

Therefore, we assessed the role of atRA in neurogenesis and its interaction with other signalling pathways. Previous work has shown the importance of atRA and Shh signalling pathways in the proliferation of NPCs in vivo (Haskell and Lamantia, 2005; Lai et al., 2003; Palma et al., 2005; Wang et al., 2005) and the Shh promoter contains RARE (Chang et al., 1997), this suggests that the Shh and RA signalling pathways might be linked in neurogenesis. In order to define this interaction further, embryonic forebrain NPCs were cultured in the presence of 100 ng/ml Shh and 0.1 μM atRA for 2 days and positive cells for the proliferation marker Ki67, and GFAP to assess neural lineage (Doetsch et al., 1999; Garcia et al., 2004) were counted. In the presence of Shh alone, proliferating cells could be detected with the proliferation marker Ki67 but not GFAP (Figs. 2A–C & P). atRA had the opposite effect, very few NPCs proliferated, and some multipolar GFAP positive cells could be identified (Figs. 2D–F & P) a characteristic of a more committed glial lineage (Garcia et al., 2004). In the presence of both atRA and Shh, there was a significant increase in proliferating cells compared to atRA alone and the majority of these were GFAP positive with a bi/unipolar morphology (Figs. 2G–I & P) a profile consistent with a neuronal progenitor phenotype (Garcia et al., 2004). In order to identify the specific RAR involved in this process we repeated the Shh supplemented cultures in the presence of the retinoid agonists. In RARα agonist and Shh treated cultures there was a significant decrease in Ki67 expressing cells compared to atRA plus Shh treated cultures and the GFAP positive cells were mainly multipolar (Figs. 2J–L & P). In contrast, there was a significant increase in proliferation in the RARβ agonist and Shh treated cultures compared to atRA treated cultures and the majority of these cells were GFAP positive bi/unipolar (Figs. 2M–O, & P). Therefore, it is RARβ signalling in combination with Shh signalling which is involved in proliferation of the NPCs and atRA predominantly activates RARβ in our culture conditions.

In order to ask if RARβ signalling is also responsible for NPC proliferation in vivo we treated adult rats (3 months old) with RAR specific agonists (5 μl of 1 μM) by daily intraventricular infusion and were concurrently injected i.p. with BrdU (50 mg/kg weight) for 7 days. The number of BrdU labelled cells in the lateral wall of the lateral ventricle of the SVZ did not significantly increase in RARα agonist treated brains compared to vehicle treated ones (Fig. 3A). In contrast, in the RARβ agonist treated brains there was a 3 fold increase in the number of BrdU labelled cells compared to controls (Fig. 3A). This increase in proliferation correlated with an increase in Shh expression as shown by in situ hybridisation (Figs. 3B, C).

We have previously shown that sequential activation of RARβ and α by receptor specific agonists leads to differentiation of NPCs into cholinergic neurons in vitro (Goncalves et al., 2005), we next asked if the same order of activation could cause endogenous adult NPCs to differentiate in the SVZ. We assessed for migrating neuroblasts by DCX labelling and cholinergic neurons by choline acetyltransferase (ChAT) labelling. In RARβ agonist treated animals there was no increase in double labelled BrdU/DCX positive cells (Fig. 4). However, when the RARα agonist was administered for 4 days subsequent to 3 days of RARβ agonist treatment, proliferating progenitor cells were induced to a neuronal fate as shown by the significant increase in the number of double labelled BrdU/DCX positive cells compared to vehicle treated. Of these BrdU positive cells, 9% differentiated into cholinergic neurons as shown by the colocalisation of ChAT expression (Fig. 4). Therefore, the same order of activation of the RARs in vivo that we
Fig. 2. The RA and Shh signalling pathways cooperate to maintain forebrain NPCs in a proliferative GFAP positive uni/bipolar state. (A–C) Shh signalling induces GFAP negative, Ki67 positive forebrain NPCs. (D–F) RA signalling induces GFAP positive, Ki67 negative forebrain NPCs. (G–I) RA and Shh signalling pathways together induce GFAP, Ki67 positive cells the majority of which are bi/unipolar. (J–L) RARα and Shh signalling induces multipolar GFAP, Ki67 negative cells. (M–O) RARβ and Shh signalling induces GFAP, Ki67 positive cells the majority of which are bi/unipolar. (P) Quantification of the cultures shown in c–q. n=3 cultures per treatment repeated 3 times and 3 fields per coverslip. *P<0.05 for Ki67 cells compared to atRA treated cultures.
Fig. 3. RARβ signalling induces proliferation of endogenous rat forebrain NPCs and expression of Shh in the SVZ. RAR specific agonists (5 μl of 1 μM) were injected via a canula into the lateral ventricle of adult rats and they were injected i.p. with BrdU (50 mg/kg weight) once a day for 7 days. (A) Quantification of number of BrdU positive cells in the SVZ. (B, C) Shh expression in the adult SVZ. In the presence of the RARβ agonist there is a significant increase in the number of proliferating NPCs compared to vehicle and RARα treated. In vehicle treated brains few if any Shh cells can be detected in the SVZ (B), in contrast the RARβ agonist induces numerous Shh expressing cells (C). n=6 animals and 5 sections per animal. *P<0.05 for BrdU compared to vehicle treated brains.

Fig. 4. Effect of retinoid signalling on differentiation of endogenous NPCs. RAR specific agonists (5 μl of 1 μM) were injected via a canula into the lateral ventricle of adult rats and they were injected i.p. with BrdU (50 mg/kg weight) once a day for 7 days. (A, B) vehicle, (C, D) RARβ agonist, (E, F) RARβ for 3 days then RARα agonist for 4 days (G, H) BrdU and ChAT expression in SVZ of rats treated with RARβ then RARα agonist. (I) Quantification of number of double labelled BrdU/DCX positive cells and BrdU/ChAT neurons. RARβ signalling does not increase the number of DCX/BrdU double labelled cells compared to vehicle. In RARβ then RARα treated animals there is a significant increase in the number of double labelled DCX/BrdU cells compared to vehicle treated brains, 9% of which differentiate into cholinergic neurons. n=6 animals and 5 sections per animal. *P<0.05 for BrdU and BrdU/DCX compared to vehicle treated brains.
have shown in vitro, is required for differentiation of the NPCs (Goncalves et al., 2005).

We next addressed the endogenous source of RARα agonists for neuronal differentiation. Overexpression of cyp26a in P19 embryonic carcinoma (EC) cells causes them to differentiate into neurons (Sonneveld et al., 1999). Furthermore, when leukaemia inhibitor factor (LIF), which maintains embryonic stem (ES) cells in a proliferative state, is withdrawn from such cultures, cyp26a is induced and the cells differentiate into neurons (Lane et al., 1999). This enzyme generates polar retinoids (Ray et al., 1997; White et al., 1996) which we have identified in the adult mouse brain (Fig. 1) and has been shown to be regulated by FGF signalling (Moreno and Kintner, 2004; Shiotsugu et al., 2004), in particular FGF-9 is involved in cholinergic differentiation (Kanda et al., 2000). To ask if this regulation occurred in forebrain NPCs, we cultured them in the presence of 0.1 μM atRA or 20 ng/ml of either FGF-9 or FGF-2, for 4 days, and assayed for cyp26a expression by RT-PCR and cholinergic differentiation by immunohistochemistry with vesicular acetylcholine transporter (VACHT). In the presence of atRA there was a 1.8 fold induction of cyp26a compared to vehicle treated controls (Fig. 5A) and some cholinergic neurons were present (Fig. 5B) whilst FGF-2 had no effect on either cyp26a expression or cholinergic differentiation (Figs. 5A, C). In contrast, in the presence of FGF-9 there was a 4 fold induction of cyp26a expression compared to control cultures (Fig. 5A) but no cholinergic neurons could be detected (Fig. 5D). This suggests that both FGF and RA signalling are required for neuronal differentiation.

Therefore, we next asked if sequential atRA and FGF signalling could induce the forebrain NPCs to differentiate into cholinergic neurons to a greater extent than atRA alone. NPCs were cultured in the presence of 0.1 μM atRA for 5 days and 20 ng/ml FGF-9 on days 3–5. Under these culture conditions there was a significant increase in the number of cholinergic neurons compared to atRA alone (Figs. 6A, F). To confirm that this was an FGF mediated response we repeated the experiment using the specific FGF inhibitor SU5402 (Sun et al., 2000) and to make sure that cyp26a was indeed important for neuronal differentiation we used: Liarozole which blocks its activity (Sonneveld et al., 1999). In the presence of either of these inhibitors neuronal cholinergic differentiation was impeded (Figs. 6B, C & F). The block in FGF signalling could be overcome when the cultures were supplemented with either 0.1 μM RARα agonist (Figs. 5D, F) or 0.1 μM 4-oxo-RA (Figs. 6E, F), suggesting that 4-oxo-RA can activate RARα which leads to cholinergic differentiation.

**Discussion**

Our data taken together suggests that a sequential activation of RARγ then α is required for the differentiation of adult forebrain NPCs in vivo as shown for cultured embryonic NPCs. (Goncalves et al., 2005), implying that similar mechanisms operate in adult and embryonic neurogenesis (reviewed in Zhao et al., 2008). One reason for the decreased neurogenesis in the adult SVZ compared to the embryo is the lack of retinoids including atRA in the forebrain as neurogenesis can be increased by agonists of RARβ and α, two receptors which are known to be expressed in the adult SVZ (Haskell and LaMantia, 2005) and which can be autoregulated by their own ligands (Leid et al., 1992). The loss of retinoid signalling has also been shown with aging in the adult brain which correlates with a decline in cognition that can be reversed by atRA (Etchamendy et al., 2001; Mingaud et al., 2008).

Whilst atRA which can activate all the RARs, causes the proliferation and differentiation of adult NPCs in vivo or in slice cultures (Haskell and LaMantia, 2005; Wang et al., 2005), we have identified here the specific RARs involved in this process. There are at least two retinoid signalling steps required for the generation of neurons from endogenous NPCs. The first step, involves the activation of RARγ which induces Shh expression (Fig. 7), a pathway known to be important for in vivo neurogenesis (Ahn and Joyner, 2005; Lai et al., 2003; Palma et al., 2005). The combination of RARγ and Shh signalling in vitro causes the NPCs to express GFAP and proliferate with a predominantly bi/unipolar morphology, which are characteristics of neuronal progenitor cells (Garcia et al., 2004) (Fig. 7) whereas RA...
signalling alone causes the NPCs to differentiate into astrocytes through RARα signalling or neurons through RARβ and α signalling (Goncalves et al., 2005). It is unlikely that the NPCs are producing the Shh themselves as we see no increase in their proliferation in the presence of the RARβ agonist alone in vitro and consistent with this Shh has not been detected in NPCs in vivo (Palma et al., 2005). The second retinoid signalling step requires the activation of RARα and leads to the expression of DCX, a microtubule-associated protein that is present in migrating neuronal precursors of the CNS (Aigner et al., 2003; Des et al., 1998; Koizumi et al., 2006) some of which differentiate into cholinergic neurons (Fig. 7). These are new neurons as they are labelled with both BrdU and ChAT rather than existing neurons expressing more ChAT due to the RARα signalling (Corcoran et al., 2004).

Shh signalling in combination with atRA (a pan agonist) can induce the uni/bipolar GFAP phenotype, this effect also occurs with the RARβ agonist suggesting that atRA preferentially activates RARβ signalling in our culture conditions (Fig. 7). The polar retinoid 4-oxo-RA can activate RARα signalling as this agonist can mimic the effect of the RARα agonist on neuronal differentiation (Fig. 7). Accordingly, previous work has shown that RARα can be activated preferentially by 4-oxo-RA rather than atRA whereas there is no preference between these agonists for RARβ activation (Idres et al., 2002). It is also interesting to note that the RARα is highly expressed in the cortex compared to the other RARs (Corcoran et al., 2004; Yamagata et al., 1994; Zetterstrom et al., 1999) and the majority of retinoids found here are of the polar type. Polar retinoids have been shown to be active compounds in other physiological pathways. In Xenopus, very little atRA can be detected, instead 4-oxoretilnaldehyde which is converted into 4-oxo-RA is the predominate retinoid (Blumberg et al., 1996) and this is involved in positional specification during Xenopus development (Pijnappel et al., 1993). In mammals 4-oxo-RA has been found to be active in skin cells (Heise et al., 2006; Reynolds et al., 1993) and is involved in alveolar regeneration (Maden, 2006).
Fig. 7. Model of retinoid signalling in forebrain NPC proliferation and differentiation. In the presence of RARβ signalling, Shh is induced and these two pathways give rise to GFAP positive uni/bipolar proliferating neuronal progenitors. FGF signalling induces the expression of Cyp26a, which breakdowns atRA into more polar metabolites, including 4-oxo-RA, which can activate RARα signalling. This leads to the NPCs becoming DCX positive neuroblasts some of which differentiate into cholinergic neurons.

The sources of 4-oxo-RA for the embryonic NPCs are the cells themselves as they express cyp26A1, this enzyme has also been shown to be expressed in ES and EC cells (Lane et al., 1999; Langton and Gudas, 2008; Ray et al., 1997; Sonneveld et al., 1999), and in the adult brain cyp26A1 is expressed in the olfactory bulb and hippocampus which are sites of neuronal differentiation (Ray et al., 1997). However, it is unclear how important these polar retinoids are as manipulations of the Cyp26 enzymes which synthesise them give contrasting results. On one hand in both ES and EC cells the presence of cyp26a is essential for differentiation (Lane et al., 1999; Langton and Gudas, 2008; Sonneveld et al., 1999). On the other hand Cyp26a null mice which are non viable and have multiple deficits characteristic of excess atRA (bu-Abed et al., 2001) can be rescued when they are crossed with heterozygous Raldh2-2 mice (Niederreither et al., 2002). This depletes their endogenous atRA levels thus removing its toxicity, suggesting that 4-oxo-RA is not essential for normal development (Niederreither et al., 2002). In agreement with this, atRA alone is sufficient for neuronal differentiation as some cholinergic neurons can be obtained, however when 4-oxo-RA signalling is used subsequent to atRA signalling many more cholinergic neurons are formed (Fig. 6).

We have further shown here that FGF signalling acts downstream of RARβ signalling (Fig. 7). FGF signalling regulates cyp26a expression, (Fig. 7), this regulation also occurs in embryonic axis formation (Shiotsugu et al., 2004) and segmental patterning during somitogenesis (Moreno and Kintner, 2004). Whilst atRA can induce cyp26a expression, this is at a lower level than FGF-9 and as such, induces cholinergic differentiation to a lower extent than sequential atRA and FGF-9 signalling. Our data also suggest that FGF signalling acts downstream of Shh signalling in the differentiation of forebrain NPCs. During development of the anterior neural tube, from which the forebrain is derived, the same order of activation occurs. Deletion of the FGF receptors 1 and 3 results in loss of ventral precursor cells whilst there is no effect on Shh expression (Gutin et al., 2006). It is unclear which FGF is involved in the developing cholinergic neurons since there are other FGF genes expressed in the developing telencephalon (Crossley et al., 2001; Maruoka et al., 1998; McWhirter et al., 1997; Xu et al., 2000). However, FGF-2 cannot regulate Cyp26a expression or drive the differentiation of the NPCs whereas FGF-9 can carry out these roles suggesting that there are different effects of FGFs on the proliferation and differentiation of NPCs.

In summary, we have shown that sequential RARβ and α signalling can lead to the proliferation and differentiation of forebrain NPCs through interaction with Shh and FGF signalling pathways. Such manipulation of the retinoid signalling pathway may lead to a therapeutic opportunity for brain repair after stroke and injury and a novel treatment for neurodegenerative diseases.

Materials and methods

In vivo procedures

Adult Wistar rats (Harlan, UK) were used for all studies. All procedures were undertaken in accordance with the UK animal’s scientific procedure Act 1986. Animals undergoing surgery were anaesthetised with an intraperitoneal (i.p.) injection of ketamine at (65 mg/kg body weight)/medetomidine (0.25 mg/kg body weight) mixture. Sterile precautions were maintained for all surgical procedures.

A brain infusion catheter (Alzet) was inserted into the lateral ventricle (Bregma coordinates: medio–lateral = −2, 5, rostro–caudal = −1, 5 and dorso–ventral = 4 mm). Animals (n=6 per group) were injected once a day for 7 days with either 5 μl of 1 μM of RARβ agonist (BMS 213309) or RARα agonist (BMS 194753). Another group was injected for 3 days with 5 μl of 1 μM of RARβ agonist then 4 days with 5 μl of 1 μM of RARα agonist (BMS). Another 6 animals were injected with vehicle for 7 days. All injections were followed by a saline flush of 6 μl. To locate proliferating cells, all animals were i.p. injected with BrdU (50 mg/kg of body weight) every day during the course of the retinoid treatment.

Animals were deeply anaesthetised with pentobarbital and perfused transcardially with 4% paraformaldehyde after saline rinse. The tissue was then gelatin embedded (10% gelatin 300 bloom from Sigma) in water fixed for 5 h with 4% PFA, this was then changed for PBS-sodium azide (1%, Sigma) and tissue was stored at 4 °C until further use. Fifty μm sagittal sections were cut on a vibratome. For quantification 5 sections of the same Bregma coordinates were used, spaced 200 μm apart. Positive cells for various markers were counted blindly in a 0.1 mm² area of the lateral ventricle of the SVZ.

Cell culture

Forebrain NPCs were isolated from gestational day 14 (E14) Fischer 344 rat embryos (crown-rump length 9–11 mm) and cultured using previously established procedures (Minger et al., 1996). The forebrain ventricular areas were removed and collected in sterile Dulbecco’s phosphate buffered saline (PBS). Tissue was incubated in 0.1% trypsin/ PBS for 30 min at 37 °C, centrifuged at 1000g and resuspended in PBS-glucose three times, then dissociated to a single cell suspension by repeated pipetting through narrowed Pasteur pipettes. Cell viability and density were determined by trypan blue exclusion and haemocytometric counting. Cells were plated at a density of 30,000 cells per well on 13 mm² glass coverslips precoated with 10 μg/ml polyornithine and 10 μg/ml laminin (Gibco) in 24-well plates (Nunc). The cells were grown in DMEM/F12 high glucose media with N2 supplement (Gibco) in 95% air/5% CO2 humidified atmosphere in the presence of 20 ng/ml FGF-2 (PetroTech) for 72 h. The media was then changed and supplemented with 0.1 μM of the appropriate retinoid agonist instead of FGF-2. The agonists used were all TRα (Sigma), 4-oxo-RA (Hoffman La Roche) and retinoid-specific agonists as described above. In addition the following compounds were used, Liarozole (10 μM) a inhibitor of cyp26a (Sonneveld et al., 1999), SU5402 (5 μM, Calbiochem) a inhibitor of FGF signalling (Sun et al.,
Graphs and statistics

Graphs were plotted using Sigma plot. Data is expressed as mean±S.E.M and statistical analysis carried out using Student’s t-test.

References


