Increased cell death and delayed development in the cerebellum of mice lacking the rev-erbAα orphan receptor

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Accepted 10 January; published on WWW 7 March 2000

SUMMARY

The rev-erbAα gene, belonging to the steroid receptor superfamily of transcription factors, is highly conserved during evolution but little is known so far about its functions in development or in adult physiology. Here, we describe genetically altered mice lacking the rev-erbAα gene. These animals do not show any obvious phenotype in either fat tissue or skeletal muscle, despite the known regulation of rev-erbAα expression during adipocyte and myotube differentiation in vitro. However, during the second week of life, the cerebellum of rev-erbAα mutants presents several unexpected abnormalities, such as alterations in the development of Purkinje cells, delay in the proliferation and migration of granule cells from the external granule cell layer and increased apoptosis of neurons in the internal granule cell layer. Interestingly, the expression pattern of rev-erbAα suggests that the abnormalities observed in the external granule cell layer could be secondary to Purkinje cell alterations. Taken together, our data underline the importance of rev-erbAα expression for the appropriate balance of transcriptional activators and repressors during postnatal cerebellar development.

Key words: Orphan nuclear receptor, Cerebellar development, Knockout mice, rev-erbAα gene

INTRODUCTION

The nuclear hormone receptors constitute a large family of transcription factors that can be broadly divided into three groups (Mangelsdorf et al., 1995). A first group includes the different steroid hormone receptors that bind as homodimers to DNA half-sites organized as inverted repeats. The second group comprises the receptors for thyroid hormone, retinoic acids and vitamin D, as well as the peroxisome-proliferator activated receptors: these receptors usually form heterodimeric complexes with the retinoid X receptor (RXR) on specific direct repeats of the AGGTCA core-binding site. The third and largest group consists of ‘orphan receptors’, i.e. proteins that by homology belong to the nuclear receptor family but for which no specific ligand has yet been identified. It is expected that some may interact with novel ligands, while others may represent constitutive activators or repressors, or factors whose activity can be modulated by post-translational modification. Orphan receptors can bind DNA either as monomers on 5′-extended half-sites (Harding and Lazar, 1993; Mc Broom et al., 1995; Wilson et al., 1993), as homodimers (Jiang et al., 1995; Mangelsdorf and Evans, 1995), or even as heterodimers with RXR (Leblanc and Stunnenberg, 1995). They are found in many different species, ranging from nematodes to vertebrates, and their conservation during evolution suggests important functions during development and/or homeostasis. This has been demonstrated by gene targeting of the SF-1, HNF4 and Nurr1 orphan receptor genes, since the corresponding null mutants die either in utero or soon after birth (Chen et al., 1994; Luo et al., 1994; Zetterström et al., 1997).

Rev-erbAα is an orphan nuclear receptor whose gene partially overlaps the thyroid hormone receptor α (TRα) gene (Lazar et al., 1989; Miyajima et al., 1989). It can bind as a monomer to a single 5′-extended core-binding site (WAWNTAGGTCA, where W = A or T), and as a dimer to a direct repeat of this element spaced by two nucleotides (Harding and Lazar, 1995). Interestingly, rev-erbAα lacks the AF-2 transactivation domain that is preserved in the C terminus of most transcription-stimulating receptors (Durand et al., 1994). In line with this, rev-erbAα does not behave as a transactivator in transfection experiments. Instead, it represses the basal level of transcription, and a transferable repression domain has been identified in the C terminus of the protein (Harding and Lazar, 1995). In addition, the E domain of rev-erbAα has been shown to specifically interact with N-CoR, the corepressor for thyroid hormone and retinoic acid receptors (Downes et al., 1996; Zamir et al., 1996). Two related orphan
receptors have been identified that also bind as monomers to the rev-erbAα binding site. One of them, rev-erbAβ, is highly related to rev-erbAα and shows a similar ability to repress the basal level of transcription (Dumas et al., 1994; Retnakaran et al., 1994). By contrast, the retinoic acid-related orphan receptor α (RORα) was found to behave as a potent constitutive transactivator when bound to this element (Forman et al., 1994), raising the interesting possibility that the opposite transcriptional effects exerted by rev-erbAαβ and RORα could provide the fine tuning needed for the expression of certain target genes.

The peculiar arrangement of the rev-erbAα gene on the opposite strand of the TRα gene has led to the suggestion that its transcription could interfere with the expression of the thyroid hormone receptor (Lazar et al., 1989). Indeed, the 3′ end of the rev-erbAα gene overlaps with the TRα2, but not with the TRα1 transcript, and it has been suggested that the presence of rev-erbAα transcripts influences the alternative splicing of TRα in vitro by an antisense mechanism (Munroe and Lazar, 1991). Since the TRα2 and TRα1 proteins are thought to be functionally antagonistic (Koenig et al., 1989), the existence of such a regulatory system could have major consequences for the cellular T3 responsiveness. However, it remains to be demonstrated whether rev-erbAα functions as a physiologically significant antisense regulator in vivo.

The high conservation of the rev-erbAα DNA binding domain during evolution suggests that it is an important regulator of gene expression. In the adult organism, rev-erbAα is expressed mainly in fat, skeletal muscle and brain, with lower levels in several other tissues (Forman et al., 1994; Lazar et al., 1989). Its expression is induced during the course of adipocyte differentiation (Chawla and Lazar, 1993), although its biological function in this process remains unclear. It has also been reported to be downregulated in vitro during myotube formation, and overexpression of the receptor in myoblasts appears to block their ability to differentiate (Downes et al., 1995). By contrast, its putative function in the nervous system is largely unknown and much remains to be discovered about the genetic pathways that are regulated by rev-erbAα. As a first step towards a better understanding of the function of this orphan receptor, we report here the deletion of the rev-erbAα gene from the mouse germ line and a first analysis of the resulting mutant animals.

MATERIALS AND METHODS

Targeting vector

To construct the rev-erbAα targeting vector, a KpnI site was first introduced in the beginning of exon 2 by site-directed mutagenesis (codons 39 and 40: GGT AGG → GGT ACC). A KpnI/HindIII β-gal/neo cassette was then inserted between this KpnI site and a BglII site located in exon 6, destroying the BglII site and bringing the β-gal coding sequence in-frame with the rev-erbAα open reading frame. In the assembled targeting vector, the 5′ and 3′ homology regions extend to BglII sites located 1.6 kb upstream and 6.4 kb downstream of the β-gal/neo cassette, respectively.

Probes

The probes used were the revα 5′ probe (a 600 b anchor-PCR product corresponding to exon 1), a 312 b PCR fragment specific for TRα1 (nt 130-441 of exon 9), a 529 b fragment derived from the α2-specific exon 10 of the TRα gene, as well as a G3PDH 450 b PCR product (Clontech).

Histochemical analysis of β-galactosidase expression

Whole-mount embryos and fresh cryostat sections of adult tissues were fixed for 10 minutes in 0.2% glutaraldehyde in PBS. After three washes in PBS, the samples were first permeabilized in PBS with 2 mM MgCl₂, 0.02% NP40 and 0.01% sodium deoxycholate for 20 minutes and then incubated from 2 hours up to overnight in the same buffer supplemented with 5 mM KFe(CN)₆, 5 mM K₃Fe(CN)₆ and 1 mg/ml 5-bromo-4-chloro-3-indole-b-D-galactoside (X-gal). Tissue sections were counterstained in Eosin, dehydrated and mounted in Pertex.

Cerebellar tissue processing

For immunohistochemistry, BrdU incorporation and in situ detection of DNA fragmentation wild-type and mutant mice were deeply anaesthetized and perfused with 5 ml of PBS followed by 30 ml of 4% paraformaldehyde in PBS. Brains were then removed, postfixed for 2 hours and cryoprotected by immersion in 10% sucrose for 48 hours at 4°C. Cryostat sections (14 µm thick) were mounted on 3-aminopropyl ethoxysilane-coated slides and stored at −20°C.

Immunohistochemistry

Sections were first incubated overnight at 4°C with an antibody against either calbindin D28k (1:500, Sigma), E. coli β-galactosidase (1:50, Cappel), p75 NGFR (1:100, a kind gift of Dr L. F. Reichardt), or antibodies against either calbindin D28k (1:500, Sigma), E. coli β-galactosidase (1:50, Cappel), p75 NGFR (1:100, Cappel), or Aldolase C/Zebbrin II (1:5, a kind gift of Dr R. Hawkes) in 3% bovine serum albumin and 0.3% Triton X-100. After washing and blocking with 10% goat serum in PBS for 1 hour, the sections were incubated for 1 hour at room temperature with a lissamine-rhodamine-donkey anti-mouse IgG (1:100, Jackson) for the calbindin D28k staining or with a rhodamine-goat anti-rabbit IgG (1:100, Dako) for the Aldolase C/Zebbrin II staining. After four washes, the slides were mounted in PBS-glycerol containing p-phenylenediamine. To detect the Aldolase C/Zebbrin II immunoreactivity, a goat horseradish peroxidase-conjugated anti-mouse IgG (1:200, Dako) was used as secondary antibody. Slides were incubated with 0.05% diaminobenzidine, 0.006% hydrogen peroxide in 0.1 M Tris, pH 7.5, dehydrated and mounted in Pertex.

Measurement of cerebellar layers

Transverse sections parallel to the axis of the folia were stained with Cresyl Violet and used to measure the size of the molecular and external granule cell layers. Images were obtained with a Sensys camera (Photometrics) attached to a Nikon Microphot FXA microscope, and were analysed using the IPLab Spectrum 3.1 image processing. Six randomly chosen fields, in the middle of the lobule, in identical anteroposterior and mediolateral coordinates for each individual, were analysed per slide in four sections per animal, in three animals per condition.

BrdU incorporation and immunohistochemical detection

Pups were subcutaneously injected with bromo-deoxy-uridine (BrdU), 50 mg/kg of body mass. 5 hours later, animals were perfused and their brains were quickly processed for immunohistochemistry, as described. Cerebellar cryostat sections were washed in PBS, treated with 2 M HCl in 70% ethanol for 10 minutes at −20° C, thereafter with 0.1 M HCl for 20 minutes at 4°C and with 2 M HCl in PBS at room temperature for 1 hour. Then, sections were treated for 15 minutes in 0.1 M boric buffer at pH 8.3, washed four times in PBS, and blocked with 10% goat serum and 2% tween in PBS at room temperature for 1 hour prior to incubation with an antibody against BrdU (Sigma 1/500) in blocking solution, at 4°C overnight. After five washes in PBS, sections were incubated with a lissamine-rhodamine-donkey antimouse IgG (1/100, Jackson) at room temperature for 1 hour and washed four times before mounting in Vectashield antifading (Vector).
In situ detection of DNA fragmentation

TUNEL stainings were performed using the in situ Apoptag-kit (Oncor) as previously described (Neveu and Arenas, 1996). For the double stainings, sections were postfixed in ethanol:acetic acid (2:1) for 5 minutes at −20°C, washed twice in PBS and equilibrated for 5 minutes at room temperature. The sections were then incubated with digoxigenin-11-dUTP and terminal deoxynucleotidyl transferase for 1 hour at 37°C, rinsed once with the working strength stop/wash buffer for 30 minutes at 37°C, washed three times with PBS and incubated overnight at 4°C with an antibody against neuron-specific enolase (Incastar) or glial fibrillary acidic protein (1:500, Sigma). Sections were then rinsed with PBS and incubated for 30 minutes at room temperature with the anti-digoxigenin-fluorescein antibody (Apoptag-kit, Oncor), and either with a rhodamine-goat anti-rabbit IgG (1:200, Jackson) for GFAP staining or with a lissamine-rhodamine-donkey anti-mouse IgG (1:100, Jackson) for GFAP staining. After four PBS washes of 15 minutes each, the slides were mounted in PBS-glycerol containing p-phenylenediamine. Cell death was quantified by counting the number of fluorescein-stained nuclei within one field, at a magnification of 40×. Only round stained nuclei were counted in a total of 24 randomly chosen fields in four different sections per cerebellum, in three animals per condition.

RESULTS

Cloning of the mouse rev-erbAα gene

Two completely different sequences have been reported as first exons for the human and rat rev-erbAα cDNAs (Lazar et al., 1989; Miyajima et al., 1989). To determine the structure of the 5′ end of the mouse gene, we performed an anchor-PCR reaction on skeletal muscle mRNA using as reverse transcription primer an oligonucleotide complementary to a sequence in the beginning of the rat second exon. The sequence of the PCR product (GenBank X86010) revealed a high homology (86% identity) with the 5′ end of the human rev-erbAα gene (not shown). In both mouse and human, the largest open reading frame of the gene starts at a consensus initiation codon found at the end of the first exon (Fig. 1A). A genomic clone encompassing gene rev-erbAα (clone 18.1.A) was subsequently retrieved from a phage library prepared with genomic DNA from mouse strain 129. A Southern blot hybridization confirmed that the first exon of the gene, as well as about 1.5 kb of upstream sequences, were included in this clone (Fig. 1A).

Targeting of the rev-erbAα gene and germ line transmission of the mutation

Embryonic stem (ES) cells were electroporated with a targeting vector in which the rev-erbAα coding sequence downstream of codon 39 was replaced by a β-galactosidase gene (Fig. 1A). Injection of cells from a correctly targeted ES clone into C57BL/6 blastocysts gave rise to chimeric males from which heterozygous mutants were derived at high frequency. The mating of heterozygous animals resulted in a Mendelian inheritance of the mutation (24.5% wild type, 50% heterozygotes, 25.5% homozygotes; N=208).

rev-erbAα and TRα expression in the brain and cerebellum of rev-erbAα mutant mice

To confirm that the gene had been successfully mutated, midbrain/forebrain and cerebellum RNA from P10 and P13 mutant animals were hybridized with the revα 5′ probe. As expected, the wild-type transcript of the gene was absent in homozygous (−/−) animals, while a 4.3 kb mRNA encoding the rev-erbAαβ-gal fusion protein was observed in mutant animals (Fig. 1B). In addition, a weaker RNA species of about 6.5 kb was detected. The fact that this band also hybridized to the mutated rev-erbAα genes. Bottom: an equivalent Northern blot was hybridized successively with a TRα1 DNA probe, a TRα2 antisense RNA probe and a G3PDH probe.

**Fig. 1.** Targeting of the rev-erbAα gene. (A) Targeting replacement vector used for electroporation (top) and map of the genomic locus containing the rev-erbAα gene and part of the overlapping TRα gene (bottom). The genomic region contained in clone 18.1.A is also indicated. BglII (Bg) and KpnI (K) restriction sites are shown. For the rev-erbAα gene, the location of the initiation codon (atg) as well as the two exons encoding the DNA binding domain (DBD) are indicated. The revα 5′ probe is represented by a solid bar. (B) Northern blot analysis of brain and cerebellum of wild-type (+/+) and mutant (−/−) P10 and P13 mice. Top: hybridization with the revα 5′ probe that recognizes transcripts derived from both the normal and the mutated rev-erbAα genes. Bottom: an equivalent Northern blot was hybridized successively with a TRα1 DNA probe, a TRα2 antisense RNA probe and a G3PDH probe.
gal coding sequence and the neomycin resistance gene. Although the brain of homozygous mice showed a mild decrease of TRα2 transcripts at P10 and P13 (Fig. 1B), as well as in the adult (not shown), the ratio of α1:α2 was not significantly modified in the cerebellum.

**Reduced fertility in rev-erbA**<sub>a</sub>** mutant females**

Mice lacking gene *rev-erbA* are viable, appear to develop normally and show neither gross anatomical defects nor any alteration in growth spurt or body weight (Fig. 2A). Importantly, although the *rev-erbA* gene has been reported to be repressed during myotube formation (Downes et al., 1995), skeletal muscle appeared to develop normally, and the analysis of histological sections of mutant soleus and extensor digitorum longus muscles revealed an apparently normal organisation in aerobic, intermediate and anaerobic fibers (Fig. 3B and data not shown). Similarly, we have not observed any gross difference in the accumulation of adipose tissue between wild-type and mutant animals despite the fact that *rev-erbA* is known to be induced during adipocyte differentiation (Chawla and Lazar, 1993). However, although homozygous males mate and reproduce normally, homozygous females are less fertile than control animals. As shown in Fig. 2B, when homozygous females were exposed to fertile males, only 32% of the matings produced normal pregnancies, compared to 75% for the wild-type and heterozygous mice (χ² test: P<0.001). Importantly, we observed that some mutant females which had had an unproductive mating were still able to have a normal pregnancy when they became older. Therefore, the mild effect observed is more probably due to a reduced fertility of the mutant females rather than to an increased proportion of sterile females among the homozygotes.

**Expression of the rev-erbA**<sub>a</sub>** gene**

We first used the β-galactosidase activity of the fusion protein to investigate the expression of gene *rev-erbA* in mutant animals. A weak but significant signal was detected during embryogenesis, first in the heart starting at 10.5 days post-coitum and 1 day later in the developing eye (Fig. 3A). Consistent with previously reported northern blot data (Jannini et al., 1992), we observed a burst in gene expression during the second week of life (data not shown). In the adult, we found expression in many cell types, including brain, liver, skeletal myotubes, cardiocytes, as well as in the smooth muscle layers surrounding the intestine and the uterus (Fig. 3B-E and data not shown). The gene appeared to be silent in testis, lung and pancreas, with the exception of the smooth muscle cells present in the walls of blood vessels and ducts (data not shown).

Interestingly, we consistently observed a much higher β-galactosidase staining in homozygous compared to heterozygous tissues, suggesting that *rev-erbA* normally represses its own expression in vivo (compare Fig. 3C,F). To substantiate this observation, we measured the β-galactosidase activity in the brains of four wild-type, +/- and -/- adult mice. Our results showed that extracts from homozygous brains had an activity of 7.7±1.4 β-galactosidase units/mg total protein, whereas +/- and wild-type extracts contained only 0.9±0.3 and 0.1±0.3 units/mg, respectively.

The β-galactosidase reporter was also used to analyze the expression of *rev-erbA* in the adult brain. Very high levels of expression were found in cerebellar and olfactory granule cells, cerebral cortex (particularly in pyramidal cells) and CA1 of the hippocampus. High levels of expression were observed in the geniculate nuclei of the thalamus, stellate cells of the cortex, cochlear nucleus, interpeduncular nucleus, superior colliculus, hippocampus, as well as in Purkinje cells of the cerebellum (Fig. 4 and data not shown). This pattern closely resembles the previously reported distribution of the *rev-erbA* transcripts in the adult brain (Kainu et al., 1996).

**Developmental regulation of rev-erbA**<sub>a</sub>** expression in the cerebellar cortex**

Interestingly, the RORα gene is disrupted in the *staggerer* mutant, which shows a severe impairment of Purkinje cell development (Hamilton et al., 1996). In view of the possible link between the *rev-erbA* repressor and the RORα transactivator (Forman et al., 1994), it was of interest to analyse in more detail the expression of gene *rev-erbA* during postnatal cerebellar development. The β-galactosidase histochemical staining of cerebellum sections first showed that the *rev-erbA* gene was silent at P3 and P7, while a strong expression was detected in the Purkinje cell layer at P11 (not shown). We then analysed the expression of the fusion protein from P10 to the adult by immunocytochemistry with a β-galactosidase-specific antibody (Fig. 4A-D). At P10, only the cell bodies and apical dendrites of Purkinje cells were fully and very strongly labelled, while very few cells in the internal granule cell layer (IGL) began to be detected over background levels. 3 days later, low levels of expression were found in a few more granule cells of the internal granule cell layer, and basket cells in the internal segment of the molecular layer.
started to be positive. At P16, the staining extended to most of the granule cells in the IGL, while intense staining was observed in most of the basket cells. Stellate cells in the external segment of the molecular layer started to be positive at P16. Finally, in the adult, most of the cells of the cerebellar cortex were found to be positive. At this stage, the strongest staining was found in the IGL, while the labelling of Purkinje cells was lower than during development. This broad pattern of expression suggested that the \textit{rev-erbA} gene could be involved in several aspects of cerebellar development.

**Persistent proliferation and delayed migration of external granule cell layer neurons**

The general morphology of the cerebellum was assessed at P16, right after the period of maximal differentiation. Cresyl Violet stained sections showed no abnormality in cerebellar foliation (Fig. 4E,F). However, we found that the external granular cell layer (EGL) of the mutant mice was thicker than in control animals. Also, the EGL between lobules VI-VII was thicker than between lobules IX-X, suggesting that the absence of \textit{rev-erbA} induces a delayed maturation of the cerebellum. We therefore analysed in more detail the postnatal development of external granule cells, by focusing on the development of the EGL in cresyl violet stained sections from P10 to the adult. We first observed that in wild-type mice, the EGL in lobules VI-VII had more cell layers than in lobules IX-X as shown by the larger size of this layer at P10 and P13 (Fig. 5A,B). Interestingly, this difference was preserved in mutant animals, although we consistently found more cell layers in the EGL of \textit{+/−} mice than in wild-type animals up to P19 (Fig. 5). Finally, the EGL disappeared and was undetectable both in wild-type and mutant adult mice.

Next we set out to determine whether the increase in the thickness of the EGL in the \textit{+/−} mice was due to a decrease in the number of cells normally dying during development or to an increase in the proliferation of precursor cells. We found that the number of TUNEL-positive cells in the EGL per mm² (mean ± s.d., \(n=3\)) was not different between wild-type and mutant mice at P10 (51.72±8.14 and 47.92±8.14, respectively), or at later stages (not shown). Similarly, the number of cells incorporating BrdU in the proliferative zone of the EGL as well as their levels of incorporation in wild-type and mutant mice was the same between P10 and P13 (not shown).
However, at P16, BrdU-labelled cells were found in the proliferative zone of the EGL of mutant mice, but not in wild-type controls (Fig. 6). Therefore, our results show that the rev-erbAα mutation extends precursor cell proliferation by 3 days (up to P16), and suggest that the enlargement of the EGL at early stages (P10-P13) is neither due to an increased proliferation of progenitors nor to an increased survival of granule cells in the EGL, but rather to a transient delay in the migration of these cells towards the IGL.

**Increased apoptosis amongst neurons from the internal granule cell layer**

We next analysed by TUNEL assay if granule neurons that were migrating through the molecular layer or that had reached the IGL survived in the rev-erbAα mouse. Although no difference could be found between wild-type and mutant animals in the molecular layer, clear differences were observed in the IGL. In normal mice, naturally occurring cell death was higher at P10 and then progressively declined to reach undetectable levels in the adult (Fig. 7A). Interestingly, in the −/− mice, we found an increased number of TUNEL-positive cells in the IGL at P10, P13, P16 and P19 (246, 385, 181 and 157% of control, respectively) but not in the adult mice (Fig. 7A, compare also C,D). Double stainings performed with antibodies against either the neuron specific enolase (NSE) or the glial fibrillary acidic protein (GFAP) showed that the TUNEL-positive cells in the IGL of wild-type and −/− mice were neurons and not astrocytes (Fig. 7B). Thus our results indicate that the deletion of the rev-erbAα gene affects the survival of IGL neurons during postnatal development.

**Alterations in the differentiation of Purkinje cells**

Since we initially observed that the rev-erbAα gene was expressed by Purkinje cells at P10, we examined the development of these cells in more detail. Measures of the thickness of the molecular layer showed no change from P10
to P13 (Fig. 8A,B). The only differences in the size of that layer were detected at P16, in lobules VI-VII and IX-X of /-/ mice, which showed a 15% and a 17% decrease, respectively, compared to wild type. However, the size of the molecular layer was normal by P19, and remained normal up to adulthood. Examination of the morphology of Purkinje cells on calbindin immunostained sections from wild-type and mutant animals showed no apparent differences in the intensity of calbindin immunostaining, neither in the number of calbindin- nor in the Cresyl Violet-positive Purkinje cells. Interestingly, measures of the length of the dendritic tree of Purkinje cells, labelled with calbindin (data not shown), gave almost identical values to those reported for the length of the molecular cell layer at p16 (Fig. 8). However, the dendritic trees of Purkinje cells were found to be clearly less elaborated in /-/ mice from P10 to P16 (Fig. 9), but not from P19 to the adult (not shown), thus suggesting a delay in the morphological maturation of Purkinje cells.

To further assess the biochemical differentiation of Purkinje cells in the rev-erbAα mutant as well as their organisation into functional compartments, we analysed the expression of Aldolase C/Zebrin II, a metabolic marker showing a well defined parasagittal banding pattern in the developing cerebellum (Hawkes and Gravel, 1991). While control animals showed the expected Aldolase C pattern in the vermis (lobule X) at P10, and in the lateral lobule VII at P13 (Fig. 10A,C), a much less developed pattern of expression was observed in mutant littermates at these stages of development (Fig. 10B,D). In rev-erbAα mutant mice, the Aldolase C parasagittal banding appears to be delayed by approximately 3 days, since a normal expression pattern was observed in the vermis and lateral lobule at P13 and P16, respectively (not shown). By contrast, the spatial and temporal patterns of expression of the p75 neurotrophin receptor (Dusart et al., 1994) and of the Kv3.3b potassium channel (Goldman-Wohl et al., 1994), also expressed in parasagittal bands of Purkinje cells, were identical in wild-type and mutant mice during the second postnatal week (data not shown). Thus, our data suggests that the deletion of the rev-erbAα gene results in a delay in the morphological differentiation of Purkinje cells as well as in their acquisition of specific biochemical aspects of their adult phenotype.

**DISCUSSION**

In this paper we describe transgenic mice lacking the rev-erbAα gene. These mice are viable, and adult animals do not show any obvious phenotype other than a reduced female fertility. However, a careful analysis revealed several defects during the postnatal development of the cerebellar cortex of mutant mice. Cerebellar granule cells of normal mice proliferate in the germinative zone of the EGL during the first 2 weeks of life and produce neurons that migrate towards the IGL, leaving their axons in the molecular layer. The EGL therefore progressively decreases in size and finally disappears in the adult. Simultaneously, Purkinje cells start to differentiate shortly after birth and develop a dendritic tree that reaches its final length in the adult. These well-characterized morphological transformations were delayed in the rev-erbAα mutant. Indeed, granule cell progenitors still proliferated at P16 in the EGL, when no proliferation could be detected in wild-
type animals. In addition, the granule cells that were produced did not migrate on time towards the IGL and therefore accumulated in the premigratory zone of the EGL. Moreover, when these cells eventually reached the IGL, they died in greater numbers than in wild-type mice. Finally, Purkinje cells of mutant mice showed a less elaborated dendritic tree during the critical differentiation period (P10-P16) and a delayed acquisition of Aldolase C expression pattern. Thus, both granule and Purkinje cell development appear to be affected by the mutation.

Intriguingly, some of these alterations are similar to those observed in hypothyroid rats, which exhibit a delay in the proliferation, migration and differentiation of granule cells, an increased cell death in the IGL, and a deficiency in the elaboration of Purkinje cell dendritic trees (Legrand, 1979). An obvious explanation would be that in the rev-erbAα mutant, the alteration of the TRα2 transcript results in an hypothyroid condition. However, the rev-erbAα mutant pups did not show any feature of hypothyroidism and had normal T3 and T4 serum levels (not shown). Moreover, in P10 and P13 mutants a mild TRα2 decrease was only found in the forebrain and midbrain (which would rather enhance the TRα1-mediated transactivation), while the TRα2/TRα1 ratio was normal in cerebellum. Therefore, our findings indicate that the alterations observed in the cerebellum of these mice are not secondary to hypothyroidism, but primarily due to the deletion of the rev-erbAα gene.

Although the mechanisms governing Purkinje cell development are not completely understood, it appears that the orphan nuclear receptor RORα plays a role in this process. RORα is disrupted in the staggerer mouse, a mutant exhibiting a severe cerebellar ataxia due to a cell-autonomous defect in the development of Purkinje cells, which are immature and reduced in number (Hamilton et al., 1996). Importantly, RORα shares the same DNA binding specificity as rev-erbAα but exerts opposite transcriptional effects since it behaves as a constitutive transactivator. In addition, RORα expression is detected in Purkinje cells starting from 14 dpc (Hamilton et al., 1996), while rev-erbAα starts to be expressed in these cells only at P10, during final stages of cerebellar development. Unlike the RORα mutant mice, the rev-erbAα-/- mice are not ataxic and their number of Purkinje cells is normal. Instead, during the second week of life they showed a transient delay in the morphological and biochemical differentiation of Purkinje cells. Hence, an intriguing possibility would be that during the second week of life, rev-erbAα allows the final maturation of Purkinje cells by negatively regulating the expression of RORα-induced genes. An obvious target for such a cross-talk is rev-erbAα itself, since its promoter contains a RORα response element (Adelmant et al., 1996). In agreement

![Fig. 8. Quantification of the size of the molecular layer in lobules VI/VII (A) and IX/X (B) of wild-type and rev-erbAα mutant mice. The values show the mean ± s.e.m. *P<0.05, Student’s t-test.](image1)

![Fig. 9. Delayed morphological differentiation of Purkinje cells in the rev-erbAα mutant mice. A calbindin immunostaining was performed at P10 and P16 on cerebellar sections from wild-type (+/+) and rev-erbAα mutant mice (-/-). Note that identical coordinates and position within the lobules were selected for analysis and photography. Bar, 25 μm.](image2)
that the lack of expression of rev-erbA. Nonetheless we cannot exclude granule cells at this time of development are secondary to therefore possible that the alterations observed in external granule neurons. The absence of signal in the cells, although a weak immunostaining was also observed in lobule X at P10 and lobule VII at p13 are indicated. Note that in the mutant mice the positive bands are either smaller, as in lobule X at P10 (B), or absent, as in lobule VII at P13 (D). Bars, 270 μm.

with this hypothesis, we found that rev-erbAα homozygous mutants had levels of β-galactosidase expression much higher than heterozygous littermates, suggesting that rev-erbAα normally represses its own expression in vivo. Another possible target for a crosstalk between the two orphan receptors could be the L7/pcp-2 gene (Oberdick et al., 1993; Smyne et al., 1991), in which a RORα binding site has recently been identified (Matsui, 1997; Schräder et al., 1996). Interestingly, the developmental regulation of L7/pcp-2 is delayed in hypothyroid rats (Strait et al., 1992), suggesting that the appropriate timing of expression of L7/pcp-2 depends on both the TR and ROR pathways. Other genes regulated by thyroid hormone during postnatal cerebellar development could also be targets for a crosstalk between rev-erbAα and ROR. For instance, this could be the case for neurotrophic factors of the neurotrophin family and their receptors, which are regulated by thyroid hormones (Lindholm et al., 1993; Neveu and Arenas, 1996) and which promote several of the developmental processes impaired by the mutation of the rev-erbAα gene. Amongst them, Neurotrophin-3 was found to regulate the differentiation of Purkinje cells and the differentiation, migration and survival of granule cells (Neveu and Arenas, 1996). However, it remains to be determined whether the Neurotrophin-3 gene is a direct target for a crosstalk between rev-erbAα, ROR and TRs.

Another interesting observation of the present study is that the increased size of the EGL as well as the increased apoptosis in the IGL of rev-erbAα mutant were detected already at P10. At this stage, the reporter activity was very high only in Purkinje cells, although a weak immunostaining was also observed in very few internal granule neurons. The absence of signal in the EGL suggests that the delay in the migration of granule cells from the EGL to the IGL is due to an extrinsic defect. It is therefore possible that the alterations observed in external granule cells at this time of development are secondary to Purkinje cell-derived signals. Nonetheless we cannot exclude that the lack of expression of rev-erbAα in the few IGL neurons detected by immunostaining could also account for the death of neurons in this layer. These findings are reminiscent of those reported in the staggerer mouse, in which initial alterations in Purkinje cells also result in secondary alterations in granule cells, including an increased cell death in the IGL (Trenkner, 1979). However, the phenotype of the staggerer and the rev-erbAα mutants differ in many other respects. For instance, Purkinje cells die only in the staggerer mice, and migration of external granule cells is only delayed in the rev-erbAα−/− mice. This suggests that the two receptors are involved in the regulation of both distinct and common developmental programs. For instance, Purkinje cell survival would be controlled by RORα, while the differentiation and/or migration of granule cells would be selectively regulated by rev-erbAα. In contrast, an appropriate balance between rev-erbAα and RORα would be necessary for a temporally correct maturation of Purkinje cells, for proliferation of EGL cells, and for survival of IGL neurons. In conclusion, rev-erbAα appears to contribute to the appropriate balance between transcriptional activators and repressors regulating the development of the cerebellum. Our results show that the mutation of the rev-erbAα gene induces alterations in the morphological and biochemical maturation of Purkinje cells and in granule cell proliferation, migration and survival. These findings underscore that rev-erbAα is required for the correct regulation of a highly specific and unique combination of cellular processes essential for the postnatal development of the cerebellum.

We thank the MouseCamp transgene facility at Karolinska Institute for generation of mice. We are grateful to Dr R. Hawke for the Aldolase C/Zebrin II antibody and to Dr N. Heintz for the Kv3.3b probe and Philippe Auquier for excellent technical assistance. Funding was obtained from the Human Frontier Science Program, Cancerfonden, Göran Gustafsson’s stiftelse, Medicinska forskningsrådet, and funds at the Karolinska Institute. P. C. was supported by the European Science Foundation (Strasbourg, France). I. N. and E. A. were supported by Medicinska forskningsrådet.

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