**staggerer phenotype in retinoid-related orphan receptor α-deficient mice**

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Communicated by William T. Greenough, University of Illinois, Urbana, IL, December 22, 1997 (received for review September 15, 1997)

**ABSTRACT** Retinoid-related orphan receptor α (RORα) is a member of the nuclear receptor superfamily. To study its physiological role we generated null-mutant mice by targeted insertion of a lacZ reporter gene encoding the enzyme β-galactosidase. In heterozygous RORα+/− mice we found β-galactosidase activity, indicative of RORα protein expression, confined to the central nervous system, skin and testis. In the central nervous system, the RORα gene is expressed in cerebellar Purkinje cells, the thalamus, the suprachiasmatic nuclei, and retinal ganglion cells. In skin, RORα is strongly expressed in the hair follicle, the epidermis, and the sebaceous gland. Finally, the peritubular cells of the testis and the epithelial cells of the epididymis also strongly express RORα. Recently, it was reported that the ataxic mouse mutant staggerer (sg/sg) is caused by a deletion in the RORα gene. The analysis of the cerebellar and the behavioral phenotype of homozygous RORα−/− mice proves identity to sg/sg mice. Although the absence of RORα causes dramatic developmental effects in the cerebellum, it has no apparent morphological effect on thalamus, hypothalamus, and retina. Similarly, testis and skin of RORα−/− mice display a normal phenotype. However, the pelage hair of both sg/sg and RORα−/− is significantly less dense and when shaved shows reluctance to regrow.

Nuclear receptors form a structurally related superfamilly of ligand-activated transcription factors (1). They are involved in several aspects of vertebrate physiology, such as development and homeostasis. Important examples are the steroid hormone receptors that regulate, in a ligand-dependent manner, specific sets of responding genes. The retinoid-related orphan nuclear receptor (ROR) α (2, 3), RORβ (4), and RORγ (5) constitute a subfamily of nuclear receptors that bind to DNA both as monomers and dimers. Distribution of RORα mRNA suggests that this receptor is widely expressed and functions in several organs including brain, heart, liver, lung, and testis; highest levels were found in peripheral blood leukocytes and skin (M.B.-A., unpublished data). RORα exists in four splicing isoforms: RORα1−4. They display different N-terminal domains causing different DNA binding site preferences (3), and they display differential expression profiles: in the thalamus there is only RORα1 mRNA; RORα4 (=RZα1) (2) transcripts are predominant in leukocytes and skin; RORα2 and RORα3 transcripts are exclusively detected in testis; and in the remaining tissues including the cerebellum there is a mixture of RORα1 and RORα4 transcripts (M.B.-A., unpublished results). In the central nervous system (CNS) RORα mRNA localizes to the cerebellar Purkinje cells (PCs), various thalamic nuclei, and, during development, to other brain areas (6, 7). To study the physiological role of this orphan receptor we generated RORα null-mutant mice by gene targeting. In the course of this work the genetic basis of the staggerer (sg) mutation in mouse was identified by positional cloning as a deletion in the RORα gene (8). The sg mutation abolishes the development of cerebellar PCs in a cell-autonomous fashion (9, 10). The resulting immature morphology and severe cerebellar ataxia (11–13) suggest an essential role of RORα in the terminal differentiation of these cells.

Here, we present data which prove that the lack of functional RORα causes a phenotype encompassing all the salient features of neurological mouse mutation sg. In addition, we show that expression of RORα is confined to the CNS, skin, and testis. Our study leads to the conclusion that the absence of RORα causes dramatic effects exclusively in the cerebellum but apparently impairs only mildly, if at all, the morphology and physiology of the remaining brain areas, skin, and testis.

**MATERIALS AND METHODS**

**Targeting the RORα Locus.** A 1.8-kb PCR-generated DNA fragment containing part of the exon encoding the second zinc finger of RORα and its 5’ flanking sequences was cloned into the pGN vector (14), via XbaI and SacII, to fuse the RORα exon with the pGN’s lacZ gene and eventually creating a RORα/β-galactosidase (β-gal) fusion. After sequencing of the insert a 1.8-kb thymidine kinase (tk) gene cassette was cloned into the XbaI site. Finally, a XhoI–NotI 6.5-kb DNA fragment derived from the RORα locus of 129/Ola mouse containing intron sequences downstream of the second zinc finger was introduced resulting in the targeting construct pRORα-koc-TK. NotI-linearized pRORα-koc-TK was electroporated into 129/Ola ES H1 cells as described (15). Chimicere mice were mated with C57BL/6 mice and offspring were genotyped by Southern hybridization of tail DNA by using a DNA fragment derived from the RORα locus upstream of the 1.8 kb XbaI–SacII DNA fragment as a probe. Genomic DNA was digested by using BamHI and size separated in a 0.8% agarose gel.

**Behavioral Tests.** Motor capability tests were performed with groups of 2- to 4-month-old wild-type RORα (+/+), heterozygous RORα knockou (+/−), homozygous RORα knockout (−/−) littermate mice, and a group of age-matched homozygous staggerer mutant mice (sg/sg).
Motor coordination test. The apparatus consisted of an experimental box (35 × 35 × 25 cm). Half a centimeter above the bottom of the box was a platform in which 36 holes, 2 cm in diameter and arranged in a 6 × 6 array, have been drilled. The animal was placed in the middle of the platform and the number of times it stumbled (one leg diving into a hole) was recorded. The test lasted 5 min. The stumbling frequency (number of stumbles/min) was calculated.

Hanging test. The muscular tone of the animal was measured by hanging it by its two anterior paws on a rod, 25 cm long, 3 mm in diameter, and located 1.5 m above a thick carpet to cushion its eventual fall. The time during which the animal kept hanging on the rod was recorded. Three trials spaced by a 3-min pause were performed and a mean score was calculated. The test was limited to 180 sec.

Equilibrium test: the nonrotated rod. The apparatus consisted of a wooden horizontal rod (50 cm long and 3 cm in diameter) covered with sticking plaster to increase roughness. It was located 80 cm above a landing platform covered with a thick sheet of soft plastic to cushion the eventual fall of the animals. The animal was placed on the middle of the rod, its body axis perpendicular to the longitudinal axis of the rod. The time the animal stayed on the rod was recorded. The trial was stopped when the animal fell or otherwise after 180 sec.

Motor learning test: the rotarod. The apparatus was the same as used for the equilibrium test. The animal was placed on the middle of the rod, its body axis perpendicular to the longitudinal axis of the rod and its head directed against the direction of rotation, so that the animal had to progress forward to maintain balance. Training consisted of 10 rotations per day at 1 rpm. We recorded how long the mice stayed on the rod and which strategy they used. The maximum score was 180 sec, because the test was limited to 3 min (16, 17). Two kinds of strategies were observed and quantified: walking and hanging. Motor synchronization learning was achieved if after 10 consecutive trials the animal managed to stay on the rod for 180 sec.

Morphological Analysis of the Cerebellum. Four ROR<sup>a</sup>-null mice, aged from 3 to 5 months, were used. Three of them were fixed by intracardiac perfusion with 4% paraformaldehyde in 0.12 M phosphate buffer (pH 7.4). Frozen frontal (two mice) and parasagittal (one mouse) sections were obtained and double immuno-labeled with mA<sub>B</sub>5113 (immunoperoxidase) detecting zebrin I and rabbit polyclonal antibodies against calbindin (fluorescein isothiocyanate-conjugated) as described (18). The antibodies were respectively gifts from R. Hawsers (University of Calgary, Calgary, Canada) and E. M. Lawson (Cambridge Research Station, Cambridge, U.K.). The fourth mouse was perfused with glutaraldehyde and paraformaldehyde (both at 1%), postfixed in osmium tetroxide, and embedded in Araldite. For light microscopy, 1-μm-thick parasagittal sections were stained with toluidine-blue. For electron microscopic analysis ultra-thin sections were either stained with lead citrate or immuno-stained with rabbit polyclonal anti-γ-aminobutyric acid (GABA) antibodies (Immunotech, Luminy, France) according to the postembedding immunogold method as described (19). The cerebella of a wild-type ROR<sup>a</sup>-null and a homozygous sg/sg mouse were also double immuno-stained with anti-zebrin I and anti-calbindin antibodies.

Electrophysiological Characterization of Cerebellar PCs. Sagittal slices, 250–300 μm thick, were prepared from the vermis of the cerebellum of 1- to 4-month-old ROR<sup>a</sup>-null and wild-type mice as described (20).

Histological Staining. Tissue was frozen in 2-methylbutane at −40°C for 5–10 min and stored at −80°C. Cryosections were cut (14 μm), air-dried on glass slides (Menzel, Braunschweig, Germany), fixed in 0.05% glutaraldehyde for 5 min at room temperature, washed with PBS, and incubated overnight at 37°C in PBS containing 4 mM MgCl<sub>2</sub>, 2 mM K<sub>2</sub>Fe(CN)<sub>6</sub>, 2 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, and 0.4 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). After washing with PBS the section were counterstained (optional) with hematoxylin/eosin and mounted.

RESULTS

Generation of Mice Lacking ROR<sup>a</sup>. We have generated mice lacking a functional ROR<sup>a</sup> gene by using a targeting vector in which a β-gal gene replaced the second zinc finger of the DNA-binding domain of ROR<sup>a</sup>. After homologous recombination the targeted gene encoded a fusion protein composed of ROR<sup>a</sup>’s N-terminal domain and first zinc finger followed by β-gal (Fig. 1A). Cross-breeding of heterozygous ROR<sup>a</sup>−/− mice yielded homozygous null-mutant ROR<sup>a</sup>−/− animals at the expected frequency of 25% as analyzed by genotyping (Fig. 1B) and easily identified by their ataxic behavior. About half of the ROR<sup>a</sup>−/− mice died shortly after weaning whereas the remaining reached adulthood and showed normal life expectancy.

ROR<sup>a</sup> Null-Mutant Mice Display Motor Deficits Nearly Identical to staggerer Mice. Global observation of ROR<sup>a</sup>−/− mice show that they exhibit a paw clasping response and a strong ataxia similar to homozygous mutant sg/sg mice (21). To assess the motor capabilities of ROR<sup>a</sup>−/− mice and to compare with sg/sg mice we quantified three parameters: the stumbling frequency, which is an indicator of the motor coordination ability; the hanging time, which indicates the degree of muscular strength; and the equilibrium capabilities. Stumbling frequency of sg/sg mice and ROR<sup>a</sup>−/− mice is higher than that of wild-type littermates, whereas hanging and equilibrium times are significantly lower (Table 1). These results demonstrate that both ROR<sup>a</sup>−/− and sg/sg mice suffer from motor coordination, hanging and equilibrium deficits (21, 22). However, sg/sg mice had lower scores in the equilibrium and hanging time tests than ROR<sup>a</sup>−/− mice (Table 1). In the motor learning test with the rotarod, ROR<sup>a</sup>+/− littermates and heterozygous ROR<sup>a</sup>+/− mice reached the maximal score of 180 sec easily in the first trial (Fig. 2A) by exclusively using a walking strategy (Fig. 2B). In contrast, ROR<sup>a</sup>−/− mice and sg/sg
mice did not reach the maximal score, even after 10 days of training (Fig. 2A), and they used principally a hanging strategy to stay on the rotarod (Fig. 2B). Thus, RORα<sup>−/−</sup> and sg/sg mice are incapable of motor synchronization learning (23, 24). However, although sg/sg mice did not improve their score, RORα<sup>−/−</sup> mice improved significantly during the 10 test days.

Anatomical Characterization of the RORα<sup>−/−</sup> Cerebellum Reveals Identical Phenocopy of staggerer. In adult mutants the cerebellum is greatly reduced in size, particularly in the intermediate region. The cytoarchitecture of the cortex is grossly altered: The molecular layer is very thin, PCs are not arranged in a monolayer (Fig. 3A and D), the granular layer is almost nonexistent and depleted of granule cells. Studies with immunomarkers to visualize either all PCs or PC compartmentation have shown that their dendrites are smaller than normal, not confined to a single plane, and devoid of spiny branchlets (Fig. 3D). Moreover, zebrin I-immunostained PCs, present in wild-type RORα<sup>+/+</sup> cerebellum (Fig. 3C), are lacking in the mutant. Thus, the phenotype resembles that of staggerer cerebellum (Fig. 3B).

The electron microscopy analysis has corroborated the lack of granule cells. The subpial region solely contains reactive astrocytic processes. The molecular layer neuropil contains dispersed synaptic islands and tiny bundles of parallel fibers, both encapsulated by various layers of thin astrocytic processes. The majority of the axon terminals are GABA-immunoreactive (Fig. 3E), and some of them are GABA-negative and exhibit the features of parallel fiber varicosities (data not shown). Many PC bodies retain their immature somatic spines which remain postsynaptic to somatic inputs. The inhibitory fibers (CF) varicosities (Fig. 3F). However, CF translocation, although only partial, has occurred in the RORα<sup>−/−</sup> cerebellum because some of the stem dendrites have spines synaptically contacted by CF varicosities (data not shown). The inhibitory synaptic investment of mutant PCs is also altered. Although axon terminals resembling basket cell axon occur (Fig. 3F), they are rare, smaller than in control cerebellum, and do not establish characteristic pinceau formations around the initial segment of PC axons (data not shown). Nevertheless, PC perikarya receive inhibitory synaptic inputs, as revealed with GABA immunocytochemistry (data not shown). Although PC perikarya abut the white matter axes of the folia, in the deeper third of the cortical gray matter mossy fiber profiles are numerous. The latter only occasionally form typical glomeruli of the simplest type because the large majority of the mossy fibers either contact a few granule cell dendrites (Fig. 3G) or remain completely surrounded by astrocytic processes.

Cerebellar PCs of RORα<sup>−/−</sup> Mice Display Electrophysiological Characteristics Identical to those of staggerer Mice. Previous studies (12, 13) have established that cerebella from adult sg/sg mice are characterized by the multiple innervation of PCs by CFs, a feature normally observed during the first 2 postnatal weeks (13, 25).

In keeping with previous observations (20), stimulation of the granular layer evoked typical all-or-none CF-mediated excited postsynaptic currents (EPSCs) (26) in 10 control brain PCs as tested in the voltage-clamp mode (Fig. 4A1). In the current-clamp mode, these responses consisted of an initial full spike followed by a plateau of depolarization with superimposed partial spikes, as in normal PCs (Fig. 4A2). In contrast, in all nine RORα<sup>−/−</sup> PCs recorded the evoked CF-EPSCs exhibited two to four steps (2.8 on average) in amplitude when the stimulus intensity was progressively increased (Fig. 4B1, B2, and C1). This stepwise variation in amplitude was observed when cells were maintained at −80 mV as well as when they were held at −10 mV or −20 mV, to inactivate sodium and calcium voltage-dependent conductance. In the latter case the ratio of the amplitude of the two steps of the CF-EPSCs was the same as that measured at −80 mV (data not shown). Yet mean rise times and mean amplitudes of the largest step of these graded CF-mediated EPSCs were not significantly different from those observed in all-or-none responses in both mutant and wild-type mice. Finally, in the current-
clamp mode the largest step of the CF responses recorded at −70 mV from the nine multiply innervated cells was very similar to that of CF responses recorded in sg ysg mice (12, 13) (Fig. 4A2).

High Resolution Mapping of RORα Distribution: Confirmations and Surprises. Adult heterozygous RORα mutant (RORα+/−) mice were examined for the distribution of β-gal activity indicative of RORα protein expression in various
shown to express significant to high levels of ROR in liver, heart, spleen, lung, and leukocytes, which have been produced spermatocytes and were putatively fertile (22).

In skin, the granular layer at the holding potential of ~80 mV was recorded in current-clamp mode at ~80 mV. (A1) Two superimposed sweeps of all-or-none CF-mediated EPSCs in normal PCs and one subthreshold response evoked by stimulation of the granular layer at the holding potential of ~80 mV. (A2) Same cell recorded in current-clamp mode at ~70 mV. (B1) Superimposed sweeps of CF-mediated EPSCs elicited in a multiply innervated RORα−/− PC held at ~80 mV by stimulating the granular layer with progressively increasing strength. (B2) Plot of amplitudes against time of CF-mediated EPSCs recorded at ~80 mV in the same cell as in B1. Note the stepwise variation of the responses with progressively increasing stimulation strength. (C1) Same as in B1 using another neuron. (C2) The cell was held in current-clamp mode at ~80 mV and the granular layer was stimulated with progressively increasing intensity.

tissues. As expected, enzyme activity was found in various areas of the brain including retinal ganglion cells, cerebellar PCs (Fig. 5 A and B) and some nuclei of the thalamus (data not shown). Moreover, our data show that RORα is also expressed in the suprachiasmatic nuclei of the hypothalamus, the testis, and skin (Fig. 5 C–F). To our surprise, other organs such as liver, heart, spleen, lung, and leukocytes, which have been shown to express significant to high levels of RORα mRNA (2, 27), did not display any detectable enzyme activity. The same expression profile was observed in the respective RORα−/− tissues—with the exception of the cerebellar PCs in which the RORα/β-gal fusion product is only scarcely expressed (data not shown).

RORα expression was observed in testis only after sexual maturation and specifically localized to peritubular cells (Fig. 5D). In addition, and already before puberty, massive expression of RORα was detected in the epithelial cell layer of the epididymis (Fig. 5E). Yet in the knockout as well as in the sg/sg mice we observed no abnormal phenotype, i.e., the animals produced spermatocytes and were putatively fertile (22).

In skin, β-gal activity localized to the epidermis, the hair follicle, and the sebaceous gland (Fig. 5F). In the epidermis RORα is expressed in suprabasal differentiating keratinocytes but not in proliferating keratinocytes of the basal generative compartment. In the growing (anagen) hair follicle, expression of RORα is confined to a discrete set of differentiating keratinocytes above the dividing cells of the hair matrix. This staining is absent from hair follicles in other hair cycle phases including the katagene, telogen, and early anagen phase (data not shown). In RORα−/− mice the anatomical and histological situation of the skin appears normal. However, the mice develop a fur that is significantly less dense lacking in particular most of the duvet hair (Fig. 5G and H). When shaved, the hair grew back only very slowly (not shown). We observed a similar behavior with sg/sg mice. However, we also noticed that a rich and balanced nourishment mitigates this phenotype.

DISCUSSION

The phenotype of the RORα−/− mice proves that the staggerer mutation is based on the functional disruption of the nuclear receptor RORα gene (8). RORα−/− and sg/sg mice exhibit the same disorders, i.e., ataxia and motor capability deficiencies. However, we also noted differences. RORα−/− mice are less asthenic, reached a better equilibrium score, and performed significantly better in the rotarod test than sg/sg mice. These discrepancies could be explained by the different genetic backgrounds of RORα−/− (C57BL/6, 129/Ola) and sg/sg mice (C57BL/6). Alternatively, the difference of the RORα gene mutations in the corresponding strains might be considered: in sg/sg mice the RORα gene is truncated at the level of the ligand binding domain leaving intact the DNA-binding domain of the encoded protein. Thus, expressed truncated staggerer RORα could still interact with its appropriate DNA binding sites but would be incapable of promoting transcription. Consequently, it could act as a dominant negative isoform and as such might exacerbate the mutant’s phenotype. In contrast, in RORα−/− mice the RORα gene is disrupted at the level of the second zinc finger and the corresponding gene product is incompetent of binding to DNA.

The abnormal anatomical phenotype of the adult RORα−/− cerebellum also corresponds to that of the staggerer mouse (Fig. 3A and B). In both, the cerebellum is agranular, PCs are ectopic, reduced in number, and exhibit atrophic cell bodies and dendritic trees that are devoid of most of their spines (9, 11, 28). Moreover, a prominent feature of the mammalian cerebellum is its organization into parasagittal compartments, characterized by the biochemical heterogeneity of their PCs. One marker of such compartments is zebrin (29) which is expressed by a subset of PCs confined to regularly occurring parasagittal bands. From all known cerebellar mutants preserving PCs into adulthood only the staggerer mouse lacks...
zebrin I-positive PCs (30). The absence of zebrin I-immuno-reactive PCs in the RORG−/− cerebellum convincingly shows that both mutations induce the same cerebellar phenotype. The analysis of the cerebellar synaptology of RORG−/− cerebellum further supports the conclusion that this cerebellum is the precise phenocopy of that of staggerer. The partial translocation of CfCs from PC bodies to stem dendrites and the absence of pericellular nests and pinceaux formations emerging from basket cell axons which characterize the synaptic investment of PCs in the RORG null mutation are salient features of staggerer PCs (31–33). The occurrence in the null mutant of mossy fibers partially or even entirely denuded of their postsynaptic granule cell dendrites at the glomeruli reproduces the staggerer condition (11, 32). Finally, similar to the behavioral and anatomical data of this study, also the electrophysiological results reveal that staggerer mutation and RORG null mutation converge on the same phenotype. Taken together, the comparative description of the phenotype of RORG−/− and sg/sg mice ultimately proves that both mutations cause a nearly identical cerebellar phenotype by affecting the same gene.

We established a high-resolution RORα protein expression map by using the lacZ reporter gene fused to the RORα gene locus. Within the CNS the expression pattern fits well with the mRNA distribution pattern based on in situ hybridization data (6, 7). In addition, we found expression in retinal ganglion cells and in the suprachiasmatic nuclei, the central part of the mammalian circadian timing system. Interestingly, there exists a report describing an arrhythmic feeding behavior in sg/sg mice (34). A careful analysis of sg/sg or RORα−/− mice with respect to their circadian behavior might reveal significant abnormalities in the biological timing system. In the periphery, RORα protein is expressed at detectable levels only in skin and testis. This is surprising in view of the distribution pattern of RORG mRNA (2, 27). In particular, peripheral blood leukocytes (T cells, B cells, neutrophils) containing highest amounts of RORG mRNA second to skin (M.B.-A., unpublished data) were negative in the β-gal assay. We hypothesize that RORα expression in those cells and tissues could be controlled at the level of translation obeying to developmental and/or physiological signals, such as apoptosis and tissue-specific differentiation programs. In sg/sg mice a delay in terminating immune responses was observed (35), suggesting a defect in regulatory feedback mechanisms caused by the lack of RORα, possibly involving macrophages (36).

The absence of RORG causes massive PC loss leading to impaired development of the cerebellum. Apparently, RORα serves vital functions for proper maturation of these cells. The regenerative growth deficiency of the pelage hair of RORα−/− mice suggests that RORα also plays an important role in terminal differentiation processes of this skin appendix. Although the lack of RORG in cerebellar PCs and in keratinocytes of adult hair follicles has clear effects, the role of RORα in epidermal keratinocytes, sebaceous gland, retina, thalamus, and testis is less obvious. There, RORα might be involved in the fine-tuning of physiological processes rather than in bringing about developmental events. Alternatively, it is conceivable that the lack of RORα in these tissues is neutralized by compensatory mechanisms during the development. The identification of genes regulated by RORα (37) will be one of the next crucial steps toward a better understanding of the mechanism of action of this orphan nuclear receptor. To this end the corresponding null-mutant animals will be of great utility.

We thank Drs. Jonathan K. C. Knowles and John F. DeLamarter for their enthusiastic support, Chris Hebert for photographic assistance, Denise Greteren for technical assistance, Laurent Potier and Roberto Lia for the animal work, and Dr. Jean-François Nicholas for helpful discussions.