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► To cite this version:

Fatiha Boukhtouche, Mohamed Doulazmi, Florence Frederic, Isabelle Dusart, Bernard Brugg, et al.. RORa, a pivotal nuclear receptor for Purkinje neuron survival and differentiation: From development to ageing. *The Cerebellum*, 2006, 5, pp.97-104. hal-00078435

HAL Id: hal-00078435

<https://hal.science/hal-00078435>

Submitted on 6 Jun 2006

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**RORa, a pivotal nuclear receptor for Purkinje neuron
survival and differentiation:
From development to ageing**

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Abstract:

ROR α (Retinoid-related Orphan Receptor) is a transcription factor belonging to the superfamily of nuclear receptors. The spontaneous *staggerer* (*sg*) mutation, which consists of a deletion in the *Rora* gene, has been shown to cause the loss of function of the ROR α protein. The total loss of ROR α expression leads to cerebellar developmental defects, particularly to a dramatic decreased survival of Purkinje cells and an early block in the differentiation process. This review focuses on recent studies which position ROR α as a pivotal factor controlling Purkinje cell survival and differentiation, from development to ageing.

Introduction

Staggerer (*Rora*^{*sg*}) is a spontaneous null mutation consisting of a deletion in the gene encoding the nuclear receptor ROR α . Homozygous *staggerer* mutant mouse (*Rora*^{*sg/sg*}), as well as transgenic mice in which the *Rora* gene has been disrupted (*Rora*^{*-/-*}), display a severe ataxic phenotype associated with a strong cerebellar hypoplasia, a consequence of the degeneration of a large majority of Purkinje cells during development and the consequent absence of virtually all granule cells (1, 2). Interestingly, the few surviving Purkinje cells display immature shapes and abnormal differentiation. Moreover, both Purkinje cell survival and differentiation have been shown to be also abnormal in aged heterozygous *staggerer* mutants (*Rora*^{*+/-sg*}), indicating a crucial role for ROR α in these processes from development to ageing. This review focuses on the recent findings which contribute to a better understanding of the role of ROR α in the cerebellar development, and particularly in the Purkinje cell survival and differentiation throughout development and ageing.

I. The nuclear receptor ROR α .

ROR α (Retinoic acid receptor related Orphan Receptor α , also called NR1F1) (3) is a member of the nuclear receptor superfamily, which includes receptors for thyroid and steroid hormones, retinoids and vitamin D (4, 5). ROR α has long been considered as an orphan receptor, constitutively active in the absence of exogenous ligand (6, 7). However, ROR α has since been shown to be activated by abundant intracellular cholesterol (8).

ROR α is composed of the characteristic nuclear receptor domains (figure 1). ROR α has an amino-terminal domain (A/B region), a conserved DNA binding domain (DBD or C region), a hinge (D region) and a ligand binding domain (LBD or E region). The LBD contains a carboxy-terminus activation function domain (AF-2), responsible for ligand dependant transcriptional activation. The DBD, composed of two zinc finger motifs and a carboxy-terminal extension (CTE), is involved in the recognition of DNA response elements. The ROR response element (RORE) sequence is composed of a 6-base pair A/T-rich region immediately preceding a consensus AGGTCA motif (9-11). ROR α interacts as a monomer with RORE sequence within the promoter regions of target genes, but ROR α is also able to bind as a homodimer at direct repeats of the RORE site separated by two base pairs, or DR2 sites (12, 13).

By a combination of promoter usage and alternative splicing, the *Rora* gene gives rise to four isoforms in human, ROR α 1, α 2, α 3 and ROR α 4 (also termed RZR α), while only α 1 and α 4 isoforms have been detected in the mouse (14-16). These isoforms differ in their N-terminal modulator region, which interacts with A/T-rich sequences of the RORE and thus permits distinct promoter recognition and transactivation properties through an identical DNA binding domain (DBD) (9, 14). Functionally active RORE have been identified in a number of putative target genes, but the functional regulation by ROR α *in vivo* remains to be

demonstrated for many of them. Micro-array analyses of the cerebellar transcriptome in *staggerer* mutants followed by chromatin immunoprecipitation experiments identified authentic ROR α target genes in the cerebellum, including genes involved in calcium signal transduction (*Pcp2*, *Pcp4*, *Itpr1*) and the mitogenic factor Sonic Hedgehog (Shh) (17).

ROR α expression.

ROR α is a widely expressed nuclear receptor. ROR α has been detected in many tissues including brain, thymus, skeletal muscle, skin, heart, vessels, liver, lung, gut, kidney tubules, whisker follicles and pancreas (15, 18-20). In the brain, *in situ* hybridization and immunohistochemical analyses revealed *Rora* expression in the cerebellum, in olfactory bulb, hippocampus, thalamus, cerebral cortex (mainly in layer IV), suprachiasmatic nuclei of the hypothalamus and retinal ganglion cells (18, 21, 22). In the cerebellum, ROR α mRNA and protein have been detected at high levels in Purkinje cells (from age E13) and at lower level in stellate and basket interneurons (figure 2) (22).

ROR α loss-of-function mutant mice.

The homozygous *staggerer* mutant mouse displays a strong ataxic phenotype due to a massive cerebellar atrophy (figure 2). The *staggerer* mutation has been identified as a deletion within the *Rora* gene that shifts the reading frame and prevents the translation of the LBD of the ROR α protein (19). The strong decrease in *Rora*^{sg} allelic mRNA (19), together with the similar cerebellar phenotype displayed by both *Rora*^{sg/sg} and *Rora*^{-/-} mutants (23), suggests that the *staggerer* mutation is a null mutation and thus leads to the loss of function of ROR α .

Studying the *staggerer* phenotype has led to the discovery of many putative functions of ROR α . From the first identification of the developmental cerebellar defect in homozygous *staggerer* mutants in 1962 (1), the role of ROR α has since been expanded beyond the cerebellum, in particular in the control of circadian rhythms through the

transcriptional regulation of Bmal1 (24-28). Moreover, outside the central nervous system, ROR α has been shown to be implicated in the development of many tissues and in the differentiation process of many cell types, and provides protection against age-related degenerative processes including osteoporosis, atherosclerosis and chronic inflammation (for review, see (29, 30)).

II. Proliferative and neuroprotective function of ROR α in the cerebellum

Mutant mice lacking functional ROR α protein (18, 19) display a massive cerebellar degeneration (1): most of the Purkinje cells degenerate (82% are lost at two months of age (23)) while granular cells are virtually all absent (31) in homozygous *staggerer* mutants. According to Vogel and collaborators, Purkinje cells die between the postnatal (P) day 0 and P5 (32).

To determine whether the effect of the *staggerer* mutation was direct (intrinsic) or indirect (extrinsic), *staggerer* \leftrightarrow wild-type chimeras were made by aggregating two embryos at the 8-cell stage of development. Analyses of these *staggerer* chimeras demonstrated that the Purkinje cell loss was a direct consequence of the cell-autonomous action of the *staggerer* mutation (33), which is consistent with the distribution of ROR α mRNA and protein in the cerebellum, where only Purkinje cells and interneurons have been shown to express ROR α (19, 22, 34).

The granule cell loss has been shown to occur as a dual consequence of the PC loss. First, the study of the developmental persistence of the external granular layer (EGL) together with the study of the post-mitotic granular cell population in *staggerer* chimeras suggested that the reduced number of granule cells due to the *staggerer* mutation were the result of both reduced granule cell genesis and increased cell death (35). These hypotheses, based on the

study of chimeras, have been confirmed recently by a molecular analysis of target genes of ROR α in the cerebellum (17). Microarray analyses of the developing *Rora*^{sg/sg} cerebellar transcriptome have shown a decreased expression of proliferation markers, while Shh has been identified as a ROR α target gene in the cerebellum (17). Quantification of BrdU incorporation revealed a decreased granular cell proliferation in *Rora*^{sg/sg} organotypic cultures compared to wild-type. Moreover, adding Shh in the mutant slice preparations could restore normal proliferation of granule cell precursors in *Rora*^{sg/sg} slices (17), confirming a crucial role of Shh in the granule cell proliferation (36). An indirect loss of almost 60% of the cells within the inferior olivary complex in *Rora*^{sg/sg} mutants has been also described (37-39).

The neurodegeneration in *Rora*^{sg/sg} mutants is accompanied by a chronic inflammatory state. Interestingly, stimulation of peripheral macrophages of *Rora*^{sg/sg} mutants by lipopolysaccharide (LPS) induces an abnormally high amount of pro-inflammatory cytokines IL-1, IL-6 and TNF α (40). In addition, *Rora*^{sg/sg} mice are more susceptible to LPS-induced airway inflammation in the lung (41), and ROR α has been reported to inhibit inflammatory responses in vascular smooth muscle cells (42). The anti-inflammatory role of ROR α might be mediated in some tissues through the direct transcriptional control of the *I-kBa* gene, an inhibitor of the NF- κ B transcription factor (42). Moreover, abnormal IL-1 β cytokine production has been also described in the *staggerer* brain after peripheral LPS treatment (43), demonstrating a general condition of hyperexcitability which could increase the neurodegeneration.

The mutation was initially described as recessive, since heterozygous *Rora*^{+/-sg} mutants are behaviourally normal, without cell loss in the young adult. However, a Purkinje cell loss of about 25 to 35% appears afterwards and mostly between 6 and 12 months of age (44-46), whereas in control, Purkinje cell loss begins at 18 months to reach 25% at 24 months of age (46). The Purkinje cell loss in the *Rora*^{+/-sg} mutant is accompanied by both granule cell

(35%) and olivary neuron (30%) degeneration (44, 47). Interestingly, although the level of *Rora*^{+/-sg} Purkinje cell survival is similar between males and females at 13 months, the time-course of the loss depends on the sex in *Rora*^{+/-sg} mutants. In males, the Purkinje cell loss starts from 1 month and continues regularly up to 13 months. In contrast, in females, Purkinje cell number remains stable up to 9 months of age, then decreases to the same number as males (46). These data indicate that *Rora*^{+/-sg} heterozygous mutants undergo a more precocious Purkinje cell loss during ageing. Moreover, unlike controls, in which the later age-related Purkinje cell loss occurs similarly between males and females, the Purkinje cell loss time-course is influenced by the sex in *Rora*^{+/-sg} animals. Half-dose loss of functional ROR α has thus revealed an influence of gender in Purkinje cell survival during ageing.

These results show that ROR α function is essential for Purkinje cell survival during development and ageing. Whereas half-dose of functional ROR α protein seems to be sufficient to allow Purkinje cell survival during development, *Rora*^{+/-sg} mutant PCs are not protected against age-associated injuries. Among these, an increased vulnerability to oxidative stress is thought to play a critical role in age-related cell death (reviewed in (48-50)). To test whether an increased ROR α expression could be neuroprotective, we have recently developed a recombinant lentiviral vector to perform ROR α overexpression in cultured neurons. The survival rate of ROR α -overexpressing cortical neurons was evaluated in response to different stressors disturbing redox homeostasis, such as A β peptide, c₂-ceramide and H₂O₂ (51). In this study, we have shown that lentiviral-mediated hROR α 1-overexpression provides neuroprotection against reactive oxygen species (ROS)-induced apoptosis. Down-regulation of Gpx1 or Prx6 by si-RNA experiments partially suppressed the ROR α -mediated neuroprotection, further demonstrating that this protection is, at least in part, mediated by an up-regulation of the anti-oxidant enzymes glutathione peroxidase 1 and peroxiredoxin 6,

which leads to a decrease of the oxidative stress in neurons. ROR α appears thus as a factor controlling the oxidative stress in neurons (51).

We can therefore propose a model in which the age-related increased Purkinje cell loss in *Rora*^{+/*sg*} animals could be linked to the loss of the anti-oxidant actions of ROR α , whereas in wild type animals the activity of ROR α might reduce the effects of oxidative stress, thus leading to a relative neuroprotection during ageing compared to that observed in the heterozygous *staggerer* mutant. However, further studies will be needed to determine whether the observed Purkinje cell loss is linked to an increased ROS production in *Rora*^{+/*sg*} mutants. Furthermore, many studies have proposed the existence of a developmental Purkinje cell death period, occurring around P3 (52-56). Interestingly, in *Rora*^{*sg*/*sg*} mice, Purkinje cell loss seems to occur during this period (32). An increased ROS production is thought to be involved in developmental cell death, such as in trophic factor starvation during development (57). We may now hypothesize that ROR α 's control of cellular oxidative damage such as those induced by trophic deprivation renders it essential for Purkinje cell survival during development.

III. ROR α , a crucial factor controlling early PC differentiation

Most of the Purkinje cells degenerate in the *Rora*^{*sg*/*sg*} cerebellum, but, interestingly, those that survive display immature features. Purkinje cells are in an embryonic state: somata appear smaller than controls (31) (figure 2), while dendrites are rudimentary and stunted, lacking distal spiny branchlets (58-60). Before their target-related cell death in the *Rora*^{*sg*/*sg*} cerebellum (2), granule cells which have been generated seem to differentiate normally. By electronic microscopy, the presence of attachment plates between Purkinje cell dendrites and parallel fibers has been described (61), suggesting that parallel fibers form transient primitive

junctions with the Purkinje cell dendritic shaft, but attachment plates then fail to develop into functional synapses (61). In contrast, qualitatively normal dendritic spines and synapses are formed with climbing fibers (31, 58-60). However, there is a failure in the developmental regression of the Purkinje cell polyinnervation by climbing fibers (62, 63). Other immature and embryonic features remain in the postnatal *Rora*^{sg/sg} cerebellum, such as the persistent expression of embryonic cell surface components (64, 65) like embryonic NCAM isoforms (66). Moreover, expression of the late Purkinje cell markers Pcp-2/L7 (19) and calmodulin (67), which normally increases during development, have been shown to be altered in *Rora*^{sg/sg} mice.

All these studies have suggested that the differentiation of the surviving *Rora*^{sg/sg} Purkinje cells is impaired. However, in *Rora*^{sg/sg}, the neurodegeneration begins just after birth, which renders identification of exact Purkinje cell differentiation abnormalities very difficult. Indeed, Purkinje cell differentiation is known to be dependent upon granular cell interaction (68-71). The differentiation of *Rora*^{sg/sg} Purkinje cells has been studied in dissociated (72) or cerebellar organotypic (73) cultures, in which the differentiation can be observed independently of the neurodegeneration. In both culture types, Purkinje cells display an early postnatal block of the differentiation process, as they did not progress beyond the embryonic shape reminiscent of their migratory morphology (72, 73). More precisely, we have shown that Purkinje cells from newborn *Rora*^{sg/sg} cerebella do not undergo the normal regression of embryonic processes; these primitive processes continuing to develop rather than regress, as happens in wild-type Purkinje cells in cerebellar slices (73) and *in vivo* (74). Indeed, Purkinje cells, like other neurons, regress their embryonic bipolar processes prior to developing their ultimate dendritic tree (74). We have further demonstrated that ROR α expression is crucial for the early steps of Purkinje cell differentiation, as lentiviral-mediated hROR α 1 overexpression in wild-type Purkinje cells led to the acceleration of the early steps of

differentiation, in particular the first step of dendritic regression (73). Moreover, hROR α 1 expression in Purkinje cells in *Rora*^{sg/sg} cerebellar slices could restore the normal differentiation progression. These results demonstrate that ROR α is a crucial factor controlling early postnatal Purkinje cell differentiation.

In addition to the differentiation abnormalities described *in vivo* in the developing cerebellum of homozygous *Rora*^{sg/sg} mice, others have been described in the ageing cerebellum of heterozygous *Rora*^{+sg} mutants (75). The effects of the ageing process are apparent in the wild-type Purkinje cell as a dendritic atrophy is observed at 24 months of age. However, in *Rora*^{+sg} mutants, there is a considerably accelerated dendritic atrophy compared to controls, which is detected as early as 4 months of age in *Rora*^{+sg} but not until 22 months in *Rora*^{+/+}. The regression of the dendritic arbor not only starts earlier in *Rora*^{+sg} than in *Rora*^{+/+}, but it is also much more extensive (75). The dendritic atrophy does not seem to be the intracellular consequence of the cell death since dendritic atrophy is still observed after 13 months of age, when the Purkinje cell number does not decrease anymore. However, dendritic atrophy could be an indirect consequence of Purkinje cell death: the resulting granular cell loss in the cortex leads to the loss of the input from the parallel fibers on Purkinje cells which is known to influence dendritic arborization. Nevertheless, in view of the demonstrated role of ROR α on the early dendritic differentiation, we can hypothesize that ROR α also plays a role in the maintenance of the dendritic arborization during ageing. Further studies will be needed to determine whether ROR α could play such a role.

Conclusion

The nuclear receptor ROR α , which plays a major role in the development of many tissues throughout the organism, appears to be a pivotal nuclear receptor for cerebellar development and ageing, in particular through its effect on Purkinje cell survival and differentiation.

During development, ROR α expression is crucial for the Purkinje cell survival and differentiation. At the level of granule cells, ROR α plays a dual role: first, it directly controls their genesis by controlling their proliferation in the EGL through Shh, and second, ROR α is indirectly necessary for their survival since granule cells undergo target-related cell death in the absence of Purkinje cells. In the adult, ROR α protects Purkinje cells against age-related deleterious effects and ROR α is involved in the maintenance of dendritic arborization of Purkinje cell during ageing.

Figure Legends

Figure 1: The ROR α protein. Above is a schematic representation of a typical nuclear receptor. Below is a depiction of the ROR α protein conformation with its interaction with its RORE promoter site. NTD: N-Terminal Domain. DBD: DNA Binding Domain. LBD: Ligand Binding Domain.

Figure 2: Cerebellar abnormalities in homozygous *staggerer* mutants. Parasagittal cerebellar sections taken from the vermis of *Rora*^{+/+} (A, C) and *Rora*^{sg/sg} (B, D) mice 2-months old. A-B: Calbindin (CaBP) immunostaining (brown) reveals the extreme hypoplasia of *Rora*^{sg/sg} cerebellum (B) compared to a control littermate (A). Scale bars correspond to 500 μ m. C-D: Immunohistochemistry of CaBP (red) and ROR α (green) reveal the expression of ROR α in both Purkinje cells and interneurons in *Rora*^{+/+} cerebellum (C), whereas no ROR α labelling is detected in the cerebellum of the *Rora*^{sg/sg} mutant mouse (D). In the *Rora*^{sg/sg} cerebellum, CaBP labelling reveals the disorganization of Purkinje cells (which are not organised in a monolayer) and the atrophic and rudimentary dendrites.

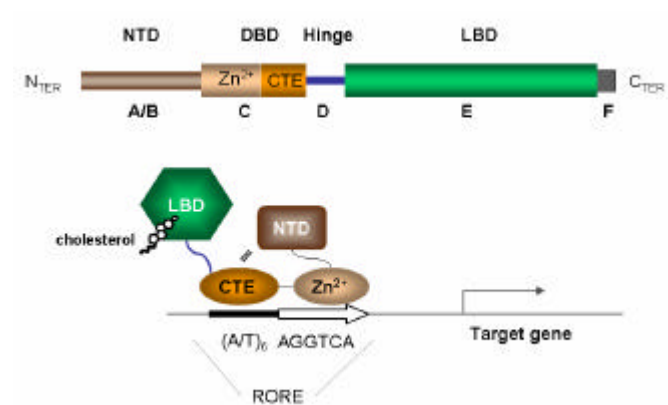


Figure 1

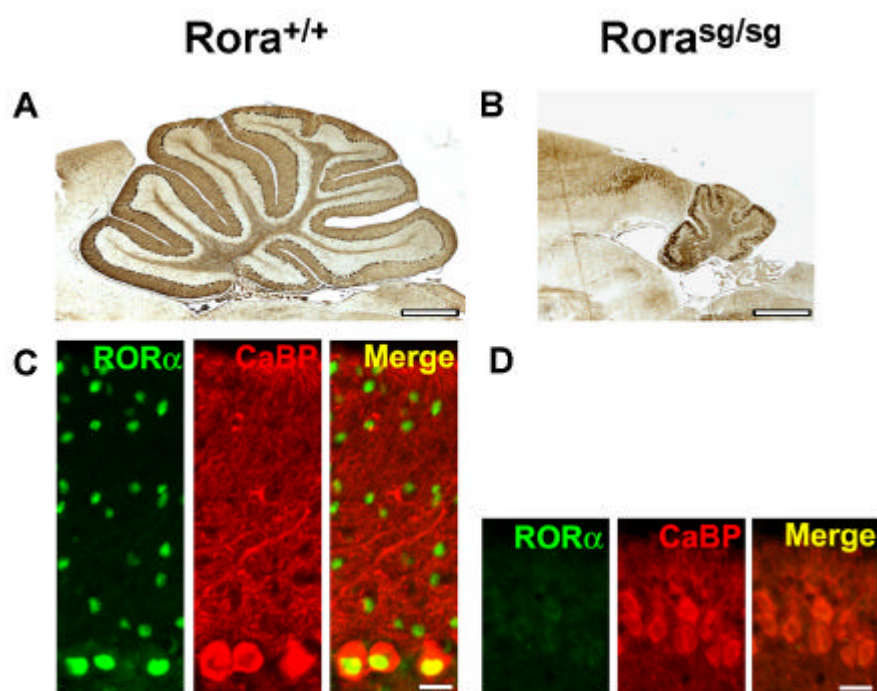


Figure 2

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