Cell-Autonomous and Non-Cell-Autonomous Neuroprotective Functions of ROR α in Neurons and Astrocytes during Hypoxia

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There is increasing evidence to suggest that the neuronal response to hypoxia is regulated through their interactions with astrocytes. However, the hypoxia-induced molecular mechanisms within astrocytes which influence neuronal death have yet to be characterized. In this study, we investigated the roles of the nuclear receptor ROR α (retinoid-related orphan receptor- α) respectively in neurons and astrocytes during hypoxia using cultures and cocultures of neurons and astrocytes obtained from ROR α -deficient mice. We found that loss of ROR α function in neuronal cultures increases neuronal death after hypoxia, suggesting a cell-autonomous neuroprotective effect of ROR α . Moreover, wild-type neurons cocultured with ROR α -deficient astrocytes are characterized by a higher death rate after hypoxia than neurons cocultured with wild-type astrocytes, suggesting that ROR α also has a non-cell-autonomous action. By using cocultures of neurons and astrocytes of different genotypes, we showed that this neuroprotective effect of ROR α in astrocytes is additive to its effect in neurons, and is mediated in part by cell-to-cell interactions between neurons and astrocytes. We also found that ROR α is upregulated by hypoxia in both neurons and astrocytes. Furthermore, our data showed that ROR α does not alter oxidative mechanisms during hypoxia but regulates hypoxic inducible factor 1 α (HIF-1 α) expression, a major regulator of hypoxia sensing, in a cell-specific manner. Indeed, the neuroprotective function of ROR α in astrocytes correlates with a downregulation of HIF-1 α selectively in these cells. Altogether, our results show that ROR α is a key molecular player in hypoxia, protecting neurons through its dual action in neurons and astrocytes.

Introduction

Hypoxia is a deficiency of available oxygen that can lead to neuronal death and cause severe brain damage. Astrocytes are intimately involved in the neuronal response to hypoxia and play an important role in many aspects of hypoxia: the regulation of glucose metabolism, transmitter release, inflammatory processes, and release of neuroprotectants (for review, see Zhao and Rempé, 2010). However, we are only beginning to understand the conditions under which these functions are neuroprotective or deleterious. Hypoxic inducible factor 1 (HIF-1) is a key element induced during hypoxia (Semenza, 2000). This transcription factor is a heterodimer of HIF-1 β , which is constitutively active, and HIF-1 α , which is stabilized and activated only during

hypoxia (Bruick and McKnight, 2001; Jaakkola et al., 2001; Lando et al., 2002). In the brain, HIF1 α production is induced by hypoxia in neurons, astrocytes, ependymal and, possibly, endothelial cells (Chávez et al., 2000). HIF-1 regulates several genes, including *Vegf* (vascular endothelial growth factor), *Nip3* (*BCL2/adenovirus E1B 19 kDa interacting protein 3*) and *Hk2* (*hexokinase 2*) (Sharp and Bernaudin, 2004). The transcription factor retinoid-related orphan receptor- α (ROR α) has been shown also to be a target of HIF-1 α (Chauvet et al., 2004; Miki et al., 2004). Moreover, ROR α expression is increased by hypoxia in various cell lines (Chauvet et al., 2002; Miki et al., 2004; Kim et al., 2008).

ROR α belongs to the nuclear receptor family and is considered to be a constitutive activator of transcription. Sterol derivatives such as cholesterol and hydroxycholesterol have recently been identified as potential ligands for ROR α (Kallen et al., 2002; Wang et al., 2010). In the brain, ROR α is expressed in neurons in the cortex, cerebellum, inferior olivary nucleus, hippocampus, thalamus, hypothalamus, olfactory bulb, and retina (Ino, 2004). We also recently reported the nuclear expression of ROR α in astrocytes, particularly in the hippocampus, cortex, and cerebellum (Journiac et al., 2009). There is a natural mutant mouse for ROR α , called *staggerer*, which carries a 122 bp deletion in the ligand-binding domain (LBD) of the *Rora* gene (Hamilton et al., 1996). The principal CNS phenotype of this mouse is extensive cerebellar neurodegeneration resulting in severe ataxia (Gold et al., 2007). In addition to its function in the cerebellum, ROR α has

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other important functions in the CNS including a neuroprotective role in cortical neurons (Boukhtouche et al., 2006). The upregulation of ROR α expression during hypoxia and its neuroprotective function raise questions about the possible role of neuronal and/or astrocyte ROR α in neuroprotective mechanisms during hypoxia in the cortex.

In this study, we focused on the function of ROR α in neurons and astrocytes following hypoxia using ROR α -deficient cultures and cocultures of neurons and astrocytes. We show that ROR α has a neuroprotective function during hypoxia not only in neurons but also through its action in astrocytes. We also demonstrate the upregulation of ROR α expression by hypoxia in cortical neurons and astrocytes. Finally, we show that ROR α selectively inhibits HIF-1 α expression in astrocytes. These results demonstrate that ROR α present in both neurons and astrocytes plays a crucial role in hypoxia.

Materials and Methods

Animals and genotyping

All efforts were made to minimize animal suffering and the number of animals used. All animal procedures were performed according to the regulations of the Comité National d'Éthique pour les Sciences de la Vie et de la Santé, in accordance with the European Communities Council Directive (86/609/EEC). Protocols were approved by the Comité régional d'éthique de Paris (file number p3/2008/026). Homozygous *staggerer* mice (*Rora*^{sg/sg}) and their control wild-type (*Rora*^{+/+}) littermates were obtained by crossing heterozygous mice (*Rora*^{+/-sg}). Mice were maintained on a common homogeneous genetic background (C57BL/6J) in our animal facilities at the Université Pierre et Marie Curie. Mice were genotyped for the *staggerer* mutation as previously described (Doumazmi et al., 1999).

Primary cortical neuron culture

Neuronal cultures were prepared from the neocortex of 14-d-old embryos of C57BL/6J (Janvier) or *staggerer* mice of either sex. Meninges were removed and the cerebral cortices were dissected. The tissue was then incubated with 0.25% trypsin-EDTA (Eurobio) and DNase I (50 μ g/ml, Sigma-Aldrich) for 10 min at 37°C. The cells were mechanically dissociated, washed three times in DMEM and plated on poly-D-lysine (0.1 mg/ml, Sigma)-coated culture wells at a density of 1.75×10^5 cells/cm². The cultures were maintained in DMEM supplemented with Glutamax, 5% heat-inactivated fetal bovine serum (FBS) (BioWest), N-2 and B-27 supplements (Invitrogen), and penicillin/streptomycin (Invitrogen). After 3 days *in vitro* (DIV 3), one third of the medium was removed and replaced with fresh medium containing 3 μ M Ara C (cytosine β -D-arabinofuranoside, Sigma) to inhibit the growth of dividing cells such as astrocytes and microglia. The cultures were maintained in a humidified incubator at 37°C under an atmosphere containing 5% CO₂. Neuronal cultures were used for experimentation on DIV 7. The purity of neuronal cultures was checked by immunolabeling with antibodies directed against neurons (mouse anti-NeuN, 1:250, Millipore Bioscience Research Reagents; anti-MAP2, 1:500, Millipore), and against astrocytes (mouse anti-GFAP-CY3, 1:1000, Sigma). Cells fixed in 4% PFA were labeled after membrane permeabilization and saturation with PBS supplemented with 0.25% Triton X-100 (Sigma), 0.2% gelatin, 0.1% sodium azide (buffer A) and 0.1 M lysine. Nuclei were stained with Hoechst 33258 (Sigma). The cultures contained >95% neurons.

Cortical astrocyte culture

Highly purified astrocyte cultures were prepared from 1- or 2-d-old pups of C57BL/6J or *staggerer* mice of either sex. Cerebral cortices were dissected and the meningeal tissue was carefully stripped off. The tissue was then incubated with 0.25% trypsin-EDTA and DNase I (50 μ g/ml) for 10 min at 37°C. The cells were mechanically dissociated, washed three times in DMEM and plated in culture wells at a density of 1 cortex/75cm². The cultures were maintained in DMEM supplemented with glucose (1 g/L), 10% heat-inactivated FBS, 1 mM L-glutamine (Seromed), 50 ng/ml gentamicin (Invitrogen) and incubated at 37°C in a humidified atmosphere

containing 5% CO₂/95% air. The medium was changed twice weekly. On DIV 7, astrocytes were treated with trypsin. For experiments on pure astrocyte cultures, cells were plated directly in 24- or six-well plates. For coculture experiments, cells were plated on glass coverslips treated with 0.5 mg/ml poly-D-lysine (P0899, Sigma) in six-well dishes. The cultures were then maintained in a humidified incubator for 1 week. The purity of astrocyte cultures was checked by immunolabeling with antibodies directed against neurons (mouse anti-NeuN), astrocytes (mouse anti-GFAP-CY3, rabbit anti-s100 Sigma) and microglial cells (rabbit anti-Iba1, Wako). Cells fixed in 4% PFA were labeled after membrane permeabilization and saturation with Buffer A and 0.1 M lysine. Nuclei were stained with Hoechst 33258 (Sigma). Contamination with other cells never exceeded 3%.

Neuron and astrocyte cocultures

Embryonic cortical *Rora*^{sg/sg} or *Rora*^{+/+} neurons were cocultured with *Rora*^{sg/sg} or *Rora*^{+/+} astrocyte monolayers. A neuronal suspension was prepared from the neocortex of 14-d-old embryos of C57BL/6J or *staggerer* mice, as described above. One day before the plating of neurons, astrocytes were maintained in neuronal medium (DMEM supplemented with Glutamax, 5% FBS, N-2 and B-27 supplements, and penicillin/streptomycin). Neurons were plated directly on the surface of the confluent astrocyte monolayers at a density of 4×10^4 cells/cm². On DIV 3 for the neurons, one third of the medium was removed and replaced with fresh medium containing 3 μ M Ara C (Sigma). The cultures were maintained in a humidified incubator at 37°C, under an atmosphere containing 5% CO₂. The cocultures were used for experimentation when the neurons had been in culture for 7 or 8 d. The purity of cocultures was checked by immunocytochemistry.

For cocultures without contact between neurons and astrocytes, we used the same protocol except that astrocytes were grown on an insert placed in the culture wells. The cell culture inserts (35–3102, Falcon) had 1 μ m pores. Neurons were plated on poly-D-lysine (0.1 mg/ml, Sigma)-coated culture wells at a density of 1.75×10^5 cells/cm².

Hypoxia treatment paradigms

Cultures were exposed to hypoxia in a triple gas incubator in which oxygen was replaced with nitrogen to achieve a concentration of 1% oxygen (Thermo Electron) for various periods of time. Hypoxia was terminated by returning the cells to normoxic conditions (reoxygenation) for various lengths of time. As a control, sister cultures maintained in normoxic conditions were analyzed in parallel.

Neurite outgrowth analysis in pure cultures and cocultures

Cortical neurons (DIV 3) cultured in 24-well dishes were transfected with the pmaxCloning vector (Amara) containing the GFP coding sequence under the control of the CMV promoter. We incubated 2 μ l of Lipofectamine 2000 (Invitrogen) in 50 μ l of DMEM (Invitrogen) for 5 min at room temperature and added an equal volume of DMEM containing 0.1 μ g of DNA. The DNA/Lipofectamine mixture was incubated at room temperature for 20 min and added to the neurons. The transfection mixture was then incubated for 90 min at 37°C, after which it was replaced with the original medium. After 48 h, cells were fixed in 4% PFA and nuclei were stained with Hoechst 33258 (GFP was visible in ~1% of the neurons). GFP-transfected neurons were observed under a Nikon Eclipse E600 microscope and photographed with a LEICA SP5 confocal microscope. All dendrites were traced with the NeuronJ plug-in of NIH ImageJ software. In all experiments, neurons were selected at random for photography and the neurites were photographed, traced and analyzed by an investigator blind to the details of the sample analyzed. Dendritic complexity was quantified by measuring the dendrite length and counting the number of dendrites for each order of branching. The primary dendrites were defined as those emanating directly from the soma. Higher-order branches were defined as those arising from the processes of the previous order. The dendrite length for a particular order of branching was calculated by averaging the lengths of all dendrites for that order of branching. The number of dendrites in a given order of branching was calculated by averaging the number of dendrites for each order of branching.

Neuronal viability assays

MTT. For experiments on neurons, cell survival was assessed with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The conversion of the yellow tetrazolium salt, MTT, to the purple formazan dye is dependent on mitochondrial activity. We added 100 μ l of a stock solution of MTT (M5655-1G, 5 mg/ml in PBS, Sigma) to 900 μ l of culture medium/well, and incubated the mixture for 4 h in a humidified atmosphere at 37°C, under an atmosphere containing 5% CO₂. The cells were then treated with 0.1 M HCl in isopropanol for 30 min. Reduced MTT levels were determined by measuring absorbance at 560 nm. The effect of hypoxia on cell viability was evaluated by comparing cultures subjected to hypoxia with sister cultures kept in normoxia.

LDH. In coculture experiments, cell death was quantified by measuring the amounts of LDH released from damaged cells into the culture medium 24 h after hypoxia according to the assay manufacturer's instructions (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega). LDH levels reflect neuronal death as astrocytes are not killed by hypoxia. Absorbance was read at 490 nm. The maximum LDH release, corresponding to almost complete cell damage, was evaluated after the lysis of all the cells. The percentage of cytotoxicity was calculated by dividing the experimental absorbance value by the absorbance for maximal LDH release, after subtracting background values due to the presence of LDH in the culture medium of cells kept in normoxia. The mean amount of LDH in normoxic cocultures was used as a control. All experiments were performed at least in triplicate and repeated five times with independent cell cultures.

Hoechst staining. After hypoxia and reoxygenation, nuclei were stained with Hoechst 33258 (Sigma). Neuronal and astrocyte viability were assessed by counting living nuclei of neurons (small nuclei with no mark of apoptosis or necrosis) and astrocytes. For each condition, three different wells were counted, three pictures per well were analyzed. Two different counters analyzed the pictures. Analysis was conducted blind to the experimental conditions. Cell survival was expressed as the percentage of living cells in hypoxic groups compared with the number of living cells in sister cultures cultivated in normoxia.

RNA purification and real-time PCR

Total RNA was extracted from cultures with TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. We checked the concentration and quality of the RNA by spectrophotometry. RNA was treated with DNaseI (Invitrogen) according to the manufacturer's instructions. Complementary DNA was synthesized from 0.5 μ g of RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Target cDNA levels were determined by real-time PCR with the Mx3005 unit (Stratagene) using SYBR Green (Abgene) to detect the amplification products. Amplification assays were performed in duplicate in 25 μ l reaction mixtures containing Absolute QPCR SYBR Green Mix (Abgene), 200 nM forward and reverse primers and cDNA. PCR was conducted over 40 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 15 s, preceded by an initial denaturation cycle at 95°C for 15 min. The efficiency of amplification was close to 100% for each set of primer, as estimated with serial dilutions of cDNA. A dissociation curve was generated for each transcript, to check the specificity of the amplification. Arbp (acidic ribosomal phosphoprotein P0) levels were used to normalize the amount of cDNA. Quantification was performed by the comparative $\Delta\Delta C_t$ method as previously described (Pfaffl, 2001). Levels relative to those in nonhypoxic cultures were calculated according to the formula $2^{-(\Delta\Delta C_t)}$.

Primers

The *Mus musculus* primer sequences used are listed in Table 1.

Nuclear protein preparation

Fractions enriched in nuclear proteins were isolated with a rapid protocol (adapted from the protocol of Rempe et al., 2007). Cultured cells were homogenized and lysed in 10 mM Tris-HCl, pH 7.6, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM AEBSEF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, Sigma) and Protease Inhibitor Cocktail (Sigma). Samples were incubated for 10 min on ice and centrifuged for 1 min at 10,000 $\times$ g; the supernatants were col-

Table 1. Primer sequences used to amplify mRNA

Gene	Forward	Reverse
<i>Rora</i>	TCCAAATCCACCTGGAAC	GAAGGTCTGCCACGTTATCTG
<i>Arbp</i>	TGCCAGCTCAGAACACTGGTCTA	GGGAGATGTTACGATGTTTCAGCA
<i>Hif1a</i>	CGGCGAAGCAAGAGTCTGA	TAGACCACCGGCATCCAGAA
<i>Vegf</i>	TGACTGCTGTGGACTTGTGT	GAATGGGTTTGTGCTGTTTC
<i>Sod1</i>	TGGGTTCCAGCTCCATCAGTA	ACCGTCTTTCCAGCAGTCA
<i>Gpx1</i>	GGGACTACACCGAGATGAACGA	ACCATTCACTCCGACTTCTCA
<i>Prdx6</i>	TGCTGGGAATAGCATGGGTA	AACCTCTGAGAAGGTGCACA
<i>Cat</i>	GGTCCAGTGCGCTGTAGA	TACGGGTGGAGCTCAGTGAA
<i>Rorb</i>	TGTGCTGCTTCAACCCATT	TCCTCTCTCAGTCAGTCGAA
<i>Nip3</i>	CCTGTCGAGTTGGGTCGG	TCGTCAGATTCACTGGGCA
<i>Hk2</i>	AGCTGGTGAGCCATCGTGGT	ATCTGTGTCGCGAGTCGG

lected (cytoplasm-enriched fraction). Pellets were resuspended in buffer containing 420 mM KCl, 1.5 mM MgCl₂, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM AEBSEF and Protease Inhibitor Cocktail (Sigma). Samples were vigorously mixed, incubated for 20 min on ice and centrifuged at 10,000 $\times$ g for 5 min. The supernatants enriched in nuclear material were subjected to four rounds of sonication for 30 s. The Bio-Rad DC protein assay was used to determine protein concentration, with bovine serum albumin as the standard.

Western blot analysis

Denatured proteins in Laemmli buffer (12.5–50 μ g) were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). Nonspecific binding was blocked with PBS/Tween 20 (PBST) supplemented with 5% nonfat dry milk, for 45 min at room temperature. Membranes were incubated overnight at 4°C in PBST supplemented with 2.5% nonfat dry milk and the following primary antibodies: goat anti-ROR α (1:1000, Santa Cruz Biotechnology), rabbit anti-HIF-1 α (NB 100-479, 1:1000, Novus Biologicals), mouse anti- β -actin (1:10,000, Sigma) antibodies. Membranes were then incubated at room temperature for 30 min in PBST-2.5% nonfat dry milk with anti-goat (1:200,000, Thermo Fisher Scientific), anti-mouse (1:50,000) or anti-rabbit (1:50,000) horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). The protein bands were detected by enhanced chemiluminescence (SuperSignal West Pico/Dura Extended Duration Substrate, Pierce). All blots were quantified by densitometry with NIH ImageJ software. Relative levels of transcription factors were determined by normalizing the density for the protein of interest with respect to a protein used as a loading control, in this case β -actin.

Statistical analysis

Statistical significance was determined with Statview software (Abacus Concepts). All the values in the text and figure legends are means $\pm$ SEM. All error bars on graphs indicate the SEM.

Results

ROR α has cell-autonomous protective functions in cortical neurons but not in astrocytes subjected to hypoxia

To determine the function of ROR α in neurons and astrocytes during hypoxia, we used primary cortical culture of neurons and astrocytes with (*Rora*^{+/+}) and without (*Rora*^{sg/sg}) endogenous ROR α . Neurons were exposed to hypoxia and were then subjected to a 24 h reoxygenation period (Fig. 1A). Hypoxia resulted in neuronal death as seen by immunostaining (Fig. 1B) and, after 14 h of hypoxia, no neuron survived (data not shown). When cortical neurons were subjected to 8 or 10 h of hypoxia followed by reoxygenation, *Rora*^{sg/sg} neurons showed moderately, but significantly lower rates of cell survival (15%) than *Rora*^{+/+} neurons ($n = 4$, Fig. 1C). After 12 h of hypoxia, neuronal survival rates were also lower when neurons lacked ROR α but the difference was not significant (Fig. 1C). The loss of ROR α function in neurons therefore increases susceptibility to hypoxia-induced neuronal death. Astrocytes are more resistant to hypoxia than

Figure 1. ROR α has a cell-autonomous neuroprotective function in neurons after hypoxia. **A**, Schematic diagram illustrating the experimental protocol: neurons (**B**, **C**) or astrocytes (**D**, **E**) (black cells) were subjected to hypoxia followed by 24 h of normoxia. **B**, Wild-type neurons were labeled with anti-MAP2 (green) antibody and nuclei were labeled with Hoechst 33258 (blue). Immunostaining shows a decrease in cell survival and the fragmentation of neurites after hypoxia (H12 + 24) not observed in neurons kept in normoxia (NH). **C**, Survival of *Rora*^{+/+} (wt) or *Rora*^{sg/sg} (sg) neurons was assessed by an MTT assay after 8–12 h of hypoxia and 24 h of reoxygenation. **D**, Wild-type astrocytes were labeled with anti-GFAP (red) antibody and nuclei were labeled with Hoechst 33258 (blue). Hypoxia did not induce the activation of cortical astrocytes. **E**, Survival of *Rora*^{+/+} (wt) or *Rora*^{sg/sg} (sg) astrocytes was assessed by an MTT assay after 24 h of hypoxia and 24 h of reoxygenation (24H). Values are the means $\pm$ SEM of 4 independent cultures. (*t* test, **p* < 0.05, ns, not significant).

neurons and cortical astrocytes remained alive even after 72 h of hypoxia followed by 24 h of reoxygenation (data not shown). After 24 h of hypoxia, all astrocytes survived and their reactivity was similar (Fig. 1D). After hypoxia, cell survival was similar in *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes indicating that the loss of ROR α function does not influence astrocyte survival during hypoxia (Fig. 1E).

ROR α has non-cell-autonomous neuroprotective functions in astrocytes subjected to hypoxia

Astrocytes play a key role in physiological and pathological processes in the CNS and are active partners of neurons. Thus, even if the loss of ROR α function in astrocytes does not influence astrocyte survival after hypoxia, it may affect neuronal survival in a non-cell-autonomous manner. We investigated the effect of the selective loss of ROR α function in astrocytes on the survival of neurons in normoxia and after hypoxia, in an *in vitro* model consisting of cocultures of cortical neurons and astrocytes. Cocultures were prepared by combining cells of different genotypes, i.e., *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes with *Rora*^{+/+} neurons (Fig. 2A). We first investigated the effect of a lack of ROR α in astrocytes on neuronal survival and growth in normal conditions. When astrocytes become reactive, they undergo major morphological modifications and one of the principal hallmarks of reactive astrocytes is the upregulation of GFAP. The inhibition of GFAP production in astrocytes has been shown to improve neuronal survival and neurite outgrowth in an *in vitro* coculture model (Desclaux et al., 2009). We therefore assessed by immunocytochemistry and Western blotting that GFAP levels were similar in *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes, in both normoxia and hypoxia (data not shown). We then quantified survival of neurons cocultured with *Rora*^{+/+} or *Rora*^{sg/sg} astrocytes in normoxia. Survival rates were similar for neurons cocultured with *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes (*n* = 4, Fig. 2B). We then investigated the effect of astrocyte genotype on neurite growth by transfecting

neurons with GFP (Fig. 2C). Astrocytes of all genotypes promoted dendritic growth, but neurons grown on ROR α -deficient astrocytes occupied a significantly larger area, had more dendrites and a greater total dendrite length (Fig. 2D,E). ROR α in astrocytes modulated the establishment and maintenance of the dendritic arbor in a non-cell-autonomous manner. This effect is specific to the function of ROR α in astrocytes as *Rora*^{+/+} and *Rora*^{sg/sg} neurons had similar branching patterns, occupied a similar area and had similar numbers of dendrites *in vitro* (data not shown). Thus the loss of ROR α in astrocytes has little effect on astrocyte reactivity and properties. Consequently, *Rora*^{sg/sg} astrocytes provide suitable support for neuronal development.

We next compared the survival of neurons grown on *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes after 6–10 h of hypoxia and reoxygenation (Fig. 2F). An analysis of MAP-2-immunostained neurons revealed that neurons grown on ROR α -deficient astrocytes had a lower neuron density and lower levels of neurite after 8 h of hypoxia than control neurons (Fig. 2F). Moreover, neuronal death rates after 6

and 8 h of hypoxia were significantly higher when astrocytes lacked ROR α (LDH experiments, *p* < 0.05, *n* = 4, Fig. 2F). After 10 h of hypoxia, neuronal death rates were higher when astrocytes lacked ROR α but the difference was not significant (Fig. 2F). We confirmed these results by quantifying neuronal survival using Hoechst. We found that neurons cocultured with mutant astrocytes had a lower survival rate (40% lower) compared with neurons cocultured with wild-type astrocytes after 8 h of hypoxia (Fig. 2F). For the following experiments, we chose to subject cocultures to 8 h of hypoxia. In conclusion, ROR α in astrocytes has a non-cell-autonomous neuroprotective effect after hypoxia. Our results suggest that ROR α has two neuroprotective effects: a cell-autonomous effect in neurons and a non-cell-autonomous effect in astrocytes.

The neuroprotective effects of ROR α in astrocytes and neurons are additive

We then investigated whether the two neuroprotective effects of ROR α in neurons and in astrocytes were additive. We used cocultures of cells with different genotypes: *Rora*^{+/+} or *Rora*^{sg/sg} astrocytes with *Rora*^{+/+} or *Rora*^{sg/sg} neurons and compared neuronal death rates after hypoxia and reoxygenation. In cocultures, ROR α deficient neurons are more susceptible to hypoxia only when astrocytes are also deficient for ROR α . After hypoxia, neuronal death rates were higher when *Rora*^{sg/sg} neurons were cocultured with *Rora*^{sg/sg} astrocytes (55% higher than for cocultures with *Rora*^{+/+} astrocytes, *n* = 5, Fig. 2G). Moreover, when both neurons and astrocytes lacked ROR α , neuronal death rates were significantly higher than for cocultures of *Rora*^{sg/sg} astrocytes and *Rora*^{+/+} neurons (30% higher, *n* = 5, Fig. 2G). In conclusion, the neuroprotective functions of ROR α in neurons and astrocytes after hypoxia are additive. Given the magnitude and the novelty of the non-cell-autonomous effect of ROR α in astrocytes, we

Figure 2. ROR α in astrocytes is neuroprotective in a non-cell-autonomous manner after hypoxia. **A–E**, The loss of ROR α in astrocytes modifies the development of the dendritic arbor of neurons in normoxia. **A**, Schematic diagram illustrating the experimental protocol: *Rora*^{+/+} (wt) neurons (black cells) were plated directly onto a monolayer of *Rora*^{+/+} (wt) or *Rora*^{sg/sg} (sg) astrocytes (as; orange and red circles). **B**, Survival rates of neurons in cocultures with *Rora*^{+/+} or *Rora*^{sg/sg} astrocytes were estimated in normoxia. **C**, Neurons grown on *Rora*^{+/+} or *Rora*^{sg/sg} astrocytes were transfected with GFP. **D**, The number of dendrites per neuron was determined. **E**, The total dendritic length per neuron was determined in each set of culture conditions. Dendrites from the individual neurons (numbers indicated in the bars) in four independent experiments were imaged and analyzed with NeuronJ software. **F**, The loss of ROR α in astrocytes increases the susceptibility of neurons to hypoxia-induced neuronal death. Schematic diagram illustrating the experimental protocol: *Rora*^{+/+} or *Rora*^{sg/sg} neurons (black cells) were plated directly onto a monolayer of *Rora*^{+/+} or *Rora*^{sg/sg} astrocytes (orange and red circles) and were subjected to hypoxia (6, 8, or 10 h) followed by 24 h of normoxia. Neuronal death rates were estimated by quantifying LDH release (left, orange). Neuronal survival rates were estimated by counting living nuclei (manual counting) (left, purple). After 8 h of hypoxia, neurons were labeled with anti-MAP2 antibody (green) and nuclei were labeled with Hoechst 33258 (blue, right). The values shown are the means $\pm$ SEM of 4 independent cultures. (*t* test, **p* < 0.05, ns, not significant). **G**, Losses of ROR α function in astrocytes and neurons have additive effects on hypoxia-induced neuronal death. Cocultures were subjected to 8 h of hypoxia followed by 24 h of normoxia. Neuronal death rates were estimated by quantifying LDH release. Values are the means $\pm$ SEM of 4 independent cultures (ANOVA, de Scheffe, **p* < 0.05).

focused on this biological function of ROR α in subsequent experiments.

Astrocyte ROR α influences neuronal susceptibility to hypoxia-induced death partly by physical contact between astrocytes and neurons

We asked whether the non-cell-autonomous neuroprotective function of ROR α involved the aberrant secretion of soluble factors by astrocytes, in an *in vitro* model based on cocultures of neurons and astrocytes without contact between the two cell types. Cocultures were prepared by combining cells of different genotypes: *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes with *Rora*^{+/+} neurons. After hypoxia and reoxygenation, the neuronal death rates were higher (25% higher) for cocultures with astrocytes lacking ROR α (*n* = 8, data not shown). However, this difference was not significant. These results suggest that ROR α has a non-cell-autonomous neuroprotective effect after hypoxia mediated

mainly by changes in the contact between neurons and astrocytes. A combination of two parameters is probably responsible for altering neuronal death rates: a difference in the contact between neurons and astrocytes and a change in the level of secretion of a soluble factor by astrocytes.

ROR α inhibits HIF-1 α expression in neuron/astrocyte cocultures

We investigated the molecular mechanism underlying the role of astrocytes in the neuroprotective function of ROR α . Oxidative stress is one of the major events leading to neuronal death after hypoxia. ROR α has been shown to protect cortical neurons against oxidative stress-induced apoptosis by increasing the expression of antioxidant proteins, thereby reducing the accumulation of reactive oxygen species (Boukhtouche et al., 2006). We thus compared the expression of various antioxidant genes in *Rora*^{+/+} and *Rora*^{sg/sg} neurons and astrocytes after hypoxia. Transcript levels for glutathione peroxidase 1,

Figure 3. Antioxidant gene expression is similar between *Rora*^{+/+} and *Rora*^{sg/sg} neurons/astrocytes after hypoxia. *Rora*^{+/+} (wt) or *Rora*^{sg/sg} (sg) neurons (**A**) and astrocytes (**B**) were subjected to hypoxia without reoxygenation. mRNA abundance for antioxidant genes was evaluated by real-time RT-PCR. Cat, Catalase; gpx1, glutathione peroxidase 1; sod1, superoxide dismutase 1; Prdx6, peroxiredoxin 6; NH, non hypoxia, H, hypoxia. Values are the means $\pm$ SEM of 4 independent cultures.

peroxiredoxin 6, catalase, and superoxide dismutase were similar in *Rora*^{+/+} and *Rora*^{sg/sg} neurons and in *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes ($n = 4$, Fig. 3*A,B*). Thus, in our model, the neuroprotective effect of ROR α during hypoxia may not be due to a regulation of antioxidant gene expression.

Another possible explanation for the neuroprotective effect of ROR α is a modulation of the HIF-1 α pathway. Indeed, HIF-1 α is key regulator of oxygen homeostasis and ROR α and HIF-1 α have been shown to interact *in vitro* (Kim et al., 2008). We investigated the regulation of HIF-1 α in cocultures of *Rora*^{+/+} or *Rora*^{sg/sg} astrocytes and *Rora*^{+/+} neurons after 8 h of hypoxia by real-time PCR (Fig. 4*A*). *Hif1a* gene transcript levels were similar in *Rora*^{+/+} and *Rora*^{sg/sg} cocultures and did not increase after hypoxia (Fig. 4*B*). Surprisingly, the post-transcriptional regulation of HIF-1 α differed considerably between *Rora*^{+/+} and *Rora*^{sg/sg} cocultures. Western blot analysis with relative quantification showed that the HIF-1 α protein was significantly more strongly induced in *Rora*^{sg/sg} cocultures ($n = 5$, $p < 0.05$, Fig. 4*C*). We checked that this increase in HIF-1 α levels was correlated with an increase in its transcriptional activity, by carrying out real time PCR to analyze the transcriptional regulation of three of its targets, *Vegf*, *Nip3*, and *Hk2*. The induction of *Vegf*, *Nip3*, and *Hk2* gene transcripts was significantly stronger in *Rora*^{sg/sg} cocultures than in control cultures, immediately after hypoxia ($n = 5$, $p < 0.05$, Fig. 4*D*). In conclusion, ROR α in astrocytes seems to inhibit the post-transcriptional activation of HIF-1 α after hypoxia.

ROR α is upregulated by hypoxia in astrocytes and neurons but inhibits HIF-1 α expression only in astrocytes

Finally, we investigated the relationship between ROR α and HIF-1 α during hypoxia in more detail and checked whether the inhibition of HIF-1 α by ROR α was a mechanism specific to astrocytes. As ROR α is a potential target of HIF-1 α (Chauvet et al., 2004), we investigated the pattern of expression of ROR α under hypoxia in primary cultures of neurons and astrocytes.

In neurons subjected to 7 h of hypoxia, *Rora* gene transcript levels were slightly but consistently upregulated (1.7 times higher than those in normoxic cells, $n = 4$, Fig. 5*A,B*). Upon reoxygenation, *Rora* transcript levels gradually returned to basal levels: after 6 h of reoxygenation, transcript levels were still 1.5 times higher than those in normoxic cells (Fig. 5*B*). Western blot experiments revealed that ROR α protein levels were also increased

by hypoxia (they were 1.6 times higher than those in normoxic cells, $n = 4$, Fig. 5*C*). Moreover, this regulation was specific to ROR α , as the level of transcripts for ROR β , another member of the ROR subfamily expressed in the cortex, was not increased by hypoxia ($n = 4$, Fig. 5*D*). We then investigated the regulation of HIF-1 α in *Rora*^{+/+} and *Rora*^{sg/sg} neurons after hypoxia by real-time PCR. Quantitative analysis of *Hif1a* gene transcripts revealed no differences between *Rora*^{+/+} and *Rora*^{sg/sg} neurons (Fig. 5*E*). *Hif1a* gene expression did not increase after hypoxia in either type of neurons (Fig. 5*E*). Similarly, the post-transcriptional regulation of HIF-1 α did not differ between *Rora*^{+/+} and *Rora*^{sg/sg} neurons. Western blots with relative quantification showed that HIF-1 α protein was similarly induced after hypoxia in *Rora*^{+/+} and *Rora*^{sg/sg} neurons ($n = 5$, Fig. 5*F*). We checked that this similar pattern of HIF-1 α expression was correlated with similar levels of transcriptional activity, by using real-time PCR to investigate the transcriptional regulation of *Vegf*, *Nip3*, and *Hk2*. We observed a similar induction of *Vegf*, *Nip3*, and *Hk2* gene transcripts in *Rora*^{+/+} and *Rora*^{sg/sg} neurons ($n = 5$, Fig. 5*G*). In conclusion, neuronal ROR α does not inhibit the post-transcriptional activation of HIF-1 α after hypoxia.

In astrocytes, real-time PCR experiments revealed a transient upregulation of the *Rora* transcript by 24 h of hypoxia (levels 2.7 times higher than those in normoxic sister cultures, $n = 4$, Fig. 6*A,B*). Upon reoxygenation, *Rora* transcript levels rapidly returned to basal levels, demonstrating that this regulation was transient. ROR β transcripts levels were not induced by hypoxia in astrocytes ($n = 4$, Fig. 6*D*). We analyzed the regulation of ROR α in nuclear extracts by Western blotting after hypoxia. ROR α protein levels were 2.6 times higher after hypoxia than in normoxic cells (Fig. 6*C*). Thus, similarly to what was shown in neurons, ROR α is upregulated at the transcriptional level by hypoxia in astrocytes. Finally, the regulation of HIF-1 α was also analyzed in *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes. As in cocultures, we observed a similar pattern of transcriptional regulation of the *Hif1a* gene in *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes, a stronger induction of the HIF-1 α protein correlated with an increase in the transcriptional induction of the *Vegf* and *Nip3* genes after hypoxia in *Rora*^{sg/sg} astrocytes than in control cultures (Fig. 6*E–G*). *Hk2* gene expression was similar between *Rora*^{+/+} and *Rora*^{sg/sg} astrocytes (Fig. 6*G*). We can therefore conclude that the differen-

tial regulation of HIF-1 α by ROR α during hypoxia is a mechanism specific to astrocytes.

Discussion

Hypoxia can have devastating consequences in the brain and the molecular mechanisms contributing to hypoxia-induced neuronal death have yet to be identified. In this study, we investigated the contribution of the nuclear receptor ROR α to hypoxia-induced mechanisms in neurons and astrocytes. We studied the consequences of the selective loss of ROR α function in either neurons or astrocytes using astrocyte/neuron cultures and cocultures. We showed that ROR α has neuroprotective properties after hypoxia through its expression both in neurons and in astrocytes (Fig. 7). We also demonstrated the upregulation of ROR α by oxygen deprivation in both neurons and astrocytes, but showed that ROR α had different functions in these two cell types. Indeed, ROR α inhibits HIF-1 α expression only in astrocytes, and this mechanism may account for the non-cell-autonomous neuroprotective function of ROR α .

Our data reveal that the loss of ROR α function in neurons increases neuronal susceptibility to hypoxia-induced death (Fig. 7A). ROR α -deficient neurons displayed correct dendritic arbor development. The increase in susceptibility to hypoxia was therefore not due to changes in neuronal development *in vitro*. Moreover, this cell-autonomous function of ROR α in neurons was not associated with a deregulation of antioxidant gene expression, contrary to what was previously shown with other stress (Boukhouché et al., 2006). The target genes of ROR α responsible for modulating neuronal survival after hypoxia have yet to be identified. The loss of ROR α function in astrocytes also leads to a marked increase in neuronal death after hypoxia in cocultures (Fig. 7B). Thus, ROR α clearly has a neuroprotective function in both neurons and astrocytes and we demonstrated that the two neuroprotective mechanisms act in an additive manner (Fig. 7C). This finding is consistent with recent studies demonstrating that astrocytes contribute to neuronal survival in many neurodegenerative or neurological disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and Rett syndrome (Lobsiger and Cleveland, 2007; Ballas et al., 2009). Astrocytes have many functions that are essential for neuronal activities (control of blood flow, release of neuroprotective substances, participation in inflammatory processes etc.). However, during pathological states, the contribution of astrocytes may become either neuroprotective or harmful. For example, during ischemia, astrocytes may increase neuronal survival and plasticity by releasing erythropoietin and ATP, by producing metalloproteinases or by secreting thrombospondin (Masuda et al., 1994; Zhao et al., 2006; Liauw et al., 2008; Lin et al., 2008). However, they may also have detrimental effects through the release of neurotoxic substances such as S-100 β or nitric oxide (NO), through the release of gluta-

Figure 4. In cocultures, ROR α inhibits HIF-1 α production after hypoxia. **A**, Schematic diagram illustrating the experimental protocol: *Rora*^{+/+} (wt) neurons (black cells) were plated directly on a monolayer of *Rora*^{+/+} (wt) or *Rora*^{sg/sg} (sg) astrocytes (as; orange or red circles) and were subjected to 8 h of hypoxia. Analyses were performed immediately upon removal from hypoxia. **B**, *Hif1a* gene transcription was analyzed by real-time RT-PCR. Transcription of the *Hif1a* gene was found to be induced similarly in *Rora*^{+/+} and *Rora*^{sg/sg} cocultures after hypoxia. **C**, Western blot analysis (top) of the HIF-1 α protein in enriched nuclear extracts and relative quantification (bottom). **D**, *Vegf*, *Nip3*, and *Hk2* gene transcription was analyzed by real-time RT-PCR. H, Hypoxia; NH, non hypoxia. Values are the means $\pm$ SEM of 4–6 independent cultures. (Mann–Whitney, * p < 0.05).

mate or through their contribution to the formation of brain edema (Endoh et al., 1994; Manley et al., 2000; Asano et al., 2005). The relative contribution of ROR α in neurons and astrocytes during hypoxia and/or ischemia *in vivo* has now to be investigated.

Astrocytes lacking ROR α may influence neuronal survival via several pathways. First, in normal conditions, neurons cocultured with mutant astrocytes survive but develop a more highly branched dendritic arbor. ROR α in astrocytes may therefore control dendritic branching. Second, during hypoxia, ROR α in astrocytes may be beneficial to neurons through a combination of several mechanisms. Our results revealed that neurons grown on mutant astrocytes have a more complex dendritic arbor. We cannot exclude the possibility that these neurons are more vulnerable and that the increased neuronal death rates observed after hypoxia are independent of hypoxia signaling in astrocytes. In cocultures without contact between neurons and astrocytes, the loss of ROR α function in astrocytes only slightly increased the rate of neuronal death. This increase in neuronal death was not significant and was only half that observed in cocultures with contact. Astrocytes lacking ROR α therefore probably influence neuronal death directly, through physical interactions with neurons, and indirectly through the release of soluble factors. ROR α -deficient astrocytes may influence neurons directly by regulating the synaptic plasticity or different molecular pathways involved in unidentified processes in neurons, resulting in an increased neuronal death after hypoxia. ROR α -deficient astrocytes may also

Figure 5. In neurons, ROR α is upregulated by hypoxia and does not prevent HIF-1 α expression after hypoxia. **A**, Schematic diagram of the experimental protocol: *Rora*^{+/+} (wt) or *Rora*^{sg/sg} (sg) neurons were subjected to 7 h of hypoxia followed by 0–24 h of normoxia. **B**, *Rora* gene transcription was analyzed by real-time RT-PCR (*t* test, **p* < 0.05). **C**, Western blot analysis (top) of ROR α protein in enriched nuclear extracts from cortical neurons and relative quantification (bottom) (*t* test, **p* < 0.05). **D**, *Rorb* gene transcription was analyzed by real-time RT-PCR. **E**, *Hif1a* gene transcription was analyzed by real-time RT-PCR. **F**, Western blot analysis (top) of HIF-1 α protein in enriched nuclear extracts and relative quantification (bottom). **G**, *Vegf*, *Nip3*, and *Hk2* gene transcription was analyzed by real-time RT-PCR. (Mann–Whitney, ns, not significant). Values are the means $\pm$ SEM of 4 independent cultures. H + 0, Hypoxia without reoxygenation, NH, non hypoxia.

have a deficiency in secretion of a protective soluble factor. One candidate is IL-6 (interleukin-6), a cytokine that can promote neuronal survival. We previously demonstrated that ROR α modulates its expression by astrocytes (Journiac et al., 2009). Alternatively, ROR α -deficient astrocytes may secrete larger amounts of factors toxic to neurons, such as NO. An analysis of the transcriptome of mutant astrocytes may help us to identify the factors involved.

Cells adapt rapidly to hypoxia, by modifying their gene expression profiles in particular. We demonstrated that, after hypoxia, ROR α gene expression increased markedly in neurons and astrocytes. The rapid induction of ROR α transcripts in these two cell types indicate that ROR α is involved in the molecular mechanisms by which cells adapt to hypoxia. ROR α expression in astrocytes returned to baseline as soon as reoxygenation occurred, but remained high for 6 h in neurons. The different time course of ROR α expression suggests that ROR α is involved in different pathways in neurons and in astrocytes. The induction of ROR α transcripts after hypoxia has been reported in various cell lines including human aortic smooth muscle cells, endothelial cells, and HepG2 cells (Besnard et al., 2002; Chauvet et al., 2002, 2004; Miki et al., 2004; Kim et al., 2008). The induction of ROR α was attributed to an HRE (hypoxia response element) in the *Rora* promoter (Chauvet et al., 2004). Indeed, the authors showed by EMSA that HIF-1 α bound to the *Rora* promoter and regulated its expression. Future studies should determine whether HIF-1 α is one of the transcription factors responsible for modulating ROR α expression in the brain.

As previously shown by Chavez et al. (2006), our data indicate that the fold induction of HIF-1 α after hypoxia is different between neurons and astrocytes. Moreover, we show here that ROR α inhibits HIF-1 α production in astrocytes at the post-transcriptional level. This inhibition is correlated with a decrease in the transcriptional induction of some HIF-1 α target genes such as *Vegf*, *Nip3*, and *Hk2*. However, *Hk2* gene expression was increased in coculture with neurons and mutant astrocytes but not in cultures with mutant astrocytes (Figs. 4D, 6G). This difference may be due to the duration of hypoxia (8 h in cocultures vs 24 h with astrocytes) or, more likely, to the different behavior of astrocytes when they are cultured with or without neurons. The inhibition of HIF-1 α expression by ROR α is specific to astrocytes because HIF-1 α levels were unaffected in mutant neurons. At first glance, our results may appear to contradict those of Kim et al., who showed that exogenous ROR α increased HIF-1 α levels and transcriptional activity in HepG2 and HeLa cells (Kim et al., 2008). These authors demonstrated that the knockdown of ROR α expression by siRNA decreased HIF-1 α activity (Kim et al., 2008). There may be several reasons for the difference between our results and those of Kim et al. (2008). Our experiments were performed in primary cultures of neurons and astrocytes and showed a cell specificity of the regulation of HIF-1 α by ROR α . We can therefore conclude that the function of ROR α during hypoxia differs between cell lines and primary cultures. Moreover, some of the experiments in the study by Kim et al.

Figure 6. In astrocytes, ROR α is upregulated by hypoxia and inhibits HIF-1 α expression after hypoxia. **A**, Schematic diagram of the experimental protocol: *Rora*^{+/+} (wt) or *Rora*^{sg/sg} (sg) astrocytes were subjected to 24 h of hypoxia followed by 0–24 h of normoxia. **B**, *Rora* gene transcription was analyzed by real-time RT-PCR. (*t* test, **p* < 0.05). **C**, Western blot analysis (top) of ROR α protein in enriched nuclear extracts from cortical astrocytes and their relative quantification (bottom) (*t* test, **p* < 0.05). **D**, *Rorb* gene transcription was analyzed by real-time RT-PCR. **E**, *Hif1a* gene transcription was analyzed by real-time RT-PCR in *Rora*^{+/+} (wt) and *Rora*^{sg/sg} (sg) astrocytes. **F**, Western blot analysis (top) of HIF-1 α protein in enriched nuclear extracts and relative quantification (bottom). **G**, *Vegf*, *Nip3*, and *Hk2* gene transcription was analyzed by real-time RT-PCR in *Rora*^{+/+} and *Rora*^{sg/sg} cells (Mann–Whitney, **p* < 0.05, ns, not significant). The values shown are the means $\pm$ SEM of 4–6 independent cultures. H + 0, Hypoxia without reoxygenation; H + 24, hypoxia and 24 h of reoxygenation; NH, non hypoxia.

(2008) included the use of melatonin to activate ROR α . This putative ligand of ROR α remains controversial and additional experiments are thus required to determine the relationship between ROR α and HIF-1 α in these cell lines (Jetten, 2009).

Could the regulation of HIF-1 α by ROR α explain the non-cell-autonomous neuroprotective function of ROR α in astrocytes? The effect of HIF-1 α on neuronal viability remains a matter of debate. For example, two groups used the same murine model to invalidate HIF-1 α expression in neurons (floxed HIF-1 mice crossed with CAM-Cre mice (Cre recombinase under the control of the calcium calmodulin-dependent kinase promoter)). They observed opposite effects after hypoxia or ischemia: a deleterious or a protective effect of HIF-1 α expression for neuronal survival (Helton et al., 2005; Baranova et al., 2007). There may be several reasons for this difference in findings. First, the type of stimulus used differed in the two studies. Second, the

Figure 7. Cell-autonomous and non-cell-autonomous neuroprotective functions of ROR α during hypoxia. **A**, ROR α -deficient neurons have increased neuronal death rates after hypoxia. **B**, The absence of ROR α in astrocytes does not modify astrocytic survival but neuronal survival after hypoxia. ROR α inhibits HIF-1 α expression in astrocytes. **C**, When both neurons and astrocytes are deficient for ROR α , the neuronal death rates are still increased after hypoxia compared with conditions A and B.

degree and cellular specificity of recombination differed between the two studies. Interestingly, HIF-1 α function may be cell-specific. Indeed, G. Vangeison et al. investigated the impact of a loss of HIF-1 α function in either neurons or astrocytes after hypoxia using neuron/astrocyte cocultures. The loss of HIF-1 α function in neurons had a deleterious effect on neurons, whereas the loss of HIF-1 α function in astrocytes had a protective effect on neurons (Vangeison et al., 2008). Thus, HIF-1 α is deleterious for neurons when produced by astrocytes and neuroprotective when produced by neurons (Vangeison et al., 2008). In our experiments, the inhibition of HIF-1 α production by ROR α in astrocytes may account for the neuroprotective function of ROR α in astrocytes.

In conclusion, in this study we provide evidence that ROR α is an important molecular player during hypoxia. Most studies investigating the molecular mechanisms underlying hypoxia-induced cell death have focused on neurons. However, astrocytes are actively involved in the response to hypoxia with some astrocyte functions increasing and others decreasing neuronal viability. This new molecular mechanism highlights the importance of astrocytes and of ROR α in hypoxia-mediated cell death.

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