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TOXICOKINETICS AND METABOLISM

Role of brain cytochrome P450 mono-oxygenases in bilirubin oxidation-specific induction and activity

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Abstract In the Crigler–Najjar type I syndrome, the genetic absence of efficient hepatic glucuronidation of unconjugated bilirubin (UCB) by the uridine 5′-diphospho-glucuronosyltransferase1A1 (UGT1A1) enzyme produces the rise of UCB level in blood. Its entry to central nervous system could generate toxicity and neurological damage, and even death. In the past years, a compensatory mechanism to liver glucuronidation has been indicated in the hepatic cytochromes P450 enzymes (Cyps) which are able to oxidize bilirubin. Cyps are expressed also in the central nervous system, the target of bilirubin toxicity, thus making them theoretically important to confer a protective activity toward bilirubin accumulation and neurotoxicity. We therefore investigated the functional induction (mRNA, EROD/MROD) and the ability to oxidize bilirubin of *Cyp1A1*, *1A2,* and *2A3* in primary astrocytes cultures obtained from two rat brain region (cortex: Cx and cerebellum: Cll). We observed that *Cyp1A1* was the Cyp isoform more easily induced by beta-naphtoflavone (βNF) in both Cx and Cll astrocytes, but oxidized bilirubin only after uncoupling by 3, 4,3′,4′-tetrachlorobiphenyl (TCB). On the contrary, *Cyp1A2* was the most active *Cyp* in bilirubin clearance without uncoupling, but its induction was confined only in Cx cells. Brain *Cyp2A3* was not inducible. In conclusion, the exposure of astrocytes to βNF plus TCB significantly enhanced Cyp1A1 mediating bilirubin clearance, improving cell viability in both regions. These results may be a relevant groundwork for the manipulation of brain Cyps as a therapeutic approach in reducing bilirubin-induced neurological damage.

Keywords Cytochrome P450 enzymes (Cyp) · Bilirubin oxidation · Kernicterus · Astrocytes · βNF · EROD/MROD

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Introduction

In the Crigler–Najjar type I syndrome (CNI), the genetic absence of efficient hepatic glucuronidation of unconjugated bilirubin (UCB) by the uridine 5′-diphospho-glucuronosyltransferase1A1 (UGT1A1) enzyme (Clarke et al. 1997) produces the rise of blood UCB level. Its entry to central nervous system (CNS) could generate toxicity and neurological damage. In the last years, several studies demonstrated the presence of a hepatic compensatory mechanism to limit this deficiency the hepatic cyotochrome P450 (Cyp) enzymes as Cyp1A1, Cyp1A2, and Cyp2A5 (in mouse, *Cyp2A3* in rat, CYP2A6 in human). Hepatic Cyps act in vitro lowering bilirubin concentration by oxidation, mainly driven by Cyp1A2 and Cyp2A5/6 in native form and Cyp1A1 after uncoupling (Zaccaro et al. 2001; De Matteis et al. 2006; Kapitulnik and Ostrow 1978; Kapitulnik and Gonzalez 1993; Abu-Bakar et al. 2005; Kapitulnik et al. 1987). In the Gunn rat suffering for hyperbilirubinemia due to a spontaneous mutation in the UGT1A1 gene, chemical stimulation of this alternative pathway produced amelioration of jaundice in vivo (Kapitulnik and Ostrow 1978; De Matteis et al. 1989). Of notice, the spontaneous hyperbilirubinemia itself modulated the hepatic Cyp1A1 and 1A2 in Gunn rats, lowering the total serum bilirubin levels in young adult animals (Kapitulnik and Gonzalez 1993).

Firstly observed by Brodersen and Bartles (1969), a bilirubin oxidative activity in the CNS was further demonstrated by Hansen, who proposed the concept of bilirubin oxidation in brain by a still unidentified enzyme called "bilirubin oxidase"(Hansen et al. 1997; Hansen 2000). The same author clearly demonstrated differences between the brain bilirubin oxidase and the commercially available "bilirubin oxidase" (1.3.3.5, from *Myrothecium verrucaria*, Cu core), supposing that brain bilirubin oxidase belonging to the cytochrome P450 enzymes family. The presence of Cyps in the brain (bCyps) is relevant due to their highly localized expression and extreme sensitivity to inducers, with the related pharmacological and toxicological implications for cerebral drug metabolism (Haining and Nichols-Haining 2007; Miksys and Tyndale 2002; Morse et al. 1998). Previous work from our laboratory in the Gunn rat demonstrated the different regional induction of Cyps (*Cyp1A1*, *1A2,* and *2A3*; mRNA, up to 70-fold) after an acute bilirubin load. It was observed that the bilirubin content was lower in those regions where the bCyps were highly induced (cerebral cortex: Cx), and as contrasted with the cerebellum (cerebellum: Cll) (Gazzin et al. 2012), substantiating a possible role of bCyps in controlling the local bilirubin concentration. Notably, this picture paralleled the topography of resistance to bilirubin-induced neurological damage (BIND). Noteworthily, the cerebellum-induced damage is landmark of the pigment toxicity in the animal models of hyperbilirubinemia, causing an evident hypoplasia of this cerebral structure, with the loss of Purkinje and granular cells (Robert et al. 2013; Bortolussi et al. 2012). In humans, the movement's disorders testify to the functional cerebellar damage (Shapiro 2010). Differently, up to now, no damage has been reported for the cerebral cortex, considered resistant to bilirubin toxicity. For these reasons, we proposed these enzymes as a possible mechanism to increase CNS bilirubin clearance and locally protect the brain from bilirubin neurotoxicity.

Considering that bCyps may possess regulation, functions, and substrate specificity different from the liver isoforms (Meyer et al. 2002; Miksys and Tyndale 2002), the aim of this study was to identify the bCyp most involved in bilirubin clearance in the CNS and their induction in terms of expression and activity. Furthermore, the ability of bCyps to protect brain cells from bilirubin toxicity was also assessed. Notably, neuronal cells are more sensible than astrocytes to bilirubin toxicity. This might be explained by the less effective defensive machinery due to a lower ability to oxidize bilirubin (Hansen and Allen 1997) together with lower activity of the UCB transmembrane transporters and an higher sensitivity to glutamate excitotoxicity (Watchko and Tiribelli 2013). Since Cyps are more prone to be modulated in astrocytes than in other cell populations of the brain (Kapoor et al. 2006; Fernandes et al. 2006), making this population the ideal target for a possible therapeutic manipulation, we decided to use primary astrocytes cultures as the model to be used in the present work.

Materials and methods

Chemicals and reagents

Dulbecco's modified Eagle medium low glucose (DMEM LG) was from Invitrogen (Life technologies Corporation, Grand Island, NY). Fetal bovine serum (FBS), trypsin from bovine pancreas, PBS (Dulbecco's phosphate-buffered saline), unconjugated bilirubin (UCB), 3(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium (MTT), dimethyl sulfoxide (DMSO), Trypan Blue powder, β-naphtoflavone (βNF), Resorufin sodium salt, 7-ethoxyresorufin (for EROD test), 7-methoxyresorufin (for MROD test), β-nicotinamide adenine dinucleotide 2′-phosphate-reduced tetrasodium salt hydrate (NADPH), fluorescamine, and 3,4,3′,4′-tetrachlorobisphenyl (TCB) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). All chemicals and solvents were of analytical grade.

Primary astrocytes culture and treatment with βNF

Wistar Han™ Rats, at two days after the birth (P2), were obtained from the animal facility of the Department of Life sciences of Trieste University (Italy). Animal care and procedures were conducted according to the guidelines approved by the Italian Law (decree 116-92) and the European Communities Council Directive 86-609-ECC. Regular communication to the National Ethic Committee was done (2009–2011). Six P2 Wistar rats were used for each astrocytes primary culture preparation (single biological repetition) as described previously (Robert et al. 2013; Booher and Sensenbrenner 1972). Cells were seeded at 1.2×10^5 cells/ml in 6-well plates or flask 75 cm^2 and cultured at 37 °C, 5 % CO_2, 90 % of humidity in a humidified incubator. Experiments were performed within 15 days in vitro (div), at 90 % of confluence. Treatments were performed in the same growth medium. βNF was selected to induce the brain cytochrome P450 enzymes, because is one of the most efficient inducer in vitro (Krusekopf et al. 2003) and because previously used in in vivo studies (Nannelli et al. 2009; Iba et al. 2003) due to its ability (as flavonoid) to pass through the brain blood barrier. Different concentrations of βNF (2.5, 5, 10, 20 µM, dissolved in DMSO; stock 20 mM) were used (Morse et al. 1998; Nannelli et al. 2009; Iba et al. 2003), and treatments were continued for 1, 6, or 24 h. Control cells were exposed to DMSO (final concentration less than 0.1 %, corresponding to the maximal concentration needed to dissolve the principle).

Viability tests

MTT and trypan blue (TB) tests were used to assess the vitality of the cells after treatment. MTT was performed exposing the cells to the MTT solution (0.5 mg/ml) in medium for 1 h in the humidified incubator, and reading the absorbance at 562 nm in a LD 400C luminescence Detector (Beckman coulter, Milan Italy). TB was performed by diluting the harvested cells 1:1 in trypan blue dye (0.4 % solution in PBS), and counting the number of blue-stained (dead) cells vs. total cells. For both assays, results were expressed as percentage in treated cells vs. controls (DMSO alone).

Quantitative real-time PCR

The mRNA expression of *Cyps* genes was analyzed by quantitative real-time PCR. Total RNA was extracted using Eurogold RNA Pure reagent (Euroclone, Milan Italy) following the producer's instructions. cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Applied biosystems, Monza Italy). For the quantitative real-time PCR, primers were designed using the Beacon designer 4.2 software (Premier Biosoft International, Palo Alto, CA, USA) on rat sequences available in GenBank (Table 1). The reaction was performed in a final volume of 15 µl in an iQ5 Bio-Rad Thermal cycler (BioRad Laboratories, Hercules, CA, USA). Briefly, 25 ng of cDNA and the corresponding gene-specific sense/antisense primers (250 nM each) were diluted in the SSo Advance SYBER green supermix (Bio-Rad Laboratories, Hercules, CA, USA). Amplification protocol was 3 min at 95 °C, 40 cycles at 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s. Specificity of the amplification was verified by a melting-curve analysis, and non-specific products of PCR were not found in any case. The relative quantification was made using the iCycleriQ software, version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) by the ΔΔCt method, taking into account the efficiencies of the individual genes and normalizing the results to the housekeeping genes (Hypoxanthine guanine phosphoribosyl transerfase: *Hprt*, Glyceraldehyde 3-phosphate dehydrogenase: *Gapdh* and *Actin*). The levels of mRNA were expressed relative to a reference sample (Bustin et al. 2009; Vandesompele et al. 2002).

Membrane fraction preparation

All procedures were performed at 4 °C. Treated astrocytes were harvested and mechanically homogenized by a glass on glass homogenizer (Dunce) in homogenization buffer (HB: Tris–HCl 10 mM; EDTA 1 mM; DTT 0.5 mM; Protein Inhibitor 0.1 mM pH:7.4). The homogenate was first centrifuged at 900 g using a Beckman Allegra 25R (Milan, Italy) to eliminate the debris. Then, the supernatant was centrifuged at 110000 g for 60 min at 4 °C using a Beckman LE-80 K Optima; rotor 70.1 Ti (Beckmann, Milan, Italy). Under these conditions, the membrane fraction we used contains microsomes (vesicles derived from the endoplasmic reticulum), nuclear membranes, and mitochondria membranes. The resulting pellet was stored in the suspension buffer (RB: 50 mM phosphate buffer with 2 mM of $MgCl_2$) at −80 °C with 20 % of glycerol till use (Meyer et al. 2002).

Enzymatic assays

The Cyp1A1 and Cyp1A2 activity was assessed by the O-dealkylations of ethoxyresorufin and methoxyresorufin

Table 1 Primer sequences designed for the mRNA quantification

Gene	Accession number	Forward	Reverse	Amplicon (bp)
Hprt	NM_012583.2	AGACTGAAGAGCTACTGTAATGAC	GGCTGTACTGCTTGACCAAG	163
Actin	NM_031144.2	GGTGTGATGGTGGGTATG	CAATGCCGTGTTCAATGG	103
Gapdh	NM_017008.	CTCTCTGCTCCTCCCTGTTC	CACCGACCTTCACCATCTTG	87
Cyp1A1	NM_012540.2	CAGGCGAGAAGGTGGATATGAC	GGTCTGTGTTTCTGACTGAAGTTG	181
Cyp1A2	NM_012541.3	GTGGTGGAATCGGTGGCTAATGTC	GGGCTGGGTTGGGCAGGTAG	175
Cyp2A3	NM_007812	ACACAGGCACCCCAGGACATC	CCAGGCTCAACGGGACAAGAAAC	99
Ugt1A1	NM_012683.2	TACACTATGAGGAAGTACC	GCAGAAAAGGATCTTGAT	100

Hprt hypoxanthine guanine phosphoribosyltranserfase, *Gapdh* glyceraldehyde 3-phosphate dehydrogenase, *Cyp1A1* cyotochrome P450 1A1, *Cyp1A2* cyotochrome P450 1A2, *Cyp2A3* cyotochrome P450 2A3, *Ugt1A1* uridine 5′-diphospho-glucuronosyltransferase 1A1 substrates (EROD and MROD, respectively) (Iba et al. 2003; Meyer et al. 2002; Liu et al. 2001; Radenac et al. 2004). Astrocytes were treated according to the method described by Iba with some modifications (Iba et al. 2003). Briefly, the cell membrane fractions were pre-incubated with 5 µM of the respective substrate in 50 mM phosphate buffer with 2 mM of $MgCl_2$ for 5 min at 37 °C. NADPH (1 µM final concentration) was added and the reaction was allowed to proceed at 37 °C in darkness measuring every 5 min the product fluorescence (for both resorufin) with the PerkinElmer Envision multilabel plate reader (excitation/emission wavelength 535/590 nm; PerkinElmer, Milan, Italy). Samples were assessed in duplicate, and the amount of resorufin produced in the samples was calculated based on a standard curve developed from a range of product (0–250 nM) concentrations in the presence of the substrate and NADPH. Protein content (used to normalize the EROD/MROD data) was measured in every single well by the addition of fluorescamine in DMSO (4 mg/ml), and resulting fluorescence assessed (excitation/emission wavelength 390 nm/460 nm; Granberg et al. 2003). The results were calculated by regression curve analysis with reference to the fluorescence generated by the standard curve of BSA (0–100 µg) in the phosphate buffer with the substrate. The results are shown in comparison with the control samples.

Bilirubin oxidation

Bilirubin clearance was followed by the decrease in absorbance at 440 nm in the PerkinElmer Envision multilabel plate reader (PerkinElmer, Milan, Italy), as previously described (De Matteis et al. 2012; Abu-Bakar et al. 2011, 2012). In brief, membrane fraction of induced and non-induced astrocytes (40 µg total protein/100µL) were added to the incubation mixture contained Tris–HCl 100 mM pH = 8.2, KCl 26 mM, EDTA 2 mM (BR oxidation buffer). Thus, bilirubin (final concentration of 15 µM) was added and mixture pre-incubated for 5 min. Finally, NADPH (final concentration 137 µM) was added to start the reaction. The coefficient of extinction (εmM = 21.6) was obtained experimentally under the same conditions. The rate of BR disappearance was expressed as pmol bilirubin disappearing/min. In the experiment with the addition of TCB, the principle [final concentration 3.42 µM, (De Matteis et al. 1989)] was added to microsomes solution together with UCB incubation, and bilirubin disappearance followed as previously described.

ROS production by βNF ± TCB exposure

Formation of H_2O_2 was determined by measuring oxidation of 2′,7′-dichlorofluorescindiacetate(DCDFDA), with procedures modified from those of Schlezinger (Schlezinger et al. 1999). Briefly, DCDFDA react with H_2O_2; the stoichiometry of the reactions is 1:1. Membrane fraction protein (50 µg) was added to wells of a 96-well plate containing the BR oxidation buffer with the addition of 10 mM HEPES and 5 µM DCDFDA. After a 15-min incubation at 37 °C, TCB (3.42 µM) was added, the reaction was initiated with NADPH (1 mM), and fluorescence was monitored over 30 min with 485/530-nm excitation/emission filters.

Cell culture exposure to βNF ± TCB and UCB

In order to substantiate the biological effect of bCyps induction in conferring protection against bilirubin, astrocytes cells were exposed to (1) DMSO alone (control); (2) βNF; and (3) βNF plus TCB. Cells were incubated (37 °C, 95 % humidity, 5 % CO2) at the optimal conditions in culture media (containing 25.6 µM albumin in FBS). Moreover, treatments with bilirubin were performed. A total of 25 µM purified UCB (Ostrow and Mukerjee 2007), dissolved in DMSO (vehicle) (Sigma-Aldrich, St. Louis, MO, USA), was diluted in culturing medium, containing 10 % FBS (containing 25.6 µM albumin), giving a free bilirubin (Bf) concentration of 140nM. This toxic value of Bf was selected based on previous data from our group (Calligaris et al. 2007; Ostrow et al. 2003; Giraudi et al. 2011). Bf concentration was assessed by peroxidase assay (Roca et al. 2006; Ahlfors 2000), and cells were exposed for 24 h. Control cells were assessed to the same DMSO amounts (0.5 % final volume) needed to produce the desired Bf. Finally, viability was assessed by MTT.

Statistical analysis

Data were analyzed with GraphPad prism 5 for Windows. For group analysis, one-way ANOVA was used. For others, statistical significance was evaluated by paired two-tailed test. A p value <0.05 was considered statistically significant. Results are expressed as mean ± SD of 4–5 independent biological repetitions.

Results

mRNA constitutive expression of *bCyps* in primary culture from cortex and cerebellum astrocytes

Cyp1A1, *1A2*, and *2A3* (and *Ugt1A1*) constitutive expression was assessed in primary culture of astrocytes derived from two different brain regions: Cx and Cll (Fig. 1). *Cyp1A1* level was significantly higher (threefold; $p < 0.05$) in cortex than cerebellum astrocytes, as already reported (Matyash and Kettenmann 2010), while no statistically relevant differences were detected for *Cyp1A2* and *Cyp2A3*

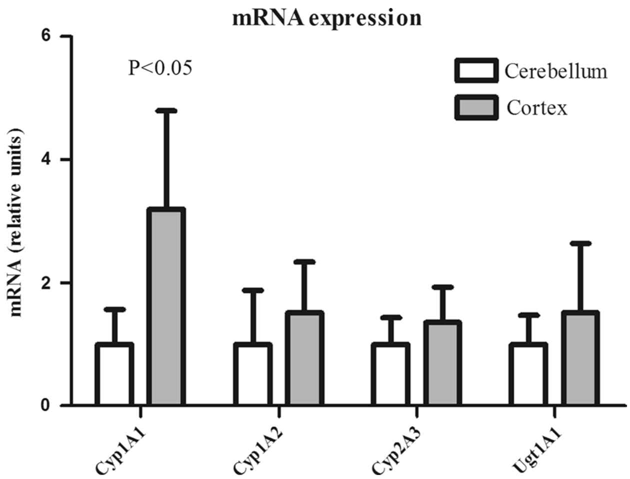

Fig. 1 Constitutive mRNA relative expression of *Cyp1A1*, *Cyp1A2*, *Cyp2A3,* and *Ugt1A1* in astrocytes from cortex and cerebellum. Three independent housekeeping genes (*Gapdh*, *Hprt*, *Actin*) were used for normalization. Relative mRNA expression levels were expressed with respect the cerebellum, corresponding to 1. Data are expressed as mean ± SD of 4–5 biological repetitions

(and *Ugt1A1)* expression between astrocytes derived from the two brain regions.

mRNA Cyps inducibility by βNF in astrocytes from cortex and cerebellum

To selectively induce the maximal up-regulation of each *bCyp* and to characterize the relative contribution in bilirubin clearance, the astrocytes were exposed to increasing concentrations of βNF (2.5, 5, 10, 20 µM) for three time intervals (1, 6, and 24 h) (Fig. 2, plus Table 2 for statistical analysis). Irrespective to the viability, all bCyps were modulated in Cx astrocytes with a maximal up-regulation of 8 times for *Cyp1A1* ($p < 0.05$), 25 times for *Cyp1A2* ($p < 0.05$), and 12 times for *Cyp2A3* ($p < 0.001$). On the contrary, in Cll astrocytes, only *Cyp1A1* mRNA level was increased by about 14 times.

Each *Cyp* showed a different kinetic and regional pattern of induction. In Cx astrocytes, *Cyp1A1* was unchanged 1 h after βNF exposure and the maximal increment was observed after 6 or 24 h of βNF exposure. No difference was observed with βNF ranging from 2.5 to 10 µM, while with 20 µM βNF for 24, the *Cyp1A1* mRNA expression returned to levels similar to 1 h. In Cll astrocytes, *Cyp1A1* was maximally up-regulated only after 24 h of exposure (about 13.5-fold). As for Cx astrocytes, when challenged with 20 µM βNF, Cyp expression returned to level similar to shorter treatments. No dose dependence was observed from 2.5 to 10 µM βNF. *Cyp1A2* mRNA was not inducible by 1 or 6 h of βNF treatments (all concentration) in Cx-derived astrocytes, but showed a dose-dependent induction after 24 h, reaching the maximal extent of mRNA with 20 µM of βNF. In Cll cells, the *Cyp1A2* mRNA modulation was marginal (about 2 times). The *Cyp2A3* was much less prone to be modulated being almost completely insensible to every dose and time of exposure, with the exception at the maximal challenging in Cx cells. Of notice, *Ugt1A1* was not changed by any concentration or time of exposure (*data not shown*).

Cell viability in astrocytes from cortex and cerebellum exposed to βNF

The cell viability was determined by two methods, trypan blue (TB: membrane integrity) and MTT (mitochondrial activity) (Fig. 3). When viability was assessed by TB, no effect was detected at any concentration and timing of βNF in both primary cultures. On the other hand, in both cortex and cerebellum-derived primary astrocytes, a toxic effect on mitochondrial functions (MTT) was observed. This effect was both time and concentration dependent reaching the 30–40 % of residual viability at the higher principle dosage (20 µM βNF) and longer exposure (24 h). This led to the exclusion of these experimental conditions in further analysis.

Optimal condition for selective bCyps enzyme activity analysis

To better characterize the brain Cyps enzymes, we selected the condition to specifically induce each individual bCyp before performing the activity tests. The optimal condition for the selective *bCyp* induction was based on both the extent of mRNA up-regulation and the cell viability. As summarized in Table 3, specific induction of *Cyp1A1* was obtained in both Cx- and Cll-derived astrocytes with 10 µM βNF after 6 and 24 h of exposure, respectively. On the contrary, a specific induction of the *Cyp1A2* isoform was not achieved. *Cyp1A2* was up-regulated 6.7-fold together with 7.8-fold of *Cyp1A1* in Cx astrocytes, while no clear induction was obtained in Cll astrocytes. *Cyp2A3* mRNA expression was not notably induced in either of the primary cultures without compromising the cell vitality, and accordingly, the specific activity test was not performed.

EROD and MROD activities under optimal condition for selective Cyps induction

Cortex astrocytes exposed to *Cyp1A1* mRNA optimal induction (see Table 3) showed a significant increase in EROD (Cyp1A1 specific) activity (fourfold $p < 0.01$) (Fig. 4); MROD activity (Cyp1A2 activity) was also slightly increased (1.4-fold, $p < 0.05$). A similar pattern was obtained for cerebellar astrocytes with an increased

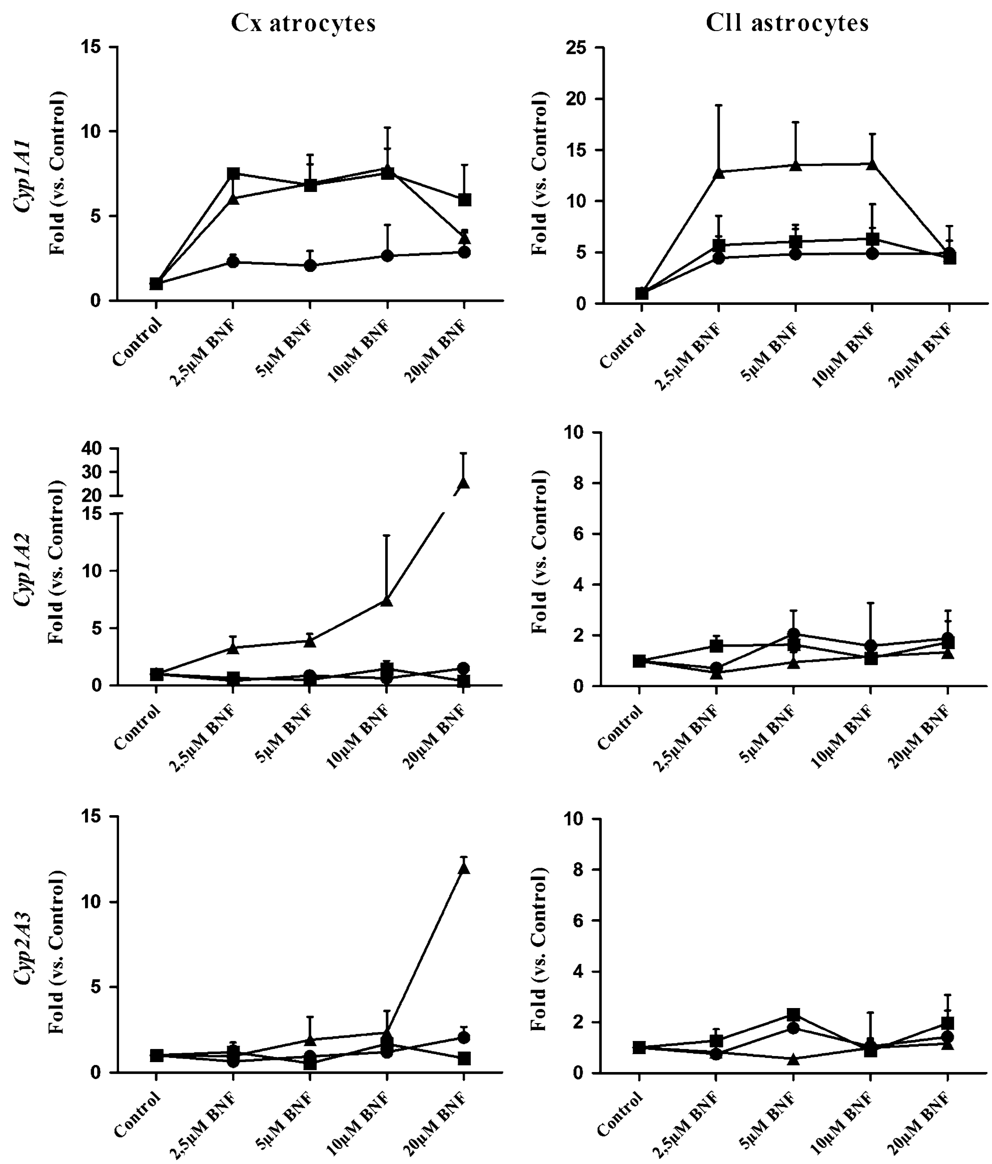

Fig. 2 mRNA relative expression of *Cyp1A1*, *Cyp1A2, and Cyp2A3* in astrocytes exposed to βNF. βNF concentration: 2.5, 5, 10, 20 µM for 1(*black circle*), 6 (*black square*), and 24 h (*black up-pointing triangle*). Relative mRNA expression levels were expressed with respect the control (cells treated only with DMSO). Three independent housekeeping genes (*Gapdh*, *Hprt*, *and Actin*) were used for normalization. Data are expressed as mean ± SD of 4–5 independent experiments. For statistical analysis, see Table 2

in both EROD (2.5-fold, $p < 0.01$, lower extent compared with Cx) and MROD test (1.5-fold, $p < 0.01$). As detailed above, a specific up-regulation of *Cyp1A2* mRNA alone was not possible (Fig. 3, Table 3). When an up-regulation of *Cyp1A2* mRNA was obtained (7.5-fold $p < 0.01$), the MROD activity was increased fivefold (Fig. 4). A concomitant *Cyp1A1* mRNA up-regulation (7.8-fold, $p < 0.05$, Table 3) was functionally associated with a 4.5-fold increase in EROD ($p < 0.01$). In Cll-derived astrocytes, no change in EROD/MROD activity was observed after induction despite the slight modulation of mRNA (Table 3).

Bilirubin oxidation

βNF treatment (alone) significantly increased bilirubin oxidation (Table 4) only under *Cyp1A2* optimal conditions in Cx-derived astrocytes (1.32-fold vs. control, $p < 0.05$), while in Cll astrocytes, a trend to inhibition was observed. The addition of TCB to cultures exposed to βNF strongly enhanced bilirubin disappearance (5.7- and 4.4-fold for Cx *Cyp1A1* and Cll *Cyp1A2* respectively, $p < 0.001$; and about fourfold for Cx *Cyp1A2* and Cll *Cyp1A1*, both $p < 0.01$) (Table 4).

ROS generation by bCyps induction by βNF ± TCB

In line with the increment in bilirubin oxidation, ROS production was only observed in Cx astrocytes after Cyp1A2 modulation without TCB addition. Inversely, TCB enhanced ROS generation by both Cyp1A1 and Cyp1A2 conditions (Fig. 5). For the case of Cyp1A2 condition in Cx, it is important to highlight again that Cyp1A1 was

Table 2 Statistical analysis of *Cyp1A1, Cyp1A2,* and *Cyp2A3* mRNA modulation by βNF

Cortex					Cerebellum				
Cyp1A1	µM βNF				*Cyp1A1*	µM βNF			
Hours	2.5	5	10	20	Hours	2.5	5	10	20
1	$p < 0.05$	ns	ns	ns	1	ns	ns	ns	ns
6	$p < 0.001$	$p < 0.05$	$p < 0.05$	$p < 0.05$	6	$p < 0.05$	<0.05	ns	$p < 0.01$
24	$p < 0.05$	$p < 0.05$	$p < 0.05$	$p < 0.01$	24	$p < 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.05$
Cyp1A2	µM βNF				*Cyp1A2*	µM βNF			
Hours	2.5	5	10	20	Hours	2.5	5	10	20
1	$p < 0.05$	ns	ns	ns	1	ns	ns	ns	ns
6	ns	$p < 0.05$	ns	$p < 0.05$	6	ns	$p < 0.05$	ns	$p < 0.05$
24	$p < 0.05$	$p < 0.01$	$p < 0.05$	$p < 0.05$	24	$p < 0.05$	ns	ns	ns
Cyp2A3	µM βNF				*Cyp2A3*	µM βNF			
Hours	2.5	5	10	20	Hours	2.5	5	10	20
1	ns	ns	ns	ns	1	$p < 0.05$	ns	ns	ns
6	ns	ns	$p < 0.05$	ns	6	ns	$p < 0.01$	ns	ns
24	ns	ns	ns	$p < 0.001$	24	ns	ns	ns	ns

One-way ANOVA study was performed. ns: not statistically relevant

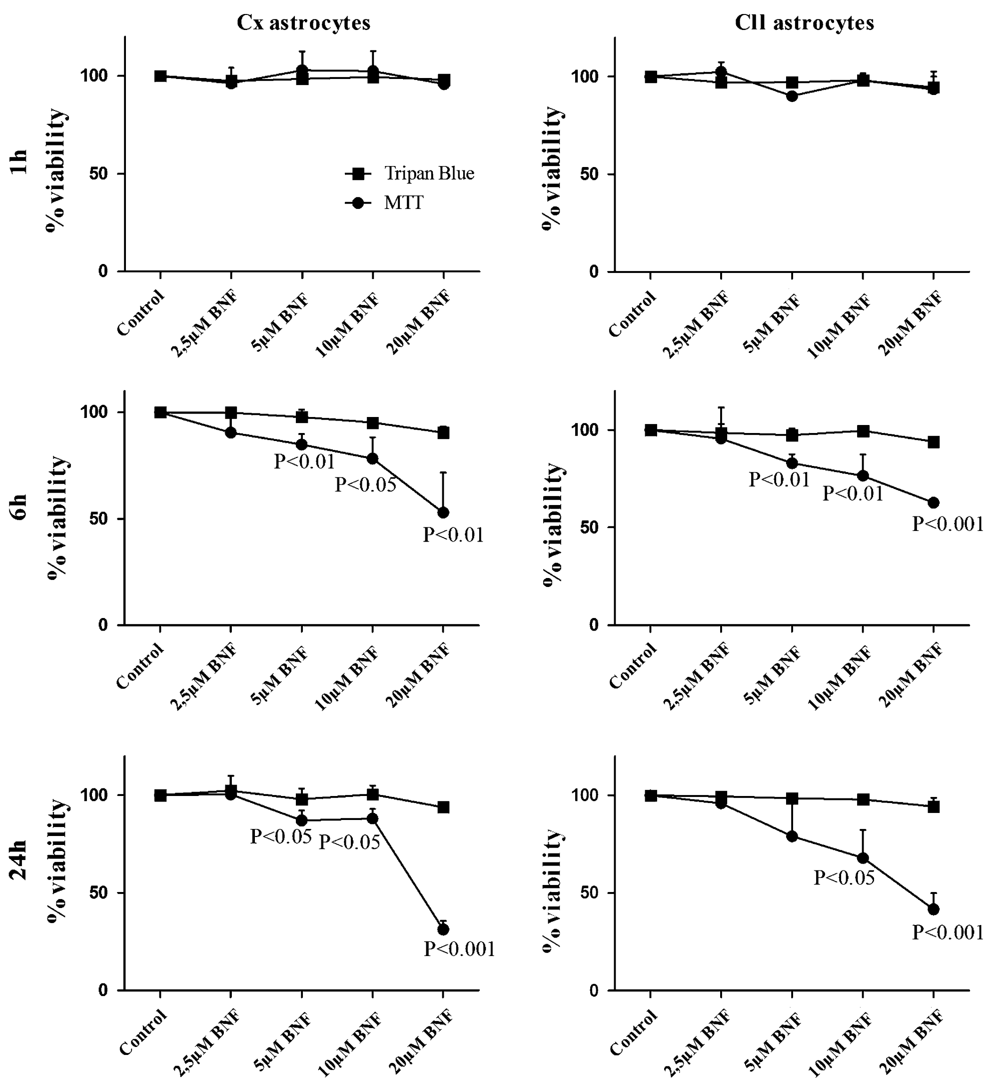

Fig. 3 Viability of astrocytes exposed to βNF. Dots (*black circle*): MTT; squares (*black squares*): trypan blue. Control cells were treated with DMSO alone. Data are expressed as mean ± SD of 4–5 independent biological repetitions

Table 3 Optimal condition for selective mRNA induction of brain *Cyps*

	Cortex			Cerebellum		
	Condition	mRNA fold change		Condition	mRNA fold change	
Cyp1A1	6 h; 10 µM βNF	7.5 ($p < 0.001$)	*Cyp1A2:* 1.44 *Cyp2A3:* 1.52	24 h; 10 µM βNF	13.65 ($p < 0.01$)	*Cyp1A2:* 1.18 *CYp2A3:* 0.99
Cyp1A2	24 h; 10 µM βNF	6.7 ($p < 0.05$)	*Cyp1A1:* 7.85 *Cyp2A3:* 2.3	6 h; 20 µM βNF	1.72	*CYp1A1:* 4.47 *Cyp2A3*: 2

Both viability and mRNA expression were considered

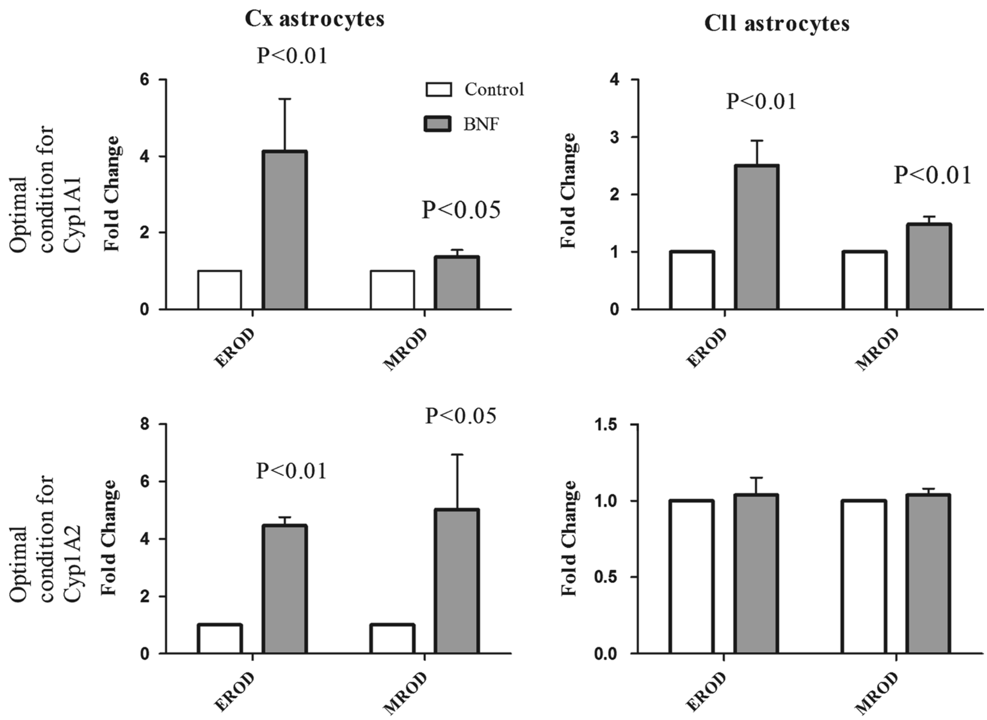

Fig. 4 Ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MROD) activities in astrocytes from Cx and Cll in control and βNF exposed cells. Tests were performed under the optimal conditions detailed in Table 3. Data are expressed as mean ± SD of 4–5 independent experiments

also induced, so Cyp1A1 might be the responsible of ROS increment by TCB addition. In Cll primary culture, a significant increment in ROS production was observed only after Cyp1A1 induction and uncoupling by TCB (Fig. 5).

Cell viability in astrocytes from cortex and cerebellum exposed to UCB after bCyps induction by βNF ± TCB

Cell viability in Cx astrocytes challenged with UCB for 24 h after *Cyp1A1* selective induction with βNF alone (Fig. 6) was decreased by about 25 %. *Cyp1A2* induction increased resistance toward UCB exposure (about of 15 %, $p < 0.05$). In Cll astrocytes, the viability was not affected by *Cyp1A1* induction and decreased ($p < 0.05$) after *Cyp1A2* induction. Pre-treatment with TCB together with βNF resulted in an improved viability in cells challenged with UCB, particularly in Cll astrocytes after *Cyp1A1* induction ($p < 0.05$), and in Cx cultures after both *Cyp1A1* and *1A2* modulation ($p < 0.05$).

Discussion

The present study investigates the role of brain Cyps in bilirubin oxidation. The data indicate that Cyp1A2 has the main intrinsic (no need for uncoupling) activity, while Cyp1A1 is the leading enzyme when uncoupled by the addition of TCB. These results confirm in the brain what previously reported for hepatic Cyps (Pons et al. 2003; Zaccaro et al. 2001; De Matteis et al. 2006, 2012). Differently from what found in the liver, however, brain Cyps are differentially induced by βNF since *Cyp2A3* is insensible to modulation and Cyp1A2 only in Cx.

A severe hyperbilirubinemia selectively damages the brain (Watchko and Tiribelli 2014), thus the possibility to reduce the pigment levels in the CNS by modulation of resident Cyps represents a promising therapeutic approach. We have reported a relevant (up to 70-fold) mRNA induction of bCyps in the cerebral cortex (resistant region) in hyperbilirubinemic Gunn rat after an acute bilirubin load

Table 4 Bilirubin disappearance

	Control	βNF	βNF + TCB	Statistic		
Initial rate of loss observed (pmol of bilirubin/min)						
Astrocytes Cx Cyp1A1	214 ± 80	259 ± 92	1,233 ± 126	ns[a]	$p < 0.001$[b]	$p < 0.001$[c]
Astrocytes Cx Cyp1A2	321 ± 143	425 ± 189	1,267 ± 395	$p < 0.05$[a]	$p < 0.01$[b]	$p < 0.001$[c]
Astrocytes Cll Cyp1A1	248 ± 89	231 ± 82	912 ± 256	ns[a]	$p < 0.01$[b]	$p < 0.001$[c]
Astrocytes Cll Cyp1A2	180 ± 96	134 ± 80	800 ± 132	ns[a]	$p < 0.001$[b]	$p < 0.001$[c]

Membrane fraction was incubated in the reaction buffer with bilirubin, and the decrease in absorbance was measured at 440 nm. Data are mean ± SD of 4–5 independent experiments. Statistical analysis when compared to [a] βNF to control sample, [b] βNF + TCB vs. βNF; [c] βNF + TCB vs. control

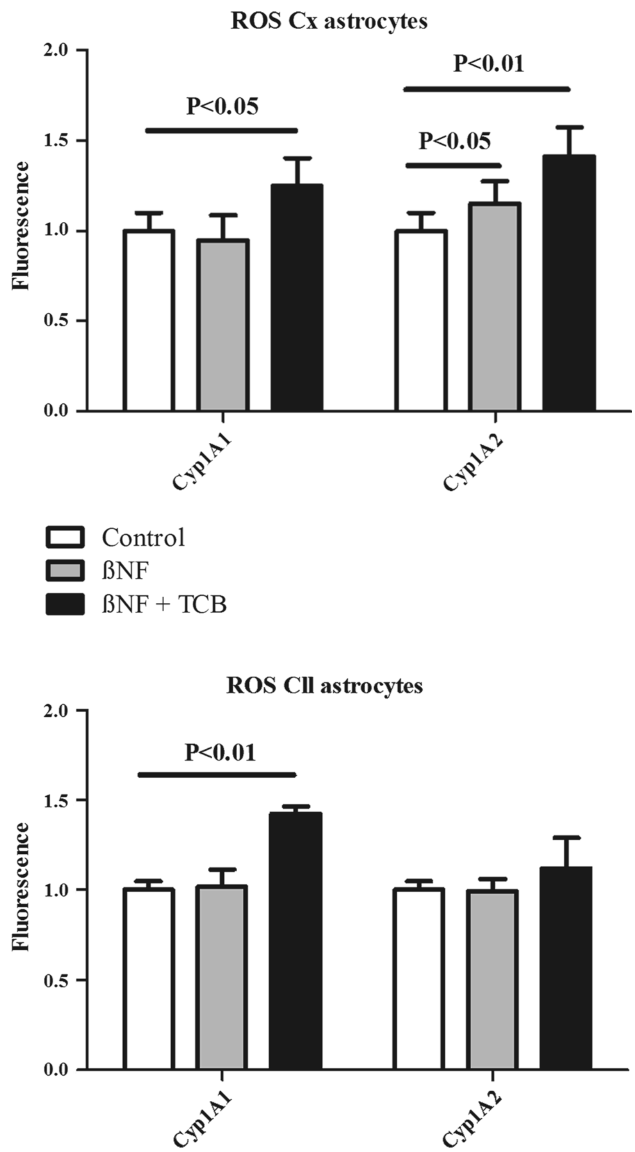

Fig. 5 ROS production in astrocytes optimal treated with βNF ± TCB. H2O2 production was assessed by the oxidation of DCDFDA to DCF. Data are expressed as mean ± SD of 4–5 independent experiments

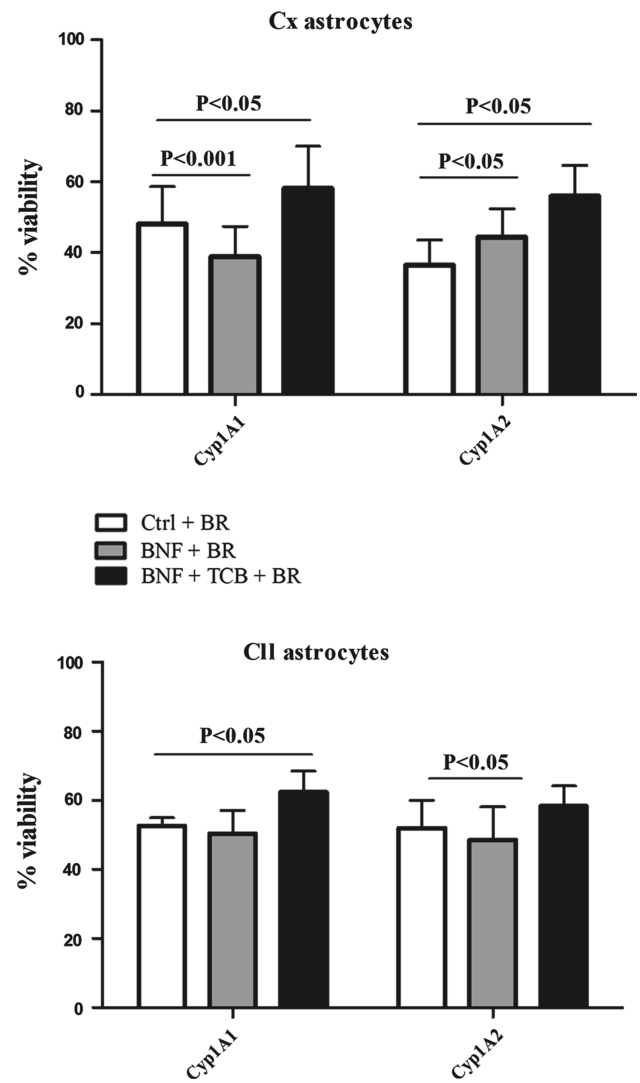

Fig. 6 Bilirubin viability challenge in Cx and Cll astrocytes induced by BNF ± TCB. Viability was assayed by MTT test. Astrocytes were pretreated under the optimal conditions of βNF with/without TCB and challenged with 25 μM of Bilirubin for 24 h. Data are expressed as mean ± SD of 4–5 independent biological repetitions

obtained by administration of sulfadimethoxine, a bilirubin–albumin displacing agent, acutely raising the tissue UCB level. On the contrary, in cerebellum (damaged region), the UCB accumulation was more consistent, enduring, and associated with a much lower bCyp (mRNA) up-regulation (Gazzin et al. 2012). The double role of bilirubin as Cyps inducer and substrate was already reported in yeast expression systems (Abu-Bakar et al. 2012) and in liver of jaundiced Gunn rat, suggesting a compensatory mechanism for bilirubin clearance in the absence of glucuronidation (Kapitulnik and Gonzalez 1993). In our model, we observed a similar pattern in which Cyps from cortex astrocytes were more easily induced compared with cerebellum (only Cyp1A1 relevant), and their capacity to oxidize bilirubin could reflect the sensibility to bilirubin toxicity.

Several in vivo and in vitro works proved the assumption that certain hepatic Cyps were able to oxidize bilirubin. Our work is in line with what reported in the hepatic model but exports this observation to the brain where bilirubin is toxic and the activity of these enzymes toward bilirubin is unexplored. We showed that Cyp1A2 in native form has a major role in bilirubin oxidation, and as in the liver, this activity is depressed by the addition of TCB (Pons et al. 2003; Zaccaro et al. 2001). The TCB "switching" mechanism largely documented for hepatic Cyp1A1 (De Matteis et al. 2002, 2006, 2012; Zaccaro et al. 2001) was confirmed also for brain Cyp1A1 in both Cll and Cx cultures, indicating that this isoform has only a limited ability to oxidize bilirubin in physiological condition, but becoming relevant after uncoupling.

Uncoupling of catalytic cycle of Cyps generate oxidative species (Schlezinger et al. 1999; Pons et al. 2003; De Matteis et al. 2012) oxidizing bilirubin to biliverdin and oxygenated dipyrroles (De Matteis et al. 2006). In this study, the induction of brain Cyps by βNF followed by uncoupling was associated with both a significant increase in ROS and bilirubin clearance (Table 4 and Fig. 5). From a biological point of view, Cyps activity stimulates the release of both potentially beneficial (biliverdin) (Dore and Snyder 1999; Kapitulnik 2004) and toxic (ROS) species (De Matteis et al. 2006; Abu-Bakar et al. 2011). In our model, cell viability was significantly improved by Cyps modulation/uncoupling in astrocytes isolated from the cortex and cerebellum. This finding may be interpreted as indication that ROS produced during UCB oxidation might not only be tolerated (Liu et al. 2001) but even beneficial, at least in the experimental condition we tested. It is important to highlight that the in vitro Cyp1A1/1A2 higher modulation (mRNA and functional) by βNF that we observed in "cortex" vs "cerebellum" was consistent with the data obtained in animals (Gazzin et al. 2012; Iba et al. 2003) where Cyps in cortex were more inducible than in cerebellum.

This differential induction, together with the differential mediated ability to oxidize bilirubin, may explain the lower sensibility to bilirubin toxicity of the cortex, in respect to the cerebellum. Additionally, the higher capacity of Cyps modulation in astrocytes may account for the reported greater sensitivity to UBC damage of neurons (Falcao et al. 2013; Brites 2012; Kapoor et al. 2006).

The present work adds important information on the dynamic of brain Cyps induction by βNF, a general Cyps inducer frequently used in vivo. The differential in regions and species modulation of bCyps we reported indicates that Cyp-mediated protection from UCB toxicity might be more difficult to reach in cerebellum than in cortex, as cerebellum astrocytes need longer exposure time and higher doses of inducer. All together, the data on brain Cyps mRNA modulation, the increase in activity toward bilirubin oxidation and improvement in cell viability, could be the basis for achieving CNS resistance to bilirubin accumulation and ensuing neurological toxicity. Moreover, these data emphasize the importance of bCyps modulation in pharmacological and toxicological implications for cerebral drug metabolism.

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Ethical standards Animal care and procedures were conducted according to the guidelines approved by the Italian Law (decree 116-92), and the European Communities Council Directive 86-609-ECC. Regular communication to the National Ethic Committee was done (2009–2011).

Conflict of interest The supporting agencies did not play any role in the study.

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