UNIVERSITÀ DEGLI STUDI DI TRIESTE

XVIII CICLO DEL DOTTORATO DI RICERCA IN

BIOMEDICINA MOLECOLARE

The organotypic brain cultures as a new tool for the study of the molecular events involved in the selective bilirubin induced regional brain damage

Settore scientifico-disciplinare: **Gastroenterologia (MED/12)**

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Dato che:

[...] le cose vere della vita non si studiano e non si imparano, ma si incontrano
[Oscar Wilde]

dedico la tesi a tutti coloro che hanno fatto parte della mia vita.

Abstract

Moderate unconjugated hyperbilirubinemia is a common condition in the first week of postnatal life. Some neonates may develop very high levels of unconjugated bilirubin (UCB), with an increase of the unbound free fraction (Bf), able to diffuse through the blood brain barrier. The clinical manifestations range from the less severe Bilirubin-Induced Neurological Damage (BIND) to a more severe chronic kernicterus. Neurological damage is characterized by variability in location and severity of symptoms, including: motor disorders and athetosis (basal ganglia and cerebellum), auditory dysfunction (inferior colliculus), memory and learning impairment (hippocampus). Amount and duration of hyperbilirubinemia are not the only parameters that influence the severity of the damages. The neurodevelopmental age at the time of insult exposition and still unexplained selective regional brain sensitivity, seem to play an important role in influencing the damages.

To investigate the reason(s) of this variability we developed organotypic brain cultures (OBCs), which preserve the architecture, cellular complexity and connection of the *in vivo* nervous tissue in an *in vitro* system. These characteristics allow the identification of the most sensible brain regions and CNS developmental stages to acute bilirubin toxicity. These data were used to evaluate alternative therapeutical strategies. Cortex (Ctx), Cerebellum (Cll), Hippocampus (Hip), Superior and Inferior collicula (SC, IC) OBCs from 2 (P2) and 8 (P8) days old rats, were produced and challenged with 70, 140 and 300nM Bf for 24Hrs. Membrane leakage, mitochondrial activity, apoptosis, glutamate excitotoxicity, inflammation, astrogliosis, oxidative stress were monitored. Finally anti-inflammatory, anti-oxidant and drugs inhibiting the glutamate channels, were screened for their ability to reverse the damage.

We highlighted a differential developmental and regional sensitivity to bilirubin. Immediately after the birth (P2), Ctx displayed the maximal damage, followed by Hip and IC (starting from 140 nM Bf). In OBCs reproducing a more developed CNS (P8), sensitivity was increased. Surprisingly 70 nM Bf, usually considered safe, was able to induce damage in three regions (Hip, IC and Ctx). On the contrary, cerebellum was not damaged by bilirubin during treatment of 24 hours, while superior colliculus confirmed its resistance to bilirubin as expected by literature data. The Hip damage was mostly mediated by glutamate excitotoxicity and inflammation,

with oxidative stress less relevant. In agreement, $MgCl_2$ and indomethacin were more effective than curcumin, unable reverting damage to control levels, objective reached by exposing the OBC to a cocktail of the aforementioned molecules. IC showed a mechanism more related to inflammation and oxidative stress, efficiently reverted by curcumin and indomethacin. On the contrary, Ctx responded well to all therapeutic approach.

Our data documented the existence of a regional and developmental differential sensitivity to bilirubin insults. Data reported suggest a complex, but somehow region-specific mechanisms of toxicity, significantly reverted by the drugs we tested. This work opens the way to a new therapeutical approach aimed in protect directly the brain.

Riassunto

L'ittero neonatale, caratterizzato da un moderato aumento della bilirubina sierica, è una condizione comune nel 60% dei neonati. Alcuni bambini possono sviluppare elevati livelli di bilirubina per un lungo periodo, favorendo l'aumento della concentrazione della frazione di bilirubina libera (Bf) non legata all'albumina e in grado di passare la barriera emato-encefalica. Il danno al cervello può essere limitato, come nell'encefalopatia da bilirubina, o progredire fino a livelli cronici e permanenti come nel kernittero. Il danno neurologico è variabile in base alla sede colpita e può interessare il comparto motorio (cervelletto), uditivo (collicoli inferiori) e cognitivo (ippocampo). Il livello di bilirubina totale e la durata dell'iperbilirubinemia sono parametri importanti per valutare la gravità della condizione patologica. In aggiunta, anche lo stadio di sviluppo del cervello dei bambini, specialmente nei prematuri, e una riportata ma ancor non spiegata differente sensibilità alla bilirubina di differenti regioni del cervello sono parametri che possono influenzare in modo rilevante la severità e le manifestazioni cliniche della patologia.

Per studiare il ruolo dello sviluppo e della differente sensibilità regionale del cervello, le colture organotipiche di cervello di ratto (che mantengono l'eterogeneità cellulare del tessuto, nella sua struttura e connettività 3D, ma che possono essere coltivate *in vitro*) sono state sviluppate e ottimizzate per lo studio della tossicità da bilirubina. Colture d'ippocampo, cervelletto, collicoli inferiore e superiore e corteccia provenienti da animali di due differenti fasi dello sviluppo post-natale (2 e 8 giorni dopo la nascita) sono state trattate con differenti concentrazioni di Bf (70, 140, 300 nM) per 24 ore. La vitalità, apoptosi, attività mitocondriale sono stati valutati per studiare la differente sensibilità delle colture. Il ruolo dello stress ossidativo, infiammazione, astrogliosi ed eccitotossicità è stato valutato per studiare i meccanismi coinvolti nel danno da bilirubina.

I risultati ottenuti mostrano differenze nella sensibilità regionale e nello stadio di sviluppo. Corteccia, e meno collicoli inferiori e ippocampo sono state le regioni più sensibili nelle colture derivate da animali di 2 giorni a partire da una Bf di 140nM. Un incremento della sensibilità alla bilirubina è stato osservato nelle stesse regioni, con ippocampo il più danneggiato, in colture di animali di 8 giorni, dove anche la concentrazione di 70 nM, considerata moderata e non tossica, è stata in grado di causare danno. Diversamente da quanto ipotizzato, il cervelletto non ha mostrato un danno significativo da bilirubina, mentre i collicoli superiori hanno confermato la loro resistenza. Il danno in ippocampo sembra essere prevalentemente causato dal rilascio di glutammato

e dall'infiammazione, come confermato dai trattamenti neuroprotettivi con $MgCl_2$ e indometacina. Una reversione del danno a livelli similari al controllo è stata tuttavia raggiunta solo esponendo le colture ad un cocktail dei tre principi farmacologici. Al contrario, la curcumina è stata il farmaco più efficace nell'inibire il danno nei collicoli inferiori. La corteccia invece non ha mostrato un meccanismo preferenziale nel danno da bilirubina, con una reversione significativa quando trattata con ciascuno dei tre farmaci.

I dati ricavati da questo lavoro hanno dimostrato una differente sensibilità regionale e nello sviluppo del cervello alla bilirubina, ed ha inoltre dimostrato la multifattorialità dei meccanismi di danno. Il lavoro ha inoltre permesso di identificare farmaci efficace nel revertire il danno al SNC, suggerendo la possibilità di un nuovo approccio terapeutico basato sulla protezione diretta del cervello.

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Abbreviations

CN-I	Crigler–Najjar syndrome type I
OBCs	Organotypic brain cultures
PBS	Phosphate-buffered saline
DMSO	Dimethyl sulfoxide
Hip	Hippocampus
Cll	Cerebellum
SC	Superior colliculi
IC	Inferior colliculi
Ctx	Cortex
Hrs	Hours
LDH	Lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PI	Propidium iodide
Tnfα	Tumor necrosis factor α
Ilβ	Interleukin 1 β
Il6	Interleukin 6
Il10	Interleukin 10
Il4	Interleukin 4
Cox2	Cyclooxygenase 2
Hmox1	Heme oxygenase 1
Srxn1	Sulfiredoxin 1
Glu	Glutamate
$MgCl_2$	Magnesium chloride

NMDA N-methyl-D-aspartate receptor

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1 Introduction

1.1 BILIRUBIN

Bilirubin, derived from Latin, *Bile*, bile and *ruber*, red, is referred to the yellow lipophilic bile pigment with a linear tetrapyrrole structure. It is derived from the catabolism of heme-containing compounds, particularly hemoglobin, muscle myoglobin but also enzymes such as cytochromes, catalases, tryptophan pyrrolase. The main biological role hypothesized for bilirubin is as antioxidant, due to the beneficial effects observed in adult individuals with highest bilirubin levels than normal, in absence of liver diseases (Gilbert Syndrome). On the contrary, excessive level of plasma bilirubin in newborn is associated with yellow skin discoloration and accumulation in brain, resulting in damages and development of disabling consequences.

1.1.1 Bilirubin metabolism

300 mg of bilirubin are produced daily[1], with a rate of 4.4 mg/kg, mainly (75-80%) derived from degradation of heme group of hemoglobin from senescent red blood cells in the reticuloendothelial system in spleen. The remaining (25%) UCB synthesis derives from heme groups of liver enzymes, excess of heme production, altered red blood cells and myoglobin[2].

The first step of heme metabolism involves the opening of heme ring and the conversion of 1 mole of heme in 1 mole of Fe^{2+}, CO and biliverdin by Heme Oxygenase 1 (HO1), consuming oxygen and NADPH. Subsequently, biliverdin is converted to bilirubin by Biliverdin Reductase (BVR), that catalyzes the oxidation of -CH= group (Figure 1.1)[3].

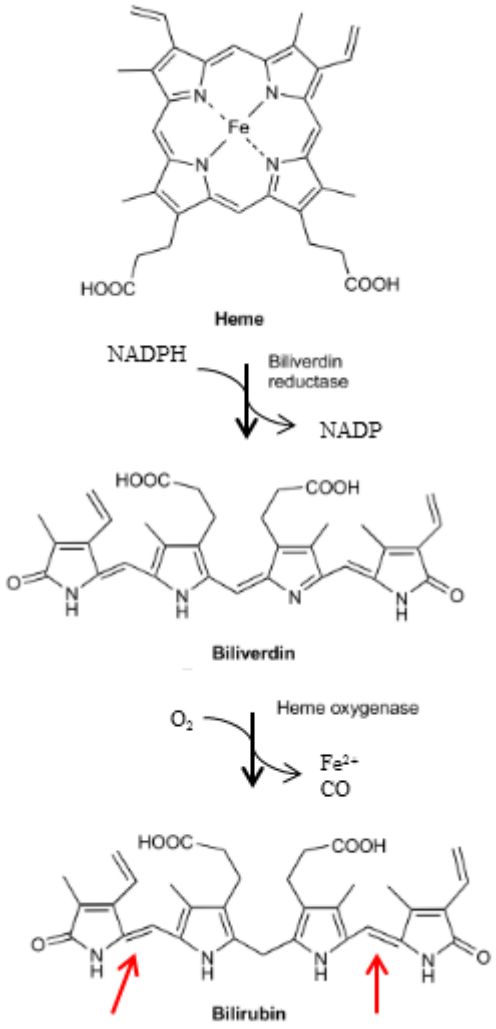

Figure 1.1 Steps of conversion of hemoglobin to bilirubin.

Conversion by heme oxygenase of hemoglobin to biliverdin, CO and Fe^{2+}, with subsequent conversion of biliverdin to unconjugated bilirubin, by biliverdin reductase. Red arrows showed double-bonds target of light of phototherapy.

The neo formed unconjugated bilirubin (UCB) is characterized of a very low solubility (<70 nM) related to the internal polar groups that cannot interact with water. For this reason, in plasma, bilirubin is transported associated with albumin[4] in adult and α-fetoprotein in newborns. In addition to its poor solubility, UCB is also poorly stable, since the double bonds in the vinyl and methene groups are readily oxidized and highly photosensitive (Figure 1.1)[3].

UCB, in complex with albumin, diffuses through the porous sinusoidal endothelium and enter into hepatocyte, probably by a combined process of transmembrane diffusion and active transport[5,6]. In hepatocytes cytoplasmic bilirubin is bound to ligandin[7] and glutathione S-transferase[8], that reduce the passive reflux of bilirubin in plasma or its interaction with cell membrane.

In the hepatocytes, the conjugation of bilirubin increases its solubility and allows elimination. Conjugation is catalyzed by the enzyme uridine diphosphate glycosyltransferase 1A1 (UGT1A1), a member of an enzyme family in the endoplasmic reticulum and nuclear envelope of hepatocytes (Figure 1.2). One or both carboxylic groups are modified by covalent attachment of polar glucuronic acid. The conjugation disrupts the internal structure and allows the release of a polar group. The polar group and the presence of hydrophilic glucuronic acid make bilirubin conjugates water-soluble. In adult humans, over 80% of the bilirubin conjugates are normally the di-glucuronide[9], whereas the mono-conjugates predominate in newborns[10].

The conjugation increases bilirubin hydrophilic properties that allow its elimination by bile excretion in the intestine. The excretion of conjugated bilirubin into bile is mediated predominantly by an active ATP-dependent process mediated by the multidrug resistance-associated protein MRP2, and also by the ATP-binding cassette efflux transporter ABCG2[11].

In the intestine, reabsorption of conjugated form is low, but it is subjected to hydrolysis by bacterial β-glucuronidases and the new unconjugated form could be reabsorbed through the enterohepatic circulation and returned to the liver. In newborn, the intestinal reabsorption could significantly contribute to the development of jaundice. The majority of the conjugated bilirubin is metabolized by anaerobic bacteria to urobilinogens, eliminated in the stool[12].

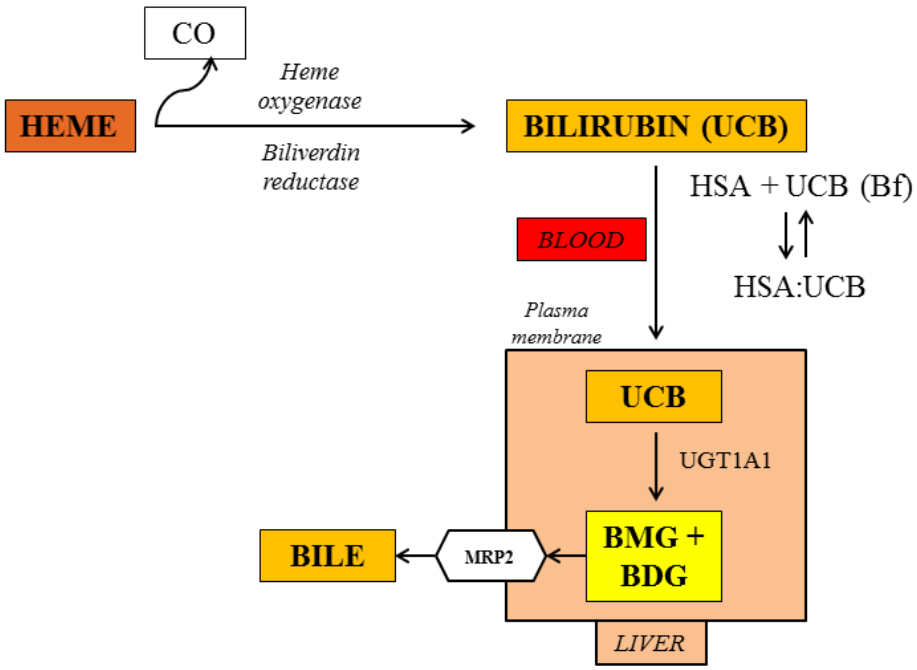

Figure 1.2 Main steps in the formation and elimination of bilirubin.

UCB: unconjugated bilirubin; Bf: free bilirubin; CO: carbon monoxide; HSA: human serum albumin; UGT1A1: uridine diphosphoglucuronosyl transferase 1A1; BMG and BDG: bilirubin monoglucuronide and diglucuronide, respectively; MRP2: Multidrug resistance related protein 2[13].

1.1.2 Free bilirubin (Bf)

The bilirubin in blood is clinically measured as Total Serum Bilirubin (TSB) and indicates the total bilirubin load, indispensable in presence of pathological value. TSB measures both the unconjugated bilirubin (UCB), defined "indirect bilirubin" and the conjugated form, called "direct bilirubin". Due to its low hydrophilicity, in blood UCB needs to be bound to albumin (ALB). This bond determines the biological effects of the pigment. The binding between bilirubin and human serum albumin (HSA) was demonstrated by Bennhold[14]. Reported association constants for HSA range from 6.7 x 10^6 M^{-1} for the high affinity site to 10^8 M^{-1} for the secondary site[15,16].

Under physiological conditions, the total albumin binding capacity is never saturated. However, as the primary binding site reaches saturation, the level of the small fraction of unbound bilirubin, called "free" bilirubin (Bf), increase quickly. Few data exist about Bf measurement in newborns. Clinically, neurological signs in fullterm newborns were observed in a Bf range of 13-46 nmol/L[17]. Free from albumin binding, Bf can enter into the tissues. Plasma albumin limits the penetration of bilirubin by two ways. First, forming a complex (ALB+UCB) larger enough to avoid the entry of albumin bound bilirubin into tissues and across the blood brain barriers. Second, the contribution of the tissues for bilirubin binding reduces the extra vassal unbound bilirubin concentration. Despite the affinity of tissues for bilirubin is highly variable and depends on their structure and lipid content, tissues must be considered a reservoir of Bf in hyperbilirubinemia[18].

If UCB concentration is higher than the albumin binding capacity, the amount of Bf increases. In this condition, the unbound molecule, that is lipophilic and smaller enough can passively diffuse across the membranes and accumulates into the tissues. This principle is now known as "the Bf theory". The first demonstration of the Bf theory was acquired to explain the outbreak of kernicterus in premature newborns treated with the antibacterial sulfisoxazole as prophylaxis for infection. At that time, kernicterus frequently occurred at total bilirubin concentrations between 12 and 18 mg/dl (considered low-middle total bilirubin, thus not dangerous), when sulfisoxazole was administered[19,20]. The drug was shown to compete with bilirubin for albumin binding sites, increasing the plasma Bf and perturbing the steady state sufficiently to increase the pigment brain entry causing toxic neuronal free bilirubin levels (acting as displacer)[21,22]. In fact, the Bf, due to its hydrophobicity, can permeate spontaneously through phospholipid bilayers of cell membrane by passive diffusion[23], passing thought the endothelial cells forming the blood-brain barrier (BBB) in either direction[24].

Moreover, all the condition perturbing the dynamic bilirubin-albumin binding equilibrium might increase the Bf. Among them: poor feeding, hypoalbuminemia (<2.5 g/dL - frequently observed in infant especially preterm); displacer assumption[25], acidosis, asphyxia, etc.

Thus the possibility of quantify the Bf should be strongly relevant in predicting the risk of developing kernicterus. A method for the quantification of Bf was developed based on the observation that the bilirubin free of the albumin binding is oxidized by horseradish peroxidase in presence of hydrogen peroxide[26]. Even if, the methodology is well accepted for simple *in vitro* cultures media, so far it doesn't work in a reproducible and easy way to perform fashion with the

more complex biological samples (blood, serum), avoiding to directly quantify the Bf in clinics. For that reason, the decision of intervention is still bases on the TSB – albumin blood ratio, and on the post-natal age of the icteric newborn (Figure 1.3)[27].

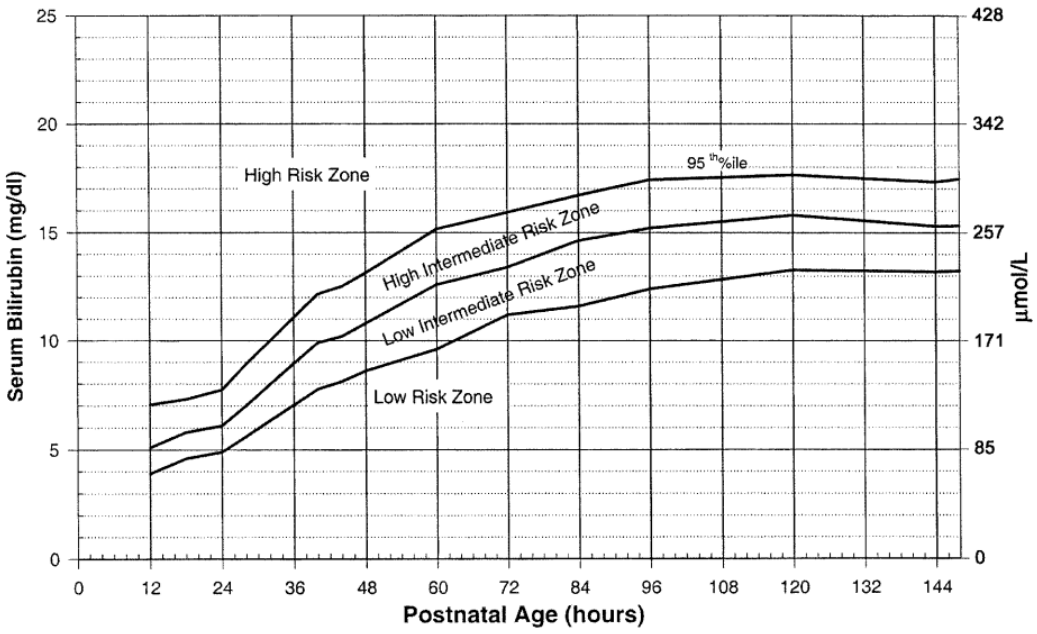

Figure 1.3 Nomogram for designation of risk of hyperbilirubinemia in newborns, based on the hour-specific serum bilirubin values[27].

1.1.3 Bilirubin in newborns

During fetal life, bilirubin production begins as early as 12 weeks' gestation. For an optimal elimination, bilirubin is retained in unconjugated form that allows passage from the fetus, through placenta, to the maternal circulation for excretion. The presence of the bacterial β-Glucuronidase in fetal bowel helps to maintain bilirubin unconjugated by hydrolyzation of the conjugated molecules of glucuronic acid[28].

At birth, the protection of the placenta is lost. At the same time, the life span of erythrocyte is shorter than in adult (70-90 days *vs.* 120 days), the conjugation of bilirubin by UGT1A1 is only the 1% of level detected in adult life (reaching adult level only after 14 weeks after birth in humans[29], as well the level of ligandin, responsible for bilirubin binding into hepatocyte is low. All

together, these conditions contribute to the physiological jaundice[30], characterized by mild to moderate elevated serum UCB levels (less than 170 μM), occurring in approximately 60% of healthy term neonates. This physiological neonatal jaundice reflects the transition from intrauterine to extrauterine bilirubin metabolism. It is considered benign, and is usually resolved by the end of the first week of life without treatment requirement[31,32].

UCB has long been considered a waste product of difficult clearance. However, accumulating evidence suggests that UCB is a potent antioxidant at very low concentration[33]. In birds, amphibians, and reptiles the biliverdin produced in the first step of heme degradation is excreted directly, while in mammals biliverdin is reduced to the non-polar bilirubin. The acquisition of this biological process could be explained by the need to protect the newborn from the oxidative stress generated by the first contact with the air oxygen[34,35].

At the contrary, when plasma UCB levels increase dramatically due to increased production or impaired clearance, hyperbilirubinemia can cause toxicity to various brain regions, leading to acute reversible bilirubin encephalopathy or progress to the irreversible chronic form kernicterus, or even death[27].

1.1.4 Main causes of severe hyperbilirubinemia

As previously explained, bilirubin uptake and elimination by the liver involves four distinct processes: uptake from the circulation, intracellular binding, conjugation, excretion. Alteration of one or more of this leads to different degree of hyperbilirubinemia, with consequent brain damages.

1.1.4.1 Rh incompatibility

Severe unconjugated hyperbilirubinemia may be determined by alteration of the hemolytic process, causing the Rh disease (also known as Rhesus incompatibility – hemolytic hyperbilirubinemia). The disease typically occurs only in second or subsequent pregnancies of Rh negative women where the fetus's father is Rh positive, leading to an Rh+ pregnancy. The disease has been virtually eliminated in high-income countries by the prophylactic use of Rh immunoglobulin given to the mother during the last trimester of pregnancy and within the first 72 h postpartum[36].

1.1.4.2 Genetic deficits of the enzyme glucose-6-phosphate dehydrogenase

Genetic deficits of the enzyme glucose-6-phosphate dehydrogenase (G6PD), that affects some 400 million people worldwide[37], contribute to the cases of neonatal hyperbilirubinemia and puts infants at risk of kernicterus. The enzyme is part of a cytoplasmic system deputy for the production of the reduced form of NADPH, involved in the maintenance of intracellular levels of reduced glutathione to protect cells from oxidative stress. Due to their activity as oxygen carriers, red blood cells are at high risk of damages by free radical. This deficit is dangerous in red blood cell that lack of other regenerating systems for NADPH, leading to increased risk of hemolysis with consequent development of hemolytic jaundice.

1.1.4.3 Genetic disorders of bilirubin metabolism: *UGT1A1*

Differently from the previous two, hyperbilirubinemia derived from genetic abnormalities of the UGT1A1 enzyme are not characterized by an excessive production of iron and carbon monoxide, but they only lead to an accumulation of unconjugated bilirubin.

1.1.4.4 Crigler-Najjar syndrome

Unconjugated bilirubin is accumulated in plasma due to an insufficient level of enzymatic conjugation in the liver. Mutation in the key enzyme *UGT1A1* leads to an autosomal recessive rare disease (0.6-1 per million live births) called Crigler-Najjar typer I syndrome (CN-1)[38–40]. Mutations in the exons 2, 3 and 4 of the gene *UGT1A1* affected all the hepatic and extrahepatic transcripts[41]. Therefore, hepatic conjugation activity is absent in patient and plasma bilirubin reaches level of 26-45 mg/mL, while the physiological concentration is 0.7-1 mg/mL. If untreated, the majority of patients die for kernicterus during the first 18 months of life. Phototherapy is of limited use in CN-I subjects, due to the increased thickening of the skin with age, the increasing of pigmentation and the decreased surface/mass ratio, strongly affecting phototherapy effectiveness. Of notice, CN-I subjects must receive 12 hours per day of phototherapy, significantly reducing the quality of their life[42]. Plasmapheresis and exchange transfusion are used in clinical practice during crisis, but the only effective treatment for CN-I patients is orthotopic liver transplantation[43]. The inactivation of the *UGT1A1* gene was also observed in a rat, called Gunn rat, which spontaneously developed

hyperbilirubinemia after birth[44]. The Gunn rats was used as animal model of hyperbilirubinemia to study neuronal effects of bilirubin due to the evident cerebellar hypoplasia observe after one week of life[45]. The original strain of Gunn rat was characterized by lethality, but in the last years, animal somehow adapted to the genetic deficit until a non-lethal hyperbilirubinemia. Hyperbilirubinemia could be experimentally exacerbated by administration of sulphadimethoxine that is able to displace bilirubin from albumin and to increase dramatically its free form in blood[46].

The variant 2 of the Crigler-Najjar syndrome, described firstly in 1962[47], is characterized by serum bilirubin of 7 to 20 mg/mL, due to a single base pair mutations in *UGT1A1* gene, with consequent substitution of a single aminoacid that decrease the activity of the enzyme to 10% of normal, without abolish completely[48]. The syndrome is characterized by lowest level of plasma bilirubin if compared with type I syndrome and by a less sever phenotype. An important difference between the two variant in term of treatment is that type 2 patients are usually respondent to phenobarbital treatment[49,50]. Patients with CNS type 2 respond with a reduction of their serum bilirubin level of about 25%, while no response is seen in type 1 patients, due to the complete abolition of the enzymatic activity.

1.1.4.5 Gilbert syndrome

A common mild and chronic form of hyperbilirubinemia was described in last century as Gilbert syndrome[51]. The syndrome is estimated to be present in 3 to 10% of general population[52] and is diagnosed for the presence of longer dinucleotide repeat, A[TA_7]TAA, in the promoter region upstream to exon 1 of UGT1A1 gene that has the sequence A[TA_6]TAA[53]. The mutation causes a reduction in UGT1A1 activity of about 70%, with variation of unconjugated plasma bilirubin from normal to 3 mg/dL, usually during fasting or illness[54]. Increased level of plasma bilirubin in Gilbert syndrome is milder than those observed in Crigler-Najjar syndromes and is associated with protective effects in term of anti-genotoxic effects[55] and reducing risk of cardiovascular diseases[56].

1.1.4.6 Conjugated Hyperbilirubinemia: Dubin Johnson and Alagille syndrome

Differently from the previously unconjugated hyperbilirubinemia, there are syndromes characterized of high level of conjugated bilirubin in plasma. Conjugated form may accumulate in plasma due to alteration of the uptake and metabolic capacity of liver cells. The rare Dubin-Johnson

syndrome is characterized also by black pigmentation of the liver in the absence of other clinical biochemical alteration. The condition showed alteration of canalicular excretion, due to the mutation in the ATP-binding domain of the gene ABCC2, observed in many patients. On the contrary, Rotor syndrome is a disorder of hepatic storage. The syndrome showed autosomal-recessive characteristic, but the molecular bases are unknown. Progressive familial intrahepatic cholestasis (PFIC) refers to a group of familial cholestatic conditions caused by defects in biliary epithelial transporters. The alteration could cause progressive liver damage. The mutation of *JAG1* gene causes Alagille syndrome, a disorder of bile duct development resulting in progressive cholestasis[57].

Indeed, chronic form of brain damages could be reached also under moderate level of total serum bilirubin (TSB) if associated with other pathologies by the opening the BBB, allowing not only Bf but also albumin bound UCB to reach the neural parenchyma (intraventricular hemorrhage and periventricular white matter injury; asphyxia; infection and inflammation)[58,59].

1.2 BILIRUBIN NEUROTOXICITY

1.2.1 Kernicterus

Historically, the term "kernicterus" was designated by Schmorl in 1904 to describe intense yellow staining of the basal ganglia and medulla oblongata in autopsy of brain newborn[60]. This historical finding perpetuates the idea of selective deposition of bilirubin in specific brain region as the main cause of the regional damages observed in the brain. This idea was first challenged by Hansen, in 2000[61]. Later on, an identical bilirubin content was measured in different regions of the brain of the Gunn rats (the spontaneous animal model of CN-I), suggesting that the dogma of a regional bilirubin deposition should be reconsidered and that the selective localization of the damage depends of a still unexplained regional selective sensitivity to toxicity[62].

The term kernicterus was used to describe neuropathology of bilirubin-induced brain injuries and its associated clinical findings. In the last decade there was the need to develop definitions for

other subtle form of bilirubin encephalopathy, usually described as bilirubin induced neurological damages (BIND). Classical kernicterus or chronic bilirubin encephalopathy is the clinical syndrome described in this chapter. BIND refers to individuals with subtle neurodevelopmental disabilities without classical findings of kernicterus. This syndrome is recognized because unknown neurological disabilities were diagnosed in patients with a history of hyperbilirubinemia. Neurological abnormalities are usually less severe than observed in classical kernicterus, allowing the introduction of the terms "subtle kernicterus" or BIND[63].

Nowadays, the American Academy of Pediatrics recommended using the term kernicterus only for the chronic and permanent signs of bilirubin damages and acute bilirubin encephalopathy for the acute effects. Jaundice newborn with signs of acute bilirubin encephalopathy became lethargic and hypotonic, followed by development moderate stupor, irritability, and hypertonia. The advanced phase, that probably became irreversible, is characterized by pronounced retrocollis-opisthotonos, apnea, fever, deep stupor to coma, seizures, and also death. Chronic signs could be preceded by acute manifestations or develop directly after very high level of serum bilirubin. Surviving infants may develop athetoid cerebral palsy, auditory dysfunction, dental-enamel dysplasia, paralysis of upward gaze, and, less often, intellectual and other handicaps[27].

Well in agreement with the symptomatology, recent histological findings in newborns died with kernicterus included apoptosis and necrosis of neurons in the cerebral cortex, basal ganglia, hippocampus, pons, and cerebral Purkinje cells and calcifications were seen in cerebral white matter[64]. Specific findings in basal ganglia were confirmed in other autopsy cases, which described edema with mild neuronal loss. Other alterations observed were selective neuronal loss with gliosis in the globus pallidus, sub-thalamic nucleus, and hippocampus[65]. Cerebellum, hippocampus (especially in third layer of the Ammon's horn, the CA3) and basal ganglia are regions commonly recognized as locations of damages in autopsy. In three children vacuolation of brain parenchyma was a common sign, related to neuronal degeneration in all the three regions described[66]. Different susceptibility of brain regions to bilirubin was confirmed also in *in vitro* and *in vivo* works, resulting in high sensitivity of hippocampus and cerebellum, and less in cortex[62,67–69]. In addition, magnetic resonance imaging (MRI) could be used to assess the damage. Abnormal increased signal intensity of T1-weighted images in the globus pallidus and subthalamic nuclei was described for the acute phase of kernicterus. MR imaging during the chronic phase displayed increased signal intensity on T2-weighted MR images of the globus pallidus, subthalamic nuclei, and hippocampus[70,71]. The brainstem auditory pathway is known to be sensitive to bilirubin

neurotoxicity[72]. Auditory measures use acoustic stimuli and auditory responses that fall into several categories. The series of characteristic peaks (labeled with Roman numerals I-V) in the response of acoustic stimuli are referred to as auditory brainstem responses (ABR). Dysfunctions in the related neural pathways cause deficits, including delayed or abnormal conduction time of neural impulses and are manifested as decreased peak latencies and increased interpeak intervals. In term neonates with hyperbilirubinemia, the wave latencies and interpeak intervals tended to be increased with the increase in the level of TSB[73]. Bilirubin has variable impact in auditory system and usually it is correlated with TSB level[74].

Kernicterus (<1 in 50000 live births) is recognized as rare disease by: Online Mendelian Inheritance in Man (OMIM#237900); European ORPHANET (ORPHA415286); NIH Genetic and Rare Diseases Information Center (GARD)[75]; The Italian Department of Health (RP0060)[40]. Nevertheless, bilirubin toxicity remains a significant problem of newborns despite recent advances in the care of jaundiced neonates. Data about rarity of kernicterus derived from registry of industrialized countries, but detailed information are lacking in low-income and middle-income countries (LMIC). A study of few years ago estimated the regional and global incidence of severe hyperbilirubinemia. The global prevalence of kernicterus was estimated in 56/100000 live birth with important difference in geographic areas. Analysis of high-income countries resulted in a prevalence of only 10/100000 live birth, while LMIC account for about 80% of the kernicterus cases with a prevalence of 73/100000[76].

As previously described, kernicterus is considered a rare consequence of hyperbilirubinemia, but some conditions contributed to its resurgence[77–79]. System failure in neonatal care during the first week of life was suggested as the possible reason of this: early discharge of the mother from the hospital during the first 72 hours of the newborn life, breastfeeding without support for optima lactation and the insufficient knowledge about the risk of severe hyperbilirubinemia. In addition, the efficacy in western countries of phototherapy, as first approach, and the availability of other medical interventions, has probably generated overconfidence in medical environment about the management of severe hyperbilirubinemia[80].

1.2.2 Molecular mechanisms of bilirubin neurotoxicity

Bilirubin could damages different brain region and it is suggested that this occurs by alteration of different biomolecular pathways. It is suggested firstly that the risk for neurological damage is related to the degree and duration of hyperbilirubinemia[81]. Moreover, it is important to understand the mechanism of toxicity to identify the key step to target to prevent the final and chronic symptoms.

Initially, bilirubin is able to be able to diffuse spontaneously through the phospholipids bilayer of the hepatocytes cell membrane[23]. The same event happens in presence of membrane of neuronal cells where unconjugated bilirubin was observed to interact with cell membranes and mitochondrial membranes[82]. This process leads to disruption of lipid polarity and fluidity, alteration of protein order, and generation of redox status. Bilirubin interacts also with mitochondrial membranes influencing lipid fluidity and cytochrome *c* release in cytoplasm[83]. The consequence of the cytochrome release is the cell death by induction of apoptosis[84]. Induction of apoptosis was observed also in granular cells from cerebellum of Gunn rat starting from the alteration of cell cycle, which could explain hypoplasia[85].

Bilirubin interaction with phospholipid membranes causes also a reduction in the activity of the Na^+-K^+-ATPase and structural modification of the glutamate NDMA receptors[86]. Bilirubin has been demonstrated to alter the glutamate pathway by excessive stimulation of glutamate release with consequent overstimulation of the receptors, a process called excitotoxicity. Glutamate is the major excitatory neurotransmitter in human central nervous system and its regulation is critical due to its important roles for neural development[87]. Improper release of glutamate and receptors activation may lead to neuronal cell death. This event was observed in primary neuronal cell cultures where bilirubin induced apoptosis through the activation of glutamate receptors; in fact the administration of the NMDA receptors antagonist MK-801, but not the AMPA receptors antagonist NBQX protects the cells from bilirubin toxicity[88]. The Gunn rat resulted more sensitive than the normobilirubinemic rat to the administration of NMDA, the agonist of the same class of glutamate receptors, and the damages caused by sulfadimethoxine treatment could be reduced by NMDA receptor antagonist MK-801[89].

The role of oxidative stress in bilirubin toxicity were investigated *in vitro* by observation of increased level of protein and lipid oxidation and by decreasing level of antioxidant systems

(glutathione)[90]. In different cell lines, bilirubin causes an accumulation of intracellular reactive oxygen species (ROS) that activate the antioxidant cell response[91,92]. Pro-oxidant effects suggested by impairment of the antioxidant enzyme in brain were observed in a mouse model of Crigler-Najjar syndrome[93]. Pro-oxidant markers consequent to bilirubin exposure were investigated also in newborns and resulted higher in infants with hyperbilirubinemia when compared with normobilirubinemic infants[94]. Another study displayed increased level of malondialdehyde in hyperbilirubinemic infants compared to healthy infants, which significantly decreased after phototherapy[95]. On the contrary, a work performed in the Gunn rat, as model of Crigler-Najjar syndrome, showed that lipid peroxidation is not the main mechanism causing BIND[96].

Bilirubin is able to alter mitochondria respiration depending on its concentration and cell target. Liver and heart cell displayed increased respiration rate at low concentration and inhibition only at high concentration, while in brain cells mitochondria activity was always inhibited[97]. Other effects in metabolic alteration were observed in brain of Gunn rat in inhibition of phosphofructokinase, a key enzyme of glycolysis[98]. The data was confirmed also in a rat model with a bilirubin infusion that displayed regional and age-dependent alteration of glucose utilization in brain[99].

The predilection of bilirubin for specific cells were tested in recent years by the measurement of viability in immortalized cell lines. One work that considered the sensibility of different tissues showed the preference of bilirubin toxicity for neuroblastoma and glioblastoma cell lines, as model of neuronal and glia cells[100]. On the contrary, liver and fibroblast cells resulted less sensitivity. In this respect, function and viability of neurons are supported by a large number of glia cells. Consequently, UCB playing its toxic effect on astrocytes, oligodendrocytes and microglia, may affect the neuronal survival. One of the first descriptions of damage of astrocytes reported swelling of perivascular astrocytes[101,102], characterized by a lower, maturation dependent, sensitivity than neurons. Indeed, astrocytes showed high glutamate release and inflammatory activation[103,104]. Microglia are resident macrophage in CNS and are known to be sensible to the presence of bilirubin[105], in turn affecting the survival of surrounding cells by the secretion of inflammatory mediators. Moreover, it was demonstrated that bilirubin induced an autophagic phenotype that is supposed to be neuroprotective, but also a pro-inflammatory phenotype with consequent cytokine release[106]. Recent *in vitro* studies clearly demonstrate that oligodendrocyte precursor cell are vulnerable to bilirubin toxicity, although less than neurons. In addition, unconjugated bilirubin limits oligodendrocyte differentiation and impairs axonal myelination *in vitro*, and demyelination

and axonal loss are reported in the cerebellum of preterm infants with kernicterus[59,107–109], in mouse model of CN-1[110] and in rats[102,111].

1.2.3 Available treatments

The Subcommittee on Hyperbilirubinemia of American Academy of Pediatrics drafted guidelines for the practical management of hyperbilirubinemia[27]. The aim of this guideline is to promote an approach that will reduce the frequency of severe neonatal hyperbilirubinemia and bilirubin encephalopathy. The global hospital policy of early discharge of the newborn form the hospital demonstrated the low knowledge of the nursery and doctors to the hyperbilirubinemia risk[79]. The key elements are summarized here:

- Establish nursery protocols for the identification and evaluation of hyperbilirubinemia.
- Measure the total serum bilirubin (TSB) on infants jaundiced in the first 24 hours.
- Recognize that preterm infants are at higher risk of developing hyperbilirubinemia.
- Provide parents with written and verbal information about newborn jaundice.
- Treat newborns, when indicated, with phototherapy or exchange transfusion.

1.2.3.1 Phototherapy

Phototherapy is the first and most common therapeutic intervention used for the treatment of hyperbilirubinemia[112]. The very first phototherapy unit incorporating an artificial light source instead of natural sunlight was developed and tested in 1958 at Rochford Hospital[113] and became of clinical use a decade later[114] (Figure 1.4). Phototherapy causes oxidation and consequent molecular rearrangement of bilirubin to produce mutant bilirubin isomers[115], which are more polar and thus excretable without conjugation. The reactions that occur with light include isomerization about the two double bonds showed in Figure 1.1, photo-oxidation, and occurrence of an intra-molecular cyclisation When blue light illuminates the skin, an amount of photons of energy are absorbed by bilirubin present in the superficial skin capillaries. The objective of the oxidation of bilirubin is to reduce rising of its plasma level and to prevent its entry and deposition in brain. Therefore, the most important intervention for the severely hyperbilirubinemic infant is to initiate phototherapy without

delay. The photoisomerization of bilirubin begins almost instantaneously when the skin is exposed to light. Phototherapy is effective (in Western countries) in reducing the circulating amounts of the pigment in newborns when applied promptly. Furthermore, it's important to remember that the ratio of bilirubin oxidation is related to the quality of the instrumentation used: wavelength into the range of 400 and 520 nm; intensity of the light; distance between the light source and the newborn; body surface area exposed to the light[116]. Phototherapy is not free of side effects; in fact they include short time alteration of thermal environment of the newborn, dehydration, alteration of electrolyte equilibrium, development of allergic diseases, retinal damages and others[117]. It is generally accepted that the side effects of neonatal therapy are rare and not serious. Bilirubin photoisomers, derived from photo-irradiation of unconjugated bilirubin, was recently studied[118]. The work demonstrated that photoisomers do not interfere in bilirubin-albumin binding, excluding any interference in Bf level, and in viability of cell lines, suggesting the absence of a direct toxic effect.

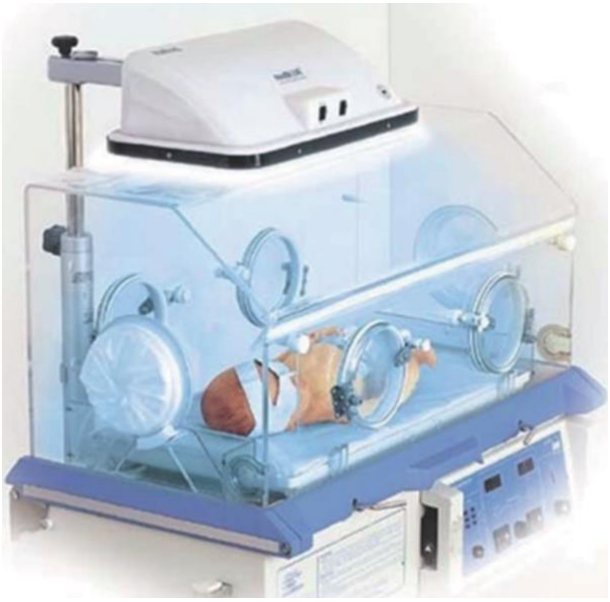

Figure 1.4 Newborn under phototherapy[116]

1.2.3.2 Exchange transfusion

Phototherapy is generally effective, but in some neonates the plasma bilirubin concentrations become dangerously high. In addition, as reported before, phototherapy is also of limited use in CN-I subjects, revealing the need of other treatment able to reduce bilirubin in blood. In these cases exchange transfusion is indicated[27]. An exchange transfusion requires that the patient's blood can be removed and replaced with "clean" blood, as demonstrated in animals and newborns[119–121]. The main disadvantages of the technique are the important side effects derived from the replacement of a large amount of blood. Complications include cardiac arrest, thrombosis of the portal vein, coagulopathies, hypoglycemia, hypocalcaemia, necrotizing enterocolitis, and transmission of infectious diseases, with a mortality rate of 0.3–2.0%[122–124].

1.2.3.3 Albumin administration

The free bilirubin theory suggested the idea of the possibility to sequester the free fraction of plasma bilirubin by administration of albumin to the newborn to prevent bilirubin entry into the brain. In addition, there was need to develop new protective treatment due the high risk of side effects of the exchange transfusion, that represent the second step to reduce plasma bilirubin after phototherapy. In 1959, newborn puppies were injected with a bilirubin solution and simultaneously with serum albumin, showing clinical improvement with increased survival and reduce tissue bilirubin accumulation[125]. The possibility to administer albumin to the newborn with hypoalbuminemia or with plasma containing molecules able to displace bilirubin from albumin was discussed in the same year[126]. Human serum albumin treatment has been used also more recently[127,128], without data about decreasing of bilirubin in brain. However, some years ago infusion of human serum albumin (HSA) resulted efficacy in decreasing of bilirubin in brain[129] when administrated in tandem with phototherapy treatment in hyperbilirubinemic Gunn rat[130].

1.2.3.4 Liver transplantation and genetic therapy

Patients with CN-I are subjected to chronic hyperbilirubinemia for entire their life, so the treatment exposed here are only temporary solutions to reduce plasma bilirubin. The only solution is the correction of the genetic error by liver transplantation or by injection of hepatocyte expressing normal level of UGT1A1, as recently tested in Gunn rats[131,132].

1.2.3.5 Alternative approaches

Alternative methods to take advantage of the clearance role of albumin were studied. Nutritional intervention was also suggested to increase plasma albumin synthesis, as demonstrated in very low weight preterm newborn fed with high dose of aminoacid and lipid when compared with standard diet[133].

1.2.3.6 A new therapeutical strategy

All the approaches previously described are focused in decreasing plasma bilirubin, but there are no sufficient data about their role in reducing efficiently and quickly bilirubin level in brain. A different field of studies is concentrated in the development of neuroprotective drugs that will be used to gain time to allow traditional therapies to be effective or as an alternative first step therapy. Till now only limited efforts in this direction have been done, mostly focused in obtaining mechanistic/bench based results. Minocycline is a semisynthetic second-generation tetracycline which exerts secondary protective effects which appear to be distinct from its anti-microbial action[134]. Minocycline possess *in vitro* anti-inflammatory, anti-excitotoxic[135,136] and anti-apoptotic effects[137,138]. More interestingly are its effects *in vivo*, in the Gunn rats, where it is able to rescue bilirubin-induced cerebellar hypoplasia[139] and auditory dysfunction[140]. The main limit of its clinical use in newborns is the presence of important side effects on bone and teeth development in child younger than 8 years. Other drugs approaches were investigated *in vitro* with the objects to inhibit mechanism suggested to be involved in bilirubin neurotoxicity: L-Carnitine[141] for excitotoxicity; Taurine[142] for intracellular calcium imbalance; MK-801 and caspase inhibitors[143] for excitotoxicity and apoptosis; modified bile acid[144,145] with multiple neuroprotective effects.

1.3 ORGANOTYPIC BRAIN CULTUREs

In vitro cell cultures are an important technique for neuroscientist for studying large quantities of homogeneous cells in an isolated environment. Cancerous or immortalized cells are used as model of specific tissue to overcome the limited capacity of cell to proliferate unlimited *in vitro*. This simple system has provided historically a unique opportunity to understand biological systems and to know all the components that make up cells and organisms. The cultures of primary dissociated neurons, astrocytes, oligodendrocytes or microglia is another important method for neuroscientist to study behavior of normal cells of CNS. However, isolated cells do not reflect the nature of the organism due to the absence of 3D environment and lack of contact with other cells that are essential for their physiological function. This is of particular importance in brain tissue, where the intrinsic connections between nerve cells are an integral part of their function and where dissociation of the tissue may impacts on its original function. There is an increased interest for research systems to mimic well the physiological interaction of the cells that are present in the tissue. In addition, pharmaceutical field need a complex model to evaluate drug activities for pre-clinical pharmacological study. Animal model is the leading model to study protective effects of drugs, but in many case they are complex, expensive and time-consuming. In addition, there are technical difficulties associated with examination or brain regions *in situ* in animal.

1.3.1 The "roller tube" organotypic brain cultures

For the reasons previously described, in neurological studies, some problems can be solved by using tissue cultures developed some decades ago and called organotypic brain cultures (OBCs – Figure 1.5). The first approach to organotypic cultures was developed by Hogue in 1947[146] and modified later by Costero and Pomerat[147]. The technique was based on a small piece of human fetal brains cultured in a tube and included in a clot prepared from coagulation of hemostatic globulin and chicken plasma, all fed by medium. This technique was described in details by Gähwiler, by using rat brain slices, and was called Roller-Tube, due to the slow rotation of the culturing systems that allow oxygenation of the slices thanks to a continuous changing of the air-liquid interface in the tubes[148]. The main advantage of this method is the reduction in thickness of the slices after few weeks under the action of the clot, from the original 300-400 um to about 50 um[149]. Thanks to the reduced volume, the accessibility of individual neurons in the slice in these cultures resembles those

prepared from dissociated cells. This is a leading technique for microscopic observation of the development of neural connectivity, analysis of fiber growth and synaptic transmission and electrophysiological studies due to the accessibility to the neurons[150,151]. On the contrary, the reduced tissue amount limits the application of the cultures method for all the purposes based on collection of biological samples (RNA, proteins), such as in the present work.

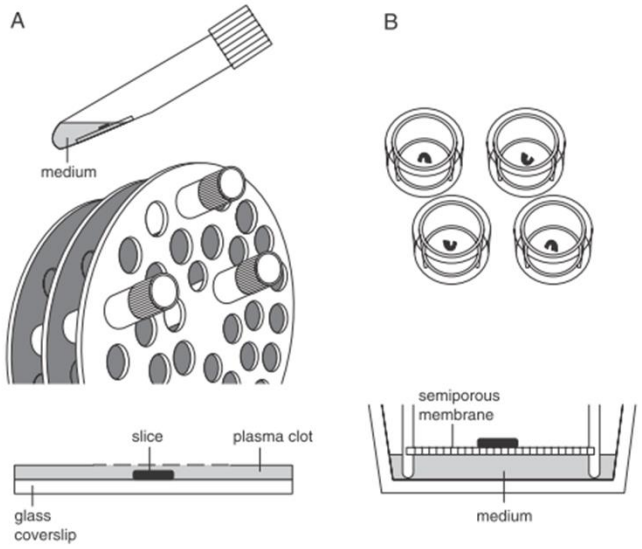

Figure 1.5 Roller-tube (A) and Interface method (B)[152]

1.3.2 "Interface" organotypic brain cultures

Twenty years ago, a relatively simple method to produce and culture OBCs was introduced by Stoppini[153] and called "interface" method. In this new system, slice prepared from explanted tissues are placed onto a semiporous membrane and fed by a small drop of medium. Slices are maintained alive in an air-liquid interface into a multi-well late in a classic 37°C, 95% humidity and 5% CO_2 incubator. Usually, slices are never fully soaked in medium but are covered with only a small film of medium at the upper surface. This technique reduced the technical requirement for production and culturing of slices. In addition, this method maintains slices in a static culture that preserve the tissue and allow the possibility to collect a good amount of sample for biomolecular analysis.

Organotypic brain cultures technique takes advantages from the properties of the neuronal tissue to survive for a long period *in vitro*. The ideas underline the development and usage of OBCs is the possibility to use cultures able to mimic the characteristics and function of the nervous tissue *in vitro*. This is supported by works that have analyzed the development of the slices during all the period of culture. Neuronal organization and morphology, and electrophysiological features of long-term cultures are similar to those observed in adult brain tissue *in vivo*[154–157].

Together with the preservation of the characteristics of the nervous tissue, one of the main advantages of OBCs is the possibility to be obtained from various brain regions. These include hippocampus[158–160], cortex[161,162], cerebellum[163,164], superior colliculus[165], inferior colliculus[166], striatum[167,168] and spinal cord[169,170]. The basic principle of the OBCs preparation is the use of tissue that possesses a regular organization along their structure that allows the production of homogenous slices.

Organotypic brain cultures were produced originally from neonatal rats of 1 to about 20 days[148,153]. The age of the animal plays a significant role in the viability of the slice culture. Viable slices are easily obtained from brain of young animal of post-natal (P) 0 to 10 days or from embryos, that results more resistant to the stress of slices preparation. There are increased evidence that healthy slices could be produced from mature animals[171,172], with increased risk of cell death and altered metabolism during culture period[162].

Despite the complexity of preparation protocol, organotypic cultures have obtained increasing success as useful alternative in simulating more *ex vivo* situations. Because they retain the cytoarchitecture of the tissue of origin, slices have evolved as one of the predominant *ex vivo* preparation used by electrophysiologists, and histologists, pharmacologists and biochemists. Slices system allow precise control of extracellular environments, so they facilitate research aiming to establish clear correlations between structure and function, as well as plasticity of neuronal interactions under different experimental conditions. In addition, it is possible to adopt these *ex vivo* models for neurotoxicity studies and for the screening of therapeutic molecules[173,174].

2 Aims

One key point related to the symptomatology of bilirubin encephalopathy is the heterogeneity in severity and appearance of neurological symptoms (auditory, motor, cognitive, etc). In addition, the presence of kernicterus at low bilirubin suggested the importance of points of vulnerability of brain development. Moreover, no attention has been dedicated till now to pharmacologically protect directly the brain, the final target of bilirubin toxicity. Interface organotypic brain cultures were selected as a good platform to test bilirubin neurotoxicity and to screen possible neuroprotective drugs.

The aims of this project are the:

- Development and optimization of organotypic brain cultures to the bilirubin induced damage field.
- Screening of the regional differential toxicity (among damaged and not damaged brain areas).
- Screening of the differential developmental toxicity.
- Individuation of the molecular mechanisms involved in bilirubin toxicity in the different regions.
- Based on such information, screening and validation drugs available to avoid the bilirubin induced brain damage.

3 Materials and Methods

3.1 Experimental plan

To provide a map of UCB target of post-natal brain during its development, and to acquire a deeper knowledge of mechanisms, as a basis for possible therapeutic intervention, we applied the interface organotypic brain cultures (OBCs) technique[148,153,175] to the study of bilirubin neurological damage. They are slices of a specific region of the brain, conserving its cellular heterogeneity and connections[149] and exhibiting synaptic plasticity and mechanisms of pathological insults comparable to what is obtained *in vivo*, but easily growth and challenged *in vitro*. Thus, they represent an ideal tool to assess *ex vivo* the effect of a compound on a specific CNS region[174,176]. In addition, the possibility to culture slices from animals at different post-natal ages, allows mimicking the CNS maturation. OBCs were prepared from hippocampus, cerebellum, inferior colliculus (pathologically involved in kernicterus)[61,177], superior colliculus and cortex dissected from 2 and 8 days old rat brains. Thus, OBCs were challenged *in vitro* with different UCB concentrations, and viability and biomolecular mechanism of damage screened. Finally, based on this information, drugs possibly already in clinical use in other neurological diseases, have been screened for their ability to reverse the damage.

3.2 Solutions

Hanks' Balanced Salt Solution (HBSS) (Sigma Aldrich, St. Louis, MO, USA): Calcium Chloride ($CaCl_2$) 0.166 g/L, Potassium Chloride (KCl) 0.97 g/L, Potassium Phosphate Monobasic (KH_2PO_4) 0.03 g/L, Magnesium Chloride ($MgCl_2$-$6H_20$) 0.21 g/L, Magnesium Sulfate ($MgSO_4$-$7H_2O$) 0.07 g/L, Sodium Chloride (NaCl) 8.00 g/L, Sodium Bicarbonate ($NaHCO_3$) 0.227 g/L, Sodium Phosphate Dibasic (Na_2HPO_4) 0.12 g/L, Glucose 1.00 g/L.

Hanks' Balanced Salt Solution (HBSS) (Sigma Aldrich, St. Louis, MO, USA): Calcium Chloride ($CaCl_2$) 0.14 g/L, Potassium Chloride (KCl) 0.40 g/L, Potassium Phosphate Monobasic

(KH_2PO_4) 0.06 g/L, Magnesium Chloride ($MgCl_2$-$6H_20$) 0.10 g/L, Magnesium Sulfate ($MgSO_4$-$7H_2O$) 0.10 g/L, Sodium Chloride (NaCl) 8.00 g/L, Sodium Bicarbonate ($NaHCO_3$) 0.35 g/L, Sodium Phosphate Dibasic (Na_2HPO_4) 0.048 g/L.

Heat-Inactivated Horse Serum (ThermoFisher Scientific, Waltham, MA, USA), Basal Medium Eagle (BME) medium (Life technologies Corporation, Grand Island, NY), Fetal Bovine Serum (FBS) (Euroclone, Milan, Italy), L-Glutamine (Life technologies Corporation, Grand Island, NY), Penicillin/Streptomycin (Life technologies Corporation, Grand Island, NY), D-Glucose (Sigma Aldrich, St. Louis, MO, USA)

3.3 "Interface" organotypic brain cultures preparation

Wistar Han^{TM} Rats, at two (P2) and eight (P8) days after birth, were obtained from the animal facility of the University of Trieste, Dept. of Life Sciences, Trieste (Italy). Animal experiments were performed according to the Italian Law (decree 87-848) and European Community directive (86-606-ECC). The study was approved by the animal care and use committee of the University of Trieste and regular communication to the competent Italian Ministry was done. Maximal effort to minimize the number of the animals used and their sufferance was done (RRR rule).

Immediately after sacrifice, brains were removed and maintained in ice cold Gey's Balanced Salt Solution plus D-Glucose 10 mg/mL (dissection medium). These experimental conditions were used to prevent damages that could affect neuronal tissue maintained in a non-physiological condition. Hippocampus (Hip), cerebellum (Cll), frontal cortex (Ctx), superior (SC) and inferior colliculi (IC) were dissected from brains and maintained in ice cold dissection medium until use (Figure 3.1). A McIIwain tissue chopper (Gomshall Surrey, U.K.) was used to cut transversely 300 (SC, IC), 350 (Hip, Ctx) and 425 (Cll) μm slices. Healthy slices were selected from the total amount of tissue prepared by the chopper for structural integrity and were maintained in ice cold dissection medium for 60 minutes to allow cleaning of cutting surfaces from preparation procedure stress. Slices were then transferred to a sterile, semi-porous Millicell-CM inserts (PICM03050, Millipore, Darmstadt, Germany), feed by 1 mL of cold medium and maintained at 37°C, 5% CO_2, 95% of

humidity in a humidified incubator. Many slices (1 to 12 maximum) were cultured in each filter depending of the objects of the experiment.

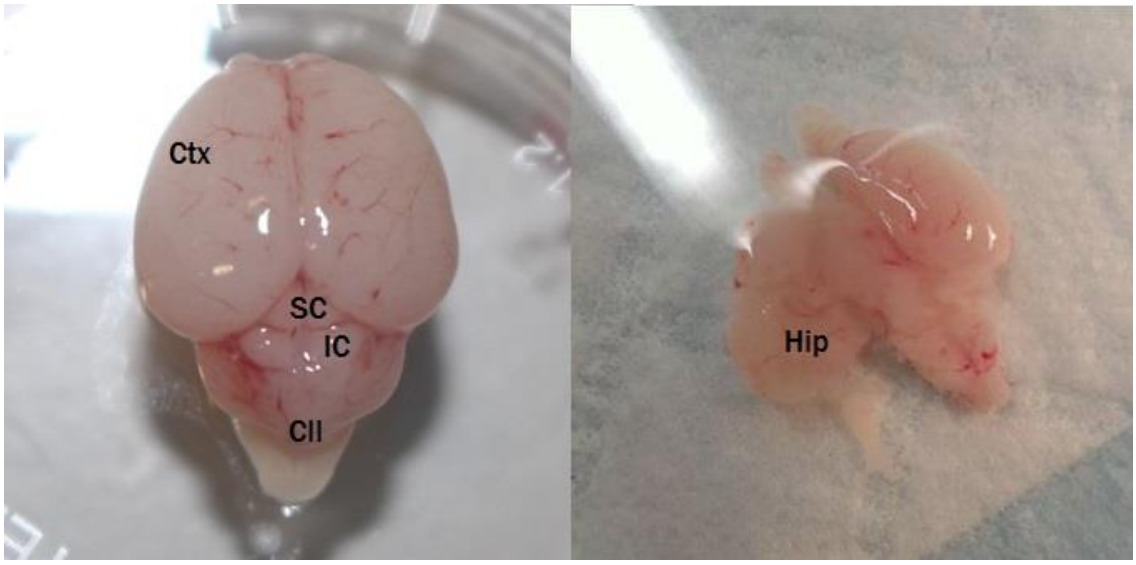

Figure 3.1 Dorsal view of a whole P8 Wistar Han™ rat brain (left) and hippocampus (right).
Ctx: Cortex; SC: Superior colliculi; IC: Inferior colliculi; Cll: Cerebellum; Hip: hippocampus.

3.4 Adapted cultures medium

Standard culture medium (50% BME medium, 25% HBSS, 25% Heat-Inactivated Horse Serum, 1% L-Glutamine, 2% Penicillin/Streptomycin, 10 mg/mL glucose)[153] was modified to allow Bf quantification. Bf adapted medium (Bf-OBCs medium) was composed by 65% of Basal Medium Eagle (BME) medium, 10% of heat-inactivated Fetal Bovine Serum (FBS), 25% of Hank's Balanced Salt Solution (HBSS), 1% L-Glutamine, 2% Penicillin/Streptomycin, 10 mg/mL D-Glucose. The medium was changed the day after cutting to help the slices to recover from the stress of the preparation protocol and every two days thereafter. Slices were maintained in culture for 10 days to test viability during a long period. For treatment, slices were maintained in culture 5 days

before administration of UCB. This time is the minimum necessary to allow recovery from the stress of slicing.

3.5 Bf treatment

Immediately after recovery, slices were challenged for 1 to 24 hours with bilirubin, based on our experience *in vitro*[69,85,178,179]. The concentration of UCB (Sigma Aldrich, St. Louis, MO, USA), dissolved in DMSO (Sigma Aldrich, St. Louis, MO, USA), required to reach the desired Bf in the OBCs medium was quantified according to protocol using a Horseradish peroxidase and hydrogen peroxide, of Roca et al[180]. The concentration of Bf is not related only to the final concentration of albumin in the medium, but also to the characteristics of the medium. The method is based on the oxidation of the bilirubin unbound to albumin by Horseradish peroxidase (Sigma Aldrich, St. Louis, MO, USA) in presence of hydrogen peroxide. Different range of [Bf]/[Albumin] were tested to obtain a mathematical relation that allow Bf determination.

UCB was purified as described by Ostrow and Mukerjee[181], divided into aliquot and stored at -20°C till use. An aliquot of UCB necessary for the experiment was dissolved in DMSO at the final concentration of 5.028 mM and the diluted to the work concentration in medium.

The toxic value of 140 nM Bf was reached adding 22.99 μM UCB (dissolved in DMSO) to our culture media. Additionally, a higher (300 nM Bf, corresponding to 34.18 uM UCB) and a lower 70 nM Bf (15.93 uM UCB) Bf concentration were used, to exacerbate the damage and individuate the precocious sensitivity threshold, respectively. Control slices were exposed to the same final concentration of DMSO needed to dissolve the UCB. For longer treatment, medium was changed and bilirubin was dissolved freshly every day. For shorter treatments, hippocampal cultures were exposed to bilirubin and DMSO for 1, 2, 4, 8, 12 or 24 hours.

3.6 Viability tests

Three viability tests were used to follow the damage induced by the Bf challenging. Each monitored a different typology of damage.

3.6.1 Lactate dehydrogenase release

The amount of total extracellular Lactate DeHydrogenase in medium **(**LDH, membrane leakage) was determined using a CytoTox-ONE™ Homogeneous Membrane Integrity Assay (G7891, Promega, Madison, WI, USA) according to the manufacturer's instructions, with minor modifications. After Bf exposure, supernatant was collected, and reaction started. The medium could be conserved also at -20°C. 100 uL of medium were incubated with 100 uL of the Reaction mix at 29°C for 5 minutes. Reaction was stopped by addition of 50 uL of Stop Solution. The fluorescence ($560_{Ex}/590_{Em}$) was determined using an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA), and the background fluorescence, derived from the medium, subtracted. LDH in challenged slices was expressed as fold respect the control.

3.6.2 Hoechst staining

Evidence of chromatin condensation, marker of cell death by apoptosis, was obtained by administration of 1 μg/mL Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) at the end of the challenging, after 3% PFA fixation[175]. Apoptotic cells were counted at 40X magnification, by a fluorescent microscopy Leica DM2000 (Leica Mycrosystems Srl, Solms, Germany) by three independent reader. At least 3 different fields were analyzed. Results were obtained as percentage of apoptotic cells related to the total number (apoptotic plus unaffected) cells in the control (100%). Final results were expressed as fold of apoptotic cells respect to the control DMSO (= 1).

3.6.3 Mitochondrial activity

Mitochondrial metabolic activity was assessed using a 1-(4, 5-dimethyltiazol-2-yl)-3, 5-diphenylformazan (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). MTT powder was dissolved in PBS at the final concentration of 5 mg/mL. 2-3 slices for each biological repetition were incubated with 0.5 mg/mL of MTT in medium at 37 °C for 1 h, harvested, and the precipitated-salt dissolved in DMSO. Absorbance was detected at 562 nm using a LD 400C luminescence Detector (Beckman coulter, Milan Italy). Results were expressed as percentage of activity related to the control (100%).

3.7 Screening of pathway of damage

3.7.1 Quantitative Real Time PCR of selected markers of bilirubin toxicity

Based on previous data from our laboratory, or publications, specific genes were selected as markers of bimolecular mechanism(s) of neuronal damage[69,92,182–184]. The mRNA expression of genes of interest was analyzed by quantitative real-time PCR. Total RNA was extracted using TRI Reagent® RNA Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA), following the producer's instructions. Complementary DNA (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 3.1). The reaction was performed in a final volume of 15 µL in an iQ5 Bio-Rad Thermal cycler (BioRad Laborato-ries, Hercules, CA, USA). Briefly, 25 ng of cDNA and the corresponding gene-specific sense/antisense primers (250 nM each, with the exception of *Cox2* and *Il1β*, 500 and 750 nM respectively) 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 modified ΔΔCt method[185], taking into account the efficiencies of the individual genes and normalizing the results to the housekeeping genes (Hypoxanthine guanine phosphoribosyl transferase: Hprt, Glyceraldehyde 3-phosphate dehydrogenase: Gapdh)[186,187]. The level of markers mRNA were expressed relative to the reference sample.

Gene	Accession number	Forward	Reverse	AL
Hprt	NM_012583.2	AGACTGAAGAGCTACTGTAATGAC	GGCTGTACTGCTTGACCAAG	163
Gapdh	NM_017008.2	CTCTCTGCTCCTCCCTGTTC	CACCGACCTTCACCATCTTG	87
Tnfα	NM_012675.2	CAACTACGATGCTCAGAAACAC	AGACAGCCTGATCCACTCC	172
Il1β	NM_031512.2	AACAAGATAGAAGTCAAGA	ATGGTGAAGTCAACTATG	137
Il6	NM_012589.1	GCCCACCAGGAACGAAAGTC	ATCCTCTGTGAAGTCTCCTCTCC	161
Il4	NM_201270.1	GCAACAAGGAACACCACGGAGAAC	CTTCAAGCACGGAGGTACATCACG	96
Il10	NM_012854.1	CCAATGGTGTCCTTTCACTTG	AACAAACTGGTCACAGCTTTC	189
Cox2	NM_017232.3	CTTTCAATGTGCAAGACC	TACTGTAGGGTTAATGTCATC	92
Hmox1	NM_012580.2	GGTGATGGCCTCCTTGTA	ATAGACTGGGTTCTGCTTGT	76
Srxn1	NM_001047858.3	AAGGCGGTGACTACTACT	TTGGCAGGAATGGTCTCT	85

Table 3.1 Primer sequences designed for the mRNA quantification.

AL: amplicon length, *Hprt* hypoxanthine guanine phosphoribosyltranserfase, *Gapdh* glyceraldehyde 3-phosphate dehydrogenase, *Tnfα* tumor necrosis factor alpha, *Il1β* interleukin 1 beta, *Il6* interleukin 6, *Il4* interleukin 4, *Il10* interleukin 10, *Cox2* cyclooxygenase 2, *Hmox1* Heme oxygenase 1, *Sxrn1* sulfiredoxin 1.

3.7.2 Glutamate quantification in culture media

Because no reliable genes to monitor glutamate excitotoxicity by quantitative Real Time PCR were individuated, the amount of extracellular glutamate was quantified using a Glutamate Assay Kit (MAK004, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's

instructions. After Bf exposure, supernatant was collected, and reaction started. 30 uL of culture medium were incubated with the reaction mix at 37°C in a 96-well plate. A standard curve of glutamate in the range of 2-10 nmol/well was prepared to quantify the total amount of glutamate released. A blank was prepared using the same reaction mix without the enzyme provided by the kit. The absorbance (450 nm), proportional to the glutamate present, was determined using an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). Glutamate release in challenged slices medium was expressed as fold respect the control.

3.7.3 Histochemical analysis of damage

3.7.3.1 Immunofluorescence

Glial fibrillary Acidic Protein (GFAP) immunofluorescence was performed to evaluate morphological changes associated with UCB exposure. Slices were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes at 4°C and then washed twice with PBS and PBS plus Glycine 0.1M (Sigma-Aldrich, St. Louis, MO, USA) to eliminate residues of paraformaldehyde. After washing, slices were pre-incubated 1 hours in 1% normal goat serum (NGS, Sigma Co., Saint Louis, Missouri, USA) and 0.1% triton-X100 (Sigma Co., Saint Louis, Missouri, USA) in PBS (blocking solution). Then, slices were incubated 2 hours at 37°C with primary antibody Polyclonal Rabbit Anti-Glial Fibrillary Acidic Protein (4.8 ug/mL, Z0334, Dako Denmark A/S) in blocking solution. Slices were then rinsed three times in PBS for 5 minutes and subsequently incubated with FITC-Conjugated Swine Anti-Rabbit secondary antibody (1:2000, F0205, Dako Denmark A/S) 2 hours at RT in blocking solution. Slices were then washed twice in PBS for 5 minutes and incubated 10 minutes at RT with Hoechst 33258 (2 ug/mL, Sigma-Aldrich, St. Louis, MO, USA), washed and mounted with Fluorescent Mounting Media (HC08, Millipore, Darmstadt, Germany). Fluorescence was detected by fluorescent microscopy Leica DM2000 (Leica Mycrosystems Srl, Solms, Germany).

3.7.3.2 Histology

At the end of the challenging, OBCs were immediately fixed in neural buffered formalin 4% (Kaltek, Padova, Italy), then embedded in paraffin. Tissues were sectioned at a thickness of 3 um, by a microtome (Microm-hm 340e- BioOptica, Milan, It), affixed on the slice and dried at 60°c for 1 hrs. Sections were stained with the hematoxylin & eosin (H&E) protocol, performed by a Leica ST5020 Multistainer (Leica Microsystem, Milano, It) to assess the presence of apoptosis, necrosis, microgliosis, edema, and cell loss. Staining was performed as follow: Hematoxylin coloration (Xylol 2x5'; Ethanol 2x4'; H_2O 1x3'; Hematoxylin 12'; H_2O 2x6'; Eosin 1x 1,30'; Ethanol 2x3'; Xylol 1x3' plus 1x2'. Histology was read by two independent pathologists, blinded to experimental design and treatment groups at the Department of Anatomy of the local Teaching Hospital of Cattinara. Pictures were collected by a D-Sight plus image digital microscope & scanner (Menarini Diagnistics, Firenze, Italy).

3.7.4 PI staining

Propidium Iodide staining was performed to localize dying cells (no discrimination between apoptosis and necrosis). To label the nuclei of dead neurons, 2 ug⁄mL PI (Sigma, Louis St, MO, USA) was added to the wells of the culture for 60 min. After incubation, digital imagines of PI fluorescence were obtained by fluorescent microscopy Leica DM2000 (Leica Mycrosystems Srl, Solms, Germany).

3.8 Neuroprotective drugs

3.8.1 Screening of neuroprotective drugs

In order to evaluate the possible biological effects of candidate drugs, hippocampal slices from P8 animals, were exposed to: 1) DMSO alone, as control; 2) 140 nM Bf; 3) drugs alone; 4) 140 nM Bf and drugs in a co-treatment, for 24 hours. Drugs used were curcumin, N-acetylcysteine

(NAC), magnesium chloride, riluzole, indomethacin, creatine[188–190]. Minocycline (60 uM, Sigma-Aldrich, St. Louis, MO, USA) was selected as golden standard, based on published *in vivo* [139,140] results demonstrating its efficacy in reverting the bilirubin induced damage.

3.8.2 Validation of selected neuroprotective drugs

In order to evaluate the biological effects of selected drugs, slices from all regions from P8 animals, were exposed to 1) DMSO alone, as control; 2) 140 nM Bf; 3) drugs alone; 4) 140 nM Bf and drugs together, for 24 hours; 5)140 nM Bf and all the drugs selected together. Drugs used were curcumin (anti-oxidant, 50 uM, C7727, Sigma-Aldrich, St. Louis, MO, USA)[191,192], magnesium chloride (glutamate channel blocker, 10 mM, M4880, Sigma-Aldrich, St. Louis, MO, USA)[193,194] and indomethacin (anti-inflammatory, 50 uM; Promedica CHIESI)[195,196].

3.9 Statistical analysis

Data was analyzed with Graph pad prism 5 for Windows (GraphPad Software). 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 three-five independent biological repetitions.

4 Results

4.1 Optimization of OBCs

Cultures medium was changed from those frequently used in literature, containing 25% horse serum. The characteristic of this serum, derived from an adult animal, do not allow the quantification of free bilirubin in medium by the peroxidase method, due to the strong intrinsic peroxidase activity and high album content[180]. On the contrary, FBS is derived from fetal animal and is characterized by low level of peroxidase activity, albumin and antibodies. To allow Bf quantification, medium composition was modified by the substitution of 25% of horse serum with 10 % of FBS, normally used for cell cultures and Bf treatment[69,92]. Viability of the slices in the Bf OBCs medium was evaluated by LDH assay, as the most used and informative technique to study viability, at least during the first 9 days of cultures, necessary for the purpose of the work.

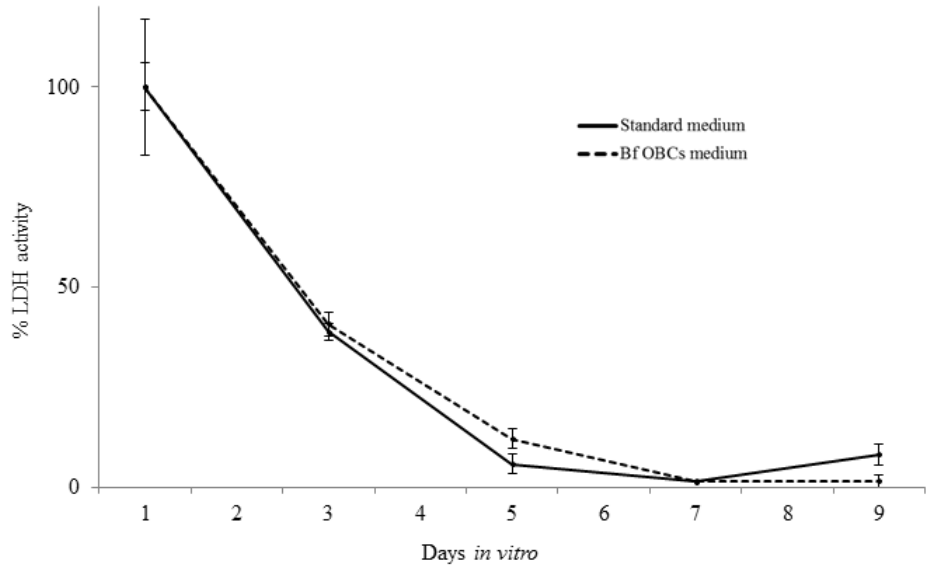

Figure 4.1 Comparison of viability recovery in the two culture medium.

Graph showing comparison of viability recovery from preparation protocol (LDH test) of slices from Ctx P8 during 10 days of cultures in usual OBCs medium (continuous line) and in the Bf adapted medium (dash line). Results are expressed as percentage (1st day = 100%).

As shown in Fig. 4.1, the organotypic brain cultures showed a relevant LDH release immediately after cutting (reflecting the slicing procedure stress), decreasing to normal levels in about 5 days (recovery time). No differences were observed between OBCs cultured in standard medium *vs.* Bf OBCs medium. No differences were detected for all regions or post-natal age of animals used (data not shown). Challenging was initiated only after OBCs recovery from slicing procedure stress, at the 6^{th} day *in vitro*.

4.2 Regional sensitivity to bilirubin toxicity

Three viability tests were used to follow the damage induced by the Bf challenging. Each monitored a different typology of damage: membrane leakage (LDH assay), apoptosis induction (Hoechst staining) and impairment of mitochondrial activity (MTT test).

4.2.1 Membranes leakage

Membrane leakage was evaluated in OBCs at the end of the 24 hours treatments with three different concentrations of Bf. Culture medium was collected and the reaction started

OBCs obtained from 2-days-old animals (Figure 4.2, left) did not show statistically relevant increase in LDH activity under 70nM Bf. 140nM Bf resulted in toxicity to Hip (3.5fold, $p<0.01$ *vs.* DMSO same region), IC (3.2fold, $p<0.01$ *vs.* DMSO same region) and Ctx (4.4fold, $p<0.001$ *vs.* DMSO same region). No effects were observed in Cll e SC. Moving to the 300nM Bf, a dose dependent effect was observed in IC and Ctx (2 and 2.5fold *vs.* 140nM Bf respectively, both $p<0.05$; and 5.9fold and 10.8fold *vs.* DMSO respectively, both $p<0.001$), with the latter showing the maximal damage. No dose effect was observed in Hip (3.8fold, $p<0.001$ *vs.* DMSO), while the maximal challenging (300nM Bf) was needed to induce membrane integrity loss in Cll (3.25fold, $p<0.01$) and SC (1.8fold, $p<0.05$).

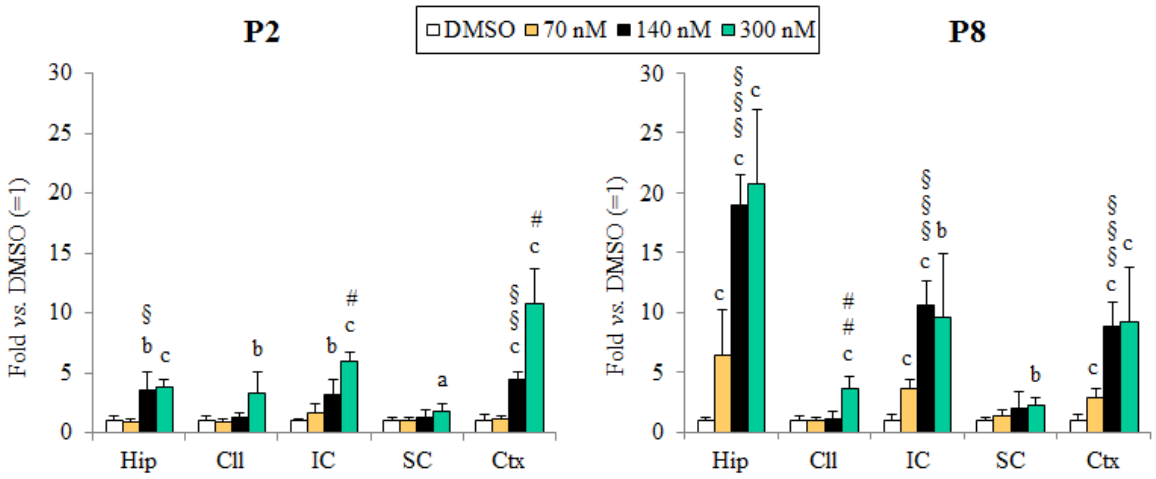

Figure 4.2 Membranes leakage (LDH) of P2 and P8 OBCs challenged with 70, 140 and 300 nM Bf. Membranes leakage is expressed as fold *vs.* control (DMSO = 1). Data are expressed as means ±S.D. of 3-5 biological repetitions. Statistical relevance: a p< 0.05, b p<0.01, c p<0.001 *vs.* DMSO; § p<0.05, §§ p<0.01, §§§ p<0.001 70 nM *vs.* 140 nM; # p<0.05, ## p<0.01, ### p<0.001 140 nM vs 300 nM.

In organotypic cultures from 8-days-old animals (Fig. 4.2, right), irrespective to the bilirubin concentration, the Hip displayed the maximal toxicity, followed by IC = Ctx. In these three regions LDH activity was statistically increased already with 24 hours challenging with 70nM Bf (6.4, 3.6 and 2.8 fold, respectively, all p<0.001). Increasing of bilirubin concentration (to 140nM) enhanced the damage in Hip, IC and Ctx (to 19, 10.6 and 8.85 fold, respectively, all p<0.001 *vs.* DMSO; all p<0.001 *vs.* 70nM Bf). No dose-dependent increase in LDH release was observed moving to the maximal challenging (300nM Bf) in Hip, IC and Ctx. Inversely, only under 24 hours of 300nM Bf, a statistically relevant changes started to be detected in Cll (3.6 fold, p<0.001) and SC (1.8 fold, p<0.01).

4.2.2 Apoptosis

Hoechst staining was used to mark all nuclei of OBCs at the end of the treatments. A representative picture of the chromatin condensation, a characteristic marker of apoptosis, is shown in Figure 4.3.

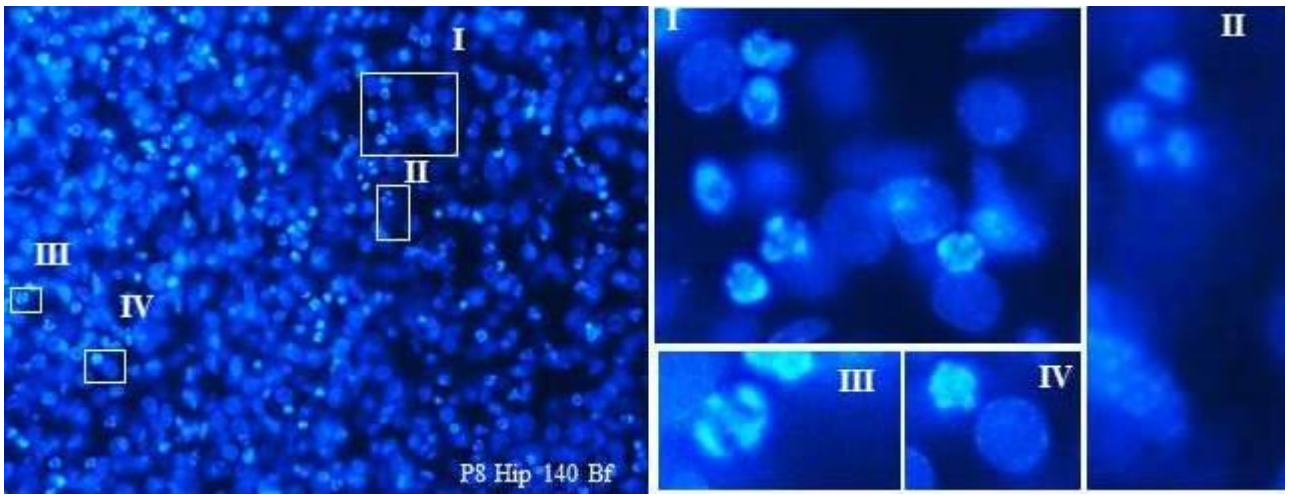

Figure 4.3 Representative images of Hoechst staining to mark apoptotic cells.
Left side, representative picture of a P8 Hip OBCs with the nuclei stained in blue by Hoechst. Right side: I, II, III and IV magnification images of single apoptotic cells.

In OBCs from 2-days-old rats (Figure 4.4, left), 70nM was already sufficient to generate a statistically relevant apoptosis only in Hip (2.16fold *vs.* control, $p<0.001$), with a clear dose effect increasing to 140nM (2.3fold *vs.* 70, $p<0.05$; $p<0.01$*vs.* DMSO) and 300nM Bf (12fold *vs.* DMSO, $p<0.001$). At 140nM Bf, apoptosis started to be significant in Ctx (2.22fold, $p<0.05$) without any increase moving to 300nM; and especially in IC (10.4fold, $p<0.01$ *vs.* control, 3.1 fold *vs.* 70nM Bf, $p<0.05$), again with no changes moving to 300nM Bf. The extreme challenging (300nM Bf) was needed to induce apoptosis in Cll (1.95fold *vs.* control, $p<0.05$) and SC (4.5fold *vs.* control, $p<0.05$).

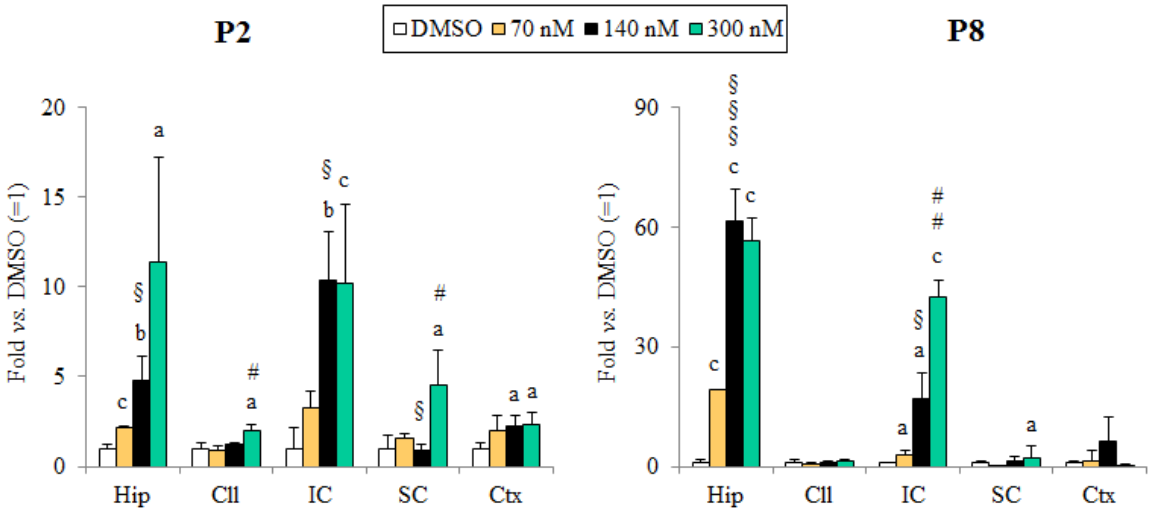

Figure 4.4 Apoptosis of P2 and P8 OBCs challenged with 70, 140 and 300 nM Bf.

Apoptosis is expressed as fold *vs.* control (DMSO = 1). Please note differences in scale bar: up-to 20 in P2 and up-to 90 in P8 preparations. Data are expressed as means ±S.D. of 3-5 biological repetitions. Statistical relevance: a p< 0.05, b p<0.01, c p<0.001 vs DMSO; § p<0.05, §§ p<0.01, §§§ p<0.001 70 nM *vs.* 140 nM; # p<0.05, ## p<0.01, ### p<0.001 140 nM *vs.* 300 nM.

In slices from more developed animals (P8 - Fig 4.4, right), hippocampus confirmed its high sensitivity to low bilirubin dose of 70nM (p<0.001), increasing at 140 = 300 nM challenging (both about 61fold *vs.* control, p<0.001, and 3.7fold *vs.* 70nM Bf, p<0.01). Inferior colliculus was also damaged after each treatments, but with a dose dependent effect from 70 (2.9fold *vs.* control, p<0.05), thought 140 (17fold *vs.* control, p<0.05 and 5.8fold *vs.* 70 nM Bf, p<0.05), to 300nM Bf (2.5fold *vs.* 140 nM, p<0.01). No significant bilirubin induced apoptosis was observed in SC, Cll and Ctx.

4.2.3 Mitochondrial activity

Despite some variation in MTT activity (Figure 4.5, left), no relevant changes were detected in P2 OBCs exposed to all Bf concentration, except for a 25% decrease (p<0.05, indication of sufferance) in SC exposed to 140nM Bf.

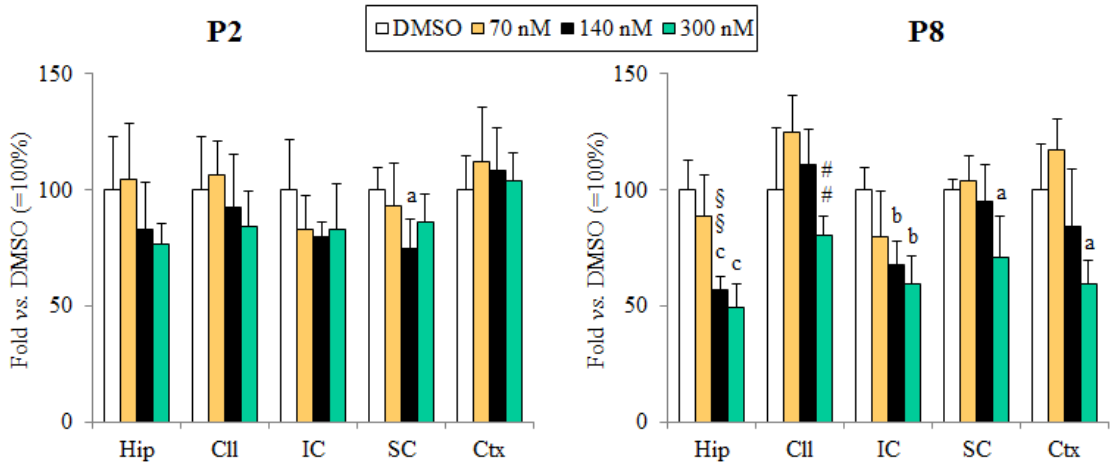

Figure 4.5 Mitochondrial activity of P2 and P8 OBCs challenged with 70, 140 and 300 nM Bf.
Mitochondrial activity is expressed as percentage *vs.* control (DMSO = 100%). Data are expressed as means ±S.D. of 3-5 biological repetitions. Statistical relevance: a p< 0.05, b p<0.01, c p<0.001 *vs.* DMSO; § p<0.05, §§ p<0.01, §§§ p<0.001 70 nM *vs.* 140 nM; # p<0.05, ## p<0.01, ### p<0.001 140 nM *vs.* 300 nM.

In P8 OBCs (Fig 4.5, right), a relevant reduction in mitochondrial metabolism was detected in Hip and IC exposed to a Bf of 140 and 300nM (Hip both concentrations p<0.001, IC both concentrations p<0.01). SC and Ctx displayed significant mitochondrial activity reduction only when exposed to the maximal dose (300nM Bf, both p<0.05). Cll was insensible to all Bf concentrations (*vs.* DMSO).

4.3 Developmental sensitivity to bilirubin toxicity

To highlight the developmental sensitivity to bilirubin toxicity, viability was expressed as ratio of P8/P2 results (Table 4.1). Ratio bigger than 1 highlights an increased developmental sensitivity in the LDH and Hoechst staining results. The opposite is for MTT activity.

LDH					
	Hip	**Cll**	**IC**	**SC**	**Ctx**
70nM	7.11 [a]	1.12	2.20 [a]	1.43	2.40 [b]
140nM	5.44 [c]	0.79	3.35 [b]	1.51	2.03 [a]
300nM	5.51 [b]	1.12	1.61	1.22	0.85
Hoechst					
70nM	8.95 [c]	0.61	0.89	0.13 [b]	0.77
140nM	12.70 [c]	0.75	1.65	1.68	2.92
300nM	4.97 [c]	0.65	4.19 [c]	0.51	0.12 [a]
MTT					
70nM	0.85	1.17	0.96	1.12	1.04
140nM	0.69 [a]	1.18	0.86	1.27	0.78
300nM	0.64 [a]	0.95	0.71	0.83	0.57 [b]

Table 4.1 P8/P2 ratio of viability tests.

LDH (upper), Hoechst (middle) and MTT (lower) viability was expressed as ratio of P8 results divided for P2 results. Ratio bigger than 1 describe an increased sensitivity during the development for LDH and Hoechst data, the opposite for the MTT test. Statistical relevance *vs.* DMSO a: $p<0.05$; b: $p<0.01$; c: $p<0.001$.

4.3.1 P8/P2 membrane leakage in developmental sensitivity

LDH (Table 4.1, upper panel) revealed a clear developmental increase in sensitivity in the hippocampus (P8 higher than P2) under all the Bf concentration used in this study ($p<0.05$, $p<0.001$ and $p<0.01$, at 70, 140 and 300nM Bf challenging, respectively). A similar behavior was also observed in the IC, but limited to the 70 and 140nM Bf concentrations ($p<0.05$ and $p<0.001$,

respectively) and Ctx ($p<0.01$ and $p<0.05$, 70 and 140nM Bf respectively). No increased developmental sensitivity to bilirubin toxicity was observed in cerebellum and superior colliculi.

4.3.2 P8/P2 apoptosis in developmental sensitivity

As shown in Table 4.1, central panel, at all bilirubin concentration, Hip sensitivity was increased in P8 *vs.* P2 OBCs (all $p<0.001$), while in IC an increase was observed only under the maximal challenging ($p<0.001$). Differently, P8 SC and Ctx were less sensible than P2 preparations to 70 and 300nM Bf, ($p<0.01$ and $p<0.05$). No developmental difference was observed in Cll.

4.3.3 P8/P2 mitochondrial activity in developmental sensitivity

Developmental sensitivity (8day after birth higher than 2day) was observed in Hip exposed to 140 and 300nM (both $p<0.05$) and in Ctx exposed to the higher concentration of 300nM ($p<0.01$). While IC, Cll and SC showed no differences between the two post-natal developmental stages (Table 4.1, lower panel).

4.4 Biomolecular mechanism involved in bilirubin toxicity

Different analysis (transcriptomic, enzymatic, and histological) were performed in OBCs challenged with bilirubin to screen the biomolecular mechanisms of damage. The screening was performed at the post-natal age showing the maximal sensitivity (P8) challenged with the well-known toxic Bf concentration (140nM).

4.4.1 Oxidative stress

The possible involvement of oxidative stress in the bilirubin induced damages was evaluated. The expression of gene activated in response to oxidative stress was measured in each region at the end of treatment and reported in Table 4.2.

	Hip	**Cll**	**IC**	**SC**	**Ctx**
Hmox1	3.94±1.54 [a]	2.31±1.31	3.43±1.40 [a]	3.29±0.67 [b]	1.98±1.15
Srxn1	17.02±2.09 [c]	3.37±1.53 [a]	7.56±4.76 [a]	13.92±3.41 [b]	3.02±0.74 [a]

Table 4.2 Analysis of marker genes expression for oxidative stress.
Values are expressed as fold of change ± standard deviation *vs.* DMSO. 3-5 biological repetitions were considered. Statistical relevance, a: $p<0.05$; b: $p<0.01$; c: $p<0.001$.

Inducible heme oxygenase 1 mRNA expression (*Hmox1*) was significantly up-regulated in Hip, IC and SC ($p<0.05$ in Hip and IC; $p<0.01$ in SC). Sulfiredoxin (*Srxn1*), responsible of the re-activation of the antioxidant peroxidases peroxiredoxins, was significantly upregulated in all regions (Hip $p<0.001$; Cll, IC and Ctx $p<0.05$; SC $p<0.01$).

4.4.2 Inflammation

The role of inflammation was investigated by analyzing the expression of pro-inflammatory cytokines gene (*Tnfα*, *Il1β* and *Il6*) and the prostaglandin-endoperoxide synthase 2 or cyclooxygenase 2 (*Cox2)*, involved in prostaglandin production. Data are showed in Table 4.3.

	Hip	Cll	IC	SC	Ctx
Tnfα	5.53±0.48 [c]	1.10±0.29	1.27±0.49	2.35±0.27 [c]	1.06±0.71
Il1β	8.40±2.14 [b]	4.31±2.54	13.69±6.82 [a]	9.32±4.18 [b]	4.10±3.00
Il6	361.31±121.43 [c]	7.03±1.12 [c]	87.99±45.59 [b]	281.89±252.88 [a]	14.36±7.32 [b]
Cox2	52.00±10.39 [c]	27.64±13.74 [a]	98.01±41.37 [b]	1.98±1.58	7.61±5.40 [a]

Table 4.3 Analysis of expression of pro-inflammatory gene.
Values are expressed as fold of change ± standard deviation *vs.* DMSO. 3-5 biological repetitions were considered. Values are fold change ± standard deviation *vs.* DMSO. Statistical relevance, a: $p<0.05$; b: $p<0.01$; c: $p<0.001$.

In Hip bilirubin treatment up-regulated the pro-inflammatory *Tnfα, Il6* (both $p<0.001$), *Il1β* ($p<0.01$) and *Cox2* ($p<0.001$). A similar pattern was detected in the IC (*Il6* and *Cox2* $p<0.01$; *Il1β* $p<0.05$). In Cll *Tnfα* or *Il1β* modulation was not observed, in spite of a *Cox2* ($p<0.05$), *Il6* ($p<0.001$) significant up-regulation. In SC and Ctx I detected the up-regulation of *Tnfα, Il1β, Il6* and *Il6* and *Cox2*, respectively.

Anti-inflammatory cytokines was also studied to evaluate the capacity of the tissue to counteract the damages. Data were showed in Table 4.4. *Il10* was upregulated in all regions with the exception of SC. On the contrary, *Il4* was overexpressed only in IC, with a non-significant trend of increased expression in Hip and Ctx.

	Hip	Cll	IC	SC	Ctx
Il10	5.02±1.03 [b]	2.69±0.64 [a]	3.18±0.37 [a]	1.12±1.21	1.90±1.21
Il4	4.96±3.30	0.75±0.89	2.55±0.98 [a]	0.42±0.26	6.65±4.78

Table 4.4 Analysis of expression of anti-inflammatory marker gene.

Values are expressed as fold of change ± standard deviation *vs.* DMSO. 3-5 biological repetitions were considered. Statistical relevance, a: $p<0.05$; b: $p<0.01$; c: $p<0.001$.

4.4.3 Glutamate release

Additionally, glutamate excitotoxicity involvement[197] was assesses by the quantification of glutamate release in the culture medium (Figure 4.6). Relevant release was detected in Hip ($p<0.01$) and IC ($p<0.05$) after bilirubin toxicity challenging.

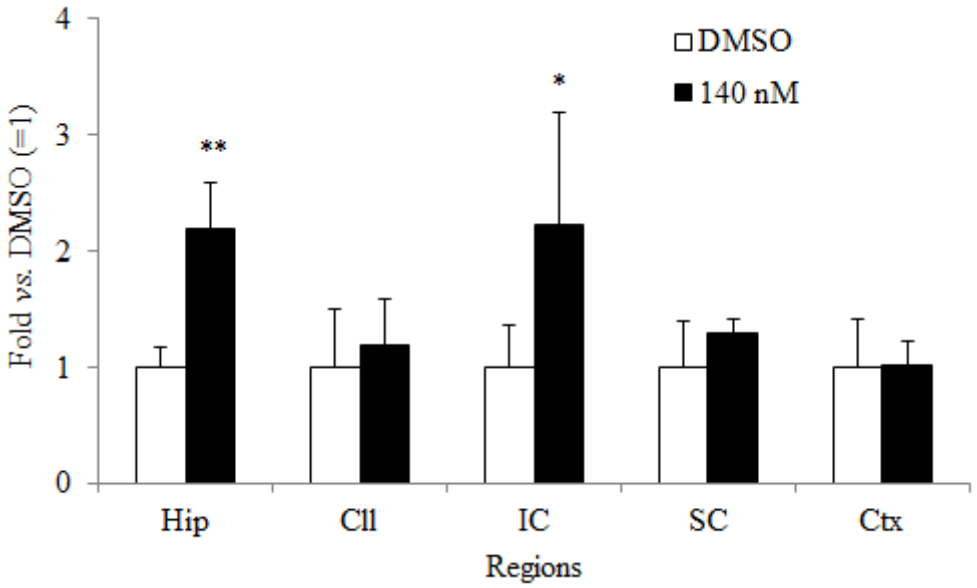

Figure 4.6 Graph representing glutamate release in medium.

Values are expressed as fold of change ± standard deviation *vs.* DMSO. 3-5 biological repetitions were considered. Statistical relevance, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

4.4.4 Immunofluorescence

Glial fibrillary acidic protein (GFAP, Figure 4.7) staining was used to visualize possible astrocytes reaction to bilirubin stimuli (astrogliosis). Representative normal astrocytic morphology and network are shown in Figure 4.7-A (P8 SC, DMSO), where the defined, continuous, and filamentous shape of the astrocyte cytoskeleton is well appreciable. No evident increased GFAP signal intensity, increased astrocytes number, or scare formation, markers of astrocytes activation (astrogliosis) were observed. In contrast, after 140nM Bf challenging, astrocytes in all section demonstrated morphological alterations, also if of different extent depending from the brain areas under analysis. The most dramatic consequences were observed in the Hip (Figure 4.7-B), presenting a clear astrocytic body and filament volume reduction, and fragmentation of dendrites (clasmatodendrosis). A lower extent of astrocytes sufferance was also observed in IC (Figure 4.7-D), Cll (Figure 4.7-C), and rarely in the SC (Figure 4.7-F). In cortex we noticed a high variability among preparations, with some biological repetitions presenting relevant damage (Figure 4.7-E1) others unaffected (Figure 4.7-E2). This technical trouble affected all the analysis of this brain area.

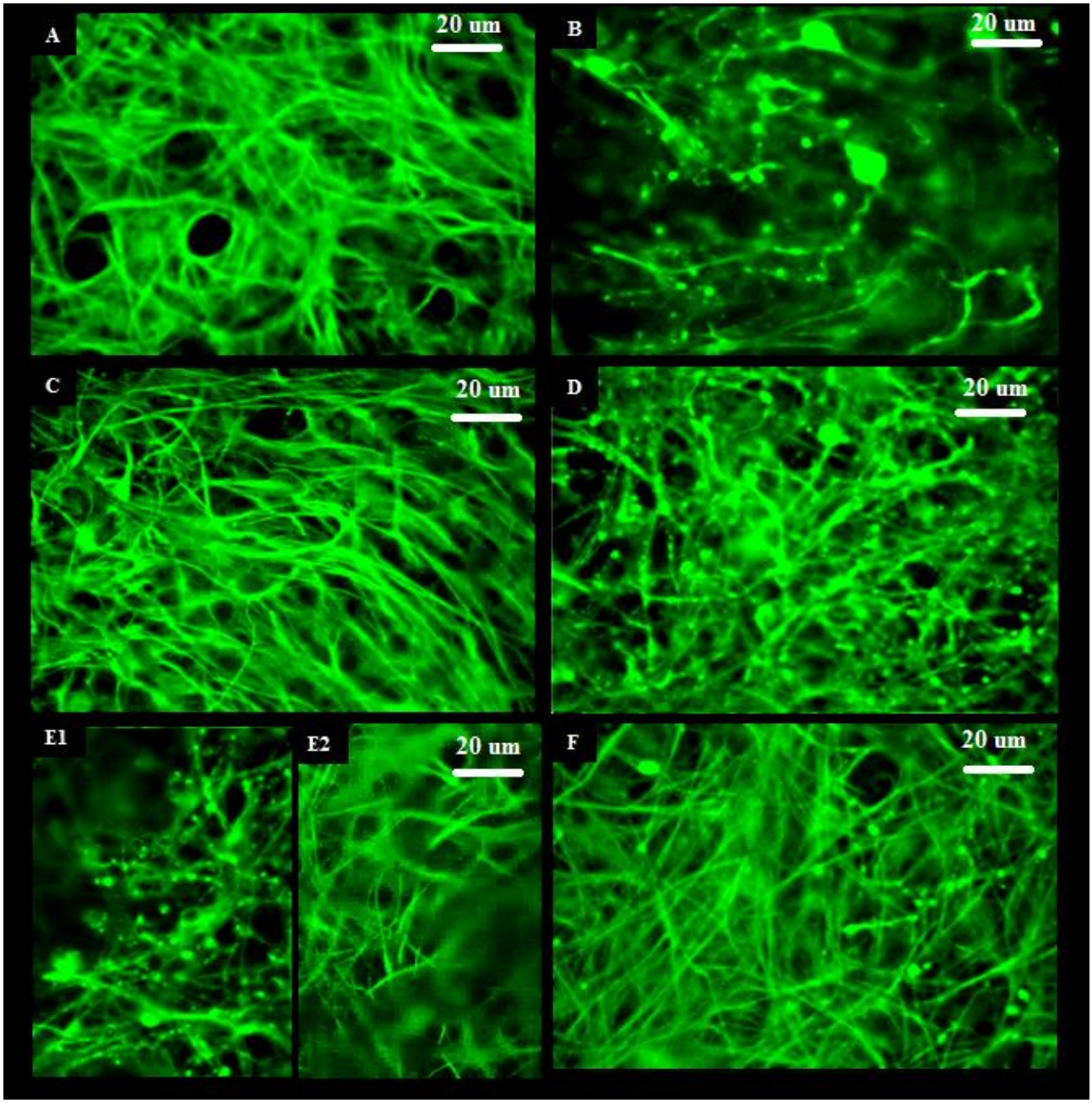

Figure 4.7 Glial fibrillary acidic protein (GFAP, green) staining of astrocytes processes on OBCs.
Representative GFAP staining in control (A) SC DMSO treated slice. Bf challenged hippocampus (B) showing the destruction of the astrocytic network in this brain structure (greens blots and dots are remnants of astrocytes). D) A relevant reaction to Bf challenging is observable in IC, while a minor degree of bilirubin toxicity is also testified in challenged Cll and SC (C and F, respectively, 140 nM Bf, P8. Variable effect is present in Ctx OBCs (E1-E2, 140 nM Bf, P8). Images are all 63X zoomed; scale bar on the images.

4.4.5 Histology

The high damage on the hippocampus was confirmed also by histological evaluation (Figure 4.8 a-b). A considerable tissue compromising with large intracellular spaces, neuronal cell loss, apoptosis and some degree of necrosis, oedema, fibrillary matrix components, presence of foam cells and microgliosis was also noticed. An important cellular death was present also in the cortex (decreased cellular density, with a general weak nuclear staining) (Figure 4.8 e-f). Similarly to Hip, but a lower extent, IC showed large intracellular spaces, apoptotic bodies and foam cells (Figure 4.8 c-d). Some degree of degeneration was detected also in the Cll OBCs, with peri-cellular edema, necrosis, and a barely detectable increase of apoptosis (Figure 4.8 g-h). The SC was only weakly changed by bilirubin exposure, presenting a negligible increase of cells with vacuoles and fibrillary matrix components with cellular debris, in respect to DMSO exposed SC OBCs (Figure 4.8 i-j). Low degree of fibrosis and apoptosis was observed also in SC OBCs treated with DMSO.

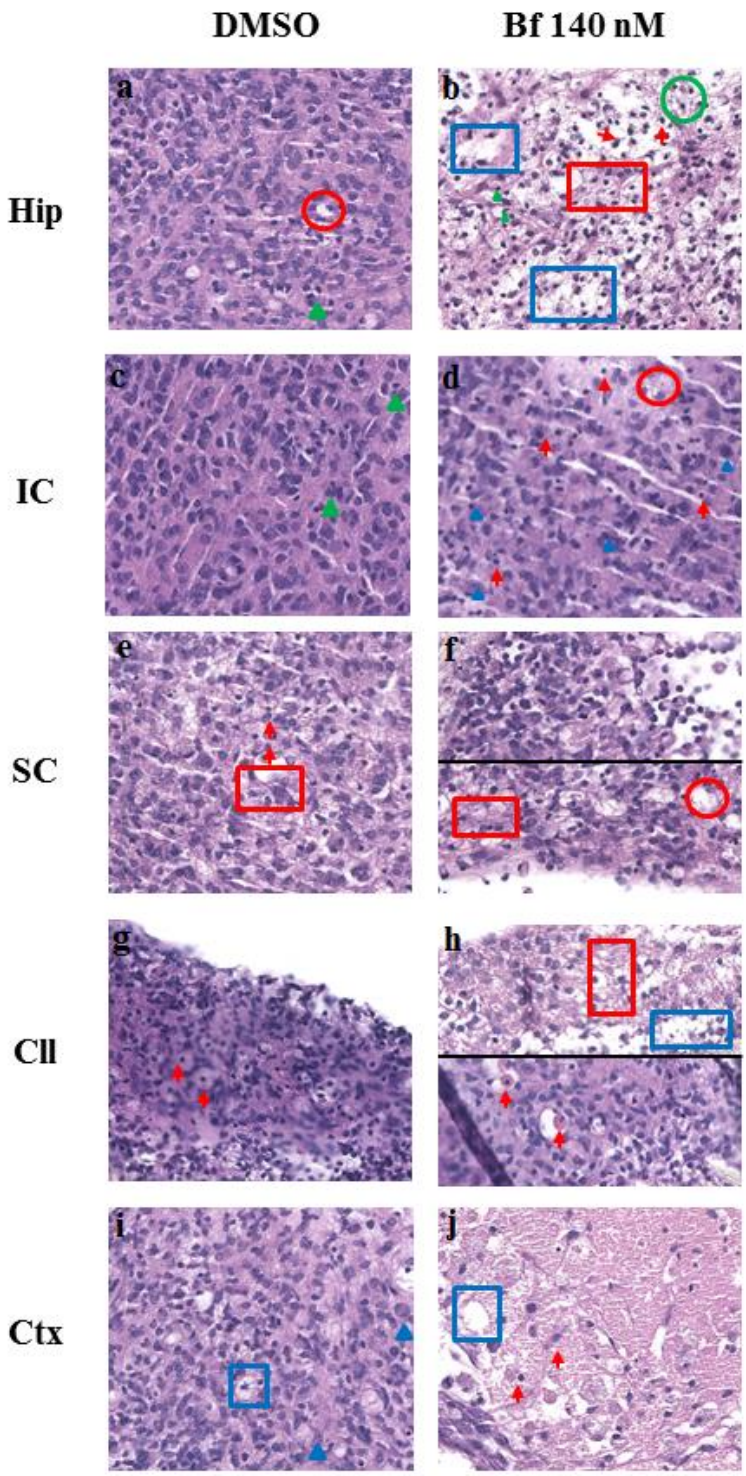

Figure 4.8 Histological analysis of OBCs treated with Bf 140 nM.

Red arrow: apoptosis. Blue triangle: swollen nuclei. Green triangle: microgliosis. Red circle: foam cells. Red square: fibrosis. Green circle: inflammation. Blue square: oedema. All images are 63x zoomed.

4.5 Additional data

4.5.1 Cerebellum: long treatment

Cerebellum is considered the landmark of bilirubin brain damages in Gunn rat, when analyzed after 9 day of life[45,62,85]. Because 24hrs of bilirubin exposure was not able to induce any relevant damage, cerebellar sensibility was tested in OBCs during a long treatment of 4 days of Bf 140 nM.

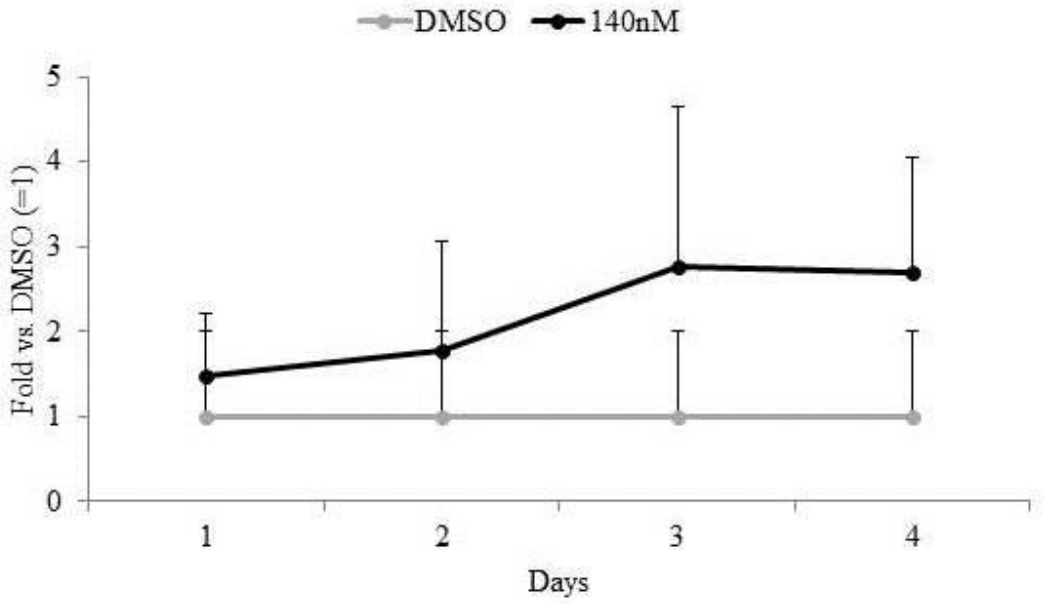

Figure 4.9 Membrane leakage (LDH) of P8 cerebellar OBCs.
Membranes leakage is expressed as fold *vs.* control (DMSO = 1). Data are expressed as means ±S.D. of 3-5 biological repetitions.

A preliminary analysis showed an increased release of LDH in medium at the 3rd and 4th days of treatment, despite that never reaching the statistical relevance (Figure 4.9).

4.5.2 PI staining

PI was used to stain dying cells (both apoptosis and necrosis – Figure 4.10). No clear images were obtained, with the exception of a strong red fluorescence of the CA3 region of the hippocampal slice from P2 rat.

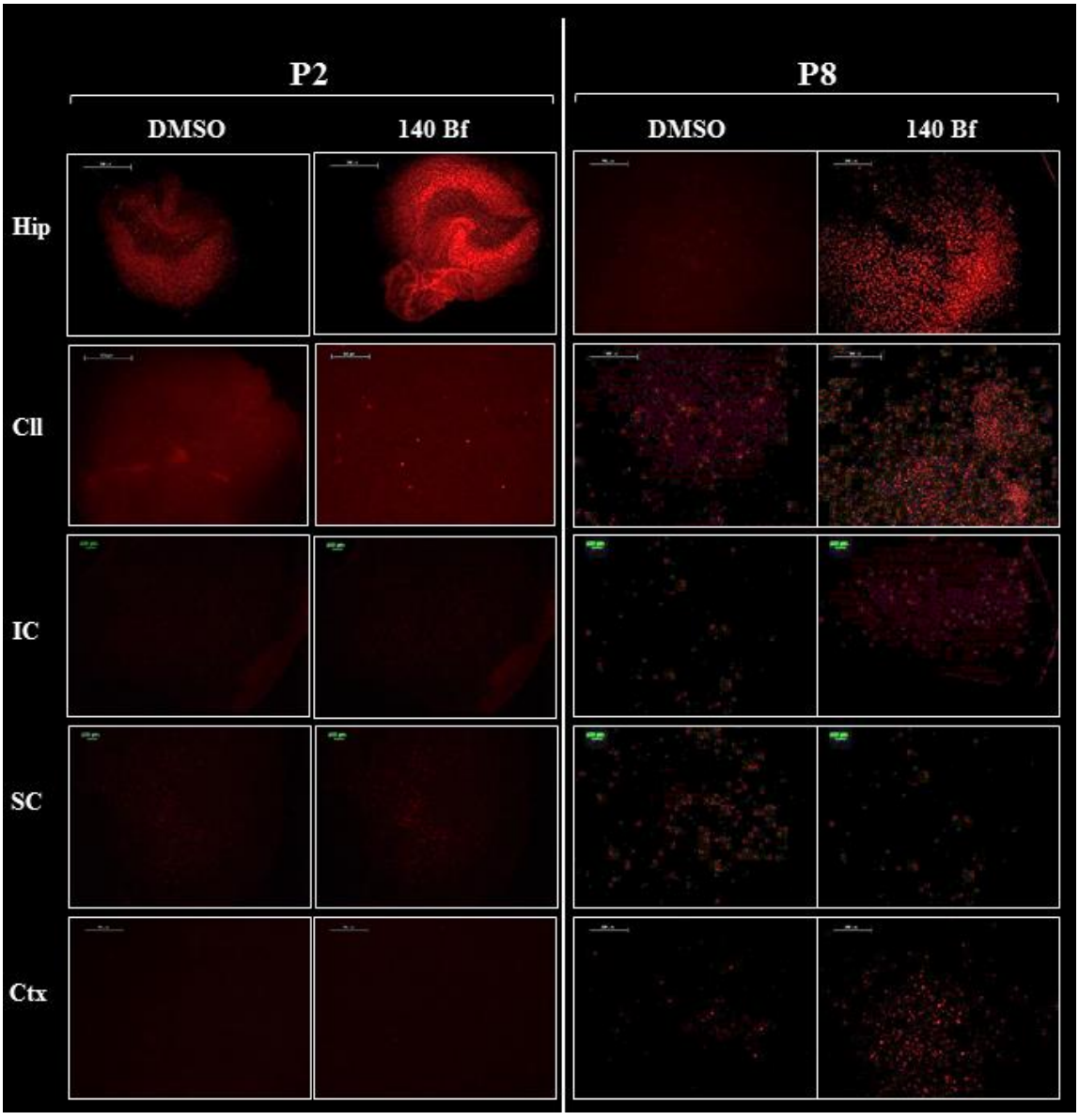

Figure 4.10 PI staining of P2 and P8 OBCs.

4.5.3 Time course characterization of hyperbilirubinemia-induced damage in rat organotypic hippocampal cultures

Complementary to the primary purpose of this work, a time course characterization of the bilirubin damages was performed in P8 hippocampal slices. The work was performed together with Michele Montrone, student of the International Master Degree in Neuroscience (University of Trieste) for its thesis. Hippocampal slices were treated for 1, 2, 4, 8, 12 and 24 hours to recognize the time of development of the mechanisms of toxicity through quantification of different biomarkers.

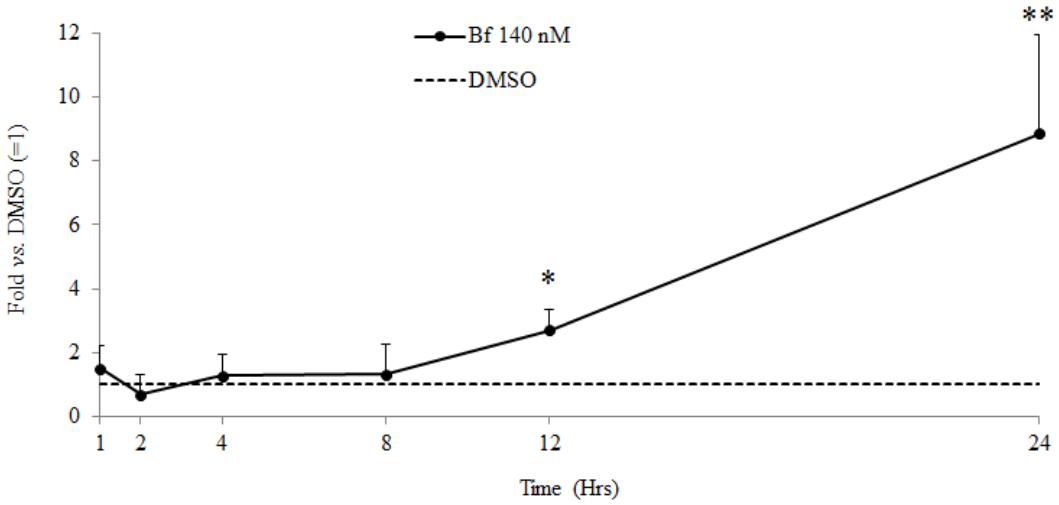

Figure 4.11 LDH release in medium after 140 nM Bf treatment.

Values are expressed as fold of change ± standard deviation *vs.* DMSO. 3-5 biological repetitions were considered. Statistical relevance, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

A relevant ($p<0.05$) LDH release was observed starting from 12 hours of treatment, increasing at the end of the following 12 hours ($p<0.01$) (Figure 4.11).

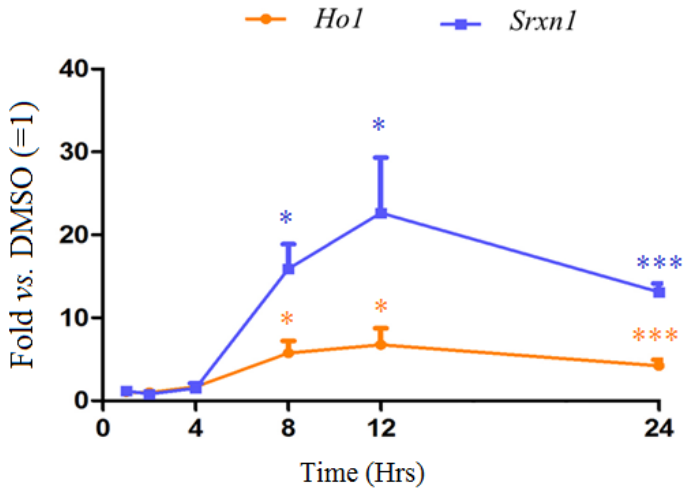

Figure 4.12 Gene expression of markers of oxidative stress after 140 nM Bf treatment.

Values are expressed as fold of change ± standard deviation *vs.* DMSO. 3-5 biological repetitions were considered. Statistical relevance, *: p<0.05; **: p<0.01; ***: p<0.001.

We observed an increased expression of *Hmox* and *Srxn1* genes (p<0.05 both) under analysis upon 8hrs of Bf exposure (Figure 4.12). Their expression reached the peak at 12 hours and then started to decay, without reaching DMSO levels also at 24 hours.

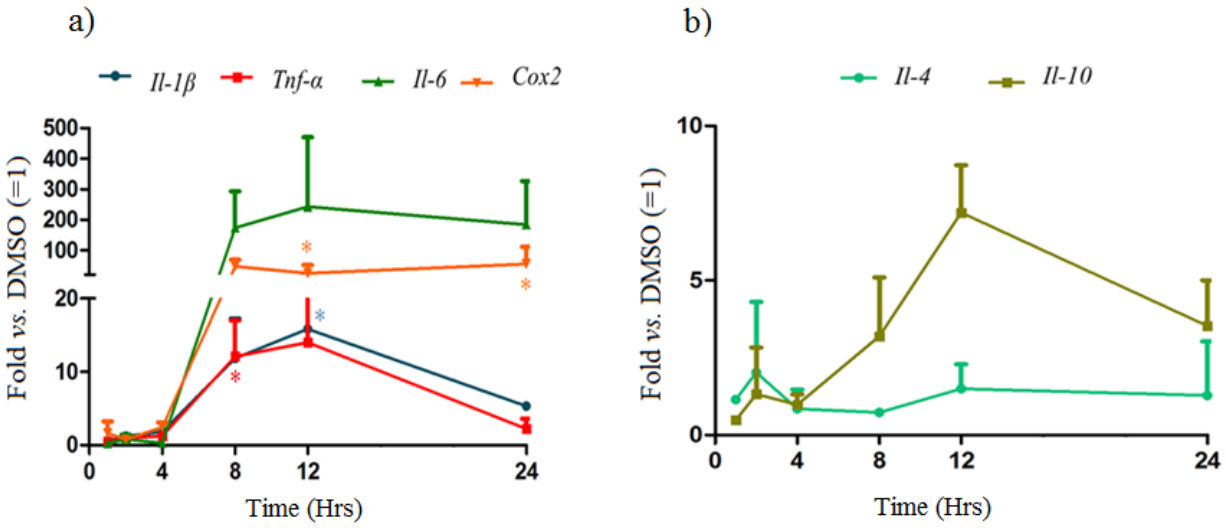

Figure 4.13 Pro- (a) and anti-inflammatory (b) genes after 140 nM Bf treatment.

Values are expressed as fold of change ± standard deviation *vs.* DMSO. 3-5 biological repetitions were considered. Statistical relevance, *: p<0.05; **: p<0.01; ***: p<0.001.

A similar trend of increased expression was also observed for inflammatory genes. Despite the high standard deviations that avoid reaching a clear statistical relevance, all inflammatory genes showed a trend of increased expression after 8 hours. All genes peaked at 12 hours and then decreased, with the exception of *Cox2* that maintain a high and significant expression until 24 hours.

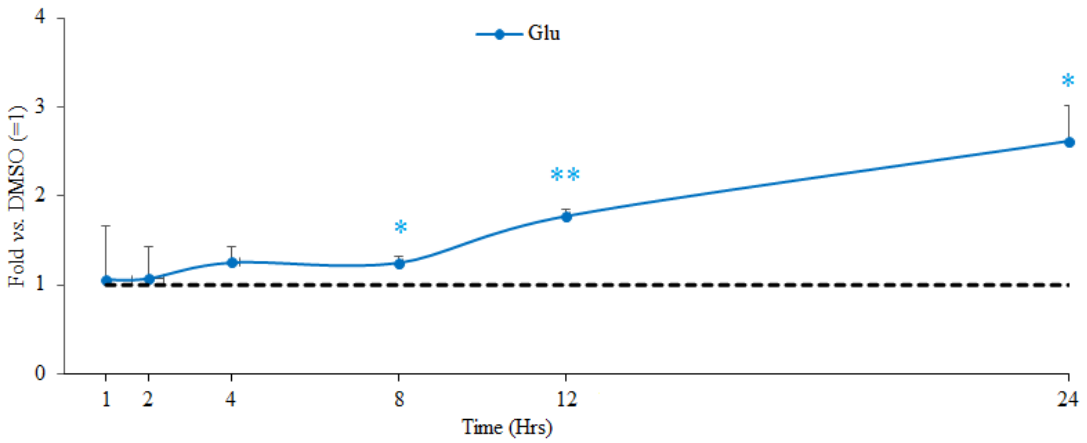

Figure 4.14 Glutamate (Glu) release in medium after 140 nM Bf treatment.

Values are expressed as fold of change ± standard deviation *vs.* DMSO. 3-5 biological repetitions were considered. Statistical relevance, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

We observed that release of glutamate (Figure 4.14) started slightly at 8 Hrs upon 140 nM UCB exposure ($p<0.05$) and increased as exposure was getting longer (12 Hrs, $p<0.01$; 24 Hrs, $p<0.05$).

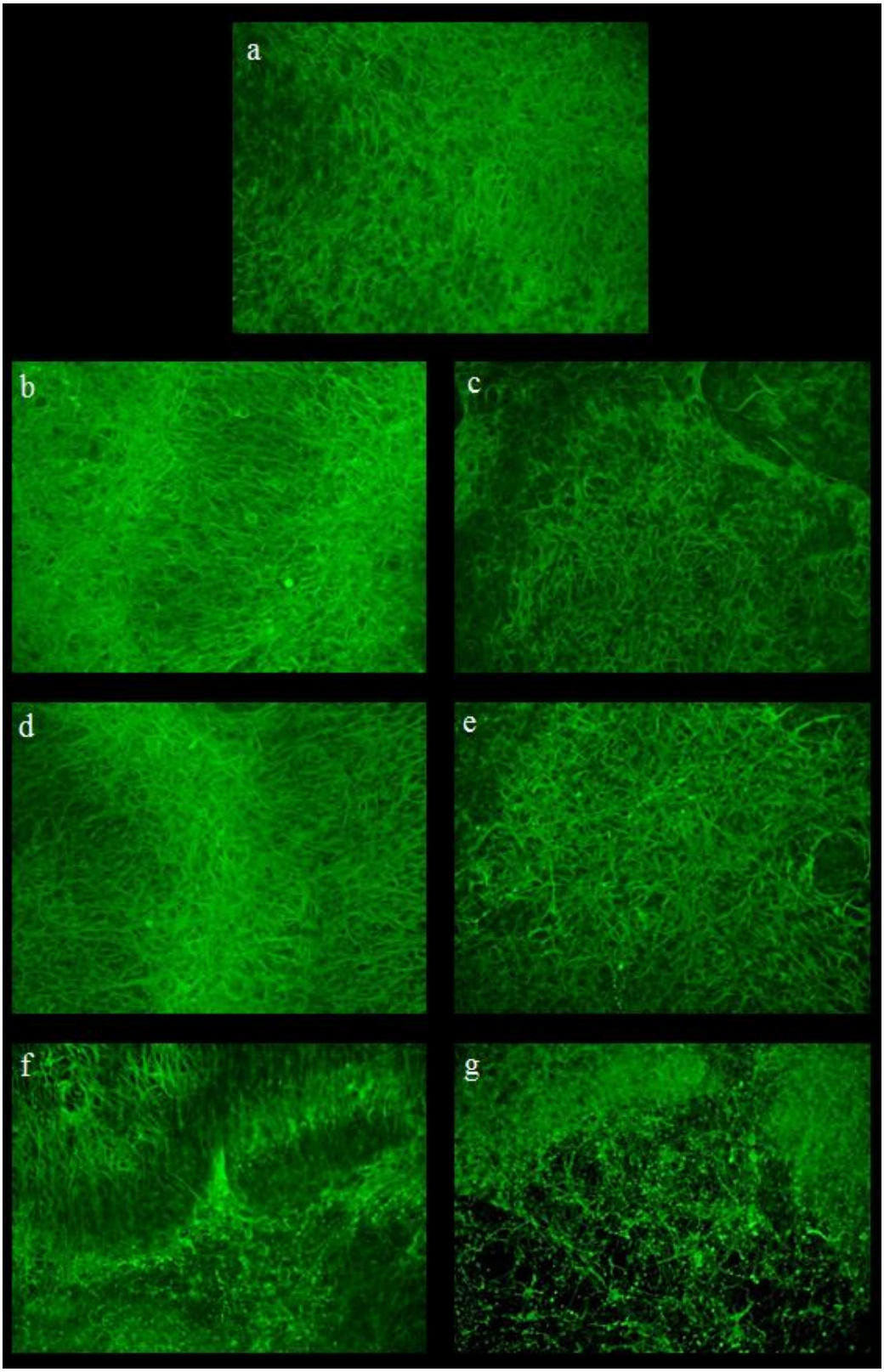

Figure 4.15 Immuno-staining against GFAP in organotypic hippocampal cultures (20x).

a) DMSO, b) 1 Hrs, c) 2 Hrs, d) 4 Hrs, e) 8 Hrs, f) 12 Hrs, g) 24 Hrs of 140 nM Bf exposure. Hrs: Hours. All images are 63x zoomed.

Preserved morphology of the distal processes of astrocytes could be observed at 1 (Figure 4.15 -b), 2 (Figure 4.15-c) and 4 (Figure 4.15-d) hours of 140nM Bf challenging, when compared with DMSO treated slices (Figure 4.15-a). After 8 hours (Figure 4.15-e) of UCB exposure the first morphological changes occur, increasing thereafter at 12 (Figure 4.15-f) and 24 hours (Figure 4.15-g), at which times GFAP staining allows to observe a clear astrocyte loss.

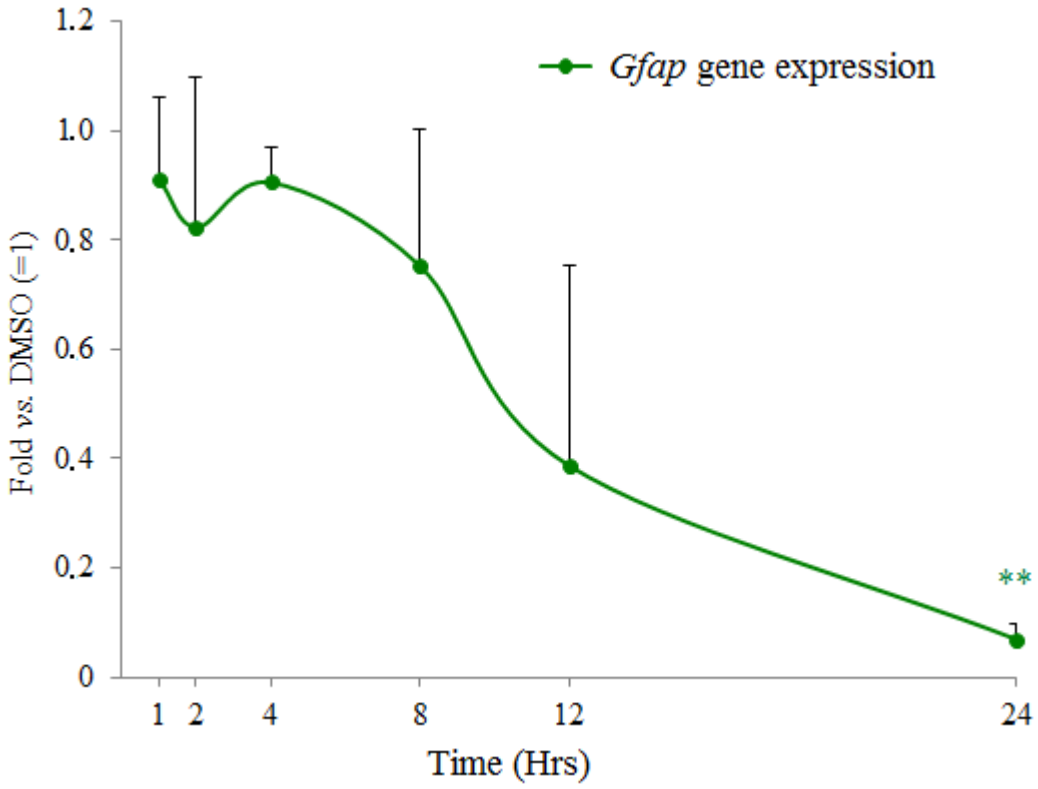

Figure 4.16 *Gfap* expression after 140 nM Bf treatment.

Values are expressed as fold of change ± standard deviation *vs.* DMSO. 3-5. Biological repetitions were considered. Statistical relevance, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

The relative *Gfap* mRNA expression presented a clear decreasing trend (Figure 4.16) since the 8 hours (0.75 fold *vs.* DMSO), and even more at 12 hours (0.38 fold) and 24 hours (0.07 fold, $p<0.01$). This data is in line with the GFAP staining trend, which showed alteration of the astrocytes morphology starting from 8 hours of treatment (Figure 4.15).

4.6 Therapeutic approach for neuroprotection

4.6.1 Screening of drugs

Finally, we used the OBCs as screening platform to identify possible neuroprotective therapeutics to test on all damaged regions. Hippocampus was used as model for a preliminary selection of the candidate drugs. LDH test was chosen to evaluate the protective effect for its maximal sensibility as demonstrated in this work.

Minocycline (golden standard), well known to avoid cerebellar hypoplasia in the Gunn rat[139,140], was effective also in protecting OBCs toward bilirubin toxicity (34%), despite never fully reverting the viability to the normal levels (DMSO).

Each molecule was tested in different concentration, starting from the data available in literature. In the present thesis, only the most effective dosage is presented.

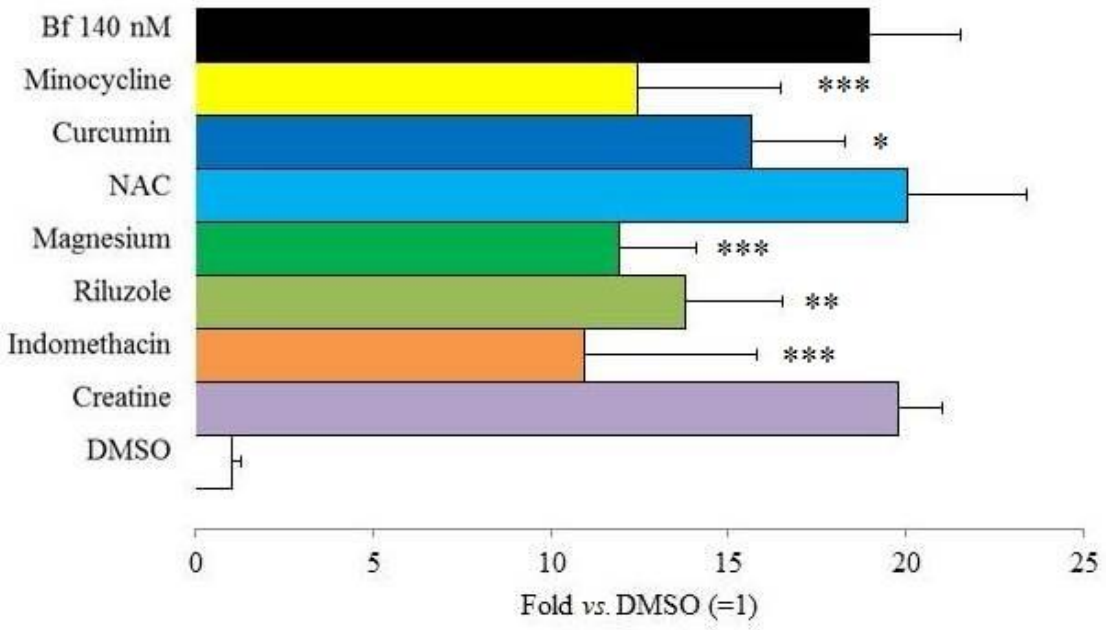

Figure 4.17 Screening of neuroprotective activity of candidate drugs.

LDH release is expressed as fold *vs.* control (DMSO = 1). Data are expressed as means ±S.D. of 3-5 biological repetitions. Statistical relevance: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

The two antioxidant drugs curcumin and N-Acetylcysteine (NAC) showed different effects (Figure 4.17). While curcumin improved the viability of about of 18% ($p<0.05$), NAC was not able to reduce the bilirubin induced damages. Based on this result, curcumin was selected for following validation experiments.

The anti-excitotoxic drugs magnesium chloride (acting as inhibitor of NMDA receptors) and riluzole (acting inhibitor of glutamate release) were able to reduce bilirubin damages. Magnesium chloride reduced the damage of 37% ($p<0.001$), while riluzole of 27% ($p<0.05$). Based on its applicability in newborns, Magnesium chloride was designated as therapeutical molecules, differently from riluzole.

The anti-inflammatory drug indomethacin reduced LDH release of 42% ($p<0.001$), while creatine, used to stimulate mitochondrial metabolism, showed no effects.

4.6.2 Neuroprotective efficacy/safety of drugs

The *ex vivo* efficacy *vs.* safety of the drugs selected during the screening (on Hip), was confirmed exposing all the regions simultaneously to 140nM Bf challenging and the principle at the defined dosage (Figure 4.18).

Curcumin, an anti-oxidant nutraceutical, was efficient in improving the viability in IC of about of 69% ($p<0.001$), of about 43% in Ctx ($p<0.01$), and only of about 18% in Hip ($p<0.05$).

Magnesium, a glutamate receptor blocker, was similarly effective in all the three damaged regions: Ctx (47%, $p<0.01$), IC (44%, $p<0.01$), and Hip (37%, $p<0.001$).

Indomethacin, an anti-inflammatory drug, increased the viability of OBCs of 63% ($p<0.01$), 60% ($p<0.001$) and 42% ($p<0.001$) in Ctx, IC and Hip, respectively.

Co-treatment was effective in IC (68%, $p<0.001$) and Ctx (52%, $p<0.01$), but not relevantly improving the already good results obtained with the single molecules. Importantly, co-treatments strongly improved viability in Hip (78%, $p<0.001$), where it represent the most effective treatment.

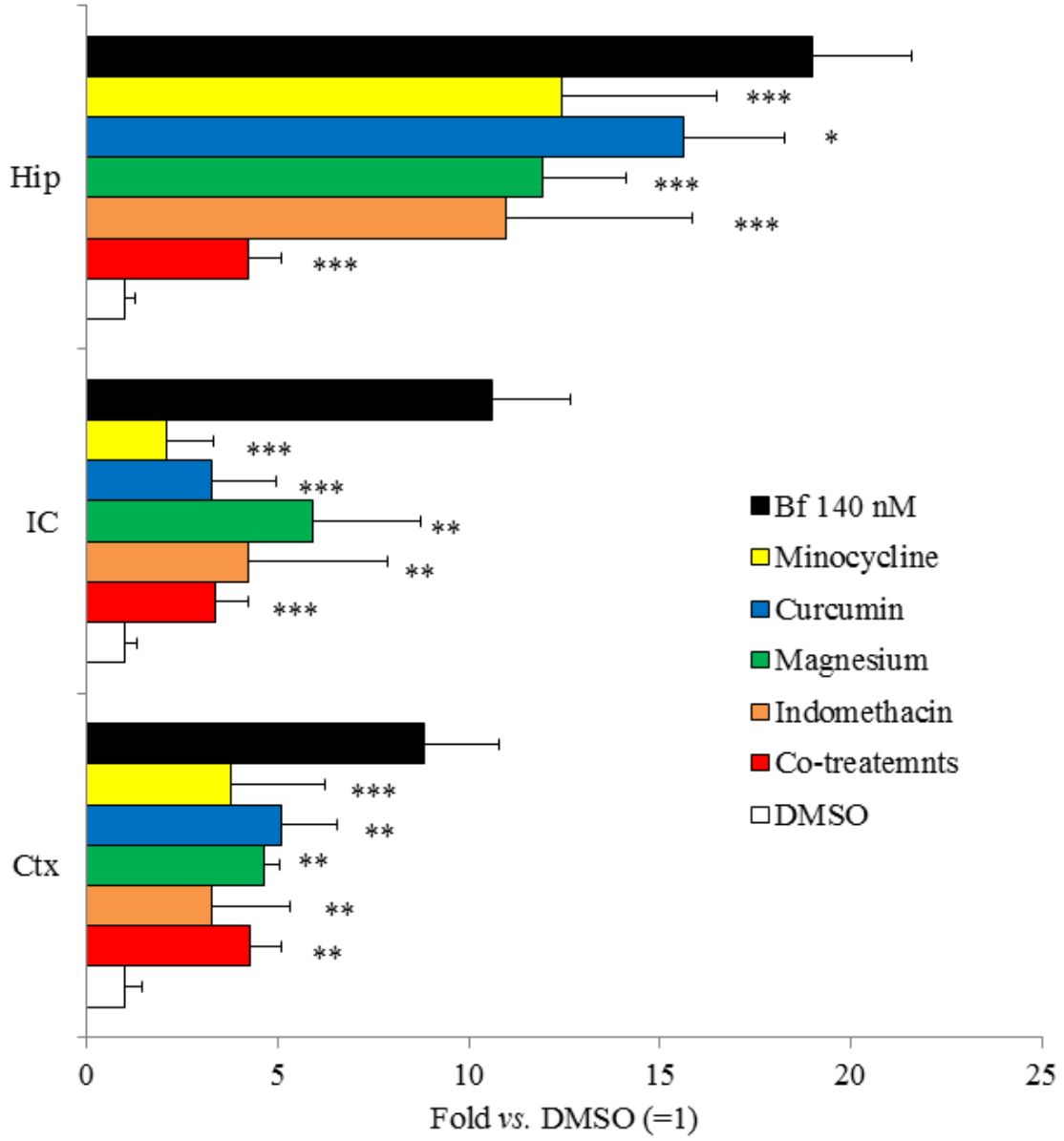

Figure 4.18 Viability improvement of therapeutic agents in co-treatment with bilirubin in Hip, IC and Ctx.

LDH release is expressed as fold *vs.* control (DMSO = 1). Data are expressed as means ±S.D. of 3-5 biological repetitions. Statistical relevance: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Single drug exposure alone (24Hrs) or in co-treatments, never increased LDH release in OBCs (data not shown), indicating safety of the dosage used in our system (Figure 4.19).

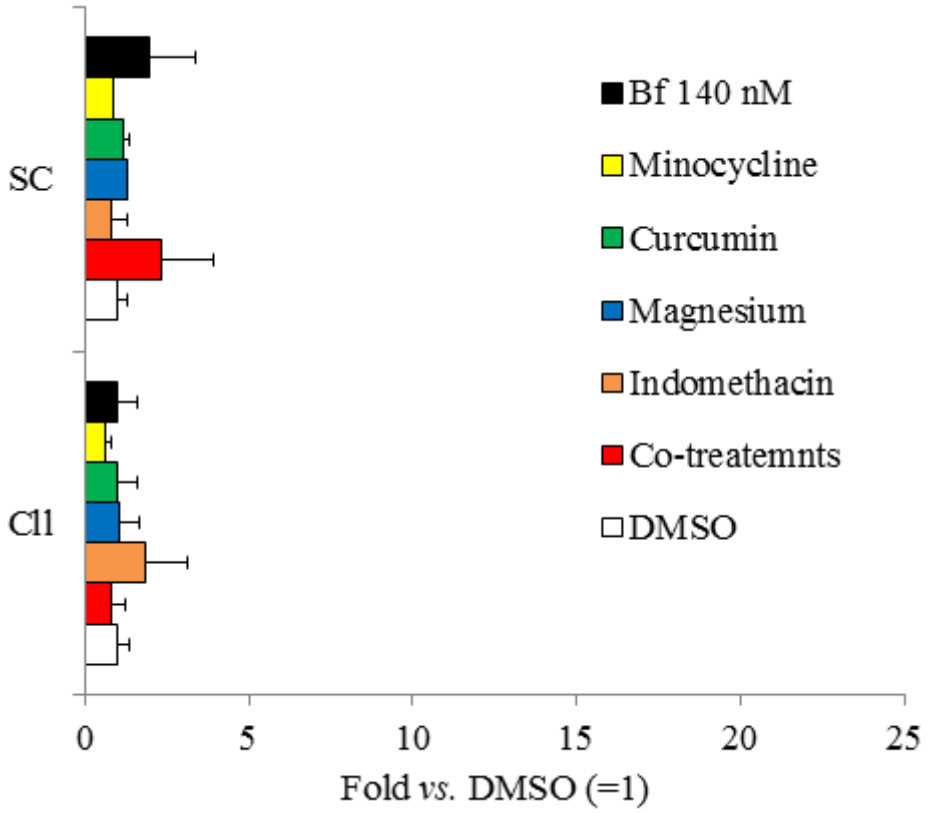

Figure 4.19 Viability of SC and Cll after exposure to therapeutic agents in co-treatment with bilirubin. LDH release is expressed as fold *vs.* control (DMSO = 1). Data are expressed as means ±S.D. of 3-5 biological repetitions.

Single drug exposure and co-treatment resulted safely in SC and Cll, undamaged by bilirubin (Figure 4.19).

5 Discussion

A resurgence of neurological consequences due to severe hyperbilirubinemia is nowadays observed in the developed countries, while this neonatal condition has never disappeared in the low- and middle-income countries, where is still a major cause of death. Main causes could be identified in a general underestimation of the risk of hyperbilirubinemia by global hospital policy, and in the failure in suitable neonatal care (early discharge from hospital, delay in seeking care for infants with hyperbilirubinemia; delay in providing appropriate treatment and/or lack in family knowledge)[198]. Especially in this circumstance, the appropriate understanding of the neurological bilirubin target of toxicity and the increased attention in direct brain protection (final target of bilirubin toxicity) are needed to avoid the possible long-term inauspicious outcomes.

By the use of the brain organotypic cultures, well reproducing *in vitr*o the CNS, we mimicked the disease and characterized the most sensible brain areas, individuating the principal mechanisms of toxicity. Un-doubtfully, the OBCs represent a step forward in respect to the cell cultures in the understanding of the mechanisms of action of bilirubin. Cell cultures lack in the reproduction of the complex environment of the neuronal tissue. Even more far from the *in vivo*, is the possibility to investigate the tropism of bilirubin for selected brain areas, as well the supposed developmental sensibility, suggested as responsible for the variability in presence and severity of symptoms[63], by a so simple model as the cell lines, despite tempted[67,100,199]. By the other side animal model are too complex, and hyperbilirubinemia quite impossible to modulate to experimental purpose.

Our OBCs, in which the inferior collicula and hippocampus emerged as the more sensible areas, well reproduced the topography of damage known in clinic, where the first diagnostic symptoms of bilirubin brain toxicity are the alterations of the auditory evoked potentials (index of IC damage)[200,201] and memory/learning deficits (Hip damage)[202–204]. Moreover, newborn autopsy findings reported yellow staining and cell death in the hippocampal region[64,65,109], and in the inferior colliculus of Gunn rats[205]. This is in agreement with our histological findings of a general cell loss, from both necrosis and apoptosis, associated with edema, fibrillary matrix development and microgliosis. These phenomena were observed in hippocampus and lower in inferior colliculus, where swollen nuclei were also observed.

Both regions, displayed glutamate release (Figure 4.6), reported leading to excitotoxicity to neuron and cellular death, through apoptosis (Figure 4.4) and necrosis (Figure 4.2)[103,197,204]. Particularly interesting is the relevant damage that occurs in astrocytes, called clasmatodendrosis. This alteration is characterized by the shortening of astrocytic process that tends to disintegrate, forming small corpuscles, cytoplasmic vacuolization and swelling (Figure 4.7 B-C)[206,207]. Astrocyte swelling may be an additional source of extracellular glutamate[208]. As reported in previous works[103,106,209,210], Hip demonstrated a clear involvement of the inflammatory pathways both in induction of cytokines expression (Table 4.3) and in microgliosis (Figure 4.8 b). Indeed, fibrosis and foam cells (Figure 4.8 b) in the brain are usually observed in presence of a pro-oxidant milieu (endocytosis of lipid-peroxidation products by macrophages), that we revealed by the analysis of *Ho1* and *Srnx1* mRNA modulation, in turn activating inflammation (*Il1β*, *Il6*, *Tnfα*, *Cox2* - Table 4.3). In this respect, it has been suggested that interleukin 1β may be related to a functional interaction between its receptor and the NMDA receptors, leading to alterations in the NMDA receptor channel-gating properties in a way that favors Ca^{2+} influx[211]. Pro-inflammatory cytokines are also able to induce *Cox2* activity that exacerbates the damage[212], in agreement with previous published works using cell cultures[144] and confirmed in the neonatal brain[213].

Importantly, by our work, we described a similar picture (presence of oxidative stress, inflammation, and glutamate excitotoxicity) also for the IC, a region usually not investigated from the "bench side", despite clinically relevant. The inferior colliculi are a key part of the auditory connectome, and a well-known target of bilirubin toxicity in clinics[205,214,215]. Its permanent damage is cause of a very disabling handicap, factually isolating from the society a baby otherwise with an intact intelligence. Similarly, P8 is a sensible age for the auditory onset in rats and it was identified as a period of high expression of metabotropic glutamate receptors[216] and increased Ca^{2+} permeability[217], if compared with newborn rats.

Surprisingly, we observed damage in the cerebral cortex, a brain area usually considered resistant to bilirubin toxicity by clinicians[177]. In our system, 70nM Bf started causing a membrane leakage in P8 slices, reaching the maximal values at 140nM, when *Il6* and *Cox2* were significantly modulated, together with *Srxn1* (but not *Ho1*). At 300nM also mitochondrial function was altered, as well as we observed a slight increase of apoptosis. The histological analysis confirmed a high degree of damages in cortex with strong decrease of cellular density and weak nuclear staining. The images obtained from histological staining of cortex are not sufficient to discuss thoroughly the effect of bilirubin, due to the complexity composition of the region. Insufficient information is

available in literature to permit an appropriate discussion of the results. Despite that, it should be considered that almost all the *in vitro* evidences about the mechanisms of toxicity triggered by bilirubin we have, have been obtained by the use of primary cultures of neurons and astrocytes isolated from the cerebral cortex[67,69,84,197,209]. Indeed, in animal models, cortical sensitivity to bilirubin was suggested also by inhibition of the phosphorylation of synapsin I[202], by histological abnormalities observed in occipital cortex, together with alteration of balance of excitatory and inhibitory neurotransmitters[68]. Even though rare, electroencephalography (EEG) measurement in newborn demonstrated that hyperbilirubinemia affects the cerebro-cortical electrical activity, in a time limited manner[218], and autopsy findings displayed apoptosis and necrosis in this area[64]. These findings suggest a potential sensitivity of the cortical area to bilirubin, probably less severe than those observed in other regions, that needs additional clinical attention to be clarified. Up to now, it has been questioned if the absence of clinical functional information about bilirubin toxicity in this CNS region depends on a general indifference toward this structure or reflecting a real finding.

Notably, OBCs from 8days old animals became sensitive, when compared to OBCs from 2days old pups, to 70nM Bf, considered a not toxic (or a threshold) concentration[31]. This finding is in line with the recent warning, reporting children recognized to have a neurological deficit, described as low bilirubin kernicterus. Elevated bilirubin in early infancy has been associated with later diagnoses of attention deficit hyperactivity disorder and autism spectrum disorder[219,220]. Accordingly to this report, moderate elevations are not considered benign, and are instead considered potential contributors to cognition problems[221].

Independently of the concentration of bilirubin used, we demonstrated the existence of a different sensitivity to bilirubin toxicity during the CNS development. The early stage after the birth (P2) was less prone to damage (Table 4.1) than only few days after (P8). This result is significant considering that if exposure to a toxicant occurs during organ development, it is more vulnerable than before or after[222]. No detailed biomolecular data are available about human post-natal brain development. In rodents, brain development starts with a "dormant period" during the first 2-3 post-natal days[223]. A dramatic increase of NMDA receptor subunits[224,225] and sensitivity of inflammatory reaction[226] are observed during the second post-natal week, together with the activation of neurogenesis, non-neuronal cells proliferation, tissues apoptosis and remodeling[223]. All those mechanisms could represent a target for bilirubin toxicity, and most of them have been observed to be affected in our *ex vivo* model. Moreover, the *in vivo* literature is consistent with our hypothesis that the target of bilirubin toxicity might be developmentally regulated. A window of vulnerability

to bilirubin toxicity have been described in animal models around at P6-10, while preceding and following CNS developmental stages resulted substantially insensible[227–229]. Interestingly, in newborns the gestational age at the time of peak of hyperbilirubinemia (usually occurring from the 4th to the 10th day after the birth) seems to drive the level of damage, from auditory to motor dysfunction[63].

This reasoning might help in explaining the most striking difference between our OBCs and the animal models of CNI and neonatal hyperbilirubinemia: the absence of damage in the cerebellum OBCs. Cerebellar hypoplasia is the hallmark of the animal models[45,62,110,230,231] and cerebellum damage was highlighted also in case report of pre-term babies died with kernicterus[109,232]. In our experimental model, the cerebellar OBCs were almost insensible to bilirubin challenge (24 hours), despite some increased expression of markers of oxidative stress (Table 4.2), inflammation (Table 4.3) and some histological alterations (oedema, necrosis and apoptosis) (Figure 4.8 g-h). This is not fully surprising, because two days are not enough to induce the cerebellar hypoplasia *in vivo*, requiring at least 8-9 days[85,228,231]. This highlights again the hypothesis that the pathway/mechanisms of bilirubin damage might be region specific, and requiring a longer time in the cerebellum. Of notice, bilirubin toxicity to cerebellar granular cells play across interference of cellular proliferation, finally leading to apoptosis, contributing to the cerebellar hypoplasia of hyperbilirubinemic Gunn rats[85]. It is believable that the acute (24 hours) protocol paradigm we used in this work was insufficient to activate the molecular events impairing cellular proliferation and reproducing the behavior observed *in vivo*. As further proof, four days of treatment with 140nM Bf increased the LDH release (>2fold) also in our organotypic cerebellar cultures (Figure 4.9). In addition, while the Bf concentration of 140 nM was the most used[69,92] and with sure toxicity in cell lines[178], no data exist about Bf concentration in newborn brain. Data from Gunn rats reported a range of brain Bf of about 71-770 nM[233], suggesting the important role played by an extreme Bf level in cerebellar damages.

Superior colliculus was the only region studied that seems to confirm the preliminary hypothesis of resistance to bilirubin toxicity. This brain region is related to the visual pathway and the absence of evident severe neurological alteration in newborns[63] makes superior colliculus a strong negative control of damage. Superior colliculus resulted sensitive in the viability tests only to the higher concentration of bilirubin, without reaching the level of damages of the most sensible regions. On the contrary, the overexpression of oxidative stress markers (Table 4.2) and pro-inflammatory genes (Table 4.3) suggested that the tissue is responsive to bilirubin, but possibly

with transient non-toxic effects. Histological finding do not allow the deeply discussion of the effects of bilirubin in SC, because vacuolization and fibrosis observed in treated slices are also reported in control (DMSO) slices, suggesting the sensitivity of the tissue to the bilirubin solvent. Clinically, visual pathway integrity was quantified by Visually Evoked Potentials (VEPs)[234]. The first study described alteration of the visual pathway reported two preterm infants with moderate serum bilirubin concentrations that showed improvement VEPs after exchange transfusion[235]. In a study recruiting 94 term infants the authors found that alterations were positively correlated with the level of maximal serum bilirubin[236]. On the contrary, a retrospective follow-up showed no differences between the group with hyperbilirubinemia regard to mean visual evoked potential latencies[237]. A more recent study, suggest again a variation of visual alteration according to the level of hyperbilirubinemia, measured by VEP[238]. This finding suggests that some of the VEP disturbances observed in the studies may be temporary and reversible. They could disappear as soon as bilirubin is removed from blood and, possibly, cleared from the brain.

One of the main aims of this work was the understanding of the region selective mechanisms of bilirubin toxicity, focused in defining the more befitting drug to protect directly the CNS parenchyma.

In agreement with a multifactorial pathway of damage in hippocampus, $MgCl_2$ (viability improvement of about 37% *vs.* Bf alone)[88,89] and indomethacin (42%)[103,210] were the most efficient. The poor role of antioxidant curcumin (18%) suggested a secondary role played by oxidative stress[96] in this brain area. Of notice in this highly damaged brain region, none of the aforementioned molecules was able to restore the viability to levels comparable to the control (DMSO). Nevertheless, both $MgCl_2$ and indomethacin equaled the protection conferred by minocycline. This result should be considered already promising because minocycline is able to totally avoid CNS damage *in vivo* [139,140]. Indeed, co-treatment significantly improved the protection (78% of recovery), clarifying that bilirubin acts simultaneously on different pathways, not necessarily connected, as proved also by the time-course study on the hippocampus (From Figure 4.11 to Figure 4.16).

Curcumin (69%, anti-oxidant) and indomethacin (60%, anti-inflammatory) treatment significantly reverted the damage in the inferior collicula, with only a slightly less efficient action of the $MgCl_2$ (44%, NMDA blocker), and no additional effects in co-treatments. Auditory deficit are the first clinical symptom of the bilirubin toxicity to the CNS[205,214,215]. Easily reverted by a prompt

phototherapy intervention, the damage assumes dramatic outcomes if not promptly faced, factually isolating from the society a baby otherwise with an intact intelligence. The great result obtained with our therapeutical scheme, might be extremely promising if confirmed *in vivo*.

In the cerebral cortex, the damage was similarly preventable by all the therapeutical approaches studied, offering the possibility to choose the best available and safe treatment.

The rationale of the drug selection preformed on this work is the nearly applicability to the newborns, therefore only drugs already in clinical use in other pathologies or tested in animal models were considered. Curcumin is the active ingredient of turmeric, and is a well-known antioxidant and anti-inflammatory of human use[239]. Curcumin reduced epilepticus events in a post-status epilepticus rat model[240]; attenuated brain edema in mice with intracerebral hemorrhage[241] and was effective against ischaemia/reperfusion insult in rat brain[192]. Magnesium is the physiological inhibitor of the NMDA family of glutamate receptors; therefore its supplementation was suggested as possible neuroprotective therapy against excitotoxic events. Magnesium, as magnesium sulfate, was tested as neuroprotective drugs in newborns[242,243], but it was excluded from this work because it is included in the list of drugs able to increase Bf level[25]. Magnesium chloride was effective in enhancing memory[244]; conferring neuroprotection in a rat model of spinal cord injury[245], and recovery of function following bilateral anterior medial cortex lesions in the rat[246]. Indomethacin is a non-steroidal anti-inflammatory drug, by inhibiting the production of prostaglandin. It is used in newborn to close patent ductus arteriosus. Indomethacine is known to be neuroprotective in an oxygen-glucose deprivation in organotypic cultures[195] and reducing microglia activation in a mouse model of focal cerebral ischemia[247]. Indeed, indomethacin could also prevent white matter injury in newborn[196]. All the drugs described are known to be able to cross the blood brain barrier to reach the brain. In addition, they are not present in the list of drugs known to alter bilirubin-albumin binding, increasing Bf level[25].

In conclusion, the adaptation of OBCs technique to the study of bilirubin brain toxicity well reproduced the findings observed *in vivo* (animal models) and in clinics, highlighting the regional and developmental sensitivity to the pigment. As such, OBCs represent a good platform for the investigation of the biomolecular events leading to the regional selective damage as well as screening new therapeutical approaches. We capitalized the research by demonstrating a multifactorial toxic action of bilirubin, with some region specificity, and we proved *ex vivo* the efficacy of a new experimental approach, aimed in protecting directly the brain from the bilirubin

induced damage, with the main advantage of gaining time allowing phototherapy to be effective in reducing serum bilirubin levels. This information may be important to suggest a specific therapeutic approach (anti-inflammatory *vs.* anti-oxidant *vs.* blocking the glutamate channels *vs.* combined), depending on the symptomatology of the patients (spy of the region with damage).

6 Conclusions

Organotypic brain cultures are a good tool to study bilirubin neurotoxicity, mimicking the *in vivo* condition, but with the possibility to control all the experimental conditions. Their characteristics overcame the limits of cell cultures and primary cell cultures and allow the possibility to study the capacity of the tissue to react to bilirubin stimuli.

Hippocampus results the most sensible region to bilirubin induced damages during the regional screening. Similar damages were observed in inferior colliculi and less in cortex. Cerebellum, the landmark of damages in Gunn rata and undamaged in OBCs, probably need more than 24 hours of Bf exposure to be damaged. Superior colliculi seem to confirm their resistance to bilirubin toxicity.

Increased sensitivity of P8 than P2 cultures was observed, especially in hippocampal slices. The identification of two temporal windows demonstrated the existence of a different sensitivity to bilirubin toxicity during the CNS development.

Glutamate release seem to play a role in damage only hippocampus and inferior colliculus. On the contrary, increased expression of inflammatory and oxidative stress markers were observed in all the five regions analyzed. The lack of damages observed in cerebellum and superior colliculus in terms of viability suggested that inflammation and oxidative stress play a different role in damages that can’t be quantified after 24 hours, if compared with the other regions tested.

Single drug treatment is able to reduce the bilirubin induced damages in all the affected regions tested, but the cocktail of drugs seem to be more effective for a future clinical approach. Drugs studied in this work could be considered for a clinical application, due to their efficacy and their simple translation to clinic.

7 Bibliography

1. Berk, P. D., Rodkey, F. L., Blaschke, T. F., Collison, H. A. & Waggoner, J. G. Comparison of plasma bilirubin turnover and carbon monoxide production in man. *J. Lab. Clin. Med.* **83,** 29–37 (1974).
2. London, I. M., West, R., Shemin, D. & Rittenberg, D. On the origin of bile pigment in normal man. *J. Biol. Chem.* **184,** 351–358 (1950).
3. Vítek, L. & Ostrow, J. D. Bilirubin chemistry and metabolism; harmful and protective aspects. *Curr. Pharm. Des.* **15,** 2869–2883 (2009).
4. Jacobsen, J. & Brodersen, R. Albumin-bilirubin binding mechanism. *J. Biol. Chem.* **258,** 6319–6326 (1983).
5. Wolkoff, A. W. Hepatocellular sinusoidal membrane organic anion transport and transporters. *Semin. Liver Dis.* **16,** 121–127 (1996).
6. Wang, P., Kim, R. B., Chowdhury, J. R. & Wolkoff, A. W. The human organic anion transport protein SLC21A6 is not sufficient for bilirubin transport. *J. Biol. Chem.* **278,** 20695–20699 (2003).
7. Wolkoff, A. W., Goresky, C. A., Sellin, J., Gatmaitan, Z. & Arias, I. M. Role of ligandin in transfer of bilirubin from plasma into liver. *Am. J. Physiol.* **236,** E638–648 (1979).
8. Vander Jagt, D. L., Dean, V. L., Wilson, S. P. & Royer, R. E. Regulation of the glutathione S-transferase activity of bilirubin transport protein (ligandin) from human liver. Enzymic memory involving protein-protein interactions. *J. Biol. Chem.* **258,** 5689–5694 (1983).
9. Fevery, J., Van Damme, B., Michiels, R., De Groote, J. & Heirwegh, K. P. Bilirubin conjugates in bile of man and rat in the normal state and in liver disease. *J. Clin. Invest.* **51,** 2482–2492 (1972).
10. Muraca, M., Rubaltelli, F. F., Blanckaert, N. & Fevery, J. Unconjugated and conjugated bilirubin pigments during perinatal development. II. Studies on serum of healthy newborns and of neonates with erythroblastosis fetalis. *Biol. Neonate* **57,** 1–9 (1990).
11. Gartung, C. & Matern, S. Molecular regulation of sinusoidal liver bile acid transporters during cholestasis. *Yale J. Biol. Med.* **70,** 355–363 (1997).
12. Ostrow, J. D. *Bile pigments and jaundice: molecular, metabolic, and medical aspects*. (Dekker, 1986).
13. McDonagh, A. F. Controversies in bilirubin biochemistry and their clinical relevance. *Semin. Fetal. Neonatal Med.* **15,** 141–147 (2010).
14. Benhold, H. *Ergeb Inn Med Kinderheilk* **42,** (1932).
15. Levine, R. L. Fluorescence-quenching studies of the binding of bilirubin to albumin. *Clin. Chem.* **23,** 2292–2301 (1977).
16. Brodersen, R. Bilirubin. Solubility and interaction with albumin and phospholipid. *J. Biol. Chem.* **254,** 2364–2369 (1979).

17. Murki, S., Kumar, P., Majumdar, S., Marwaha, N. & Narang, A. Risk factors for kernicterus in term babies with non-hemolytic jaundice. *Indian Pediatr.* **38,** 757–762 (2001).
18. Wennberg, R. P. The blood-brain barrier and bilirubin encephalopathy. *Cell. Mol. Neurobiol.* **20,** 97–109 (2000).
19. Silverman, W. A., Andersen, D. H., Blanc, W. A. & Crozier, D. N. A Difference in Mortality Rate and Incidence of Kernicterus Among Premature Infants Allotted to Two Prophylactic Antibacterial Regimens. *Pediatrics* **18,** 614–625 (1956).
20. Harris, R. C., Lucey, J. F. & Maclean, J. R. Kernicterus in premature infants associated with low concentrations of bilirubin in the plasma. *Pediatrics* **21,** 875–884 (1958).
21. Ahlfors, C. E. Unbound bilirubin associated with kernicterus: a historical approach. *J. Pediatr.* **137,** 540–544 (2000).
22. Ahlfors, C. E. Bilirubin-albumin binding and free bilirubin. *J. Perinatol. Off. J. Calif. Perinat. Assoc.* **21 Suppl 1,** S40–42; discussion S59–62 (2001).
23. Zucker, S. D., Goessling, W. & Hoppin, A. G. Unconjugated bilirubin exhibits spontaneous diffusion through model lipid bilayers and native hepatocyte membranes. *J. Biol. Chem.* **274,** 10852–10862 (1999).
24. Diamond, I. & Schmid, R. Experimental bilirubin encephalopathy. The mode of entry of bilirubin-14C into the central nervous system. *J. Clin. Invest.* **45,** 678–689 (1966).
25. Strauss, K. A. *et al.* Management of hyperbilirubinemia and prevention of kernicterus in 20 patients with Crigler-Najjar disease. *Eur. J. Pediatr.* **165,** 306–319 (2006).
26. Wennberg, R. P., Rasmussen, L. F., Ahlfors, C. E. & Valaes, T. Mechanized determination of the apparent unbound unconjugated bilirubin concentration in serum. *Clin. Chem.* **25,** 1444–1447 (1979).
27. American Academy of Pediatrics Subcommittee on Hyperbilirubinemia. Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. *Pediatrics* **114,** 297–316 (2004).
28. Reiser, D. J. Neonatal jaundice: physiologic variation or pathologic process. *Crit. Care Nurs. Clin. North Am.* **16,** 257–269 (2004).
29. Kawade, N. & Onishi, S. The prenatal and postnatal development of UDP-glucuronyltransferase activity towards bilirubin and the effect of premature birth on this activity in the human liver. *Biochem. J.* **196,** 257–260 (1981).
30. Levi, A. J., Gatmaitan, Z. & Arias, I. M. Deficiency of hepatic organic anion-binding protein, impaired organic amnion uptake by liver and 'physiologic' jaundice in newborn monkeys. *N. Engl. J. Med.* **283,** 1136–1139 (1970).
31. Ostrow, J. D., Pascolo, L. & Tiribelli, C. Reassessment of the unbound concentrations of unconjugated bilirubin in relation to neurotoxicity in vitro. *Pediatr. Res.* **54,** 98–104 (2003).

32. Brito, M. A., Silva, R. F. M. & Brites, D. in *New Trends in Brain Research* 1–38 (Nova Science Publishers, Inc., 2006).
33. Doré, S. *et al.* Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2445–2450 (1999).
34. Stocker, R., Yamamoto, Y., McDonagh, A. F., Glazer, A. N. & Ames, B. N. Bilirubin is an antioxidant of possible physiological importance. *Science* **235,** 1043–1046 (1987).
35. Wagner, K.-H. *et al.* Looking to the horizon: the role of bilirubin in the development and prevention of age-related chronic diseases. *Clin. Sci. Lond. Engl. 1979* **129,** 1–25 (2015).
36. Zipursky, A. & Bhutani, V. K. Impact of Rhesus disease on the global problem of bilirubin-induced neurologic dysfunction. *Semin. Fetal. Neonatal Med.* **20,** 2–5 (2015).
37. Cappellini, M. D. & Fiorelli, G. Glucose-6-phosphate dehydrogenase deficiency. *Lancet Lond. Engl.* **371,** 64–74 (2008).
38. Crigler, J. F. & Najjar, V. A. Congenital familial nonhemolytic jaundice with kernicterus. *Pediatrics* **10,** 169–180 (1952).
39. Crigler-Najjar Type 1 syndrome in the NIH Genetic and Rare Diseases Information Center. at <https://rarediseases.info.nih.gov/gard/47/crigler-najjar-syndrome-type-1/Resources/1>
40. List of Rare Diseases of the Italian Department of Health. at <www.salute.gov.it/portale/malattieRare/documenti/elenco.pdf>
41. Strassburg, C. P. Hyperbilirubinemia syndromes (Gilbert-Meulengracht, Crigler-Najjar, Dubin-Johnson, and Rotor syndrome). *Best Pract. Res. Clin. Gastroenterol.* **24,** 555–571 (2010).
42. Sampietro, M. & Iolascon, A. Molecular pathology of Crigler-Najjar type I and II and Gilbert's syndromes. *Haematologica* **84,** 150–157 (1999).
43. van der Veere, C. N. *et al.* Current therapy for Crigler-Najjar syndrome type 1: report of a world registry. *Hepatol. Baltim. Md* **24,** 311–315 (1996).
44. Gunn, C. H. HEREDITARY ACHOLURIC JAUNDICE in a New Mutant Strain of Rats. *J. Hered.* **29,** 137–139 (1938).
45. Conlee, J. W. & Shapiro, S. M. Development of cerebellar hypoplasia in jaundiced Gunn rats: a quantitative light microscopic analysis. *Acta Neuropathol. (Berl.)* **93,** 450–460 (1997).
46. Rose, A. L. & Wisniewski, H. Acute bilirubin encephalopathy induced with sulfadimethoxine in Gunn rats. *J. Neuropathol. Exp. Neurol.* **38,** 152–164 (1979).
47. Arias, I. M. Chronic unconjugated hyperbilirubinemia without overt signs of hemolysis in adolescents and adults. *J. Clin. Invest.* **41,** 2233–2245 (1962).
48. Kadakol, A. *et al.* Genetic lesions of bilirubin uridine-diphosphoglucuronate glucuronosyltransferase (UGT1A1) causing Crigler-Najjar and Gilbert syndromes: correlation of genotype to phenotype. *Hum. Mutat.* **16,** 297–306 (2000).

49. Arias, I. M., Gartner, L. M., Cohen, M., Ezzer, J. B. & Levi, A. J. Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency. Clinical, biochemical, pharmacologic and genetic evidence for heterogeneity. *Am. J. Med.* **47,** 395–409 (1969).
50. Ito, T. *et al.* Phenobarbital following phototherapy for Crigler-Najjar syndrome type II with good fetal outcome: a case report. *J. Obstet. Gynaecol. Res.* **27,** 33–35 (2001).
51. Gilbert, A. & Lereboullet, P. La cholemia simple familiale. *Semaine Med* **21,** 241–3 (1901).
52. Owens, D. & Evans, J. Population studies on Gilbert's syndrome. *J. Med. Genet.* **12,** 152–156 (1975).
53. Bosma, P. J. *et al.* The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N. Engl. J. Med.* **333,** 1171–1175 (1995).
54. Watchko, J. F. & Lin, Z. Exploring the genetic architecture of neonatal hyperbilirubinemia. *Semin. Fetal. Neonatal Med.* **15,** 169–175 (2010).
55. Wallner, M. *et al.* Anti-genotoxic potential of bilirubin in vivo: damage to DNA in hyperbilirubinemic human and animal models. *Cancer Prev. Res. Phila. Pa* **6,** 1056–1063 (2013).
56. Schwertner, H. A. & Vítek, L. Gilbert syndrome, UGT1A1*28 allele, and cardiovascular disease risk: possible protective effects and therapeutic applications of bilirubin. *Atherosclerosis* **198,** 1–11 (2008).
57. Chowdhury, N. R., Arias, I. M., Wolkoff, A. W. & Chowdhury, J. R. in *The Liver: Biology and Pathobiology, Fourth Edition* (Lippincott Williams & Wilkin, 2001).
58. Okumura, A. *et al.* Kernicterus in Preterm Infants. *Pediatrics* **123,** e1052–e1058 (2009).
59. Watchko, J. F. & Maisels, M. J. The enigma of low bilirubin kernicterus in premature infants: why does it still occur, and is it preventable? *Semin. Perinatol.* **38,** 397–406 (2014).
60. Schmorl, C. Zur Kenntnis des ikterus neonatorum, insbesondere der dabei auftretenden gehirnvera¨nderungen. *Verh Dtsch Pathol Ges* **6,** 109–115 (1904).
61. Hansen, T. W. Pioneers in the scientific study of neonatal jaundice and kernicterus. *Pediatrics* **106,** E15 (2000).
62. Gazzin, S. *et al.* Bilirubin accumulation and Cyp mRNA expression in selected brain regions of jaundiced Gunn rat pups. *Pediatr. Res.* **71,** 653–660 (2012).
63. Shapiro, S. M. Chronic bilirubin encephalopathy: diagnosis and outcome. *Semin. Fetal. Neonatal Med.* **15,** 157–163 (2010).
64. Zangen, S. *et al.* Fatal kernicterus in a girl deficient in glucose-6-phosphate dehydrogenase: a paradigm of synergistic heterozygosity. *J. Pediatr.* **154,** 616–619 (2009).
65. Hachiya, Y. & Hayashi, M. Bilirubin encephalopathy: a study of neuronal subpopulations and neurodegenerative mechanisms in 12 autopsy cases. *Brain Dev.* **30,** 269–278 (2008).
66. Palmela, I., Pereira, P., Hayashi, M., Brites, D. & Brito, M. A. Histological Findings in the Kernicterus-Associated Vulnerable Brain Regions are Linked to neurodegeneration, Alterations in

Astrocyte and Pericyte Distribution, and Vascular Modifications. *Int. J. Pathol. Clin. Res.* **1,** 003 (2015).

67. Vaz, A. R. *et al.* Selective vulnerability of rat brain regions to unconjugated bilirubin. *Mol. Cell. Neurosci.* **48,** 82–93 (2011).
68. Hu, W. *et al.* Ex vivo (1)H nuclear magnetic resonance spectroscopy reveals systematic alterations in cerebral metabolites as the key pathogenetic mechanism of bilirubin encephalopathy. *Mol. Brain* **7,** 87 (2014).
69. Gambaro, S. E., Robert, M. C., Tiribelli, C. & Gazzin, S. Role of brain cytochrome P450 mono-oxygenases in bilirubin oxidation-specific induction and activity. *Arch. Toxicol.* (2014). doi:10.1007/s00204-014-1394-4
70. Oakden, W. K., Moore, A. M., Blaser, S. & Noseworthy, M. D. 1H MR spectroscopic characteristics of kernicterus: a possible metabolic signature. *AJNR Am. J. Neuroradiol.* **26,** 1571–1574 (2005).
71. Katar, S., Akay, H. O., Taskesen, M. & Devecioglu, C. Clinical and cranial magnetic resonance imaging (MRI) findings of 21 patients with serious hyperbilirubinemia. *J. Child Neurol.* **23,** 415–417 (2008).
72. Shapiro, S. M. & Popelka, G. R. Auditory impairment in infants at risk for bilirubin-induced neurologic dysfunction. *Semin. Perinatol.* **35,** 162–170 (2011).
73. Jiang, Z. D. & Wilkinson, A. R. Impaired function of the auditory brainstem in term neonates with hyperbilirubinemia. *Brain Dev.* **36,** 212–218 (2014).
74. Akinpelu, O. V., Waissbluth, S. & Daniel, S. J. Auditory risk of hyperbilirubinemia in term newborns: a systematic review. *Int. J. Pediatr. Otorhinolaryngol.* **77,** 898–905 (2013).
75. Kernicterus in the NIH Genetic and Rare Diseases Information Center. at <https://rarediseases.info.nih.gov/gard/6830/kernicterus/Resources/1>
76. Bhutani, V. K. *et al.* Neonatal hyperbilirubinemia and Rhesus disease of the newborn: incidence and impairment estimates for 2010 at regional and global levels. *Pediatr. Res.* **74 Suppl 1,** 86–100 (2013).
77. Manning, D., Todd, P., Maxwell, M. & Jane Platt, M. Prospective surveillance study of severe hyperbilirubinaemia in the newborn in the UK and Ireland. *Arch. Dis. Child. Fetal Neonatal Ed.* **92,** F342–346 (2007).
78. Bjerre, J. V., Petersen, J. R. & Ebbesen, F. Surveillance of extreme hyperbilirubinaemia in Denmark. A method to identify the newborn infants. *Acta Paediatr. Oslo Nor. 1992* **97,** 1030–1034 (2008).
79. Bhutani, V. K. & Johnson, L. Kernicterus in the 21st century: frequently asked questions. *J. Perinatol. Off. J. Calif. Perinat. Assoc.* **29 Suppl 1,** S20–24 (2009).
80. Bhutani, V. K. & Johnson, L. Synopsis report from the pilot USA Kernicterus Registry. *J. Perinatol. Off. J. Calif. Perinat. Assoc.* **29 Suppl 1,** S4–7 (2009).

81. Stevenson, D. K., Vreman, H. J. & Wong, R. J. Bilirubin production and the risk of bilirubin neurotoxicity. *Semin. Perinatol.* **35,** 121–126 (2011).
82. Rodrigues, C. M. P. *et al.* Perturbation of membrane dynamics in nerve cells as an early event during bilirubin-induced apoptosis. *J. Lipid Res.* **43,** 885–894 (2002).
83. Rodrigues, C. M. P., Solá, S., Brito, M. A., Brites, D. & Moura, J. J. G. Bilirubin directly disrupts membrane lipid polarity and fluidity, protein order, and redox status in rat mitochondria. *J. Hepatol.* **36,** 335–341 (2002).
84. Rodrigues, C. M. P., Solá, S. & Brites, D. Bilirubin induces apoptosis via the mitochondrial pathway in developing rat brain neurons. *Hepatol. Baltim. Md* **35,** 1186–1195 (2002).
85. Robert, M. C. *et al.* Alterations in the cell cycle in the cerebellum of hyperbilirubinemic Gunn rat: a possible link with apoptosis? *PloS One* **8,** e79073 (2013).
86. Hoffman, D. J., Zanelli, S. A., Kubin, J., Mishra, O. P. & Delivoria-Papadopoulos, M. The in vivo effect of bilirubin on the N-methyl-D-aspartate receptor/ion channel complex in the brains of newborn piglets. *Pediatr. Res.* **40,** 804–808 (1996).
87. Suzuki, M. *et al.* Glutamate enhances proliferation and neurogenesis in human neural progenitor cell cultures derived from the fetal cortex. *Eur. J. Neurosci.* **24,** 645–653 (2006).
88. Grojean, S., Koziel, V., Vert, P. & Daval, J. L. Bilirubin induces apoptosis via activation of NMDA receptors in developing rat brain neurons. *Exp. Neurol.* **166,** 334–341 (2000).
89. McDonald, J. W., Shapiro, S. M., Silverstein, F. S. & Johnston, M. V. Role of glutamate receptor-mediated excitotoxicity in bilirubin-induced brain injury in the Gunn rat model. *Exp. Neurol.* **150,** 21–29 (1998).
90. Brito, M. A. *et al.* Bilirubin injury to neurons: contribution of oxidative stress and rescue by glycoursodeoxycholic acid. *Neurotoxicology* **29,** 259–269 (2008).
91. Cesaratto, L. *et al.* Bilirubin-induced cell toxicity involves PTEN activation through an APE1/Ref-1-dependent pathway. *J. Mol. Med. Berl. Ger.* **85,** 1099–1112 (2007).
92. Qaisiya, M., Coda Zabetta, C. D., Bellarosa, C. & Tiribelli, C. Bilirubin mediated oxidative stress involves antioxidant response activation via Nrf2 pathway. *Cell. Signal.* **26,** 512–520 (2014).
93. Bortolussi, G. *et al.* Impairment of enzymatic antioxidant defenses is associated with bilirubin-induced neuronal cell death in the cerebellum of Ugt1 KO mice. *Cell Death Dis.* **6,** e1739 (2015).
94. Davutoglu, M. *et al.* Oxidative stress and antioxidant status in neonatal hyperbilirubinemia. *Saudi Med. J.* **29,** 1743–1748 (2008).
95. Sarici, D. *et al.* Investigation on Malondialdehyde, S100B, and Advanced Oxidation Protein Product Levels in Significant Hyperbilirubinemia and the Effect of Intensive Phototherapy on these Parameters. *Pediatr. Neonatol.* (2014). doi:10.1016/j.pedneo.2014.06.006

96. Daood, M. J., Hoyson, M. & Watchko, J. F. Lipid peroxidation is not the primary mechanism of bilirubin-induced neurologic dysfunction in jaundiced Gunn rat pups. *Pediatr. Res.* **72,** 455–459 (2012).
97. Mustafa, M. G., Cowger, M. L. & King, T. E. Effects of bilirubin on mitochondrial reactions. *J. Biol. Chem.* **244,** 6403–6414 (1969).
98. Katoh-Semba, R. Studies on cellular toxicity of bilirubin: effect on brain glycolysis in the young rat. *Brain Res.* **113,** 339–348 (1976).
99. Roger, C., Koziel, V., Vert, P. & Nehlig, A. Effects of bilirubin infusion on local cerebral glucose utilization in the immature rat. *Brain Res. Dev. Brain Res.* **76,** 115–130 (1993).
100. Ngai, K. C., Yeung, C. Y. & Leung, C. S. Difference in susceptibilities of different cell lines to bilirubin damage. *J. Paediatr. Child Health* **36,** 51–55 (2000).
101. Chen, H. C., Tsai, D. J., Wang, C. H. & Chen, Y. C. An electron microscopic and radioautographic study on experimental kernicterus. I. Bilirubin transport via astroglia. *Am. J. Pathol.* **56,** 31–58 (1969).
102. Silberberg, D. H. & Schutta, H. S. The effects of unconjugated bilirubin and related pigments on cultures of rat cerebellum. *J. Neuropathol. Exp. Neurol.* **26,** 572–583 (1967).
103. Falcão, A. S., Fernandes, A., Brito, M. A., Silva, R. F. M. & Brites, D. Bilirubin-induced immunostimulant effects and toxicity vary with neural cell type and maturation state. *Acta Neuropathol. (Berl.)* **112,** 95–105 (2006).
104. Amit, Y. & Brenner, T. Age-dependent sensitivity of cultured rat glial cells to bilirubin toxicity. *Exp. Neurol.* **121,** 248–255 (1993).
105. Gordo, A. C. *et al.* Unconjugated bilirubin activates and damages microglia. *J. Neurosci. Res.* **84,** 194–201 (2006).
106. Silva, S. L. *et al.* Features of bilirubin-induced reactive microglia: from phagocytosis to inflammation. *Neurobiol. Dis.* **40,** 663–675 (2010).
107. Barateiro, A. *et al.* Unconjugated bilirubin restricts oligodendrocyte differentiation and axonal myelination. *Mol. Neurobiol.* **47,** 632–644 (2013).
108. Barateiro, A., Domingues, H. S., Fernandes, A., Relvas, J. B. & Brites, D. Rat cerebellar slice cultures exposed to bilirubin evidence reactive gliosis, excitotoxicity and impaired myelinogenesis that is prevented by AMPA and TNF-α inhibitors. *Mol. Neurobiol.* **49,** 424–439 (2014).
109. Brito, M. A. *et al.* Cerebellar axon/myelin loss, angiogenic sprouting, and neuronal increase of vascular endothelial growth factor in a preterm infant with kernicterus. *J. Child Neurol.* **27,** 615–624 (2012).
110. Bortolussi, G. *et al.* Rescue of bilirubin-induced neonatal lethality in a mouse model of Crigler-Najjar syndrome type I by AAV9-mediated gene transfer. *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.* **26,** 1052–1063 (2012).

111. Schutta, H. S. & Johnson, L. Electron microscopic observations on acute bilirubin encephalopathy in Gunn rats induced by sulfadimethoxine. *Lab. Investig. J. Tech. Methods Pathol.* **24,** 82–89 (1971).
112. Vreman, H. J., Wong, R. J. & Stevenson, D. K. Phototherapy: current methods and future directions. *Semin. Perinatol.* **28,** 326–333 (2004).
113. Cremer, R. J., Perryman, P. W. & Richards, D. H. Influence of light on the hyperbilirubinaemia of infants. *Lancet Lond. Engl.* **1,** 1094–1097 (1958).
114. Lucey, J., Ferriero, M. & Hewitt, J. Prevention of hyperbilirubinemia of prematurity by phototherapy. *Pediatrics* **41,** 1047–1054 (1968).
115. McDonagh, A. F. & Lightner, D. A. Phototherapy and the photobiology of bilirubin. *Semin. Liver Dis.* **8,** 272–283 (1988).
116. Stokowski, L. A. Fundamentals of phototherapy for neonatal jaundice. *Adv. Neonatal Care Off. J. Natl. Assoc. Neonatal Nurses* **11,** S10–21 (2011).
117. Xiong, T., Qu, Y., Cambier, S. & Mu, D. The side effects of phototherapy for neonatal jaundice: what do we know? What should we do? *Eur. J. Pediatr.* **170,** 1247–1255 (2011).
118. Jasprova, J. *et al.* The Biological Effects of Bilirubin Photoisomers. *PloS One* **11,** e0148126 (2016).
119. Sellier, A. L. *et al.* Successful plasmapheresis for acute and severe unconjugated hyperbilirubinemia in a child with crigler najjar type I syndrome. *JIMD Rep.* **2,** 33–36 (2012).
120. Schreuder, A. B. *et al.* Optimizing exchange transfusion for severe unconjugated hyperbilirubinemia: studies in the Gunn rat. *PloS One* **8,** e77179 (2013).
121. Chitty, H. E., Ziegler, N., Savoia, H., Doyle, L. W. & Fox, L. M. Neonatal exchange transfusions in the 21st century: a single hospital study. *J. Paediatr. Child Health* **49,** 825–832 (2013).
122. Keenan, W. J., Novak, K. K., Sutherland, J. M., Bryla, D. A. & Fetterly, K. L. Morbidity and mortality associated with exchange transfusion. *Pediatrics* **75,** 417–421 (1985).
123. Jackson, J. C. Adverse events associated with exchange transfusion in healthy and ill newborns. *Pediatrics* **99,** E7 (1997).
124. Patra, K., Storfer-Isser, A., Siner, B., Moore, J. & Hack, M. Adverse events associated with neonatal exchange transfusion in the 1990s. *J. Pediatr.* **144,** 626–631 (2004).
125. Bowen, W., Porter, E. & Waters, W. The protective action of albumin in bilirubin toxicity in newborn puppies. *AMA J. Dis. Child.* **98,** 568 (1959).
126. Odell, G. B. The dissociation of bilirubin from albumin and its clinical implications. *J. Pediatr.* **55,** 268–279 (1959).
127. Caldera, R. *et al.* [The effect of human albumin in association with intensive phototherapy in the management of neonatal jaundice]. *Arch. Fr. Pédiatrie* **50,** 399–402 (1993).
128. Hosono, S. *et al.* Effects of albumin infusion therapy on total and unbound bilirubin values in term infants with intensive phototherapy. *Pediatr. Int. Off. J. Jpn. Pediatr. Soc.* **43,** 8–11 (2001).

129. Zelenka, J. *et al.* Highly sensitive method for quantitative determination of bilirubin in biological fluids and tissues. *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.* **867,** 37–42 (2008).
130. Cuperus, F. J. C. *et al.* Beyond plasma bilirubin: the effects of phototherapy and albumin on brain bilirubin levels in Gunn rats. *J. Hepatol.* **58,** 134–140 (2013).
131. Lysy, P.-A. *et al.* Liver cell transplantation for Crigler-Najjar syndrome type I: update and perspectives. *World J. Gastroenterol. WJG* **14,** 3464–3470 (2008).
132. Chen, Y. *et al.* Amelioration of Hyperbilirubinemia in Gunn Rats after Transplantation of Human Induced Pluripotent Stem Cell-Derived Hepatocytes. *Stem Cell Rep.* **5,** 22–30 (2015).
133. Vlaardingerbroek, H. *et al.* Albumin synthesis in very low birth weight infants is enhanced by early parenteral lipid and high-dose amino acid administration. *Clin. Nutr. Edinb. Scotl.* (2015). doi:10.1016/j.clnu.2015.04.019
134. Kielian, T. *et al.* Minocycline modulates neuroinflammation independently of its antimicrobial activity in staphylococcus aureus-induced brain abscess. *Am. J. Pathol.* **171,** 1199–1214 (2007).
135. Tikka, T., Fiebich, B. L., Goldsteins, G., Keinanen, R. & Koistinaho, J. Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. *J. Neurosci. Off. J. Soc. Neurosci.* **21,** 2580–2588 (2001).
136. Tikka, T. M. & Koistinaho, J. E. Minocycline provides neuroprotection against N-methyl-D-aspartate neurotoxicity by inhibiting microglia. *J. Immunol. Baltim. Md 1950* **166,** 7527–7533 (2001).
137. Chen, M. *et al.* Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. *Nat. Med.* **6,** 797–801 (2000).
138. Du, Y. *et al.* Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 14669–14674 (2001).
139. Lin, S. *et al.* Minocycline blocks bilirubin neurotoxicity and prevents hyperbilirubinemia-induced cerebellar hypoplasia in the Gunn rat. *Eur. J. Neurosci.* **22,** 21–27 (2005).
140. Geiger, A. S., Rice, A. C. & Shapiro, S. M. Minocycline blocks acute bilirubin-induced neurological dysfunction in jaundiced Gunn rats. *Neonatology* **92,** 219–226 (2007).
141. Tastekin, A., Gepdiremen, A., Ors, R., Buyukokuroglu, M. E. & Halici, Z. Protective effect of L-carnitine against bilirubin-induced neuronal cell death. *Brain Dev.* **28,** 436–439 (2006).
142. Zhang, B., Yang, X. & Gao, X. Taurine protects against bilirubin-induced neurotoxicity in vitro. *Brain Res.* **1320,** 159–167 (2010).
143. Hankø, E., Hansen, T. W. D., Almaas, R., Paulsen, R. & Rootwelt, T. Synergistic protection of a general caspase inhibitor and MK-801 in bilirubin-induced cell death in human NT2-N neurons. *Pediatr. Res.* **59,** 72–77 (2006).

144. Silva, S. L. *et al.* Neuritic growth impairment and cell death by unconjugated bilirubin is mediated by NO and glutamate, modulated by microglia, and prevented by glycoursodeoxycholic acid and interleukin-10. *Neuropharmacology* **62,** 2398–2408 (2012).
145. Palmela, I. *et al.* Hydrophilic bile acids protect human blood-brain barrier endothelial cells from disruption by unconjugated bilirubin: an in vitro study. *Front. Neurosci.* **9,** 80 (2015).
146. Hogue, M. J. Human fetal brain cells in tissue cultures; their identification and motility. *J. Exp. Zool.* **106,** 85–107 (1947).
147. Costero, I. & Pomerat, C. M. Cultivation of neurons from the adult human cerebral and cerebellar cortes. *Am. J. Anat.* **89,** 405–467 (1951).
148. Gähwiler, B. H. Organotypic monolayer cultures of nervous tissue. *J. Neurosci. Methods* **4,** 329–342 (1981).
149. Gähwiler, B. H., Capogna, M., Debanne, D., McKinney, R. A. & Thompson, S. M. Organotypic slice cultures: a technique has come of age. *Trends Neurosci.* **20,** 471–477 (1997).
150. Steensen, B. H., Nedergaard, S., Østergaard, K. & Lambert, J. D. C. Electrophysiological characterization of dopaminergic and non-dopaminergic neurones in organotypic slice cultures of the rat ventral mesencephalon. *Exp. Brain Res.* **106,** 205–214 (1995).
151. Raineteau, O., Rietschin, L., Gradwohl, G., Guillemot, F. & Gähwiler, B. H. Neurogenesis in hippocampal slice cultures. *Mol. Cell. Neurosci.* **26,** 241–250 (2004).
152. Gähwiler, B. H., Thompson, S. M. & Muller, D. in *Current Protocols in Neuroscience* (John Wiley & Sons, Inc., 2001). at <http://onlinelibrary.wiley.com/doi/10.1002/0471142301.ns0611s09/abstract>
153. Stoppini, L., Buchs, P. A. & Muller, D. A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods* **37,** 173–182 (1991).
154. Gähwiler, B. H. Slice cultures of cerebellar, hippocampal and hypothalamic tissue. *Experientia* **40,** 235–243 (1984).
155. Buchs, P. A., Stoppini, L. & Muller, D. Structural modifications associated with synaptic development in area CA1 of rat hippocampal organotypic cultures. *Brain Res. Dev. Brain Res.* **71,** 81–91 (1993).
156. Frotscher, M., Zafirov, S. & Heimrich, B. Development of identified neuronal types and of specific synaptic connections in slice cultures of rat hippocampus. *Prog. Neurobiol.* **45,** 143–164 (1995).
157. Vanderklish, P., Saido, T. C., Gall, C., Arai, A. & Lynch, G. Proteolysis of spectrin by calpain accompanies theta-burst stimulation in cultured hippocampal slices. *Brain Res. Mol. Brain Res.* **32,** 25–35 (1995).
158. McCutchen, M. E., Bramham, C. R. & Pozzo-Miller, L. D. Modulation of neuronal calcium signaling by neurotrophic factors. *Int. J. Dev. Neurosci. Off. J. Int. Soc. Dev. Neurosci.* **20,** 199–207 (2002).
159. Ebrahimi, F. *et al.* Analyses of neuronal damage in excitotoxically lesioned organotypic hippocampal slice cultures. *Ann. Anat. Anat. Anz. Off. Organ Anat. Ges.* **192,** 199–204 (2010).

160. Miller, A. P. *et al.* Effects of blast overpressure on neurons and glial cells in rat organotypic hippocampal slice cultures. *Front. Neurol.* **6,** 20 (2015).
161. Shirakawa, H., Katsuki, H., Kume, T., Kaneko, S. & Akaike, A. Aminoglutethimide prevents excitotoxic and ischemic injuries in cortical neurons. *Br. J. Pharmacol.* **147,** 729–736 (2006).
162. Staal, J. A., Alexander, S. R., Liu, Y., Dickson, T. D. & Vickers, J. C. Characterization of cortical neuronal and glial alterations during culture of organotypic whole brain slices from neonatal and mature mice. *PloS One* **6,** e22040 (2011).
163. Bouslama-Oueghlani, L., Wehrlé, R., Sotelo, C. & Dusart, I. The developmental loss of the ability of Purkinje cells to regenerate their axons occurs in the absence of myelin: an in vitro model to prevent myelination. *J. Neurosci. Off. J. Soc. Neurosci.* **23,** 8318–8329 (2003).
164. Mancini, J. D., Autio, D. M. & Atchison, W. D. Continuous exposure to low concentrations of methylmercury impairs cerebellar granule cell migration in organotypic slice culture. *Neurotoxicology* **30,** 203–208 (2009).
165. Jost, B., Grabert, J., Patz, S., Schmidt, M. & Wahle, P. GABAC receptor subunit mRNA expression in the rat superior colliculus is regulated by calcium channels, neurotrophins, and GABAC receptor activity. *Brain Cell Biol.* **35,** 251–266 (2006).
166. Hafidi, A., Sanes, D. H. & Hillman, D. E. Regeneration of the auditory midbrain intercommissural projection in organotypic culture. *J. Neurosci. Off. J. Soc. Neurosci.* **15,** 1298–1307 (1995).
167. Lyng, G. D., Snyder-Keller, A. & Seegal, R. F. Dopaminergic development of prenatal ventral mesencephalon and striatum in organotypic co-cultures. *Brain Res.* **1133,** 1–9 (2007).
168. Neely, M. D., Schmidt, D. E. & Deutch, A. Y. Cortical regulation of dopamine depletion-induced dendritic spine loss in striatal medium spiny neurons. *Neuroscience* **149,** 457–464 (2007).
169. Shichinohe, H. *et al.* Bone marrow stromal cells promote neurite extension in organotypic spinal cord slice: significance for cell transplantation therapy. *Neurorehabil. Neural Repair* **22,** 447–457 (2008).
170. Tolosa, L., Mir, M., Asensio, V. J., Olmos, G. & Lladó, J. Vascular endothelial growth factor protects spinal cord motoneurons against glutamate-induced excitotoxicity via phosphatidylinositol 3-kinase. *J. Neurochem.* **105,** 1080–1090 (2008).
171. Leutgeb, J. K., Frey, J. U. & Behnisch, T. LTP in cultured hippocampal-entorhinal cortex slices from young adult (P25-30) rats. *J. Neurosci. Methods* **130,** 19–32 (2003).
172. Tom, V. J., Doller, C. M., Malouf, A. T. & Silver, J. Astrocyte-associated fibronectin is critical for axonal regeneration in adult white matter. *J. Neurosci. Off. J. Soc. Neurosci.* **24,** 9282–9290 (2004).
173. Noraberg, J. *et al.* Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. *Curr. Drug Targets CNS Neurol. Disord.* **4,** 435–452 (2005).
174. Sundstrom, L., Morrison, B., Bradley, M. & Pringle, A. Organotypic cultures as tools for functional screening in the CNS. *Drug Discov. Today* **10,** 993–1000 (2005).

175. Lossi, L., Alasia, S., Salio, C. & Merighi, A. Cell death and proliferation in acute slices and organotypic cultures of mammalian CNS. *Prog. Neurobiol.* **88,** 221–245 (2009).
176. Holopainen, I. E. Organotypic hippocampal slice cultures: a model system to study basic cellular and molecular mechanisms of neuronal cell death, neuroprotection, and synaptic plasticity. *Neurochem. Res.* **30,** 1521–1528 (2005).
177. Shapiro, S. M. Definition of the clinical spectrum of kernicterus and bilirubin-induced neurologic dysfunction (BIND). *J. Perinatol. Off. J. Calif. Perinat. Assoc.* **25,** 54–59 (2005).
178. Calligaris, S. D. *et al.* Cytotoxicity is predicted by unbound and not total bilirubin concentration. *Pediatr. Res.* **62,** 576–580 (2007).
179. Giraudi, P. J., Bellarosa, C., Coda-Zabetta, C. D., Peruzzo, P. & Tiribelli, C. Functional induction of the cystine-glutamate exchanger system Xc(-) activity in SH-SY5Y cells by unconjugated bilirubin. *PloS One* **6,** e29078 (2011).
180. Roca, L. *et al.* Factors affecting the binding of bilirubin to serum albumins: validation and application of the peroxidase method. *Pediatr. Res.* **60,** 724–728 (2006).
181. Ostrow, J. D. & Mukerjee, P. Solvent partition of 14C-unconjugated bilirubin to remove labeled polar contaminants. *Transl. Res. J. Lab. Clin. Med.* **149,** 37–45 (2007).
182. Zhang, S.-J. *et al.* Nuclear calcium signaling controls expression of a large gene pool: identification of a gene program for acquired neuroprotection induced by synaptic activity. *PLoS Genet.* **5,** e1000604 (2009).
183. Peng, Z. F. *et al.* Multifaceted role of nitric oxide in an in vitro mouse neuronal injury model: transcriptomic profiling defines the temporal recruitment of death signalling cascades. *J. Cell. Mol. Med.* **16,** 41–58 (2012).
184. Chen, M. J. *et al.* Gene profiling identifies commonalities in neuronal pathways in excitotoxicity: evidence favouring cell cycle re-activation in concert with oxidative stress. *Neurochem. Int.* **62,** 719–730 (2013).
185. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29,** e45 (2001).
186. Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3,** RESEARCH0034 (2002).
187. Bustin, S. A. *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **55,** 611–622 (2009).
188. Palace, J. Neuroprotection and repair. *J. Neurol. Sci.* **265,** 21–25 (2008).
189. Chaturvedi, R. K. & Beal, M. F. Mitochondria targeted therapeutic approaches in Parkinson's and Huntington's diseases. *Mol. Cell. Neurosci.* **55,** 101–114 (2013).

190. Tataranno, M. L., Perrone, S., Longini, M. & Buonocore, G. New antioxidant drugs for neonatal brain injury. *Oxid. Med. Cell. Longev.* **2015,** 108251 (2015).
191. Lim, G. P. *et al.* The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. *J. Neurosci. Off. J. Soc. Neurosci.* **21,** 8370–8377 (2001).
192. Ghoneim, A. I., Abdel-Naim, A. B., Khalifa, A. E. & El-Denshary, E. S. Protective effects of curcumin against ischaemia/reperfusion insult in rat forebrain. *Pharmacol. Res.* **46,** 273–279 (2002).
193. Crowther, C. A., Hiller, J. E., Doyle, L. W., Haslam, R. R. & Australasian Collaborative Trial of Magnesium Sulphate (ACTOMg SO4) Collaborative Group. Effect of magnesium sulfate given for neuroprotection before preterm birth: a randomized controlled trial. *JAMA* **290,** 2669–2676 (2003).
194. Westermaier, T. *et al.* Magnesium treatment for neuroprotection in ischemic diseases of the brain. *Exp. Transl. Stroke Med.* **5,** 6 (2013).
195. Chechneva, O., Dinkel, K., Cavaliere, F., Martinez-Sanchez, M. & Reymann, K. G. Anti-inflammatory treatment in oxygen-glucose-deprived hippocampal slice cultures is neuroprotective and associated with reduced cell proliferation and intact neurogenesis. *Neurobiol. Dis.* **23,** 247–259 (2006).
196. Miller, S. P. *et al.* Prolonged indomethacin exposure is associated with decreased white matter injury detected with magnetic resonance imaging in premature newborns at 24 to 28 weeks' gestation at birth. *Pediatrics* **117,** 1626–1631 (2006).
197. Falcão, A. S., Fernandes, A., Brito, M. A., Silva, R. F. M. & Brites, D. Bilirubin-induced inflammatory response, glutamate release, and cell death in rat cortical astrocytes are enhanced in younger cells. *Neurobiol. Dis.* **20,** 199–206 (2005).
198. Olusanya, B. O., Ogunlesi, T. A. & Slusher, T. M. Why is kernicterus still a major cause of death and disability in low-income and middle-income countries? *Arch. Dis. Child.* **99,** 1117–1121 (2014).
199. Silva, R. F. M., Rodrigues, C. M. P. & Brites, D. Rat cultured neuronal and glial cells respond differently to toxicity of unconjugated bilirubin. *Pediatr. Res.* **51,** 535–541 (2002).
200. Agrawal, V. K., Shukla, R., Misra, P. K., Kapoor, R. K. & Malik, G. K. Brainstem auditory evoked response in newborns with hyperbilirubinemia. *Indian Pediatr.* **35,** 513–518 (1998).
201. Sharma, P. *et al.* Brainstem evoked response audiometry (BAER) in neonates with hyperbilirubinemia. *Indian J. Pediatr.* **73,** 413–416 (2006).
202. Hansen, T. W., Bratlid, D. & Walaas, S. I. Bilirubin decreases phosphorylation of synapsin I, a synaptic vesicle-associated neuronal phosphoprotein, in intact synaptosomes from rat cerebral cortex. *Pediatr. Res.* **23,** 219–223 (1988).
203. Zhang, L., Liu, W., Tanswell, A. K. & Luo, X. The effects of bilirubin on evoked potentials and long-term potentiation in rat hippocampus in vivo. *Pediatr. Res.* **53,** 939–944 (2003).
204. Song, S., Hu, Y., Gu, X., Si, F. & Hua, Z. A novel newborn rat kernicterus model created by injecting a bilirubin solution into the cisterna magna. *PloS One* **9,** e96171 (2014).

205. Spencer, R. F., Shaia, W. T., Gleason, A. T., Sismanis, A. & Shapiro, S. M. Changes in calcium-binding protein expression in the auditory brainstem nuclei of the jaundiced Gunn rat. *Hear. Res.* **171,** 129–141 (2002).
206. Friede, R. L. & Van Houten, W. H. Relations between postmortem alterations and glycolytic metabolism in the brain. *Exp. Neurol.* **4,** 197–204 (1961).
207. Hulse, R. E., Winterfield, J., Kunkler, P. E. & Kraig, R. P. Astrocytic clasmatodendrosis in hippocampal organ culture. *Glia* **33,** 169–179 (2001).
208. Xu, J. *et al.* Glutamate-induced exocytosis of glutamate from astrocytes. *J. Biol. Chem.* **282,** 24185–24197 (2007).
209. Fernandes, A. *et al.* Inflammatory signalling pathways involved in astroglial activation by unconjugated bilirubin. *J. Neurochem.* **96,** 1667–1679 (2006).
210. Liaury, K. *et al.* Morphological features of microglial cells in the hippocampal dentate gyrus of Gunn rat: a possible schizophrenia animal model. *J. Neuroinflammation* **9,** 56 (2012).
211. Ali, D. W. & Salter, M. W. NMDA receptor regulation by Src kinase signalling in excitatory synaptic transmission and plasticity. *Curr. Opin. Neurobiol.* **11,** 336–342 (2001).
212. Holopainen, I. E. Seizures in the developing brain: cellular and molecular mechanisms of neuronal damage, neurogenesis and cellular reorganization. *Neurochem. Int.* **52,** 935–947 (2008).
213. Thornton, C. *et al.* Molecular mechanisms of neonatal brain injury. *Neurol. Res. Int.* **2012,** 506320 (2012).
214. Jew, J. Y. & Williams, T. H. Ultrastructural aspects of bilirubin encephalopathy in cochlear nuclei of the Gunn rat. *J. Anat.* **124,** 599–614 (1977).
215. Shaia, W. T. *et al.* Immunohistochemical localization of calcium-binding proteins in the brainstem vestibular nuclei of the jaundiced Gunn rat. *Hear. Res.* **173,** 82–90 (2002).
216. Martinez-Galan, J. R., Perez-Martinez, F. C. & Juiz, J. M. Signalling routes and developmental regulation of group I metabotropic glutamate receptors in rat auditory midbrain neurons. *J. Neurosci. Res.* **90,** 1913–1923 (2012).
217. Caicedo, A., Kungel, M., Pujol, R. & Friauf, E. Glutamate-induced Co2+ uptake in rat auditory brainstem neurons reveals developmental changes in Ca2+ permeability of glutamate receptors. *Eur. J. Neurosci.* **10,** 941–954 (1998).
218. Gürses, D., Kiliç, I. & Sahiner, T. Effects of hyperbilirubinemia on cerebrocortical electrical activity in newborns. *Pediatr. Res.* **52,** 125–130 (2002).
219. Scheidt, P. C., Mellits, E. D., Hardy, J. B., Drage, J. S. & Boggs, T. R. Toxicity to bilirubin in neonates: infant development during first year in relation to maximum neonatal serum bilirubin concentration. *J. Pediatr.* **91,** 292–297 (1977).

220. Rubin, R. A., Balow, B. & Fisch, R. O. Neonatal serum bilirubin levels related to cognitive development at ages 4 through 7 years. *J. Pediatr.* **94,** 601–604 (1979).
221. Koziol, L. F., Budding, D. E. & Chidekel, D. Hyperbilirubinemia: subcortical mechanisms of cognitive and behavioral dysfunction. *Pediatr. Neurol.* **48,** 3–13 (2013).
222. Rice, D. & Barone, S. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ. Health Perspect.* **108 Suppl 3,** 511–533 (2000).
223. Bandeira, F., Lent, R. & Herculano-Houzel, S. Changing numbers of neuronal and non-neuronal cells underlie postnatal brain growth in the rat. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 14108–14113 (2009).
224. Miyamoto, K. *et al.* Involvement of enhanced sensitivity of N-methyl-D-aspartate receptors in vulnerability of developing cortical neurons to methylmercury neurotoxicity. *Brain Res.* **901,** 252–258 (2001).
225. Haberny, K. A. *et al.* Ontogeny of the N-methyl-D-aspartate (NMDA) receptor system and susceptibility to neurotoxicity. *Toxicol. Sci. Off. J. Soc. Toxicol.* **68,** 9–17 (2002).
226. Galic, M. A. *et al.* Postnatal inflammation increases seizure susceptibility in adult rats. *J. Neurosci. Off. J. Soc. Neurosci.* **28,** 6904–6913 (2008).
227. Keino, H. *et al.* Mode of prevention by phototherapy of cerebellar hypoplasia in a new Sprague-Dawley strain of jaundiced Gunn rats. *Pediatr. Neurosci.* **12,** 145–150 (1985).
228. Keino, H. & Kashiwamata, S. Critical period of bilirubin-induced cerebellar hypoplasia in a new Sprague-Dawley strain of jaundiced Gunn rats. *Neurosci. Res.* **6,** 209–215 (1989).
229. Lee, Y.-K., Daito, Y., Katayama, Y., Minami, H. & Negishi, H. The significance of measurement of serum unbound bilirubin concentrations in high-risk infants. *Pediatr. Int. Off. J. Jpn. Pediatr. Soc.* **51,** 795–799 (2009).
230. Mikoshiba, K., Kohsaka, S., Takamatsu, K. & Tsukada, Y. Cerebellar hypoplasia in the Gunn rat with hereditary hyperbilirubinemia: immunohistochemical and neurochemical studies. *J. Neurochem.* **35,** 1309–1318 (1980).
231. Bortolussi, G. *et al.* Age-dependent pattern of cerebellar susceptibility to bilirubin neurotoxicity in vivo in mice. *Dis. Model. Mech.* **7,** 1057–1068 (2014).
232. Tabarki, B., Khalifa, M., Yacoub, M., Tlili, K. & Essoussi, A. S. Cerebellar symptoms heralding bilirubin encephalopathy in Crigler-Najjar syndrome. *Pediatr. Neurol.* **27,** 234–236 (2002).
233. Daood, M. J. & Watchko, J. F. Calculated in vivo free bilirubin levels in the central nervous system of Gunn rat pups. *Pediatr. Res.* **60,** 44–49 (2006).
234. Creel, D. J. in *Webvision: The Organization of the Retina and Visual System* (eds. Kolb, H., Fernandez, E. & Nelson, R.) (University of Utah Health Sciences Center, 1995). at <http://www.ncbi.nlm.nih.gov/books/NBK107218/>

235. Chin, K. C., Taylor, M. J. & Perlman, M. Improvement in auditory and visual evoked potentials in jaundiced preterm infants after exchange transfusion. *Arch. Dis. Child.* **60,** 714–717 (1985).
236. Chen, Y. J. & Kang, W. M. Effects of bilirubin on visual evoked potentials in term infants. *Eur. J. Pediatr.* **154,** 662–666 (1995).
237. Ozmert, E. *et al.* Long-term follow-up of indirect hyperbilirubinemia in full-term Turkish infants. *Acta Paediatr. Oslo Nor. 1992* **85,** 1440–1444 (1996).
238. Hou, C., Norcia, A. M., Madan, A. & Good, W. V. Visuocortical function in infants with a history of neonatal jaundice. *Invest. Ophthalmol. Vis. Sci.* **55,** 6443–6449 (2014).
239. Aggarwal, B. B. & Harikumar, K. B. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. *Int. J. Biochem. Cell Biol.* **41,** 40–59 (2009).
240. Jiang, Z. *et al.* Protection against cognitive impairment and modification of epileptogenesis with curcumin in a post-status epilepticus model of temporal lobe epilepsy. *Neuroscience* **310,** 362–371 (2015).
241. Wang, B. *et al.* Curcumin attenuates brain edema in mice with intracerebral hemorrhage through inhibition of AQP4 and AQP9 expression. *Acta Pharmacol. Sin.* **36,** 939–948 (2015).
242. Levene, M. *et al.* Acute effects of two different doses of magnesium sulphate in infants with birth asphyxia. *Arch. Dis. Child. Fetal Neonatal Ed.* **73,** F174–177 (1995).
243. Ichiba, H. *et al.* Randomized controlled trial of magnesium sulfate infusion for severe birth asphyxia. *Pediatr. Int. Off. J. Jpn. Pediatr. Soc.* **44,** 505–509 (2002).
244. Wilmott, L. A. & Thompson, L. T. Sex- and dose-dependent effects of post-trial calcium channel blockade by magnesium chloride on memory for inhibitory avoidance conditioning. *Behav. Brain Res.* **257,** 49–53 (2013).
245. Kwon, B. K. *et al.* Magnesium chloride in a polyethylene glycol formulation as a neuroprotective therapy for acute spinal cord injury: preclinical refinement and optimization. *J. Neurotrauma* **26,** 1379–1393 (2009).
246. Hoane, M. R., Knotts, A. A., Akstulewicz, S. L., Aquilano, M. & Means, L. W. The behavioral effects of magnesium therapy on recovery of function following bilateral anterior medial cortex lesions in the rat. *Brain Res. Bull.* **60,** 105–114 (2003).
247. Bok, S. *et al.* In vivo imaging of activated microglia in a mouse model of focal cerebral ischemia by two-photon microscopy. *Biomed. Opt. Express* **6,** 3303–3312 (2015).

8 Publications

8.1 Peer reviewed papers

Jasprova J, **Dal Ben M**, Vianello E, Goncharova I, Urbanova M, Vyroubalova K, Gazzin S, Tiribelli C, Sticha M, Cerna M, Vitek L.; "The biological effects of bilirubin photoisomers" PLoS One. 2016 Feb 1;11(2):e0148126. doi: 10.1371/journal.pone.0148126.

Dal Ben M, Bottin C., Zanconati F, Tiribelli C, Gazzin S. "Brain regional and developmental sensitivity in hyperbilirubinemia: efficacy of a new therapeutical approach for brain protection" In preparation.

Marin V, Rosso N, **Dal Ben M**, Raseni A, Boschelle M, Degrassi C, Nemeckova I, Nachtigal P, Avellini C, Tiribelli C, Gazzin S. "*A juvenile model for study the progression to nonalcoholic steatohepatitis toward of non-alcoholic fatty liver disease tacking care of the sexual differences***".** Submitted.

8.2 Congresses publications (abstracts)

Marin V, Rosso N, **Dal Ben M**, Raseni A, Degrassi C, Tiribelli C, Gazzin S. A novel mouse model for the study of pediatric Non-Alcoholic Fatty Liver Disease (NAFLD). Hepatology October 2015 - Vol 62, Supplement S1 Pages iiA–vA, 2A–1378A.

V. Marin, S.E. Gambaro, **M. Dal Ben**, A. Raseni, C. Degrassi, C. Tiribelli, S. Gazzin, N. Rosso. "*Therapeutic approach to NAFLD/NASH: is Silimarin able to ameliorate liver injury?"* Digestive and Liver Disease 2015, 47(3). Abstracts of the A.I.S.F. – Italian Association for the Study of the Liver – Monothematic Conference 2015 "NASH: hepatic, oncologic and cardiovascular disease" Modena, 8–10 October 2015

V. Marin, N. Rosso, **M. Dal Ben**, A. Raseni, C. Degrassi, C. Tiribelli, S. Gazzin; "Effect of age and gender in the progression of NAFLD towards NASH in a juvenile mice model". Digestive and Liver Disease 2015, 47(3). Abstracts of the A.I.S.F. – Italian Association for the Study of the Liver – Monothematic Conference 2015 "NASH: hepatic, oncologic and cardiovascular disease" Modena, 8–10 October 2015

Dal Ben M, Tiribelli C, Gazzin S; "*Regional and developmental screening of bilirubin toxicity: hippocampus is the most sensible region*". Journal of Pediatric and Neonatal Individualized Medicine 2015;4(2):e040206. Selected Abstracts of the 1st Congress of joint European Neonatal Societies (jENS 2015).

Marin V, Rosso N, **Dal Ben M**, Raseni A, Degrassi C, Tiribelli C, Gazzin S. "*Characterization of an in vivo model of juvenile non-alcoholic fatty liver disease***".** Journal of Hepatology 2015, 62 (Suppl 2):S711-S712. Abstracts of The International Liver Congress™ 2015 – 50th Annual meeting of the European Association for the Study of the Liver

Marin V, Gazzin S, **Dal Ben M**, Raseni A, Degrassi C, Tiribelli C, Rosso N. *"Progression from NAFLD to NASH: Gender does really matter?"* Journal of Hepatology 2015, 62 (Suppl 2):S707. Abstracts of The International Liver Congress™ 2015 – 50th Annual meeting of the European Association for the Study of the Liver.

Dal Ben M, Gazzin S, Tiribelli C. "*Neonatal hyperbilirubinemia*"**.** Italian Journal of Pediatrics 2014 40(Suppl 2):A10.

8.3 Oral presentations at international congresses

Dal Ben M, Tiribelli C, Gazzin S; "*Regional and developmental screening of bilirubin toxicity: hippocampus is the most sensible region*". Abstract selected for short oral presentation at 1st jENS 2015 - Budapest (Hungary)

Dal Ben M, Tiribelli C, Gazzin S; "*Discovery of regional and developmental brain pattern of sensibility to bilirubin damage in an organotypic culture screening*". DOTYR2015 - Trieste (Italy)