# Bilirubin and Brain Toxicity

by

Erik Hankø

Department of Pediatric Research, Rikshospitalet-Radiumhospitalet Medical Center, and University of Oslo, N-0027 Oslo, Norway

The Medical Faculty,
University of Oslo

*Series of dissertations submitted to the*
*Faculty of Medicine, University of Oslo*
*No. 630*

ISBN 978-82-8072-733-6



Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2008.

Produced in co-operation with Unipub AS.
The thesis is produced by Unipub AS merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

*Unipub AS is owned by*
*The University Foundation for Student Life (SiO)*

## CONTENTS

## ACKNOWLEDGEMENTS

This work was carried out at the Department of Medical Biochemistry, the University of Oslo and the Department of Pediatric Research, Rikshospitalet-Radiumhospitalet Medical Center, Faculty of Medicine, University of Oslo during the years 1998 to 2006. From 1998 to 2003 I was financed by the University of Oslo as a researcher and lecturer for medical students in pediatrics at Rikshospitalet-Radiumhospitalet Medical Center. The first study was planned and performed in close cooperation with my supervisor, Professor Thor Willy Ruud Hansen. Ruud Hansen generously invited me to complete the first study which had been initiated in collaboration with Jon F Watchko in their laboratories in Pittsburgh.

The three other studies were performed with my second supervisor, Dr. Terje Rootwelt and his research group at the Department of Pediatric Research. Dr Rootwelt's inspiring feed back and guidance during the period from 2001 to 2006 combined with Professor Ruud Hansen's knowledge of the scientific field of bilirubin made this an exciting time. In my work on the NT2-N neurons I was given freedom to experiment and to learn from mistakes, and to take my own ideas into the laboratory. This made this achievement most valuable to me. I am also grateful to Professor Ivar Walaas at the Institute of Medical Biochemistry, who introduced me to enzyme kinetics, although this work could not be included in the thesis. My supervisors' generous guidance, support and patience are highly appreciated.

I am also most grateful to the other members of the group; Julie K. Lindstad, Arild Holth and Cecilie Kåsi who cultured the NT2-N neurons with a gentle touch and provided excellent conditions for the experiments; Runar Almaas who passed on his laboratory skills and provided critical scientific feedback; and Elisabeth Frøyland for cheerful support and for doing her best to keep me organized. I would also like to thank Professor Ola Didrik Saugstad Director at the Department of Pediatric Research for letting me work in a stimulating environment with many skilful people who all have contributed to this work. Also, the generous contributions from Frode Fonnum, Ragnhild Paulsen, Christine Gran, Carola Rosseland, Luisa Luna, Kirsten Grundt, Anne Karine Østvold, Jon Berg-Johnsen, and Elin Kampenhaug are not forgotten.

I would also like to express my gratitude to my teacher colleagues at Ullevål University Hospital and Rikshospitalet-Radiumhospitalet Medical Center and the medical students in pediatrics who gave me the opportunities and the challenges to develop my academic skills. My special thanks go to Professor Bengt Frode Kase for his cooperation and support in developing teaching programs and for listening when I needed his advice, and to Øystein Riise and Kai Handeland with whom I happily shared office the last three years of this work.

This work was supported financially by the University of Oslo, which generously extended my position, and grants from The University Anesthesia and Critical Care Medical Foundation, Pittsburgh, Children's Hospital of Pittsburgh, the Nansen Fund, Norwegian Research Council, Anders Jahres Foundation, the Blix Family Foundation, the Laerdal Foundation for Acute Medicine, the Odd Fellow Foundation, and the Sønneland Foundation.

My deepest felt gratitude and love goes to my mother and father who both passed away during the work, and to Unni, Johannes, and Tomine for their support and understanding.

## PUBLICATIONS INCLUDED IN THIS THESIS

1. Hankø E, Tommarello S, Watchko JF, Hansen TW. Administration of drugs known to inhibit P-glycoprotein increases brain bilirubin and alters the regional distribution of bilirubin in rat brain. Pediatric Research 2003; 54:441-445

2. Hankø E, Hansen TWR, Almaas R, Lindstad J, Rootwelt T. Bilirubin induces apoptosis and necrosis in human NT2-N neurons. Pediatric Research 2005; 57:179-184

3. Hankø E, Hansen TWR, 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. Pediatric Research 2006; 59:72-77

4. Hankø E, Hansen TWR, Almaas R, Rootwelt T. Recovery after short term bilirubin exposure in human NT2-N neurons. Brain Res. 2006; 1103;1:56-64

## ABBREVIATIONS

AIF - Apoptosis inducing factor

ATP - adenosine triphosphate

BAEP - brain auditory evoked potential

BBB - blood-brain barrier

BSA - bovine serum albumin

CAD - caspase activated DNase

EH - ethidium homodimer

HI - hypoxic ischemic

HIE - hypoxic ischemic encephalopathy

HSA - human serum albumin

LDH - lactate dehydrogenase

MDR - multidrug resistance

MRP - multi drug resistance associated protein

MR - molar ratio

MTT - 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide

NMDA - N-methyl-D-aspartate

NGF - neuronal growth factor

OATp - organic anion transport protein

PARP - poly (ADP-ribose) polymerase

P-gp - Phospho-glycoprotein

RA - retinoic acid

TSB - total serum bilirubin

zDEVD.FMK - z-asp-glu-val-asp-fluoromethyl ketone (caspase-3 inhibitor)

zVAD.FMK - z-val-ala-asp-fluoromethyl ketone (general caspase inhibitor)

# 1. INTRODUCTION

## 1.1 Hyperbilirubinemia, neonatal jaundice and kernicterus

*Hyperbilirubinemia* is defined as an abnormally high concentration of bilirubin in the circulating blood. A wide definition of hyperbilirubinemia in the newborn is the one of a *total serum bilirubin (TSB)* concentration in excess of the normal adult upper limit of 22 to 26 µmol/L (Gartner, 1994). As the TSB level of healthy term infants peaks at approximately 100 µmol/L, a more useful definition of hyperbilirubinemia might be a TSB level that exceeds the 95th percentile for the infants age in hours (Maisels, 2006). In the newborn, bilirubin in the blood stream and tissues is almost exclusively present as *unconjugated bilirubin***.** This is converted to *conjugated bilirubin* in the liver and excreted into bile. Unconjugated bilirubin may exist in the blood stream as *albumin-bound bilirubin* or as bilirubin not bound to albumin, labelled *free or unbound bilirubin*. In the newborn a visible yellowish staining of the sclera and skin, *icterus or neonatal jaundice*, results from an increase in TSB beyond 80 - 100µmol/L (Gartner, 1994), which is present in almost two thirds of all newborns during the first week of life (Maisels, 1999). This so-called physiologic jaundice of the newborn is a transient phenomenon caused by a set of normal developmental features during the newborn period.

Bilirubin is toxic to the central nervous system and may cause a sequence of neurological symptoms and signs labelled *acute bilirubin encephalopathy.* A considerable proportion of these infants develops *chronic bilirubin encephalopathy,* a clinical tetrade consisting of movement disorders, auditory dysfunction, oculomotor impairments, and dental enamel hypoplasia of the deciduous teeth (Volpe, 2003). The clinical sequel in these infants correspond to the pathologic-anatomic localisation of the lesions in the basal ganglia, auditory brainstem nuclei, and brainstem oculomotor nuclei (Volpe, 2003). The bilirubin staining of the basal nuclei in the brain has given rise to the term *kernicterus* (Schmorl, 1904). Although kernicterus is a pathologic-anatomic term, it is often used to describe the associated clinical findings. There is evidence that less severe hyperbilirubinemia can produce more subtle, chronic encephalopathy referred to as bilirubin-induced neuronal dysfunction (BIND) (Johnson *et al.*, 2002). A BIND scoring scale has been proposed as a tool to objectify the neonatal neurological exam, however, this BIND score has not been validated (Shapiro, 2005). In the present thesis kernicterus is used as the term to describe the whole spectrum of permanent bilirubin-induced clinical sequel. For “unconjugated bilirubin”, “bilirubin” is used.

### 1.2 A brief review of the history of kernicterus

Kernicterus was long considered to be a specific sequel of Rh immunization. Convincing evidence of an association between hyperbilirubinemia and kernicterus was first reported in the early 1950`s, when Hsia *et al* introduced guidelines based on serum bilirubin levels for the management of infants with Rh immunization (Hsia *et al.*, 1952;Hsia *et al.*, 1953). The authors stated that kernicterus was unlikely to occur when the TSB remained below 340 µmol/L (20 mg/100 ml), which remains an important perception in the treatment of hyperbilirubinemia in the term infant. However, observations from 1950's reporting that premature birth was associated with increased risk of kernicterus, even in the absence of extreme hyperbilirubinemia ("low bilirubin kernicterus"), suggested a more complex relationship between hyperbilirubinemia and kernicterus (Harris *et al.*, 1958).

The prevention of Rh (D) sensitization in Rh-negative mothers by postpartum prophylaxis with Rh immunoglobulin greatly reduced the incidence of kernicterus (Volpe JJ, 2003). Exchange transfusion to reduce the level of unconjugated bilirubin in the bloodstream was introduced by Wallerstein in 1946 (Wallerstein, 1946). In the 1950's an English nurse observed that the skin of a jaundiced infant who had been exposed to sunlight had faded, leading to the discovery of the principle of photo-conversion of bilirubin (Cremer *et al.*, 1958) and to the introduction of phototherapy in the 1960's. With improved neonatal care, the prevention of Rh (D) sensitization, and the possibility to detect and treat infants at risk, kernicterus almost disappeared in the developed world during the 1970's. A declining number of cases of kernicterus were reported in preterm babies, and very few cases of kernicterus were reported in term or near term infants in the 1970's and 1980's (Ebbesen, 2000;Hansen, 2000b;Johnson *et al.*, 2002).

Recently there has been an apparent resurgence of kernicterus in the industrialized world. In Denmark no cases of kernicterus were reported from the early 1970's till 1994, but between 1994 and 1998 six cases were reported (Ebbesen, 2000). In response to anecdotal reports of kernicterus in term and near term infants a Pilot Kernicterus Register for the US was established in 1992 (Johnson *et al.*, 2002). By 2002, 90 infants had been enrolled in the registry, and by 2004 125 cases had been identified (Bhutani *et al.*, 2004). The reason for this resurgence of kernicterus is uncertain, but both early postnatal discharge and loss of concern about jaundice in full term infants may be implicated (Brown & Johnson, 1996;Ebbesen, 2000). It should be kept in mind that outside the industrialized world the picture is different. In parts of sub-Saharan Africa kernicterus has been reported in up to 20 % of severely jaundiced infants born outside hospital (Ahmed *et al.*, 1995). In children with

cerebral palsy the proportion of children thought to be suffering from the effects of kernicterus has been estimated to be as high as 50 % (2 - 50 %) in third world countries (Egdell & Stanfield, 1972).

### 1.3 The clinical problem

Hyperbilirubinemia is the most commonly treated condition in newborn medicine. In a survey by Atkinson *et al* approximately 7 % of all newborns met the criteria for phototherapy (Atkinson *et al.*, 2003). In Canada severe hyperbilirubinemia is the most common cause of neonatal readmission to hospital (Sgro *et al.*, 2006). In otherwise healthy infants without predisposing factors for hyperbilirubinemia TSB levels as high as 340 - 450 μmol/L can occur due to physiologic processes during the first days of life (Gartner, 1994), and in most cases the underlying cause of hyperbilirubinemia can not be identified (Sgro *et al.*, 2006;Tiker *et al.*, 2006). 31 % of the infants enrolled in the Pilot Kernicterus Register had no detectable cause for their hyperbilirubinemia and were classified as idiopatic (Johnson *et al.*, 2002). Still, most otherwise healthy infants with TSB levels exceeding 340 μmol/L are not likely to develop cerebral damage even in the absence of treatment (Bengtsson & Verneholt, 1974;Killander *et al.*, 1963). Hence, a TSB level exceeding 340 μmol/L in an otherwise healthy, term infant, is a poor marker to identify newborns at risk of developing kernicterus.

Long duration of hyperbilirubinemia has been reported to be an important risk factor for developing kernicterus (Devries *et al.*, 1985;Ozmert *et al.*, 1996), however, duration of exposure is not included in current treatment guidelines. More specific and clinically useful markers of the risk of subsequent kernicterus such as the level of unbound or free bilirubin, which is supposed to cross the blood-brain barrier (BBB) more readily than albumin-bound bilirubin (Ahlfors, 2000;Wennberg, 2000), or bilirubin-induced changes in brainstem auditory evoked potential (BAEP) (Shapiro, 1988;Shapiro, 2003) have been proposed. To date, the usefulness of these markers in determining which infants are at risk of developing kernicterus is uncertain.

At physiologic and slightly higher concentrations (low nanomolar concentrations) bilirubin is considered to be protective against oxidative stress (Baranano *et al.*, 2002;Dore *et al.*, 1999). The antioxidant properties have been associated with a decreased risk of atherosclerotic disease (Sedlak & Snyder, 2004). It has repeatedly been claimed that hyperbilirubinemia is a necessary, but not sufficient condition to cause kernicterus. Additional risk factors may include diseases or disorders that increase the entry of bilirubin

into the brain such as asphyxia (Lucey *et al.*, 1964) or meningitis (Pearlman *et al.*, 1980). Also, the concomitant presence of factors such as hypoglycemia, metabolic aberrations or infection may enhance the susceptibility of the brain to bilirubin (Volpe, 2003). However, in a review of 123 cases of kernicterus in infants ≥ 34 weeks gestation reported between 1955 and 2001, 35 cases had no evidence of co-morbid factors (Ip *et al.*, 2004a). Volpe states that "although the essential toxicity of bilirubin has been questioned, on the balance the weight of available data favours the notion that bilirubin per se is injurious to neurons" (Volpe, 2003). Recent findings suggest that even moderate hyperbilirubinemia may result in subtle, but possibly permanent, minor motor impairments (Grimmer *et al.*, 1999;Ozmert *et al.*, 1996;Soorani-Lunsing *et al.*, 2001). Minor motor impairments could, however, not be confirmed in a recent study in infants with TSB levels exceeding 428 μmol/L (Newman *et al.*, 2006).

Over the last two to three decades, changes in the standards of neonatal care such as an increase in the practice of breast feeding and early hospital discharge have taken place. Data from the Pilot Kernicterus Registry found that of 61 neonates with acute bilirubin encephalopathy during the first week of life, 59 were breast fed and 24 (39 %) had a weight loss of >10 % of birth weight on admission (Johnson *et al.*, 2002). A possible causal relationship between breastfeeding and kernicterus is not settled, but breastfeeding is associated with enhanced enterohepatic circulation of bilirubin and relative caloric deprivation (Watchko, 2005b).

To summarize, there are no reliable, specific markers to identify which jaundiced infant is at risk of developing kernicterus. While clinical efforts such as pre-discharge risk assessment and close follow-up monitoring after discharge hold promise for avoiding serious hyperbilirubinemia and kernicterus, important issues concerning bilirubin neurotoxicity await further clarification. These issues include 1) the relative contribution of albumin-bound and -unbound bilirubin to bilirubin toxicity, 2) the mechanism by which bilirubin enters the brain, 3) the preference of bilirubin for certain areas of the brain like the basal nuclei and the cerebellum, 4) whether bilirubin is, per se neurotoxic, or co-morbid factors are necessary to induce neurotoxicity, and 5) the molecular mechanisms by which bilirubin exerts its toxicity. As summarized by Ip *et al* in a comment on a recent evidence-based review of neonatal hyperbilirubinemia: "Additional understanding of bilirubin neurotoxicity is clearly needed and may be forthcoming from basic science investigations" (Ip *et al.*, 2004b).

# 2. ORIGIN, PHYSICAL PROPERTIES, AND KINETICS OF BILIRUBIN

## 2.1 Bilirubin metabolism

Bilirubin is formed in the organism as the end product of heme catabolism. The heme porphyrin ring is oxidized, producing carbon monoxide, iron and biliverdin. Biliverdin is then reduced to bilirubin primarily in the reticuloendothelial system (Maisels, 1999). In the blood stream bilirubin appears as a weak dianion that is almost insoluble in aqueous solutions at physiological pH, and to a large extent bound to albumin in the circulation (Brodersen, 1979;Lightner *et al.*, 1986). Reaching the parenchymal cells of the liver, bilirubin, but not albumin, crosses the cell membrane of the hepatocyte (Maisels, 1999). In the hepatozyte unconjugated bilirubin is converted to the water-soluble conjugated bilirubin by uridine diphosphoglucuronosyl transferase (UDPGT) and excreted into the bile (Maisels, 1999). Total bilirubin production per kg body weight during the first few days of life is about twice that of adults. As UDPGT activity is insufficient to handle the bilirubin load during these first days, bilirubin levels almost invariably increase. Due to a rapid increase in UDPGT activity, probably due to induction by bilirubin itself, bilirubin elimination from the circulation increases and serum bilirubin levels then fall (Maisels, 1999).

## 2.2 Structure and solubility of bilirubin

Spectroscopic studies have revealed that the bilirubin molecule is not planar as originally assumed, but rather has a folded ridge tail shape (Boiadjiev *et al.*, 2004;Lightner *et al.*, 1986). Stability is provided by intra-molecular hydrogen bonds which are responsible for its hydrophobicity, making it unexcretable in normal hepatic metabolism (Boiadjiev *et al.*, 2004). Phototherapy induces alterations in the structure of the bilirubin molecule principally by breaking the intra-molecular hydrogen bond of one of the propionic acids, causing the lipophilicity of the molecule to decrease and to allow intact excretion by the liver (Lightner & McDonagh, 2001).

While the structure of the bilirubin molecule is well established, its aggregation is less well characterized. The solubility of bilirubin has been estimated to be as low as 7 nmol/L based on bilirubin crystals in water (Brodersen, 1979) or 66 nmol/L based on its partitioning between water and chloroform (Hahm *et al.*, 1992). It has been claimed that the higher value is more consistent with the values reported from serum of infants (Ostrow *et al.*, 1994). It is, however, possible that this high value may be erroneous due to the experimental

techniques used in these studies (Boiadjiev *et al.*, 2004;Lightner *et al.*, 1986). Under physiological conditions bilirubin does not aggregate in the blood due to its binding to albumin in the blood stream.

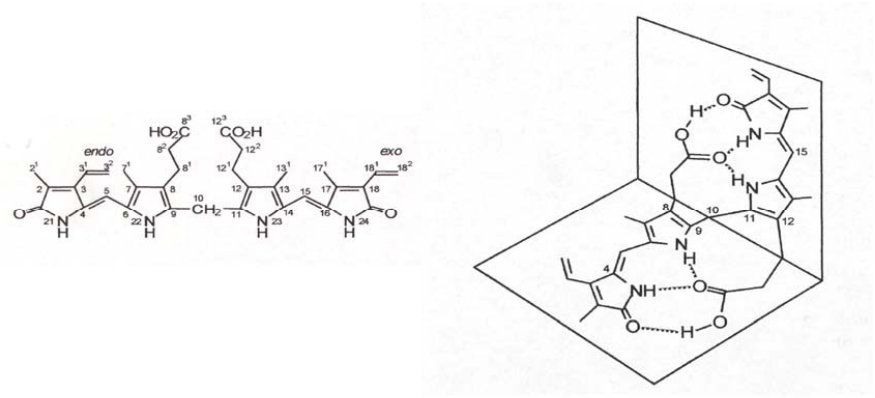


Figure 1. Bilirubin structure: Left: linear representation with conventional numbering system; Right: the energetically more stable structure shaped like a ridge-tile (modified from Boiadjiev *et al.*, 2004).

**2.3 Bilirubin, albumin, and the blood-brain barrier**

Albumin has a binding affinity for bilirubin of $10^7$-$10^8$ at its primary binding site for bilirubin, and one or two additional binding sites with much lower binding affinity (Brodersen, 1979;Lightner *et al.*, 1986). Only bilirubin which is not bound to albumin enters the brain by crossing an intact blood-brain barrier (BBB) (Maisels, 1999). Whether the fraction of bilirubin which actually enters the brain is derived exclusively from the fraction of unbound bilirubin or also from albumin-bound bilirubin in the blood stream is not finally settled. Even though the concentrations of unbound bilirubin rarely exceed 60 nM/L, even in jaundiced serum containing 100-300 µM/L TSB, it may be the principal driving force to achieve equilibrium between the different compartments (Wennberg, 1988;Wennberg, 2000). In the case of a rapid increase of bilirubin, as seen in the newborn infant, albumin serves as a biological buffer against bilirubin encephalopathy. Under certain conditions like excessive production of bilirubin due to hemolysis (Maisels, 1999;Volpe, 2003), in the presence of binding competitors for the bilirubin binding site of albumin (McDonald *et al.*, 1998;Silvermann *et al.*, 1956), or reduced affinity of albumin to bilirubin as seen in sick and

preterm infants (Cashore, 1980), the binding capacity of albumin for bilirubin may be overwhelmed, allowing increased amounts of unbound bilirubin to enter the brain.

The permeability of the BBB to bilirubin may increase in certain diseases like meningitis (Pearlman *et al.*, 1980) or asphyxia (Lucey *et al.*, 1964). Opening of the BBB for bilirubin has been achieved by experimental induction of hypercarbia (Bratlid *et al.*, 1984;Wennberg *et al.*, 1993) or hyperosmolarity (Ives *et al.*, 1989;Wennberg *et al.*, 1991). A study by Hansen *et al* examined the uptake of bilirubin and albumin in different brain regions in young adult rats during short term (1 h) hyperbilirubinemia, and concomitant administration of either a displacing agent (sulfisoxazole), or hypercarbia, or hyperosmolarity. Brain/serum bilirubin ratios increased by 58 % (sulfisoxazole), 70 % (hypercarbia) and 39 % (hyperosmolarity), but there were no significant inter-regional differences in brain uptake in any of the study groups (Hansen *et al.*, 1989). Other investigators have found that osmotic opening of the BBB in the rat leads to somewhat higher bilirubin concentrations in the basal ganglia than other parts of the brain, but the general tendency was that bilirubin staining of the brain was more diffuse than that of the typical kernicteric staining pattern seen at autopsy of the newborn infant (Levine *et al.*, 1982).

**2.4 The blood-brain barrier and membrane transport proteins**

The BBB restricts the access of hydrophilic compounds, and only lipophilic drugs and endogenous compounds can enter the brain by passive diffusion (Fricker & Miller, 2004). However, many lipophilic substances such as bilirubin exhibit an unexpectedly low concentration in the brain, suggesting additional mechanisms operating at the BBB (Schinkel & Jonker, 2003;Tamai & Tsuji, 2000). Many of these compounds are substrates for phospho-glycoprotein (P-gp), a membrane transport protein that regulates the entry and efflux of substrates across cell membranes in many tissues (Lin & Yamazaki, 2003;Schinkel & Jonker, 2003).

P-gp is a member of the ATP-binding family of membrane transporters. Three isoforms of this protein have been identified in rodents (mdr1a, mdr1b and mdr2) and two isoforms have been identified in humans (mdr1 and mdr2) (Fricker & Miller, 2004). Studies on mdr1a and mdr1a/1b knockout mice suggest that the brain is more sensitive to the changes in P-gp function than other tissues (Schinkel *et al.*, 1995). In the brain P-gp exerts its function in the capillary vessels by pumping back the substrate upon entry at the luminal membrane (facing the blood stream) (Beaulieu *et al.*, 1997;Higgins & Gottesman, 1992;Tamai & Tsuji,

2000), and in astrocytes (Gennuso *et al.*, 2004;Pardridge, 1998). Pharmacological inhibition of P-gp has been used in, for example, cancer treatment to enhance the influx of anti cancer drugs into cancer cells (Bellamy *et al.*, 1988;Slater *et al.*, 1986). Unconjugated bilirubin has been shown to be a substrate for P-gp (Jette *et al.*, 1995), raising the question as to whether drug-induced inhibition of P-gp may be a mechanism of clinical relevance for bilirubin kinetics at the BBB in the newborn infant. In addition to P-gp, transport membrane proteins expressed at the BBB also include the multi drug resistance-associated protein (MRP) and the organic anion transport proteins (OATp) (Bart *et al.*, 2000;Fricker & Miller, 2004). Bilirubin may serve as a substrate for both MRP (Falcao *et al.*, 2007), and OATp (Cui *et al.*, 2001).

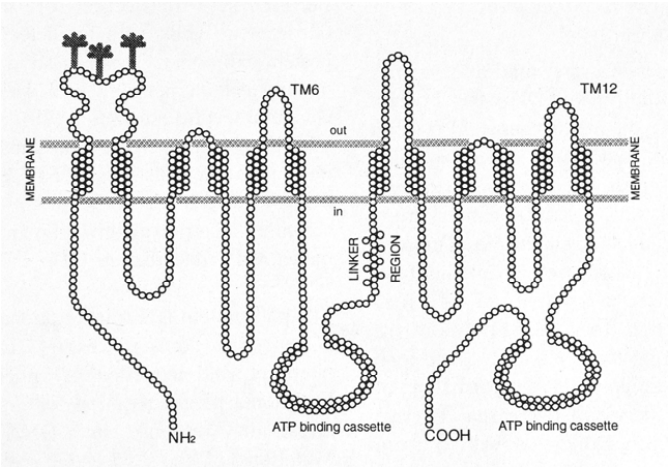


Figure 2. Schematic drawing of P-gp demonstrating two homologous halves with six trans-membrane regions and a nucleotide binding domain each connected with a linker region (shown by courtesy of Gustav Lehne).

**2.5 Bilirubin interaction with cell membranes**

Bilirubin diffuses spontaneously through isolated phospholipid bilayers (Hayward *et al.*, 1986;Zucker *et al.*, 1999). In cultured hepatoblastoma cells bilirubin crosses the cell membrane by a mechanism involving spontaneous diffusion (Zucker & Goessling, 2000). It is likely that a similar phenomenon exists in neurons, but spontaneous diffusion of bilirubin over the neuronal plasma membrane has never been shown. Eriksen *et al* found that bilirubin interaction with phospholipids takes place through three different steps. In the first step the

bilirubin dianion attaches reversibly to phospholipids, in the second step the bilirubin-phospholipid complex reversibly takes up $H^+$ to form the bilirubin acid-phospholipids complex, and in the third step the bilirubin acid aggregates attach to the phospholipids. At pH values > 8.2 only the first step is observed, however, at physiologic and acidotic pH values the total process is completed within 1 min (Eriksen *et al.*, 1981). These observations were supported and further extended in rat synaptosomal plasma membranes (Vazquez *et al.*, 1988). A study on a human neuroblastoma cell line was not consistent with a simple diffusion of bilirubin over the cell membrane, but rather with a multi-step binding mechanism as proposed by both Eriksen and Vazquez (Amit *et al.*, 1990). However, rat brain homogenates showed elevated intracellular concentrations of bilirubin in the myelin fraction of cellular organelles, especially in the mitochondria, ribosomes and cytoplasm after increasing the BBB permeability for bilirubin by the induction of hyperosmolarity, strongly suggesting that bilirubin does enter the interior of neurons (Hansen *et al.*, 2001).

**2.6 Bilirubin elimination from the brain**

Bilirubin is readily removed from the brain. In a rat model where the BBB was opened by hyperosmolarity, the half life of bilirubin in the brain has ranged from 1.7 h (Levine *et al.*, 1985), to 38 min (Hansen, 1996), and was found to be13 min in a model of opening of the BBB by hypercarbia (Hansen, 1996). In a different model with intact BBB half life was found to be approximately 20 minutes (Hansen & Cashore, 1995). In the two latter studies, elimination did not differ substantially within brain regions (Hansen & Cashore, 1995;Hansen, 1996). Hence, studies of bilirubin clearance from rat brain regions have not contributed to our understanding of the phenomenon of the kernicteric staining pattern. The mechanism by which bilirubin is eliminated from the brain is not known. Bilirubin may cross the BBB either by diffusion or an active transport mechanism back into the circulation (Levine *et al.*, 1985) or into the cerebrospinal fluid (Lee & Hsia, 1959). Also, bilirubin may be detoxified in the brain by mitochondrial oxidation into non-toxic products (Brodersen & Bartels, 1969;Hansen & Allen, 1996a;Hansen, 2000a), however, the clinical relevance of this mechanism is not known. Taken together, neither studies on bilirubin entry into the brain, nor the elimination of bilirubin from the brain have satisfactorily explained the preference for bilirubin accumulation in certain areas of the brain as seen at autopsy of kernicteric infants.

# 3. TOXICITY OF BILIRUBIN

## 3.1 A case of kernicterus

In 1907 Beneke gave a detailed description of the clinical features of fatal bilirubin-induced encephalopathy in the ”Münchener Medizinische Wochenschrift” (Beneke, 1907).
“The cases are a pair of twins born prematurely (approximately 6 weeks before term) due to an intensely frightening event to the mother. The mother gave birth to healthy twins nine years earlier. Later a baby boy was born, but died under similar circumstances as the actual twins. Two further pregnancies led to the death of the fetus without detectable cause. The two latest-born twins displayed jaundice remarkably early, already after twelve hours. They appeared otherwise healthy. Twenty hours after birth, the first-born twin developed light seizures (jerking of the extremities); 36 h post partum the doctor observed tonic seizures of the extremities and the trunk. Both children drank without difficulties from the spoon until shortly before their death. The first-born died at the age of 48 h; the younger with similar symptoms 6 h later”.

In the autopsy description of the first twin Beneke wrote: “Generally the brain is only faintly icteric, the cortex no stronger than the medulla. On the other hand, periventricular tissues such as the ependymym, the telae and the plexus choroideus appear clear yellowish. The thalami are not obviously stained, while the dentate nuclei are somewhat stronger yellow. The nuclei of the horn of ammon and the luys bodies on both sides are intensely yellow and are sharply distinct from the surroundings. The cortex of the cerebellum is in part stronger yellow than the cortex of the cerebrum … . The strongest colouring is found in the nuclei of medulla oblongata, especially the nuclei of the trigeminal and acoustic nerves in their whole length….” and further “..the peculiar localisation in certain nuclei suggests a specific vulnerability to bilirubin for these regions of the brain”.

These fatal cases of bilirubin encephalopathy were most likely due to Rh immunization leading to excessive intravascular hemolysis and increase in serum bilirubin levels after birth. Prematurity may also have increased the risk of kernicterus.

## 3.2 Clinical features in bilirubin encephalopathy

Acute bilirubin encephalopathy, as described above, does not invariably lead to death, but may cause persistent neurological disabilities in survivors. The sequel of kernicterus reflects the regional distribution of neuronal injury which is most prominent in the basal ganglia, and

in the cochlear and oculomotor nuclei. Clinical features include extrapyramidal movement disorders like dystonia and athethosis, gaze palsies, and dental dysplasia of baby teeth (Maisels, 1999;Volpe, 2003). Auditory abnormalities, which are both central and peripheral in origin, are practically invariably present, and may sometimes be the only apparent sequel (Johnson *et al.*, 2002). Severe involvement of cerebral cortical neurons is uncommon in kernicterus, but several investigators report intellectual deficits, although rarely in the mentally retarded range (Day & Haines, 1954;Nilsen *et al.*, 1984;Ozmert *et al.*, 1996).

### 3.3 Subtle bilirubin encephalopathy in healthy term infants

Several investigations indicate that moderate degrees of hyperbilirubinemia may be associated with an increase in minor neurological dysfunction. In the Collaborative perinatal project where more than 41 000 singletons were enrolled, minor motor abnormalities increased in a stepwise fashion from 14.9 % in those with TSB levels ≤ 171 μmol/L to 22.4 % in those with TSB levels ≥ 342 μmol/L at the age of 7 years (Newman & Klebanoff, 1993). Grimmer *et al* found that non-hemolytic, apparently healthy term infants with TSB levels in excess of 342 μmol/L scored worse on a choreiform dyskinesia scale than controls (TSB ≤ 205 μmol/L) at four years of age (Grimmer *et al.*, 1999). In another study of infants with TSB levels of 233 - 444 μmol/L hypotonia or mild abnormalities in muscle tone-regulation in combination with significant postural and reflex dysfunction was found in 50 % (versus 10 % in the control group) at 12 months of age (Soorani-Lunsing *et al.*, 2001). The sample sizes of the two later studies were small, and the clinical importance of theses findings is uncertain (Bhutani, 2001;Hintz *et al.*, 2001;Maisels & Newman, 2001). A recent, better designed study including 140 newborns with TSB levels exceeding 428 μM/L found no differences in the proportion of abnormal neurological findings or behavioural problems compared to a matched control group at the age of five years (Newman *et al.*, 2006).

### 3.4 Reversibility of impaired neuronal function in infants with hyperbilirubinemia

The risk of prominent neurological abnormalities may correlate not only with the degree of hyperbilirubinemia, but also with its duration (Devries *et al.*, 1985;Ozmert *et al.*, 1996). Clinical observations suggest that hyperbilirubinemia in infants may alter neonatal behaviour and responsiveness, but that these behavioural alterations normalize when hyperbilirubinemia subsides (Paludetto *et al.*, 2002). Disturbances in brain auditory evoked potential (BAEP) in

severely hyperbilirubinemic newborns are promptly improved after exchange transfusions (Nwaesei *et al.*, 1984), and with modulation of pCO2 in primates (Wennberg *et al.*, 1993). A similar phenomenon has been demonstrated in a Gunn rat model of bilirubin encephalopathy (Shapiro, 1988;Shapiro, 1993). These phenomena are believed not necessarily to be associated with brain damage. However, changes in BAEP may also indicate damage in the auditory brainstem (Conlee & Shapiro, 1991;Shapiro & Conlee, 1991).

### 3.5 The neuropathology of kernicterus

The patterns by which yellow staining of the brain occur in the presence of elevated levels of bilirubin, have been summarized by Mamdouha Ahdab-Barmada and include, 1) diffuse yellow staining of areas that lack blood brain barrier, including leptomeninges, ependyma, choroid plexuses and cerebrospinal fluid, 2) diffuse yellow staining in areas where blood brain barrier integrity has become compromised by conditions like hypoxic ischemic encephalopathy (HIE), periventricular leukomalacia, ischemic cerebral infarcts, or traumatic lesions, 3) classical kernicterus (as described by Beneke) with the typical staining of specific neuronal groups including the globus pallidus, the subthalamic nuclei, the hippocampus, the oculomotor nuclei, the cranial nerve nuclei, and other nuclei in the lower central part of the brain (Ahdab-Barmada, 2000).

Bilirubin-induced neuronal cell death is typically described as necrosis (Ahdab-Barmada, 2000;Watchko, 2005a). However, neuropathological investigations on bilirubin encephalopathy largely predate the recognition of apoptosis. A recent study on severely hyperbilirubinemic infants suggested a more prominent role of apoptotic cell death in bilirubin-induced injury than in HIE, based on differences in the diffusion weighted magnetic resonance imaging (Groenendaal *et al.*, 2004). In 7 day old hyperbilirubinemic Gunn rats subjected to sulfadimethoxine to enhance brain bilirubin, light microscopic investigations indicated apoptotic death in addition to necrosis in neurons (McDonald *et al.*, 1998). To date, no further investigations have investigated bilirubin-induced apoptosis *in vivo.* However, several recent *in vitro* studies strongly suggest that apoptosis may play a prominent role in bilirubin-induced cell death.

### 3.6 Experimental studies on bilirubin toxicity

Animal models using disruption of the BBB to induce entry of bilirubin into the brain have shown that bilirubin induces a decline in brain energy metabolites (Wennberg *et al.*,

1991;Ives *et al.*, 1989). In the above-mentioned study by Groenendal abnormally high lactate values could only be demonstrated in the only infant who later developed severe athetoid cerebral palsy (Groenendaal *et al.*, 2004). Bilirubin-induced ATP depletion has also been demonstrated in cultured neurons (Cowger, 1971). Whether energy depletion is due to direct effects of bilirubin on mitochondrial oxidative phosphorylation (Day, 1954) has later been questioned (Diamond & Schmid, 1967;Katoh *et al.*, 1975).

Karp reviewed a number of *in vitro* studies that predominantly showed inhibitory effects of bilirubin on 28 different enzymes, grouped into respiratory and oxidative phosphorylation, carbohydrate metabolism, tricarboxylic acid cycle metabolism, amino acid and protein metabolism, and lipid metabolism (Karp, 1979). Reversible phosphorylation and de-phosphorylation of proteins is a key mechanism for regulation of cellular functions (Walaas & Greengard, 1991). Inhibition of protein phosphorylation by bilirubin has been shown in newborn rabbits *in vivo* (Morphis *et al.*, 1982), and *in vitro* in the synaptic vesicle associated protein synapsin I in isolated nerve terminals (Hansen *et al.*, 1988), and in cell free systems (Hansen *et al.*, 1996;Sano *et al.*, 1985). In apparent contradiction to these studies, bilirubin induced hyper-phosphorylation of MAP kinases in cerebellar granule neurons. These MAP kinases are supposed to play a role in bilirubin-mediated apoptosis (Lin *et al.*, 2003).

# 4. NECROSIS, APOPTOSIS AND EXCITOTOXICITY

## 4.1 Definitions and concepts

The classical concept of toxic action on cell homeostasis is the one of a dose-dependent progression from reversible adaptive changes in cellular metabolism to a major and irreversible collapse of cellular homeostasis resulting in disintegration of the cellular membrane and cell death seen as *necrosis.* Over the last 30 years it has become evident that cells of multi-cellular organisms have the ability to activate an internal cell death program which has been labelled *programmed cell death.* Programmed cell death may be identified primarily due to cellular morphological changes referred to as *apoptosis*. In this thesis both the phenomenon of programmed cell death and its morphological consequences will be referred to as apoptosis. The term *excitotoxicity* refers to the ability of glutamate and structurally related amino acids to induce cellular damage mediated by glutamate sensitive receptors on the cell surface (Choi, 1992).

After the recognition of apoptosis, Wyllie put forward two postulates concerning non-necrotic cell death; that apoptosis is induced by insults of lesser amplitude than those causing necrosis in the same cell, and that apoptosis is more prone to occur in cells primed for apoptosis (Wyllie, 1987). The two classical types of cell demise can occur simultaneously in tissues or cell cultures exposed to the same stimulus, and the magnitude of the same insult decides the prevalence of different forms of cell death (Bonfoco *et al.*, 1995;Nicotera *et al.*, 1999). After a given insult the metabolic state of the cell is of vital importance for the cell's decision to survive or undergo apoptotic or necrotic cell death (Ankarcrona *et al.*, 1995;Nicotera & Leist, 1997). Typically, necrotic cell death is an early event in a subpopulation of cells, while apoptosis is a slower process (Ankarcrona *et al.*, 1995;Castoldi *et al.*, 2000;Ding *et al.*, 2000).

## 4.2 Caspase-dependent apoptosis

Various apoptotic stimuli like activation of receptors on the cell surface, serum deprivation, ionizing radiation, or toxic stimuli, may trigger the activation of apoptosis (Widlak & Garrard, 2005). The core biochemical apoptotic pathway is executed by a family of cysteine proteases, so-called caspases, which are activated during the progress of the apoptotic process. They are synthesized as inactive pro-enzymes which are activated by proteolytic cleavage (for reviews see Degterev *et al.*, 2003;Earnshaw *et al.*, 1999). To date at least

fourteen caspases have been identified, of which at least seven contribute to cell death (Chang & Yang, 2000). Caspases execute their role as cell killers by carrying out the limited cleavage of over 100 cellular proteins at the site of key aspartic residues leading to the orderly disassembly of the cell and the morphological features of apoptosis (Earnshaw *et al.*, 1999;Ryan & Salvesen, 2003). The execution of caspase-mediated apoptosis is the result of a cascade of events resulting in the activation of the executioner caspases -3, -6, or -7, where caspase-3 has been identified as a major executioner caspase (Nicholson *et al.*, 1995;Racke *et al.*, 2002). Caspase-mediated apoptosis is an energy requiring process, and a sufficient ATP concentration must be sustained throughout the apoptotic execution for the final morphological and biochemical features to occur (Leist & Nicotera, 1997;Nicotera *et al.*, 2000).

The caspase-dependent apoptotic signalling pathways can be divided into two categories; cell surface sensor mediated (extrinsic) and intracellular sensor mediated (intrinsic or mitochondrial). In the intrinsic pathway an apoptotic insult induces permeabilization of the mitochondrial membrane resulting in the release of cytochrome c from the mitochondria to the cytosol (Earnshaw *et al.*, 1999;Zou *et al.*, 1997). Cytochrome c combines with the protease activating factor (Apaf-1), procaspase-9, and ATP to form the apoptosome complex, which activates caspase-9 (Degterev *et al.*, 2003;Polster & Fiskum, 2004). The activation of caspase-9 leads to the proteolytic activation of the effector caspases caspase-3 and (to a lesser extent) caspase-7 (Degterev *et al.*, 2003;Polster & Fiskum, 2004). Cytochrome c-release and apoptosome formation is regulated through a complex and incompletely understood interplay of pro-apoptotic and anti-apoptotic factors, many of which belong to the Bcl-2 family of proteins (Degterev *et al.*, 2003;Zamzami *et al.*, 1998). Activation of PARP, a cell defence mechanism during apoptosis, is an energy requiring process leading to a decrease in intracellular ATP levels. Caspase-3 cleaves PARP terminating PARP activity, which may be viewed as an attempt to preserve sufficient energy to undergo apoptosis (Degterev *et al.*, 2003).

In the extrinsic pathway extra-cellular ligands bind to cell death receptors, many of which belong to the tumor necrosis factor receptor (TNFR) super family of receptors. These receptors have a common intracellular sequence known as the death domain which through binding of the ligand (e. g. TNF-α, or FAS) promotes recruitment of adapter proteins that result in the formation of a death inducing signalling complex (DISC). Recruitment of procaspase-8 to this complex promotes its transition to the active form. Executioner caspases such as caspase-3 may be activated by caspase-8 in a direct fashion, or, for more efficient

killing of cells, this process may be amplified at the mitochondria by a mechanism involving pro-apoptotic members of the Bcl-2 family (Degterev *et al.*, 2003;Polster & Fiskum, 2004).

Both intrinsic and extrinsic apoptotic pathways are regulated by the interplay of multiple signalling pathways, such as the mitogen activated protein kinases (MAPK) which are activated by various environmental stresses. They mediate their function by phosphorylation of different kinase systems such as the jnk and p38 signalling pathways. These may either amplify the stress signal leading to enhanced apoptosis or reduce apoptosis by the activation of apoptosis inhibitors (for review see Mehta *et al.*, 2006)

**4.3 Caspase inhibitors and caspase-independent apoptosis**

The action of caspases may be blocked by endogenous substances such as free oxygen radicals (Hampton & Orrenius, 1998), by the influence of exogenous caspase inhibitors such zVAD.FMK (Volbracht *et al.*, 2001), or by the lack of intracellular ATP (Leist *et al.*, 1997;Nicotera *et al.*, 1998). Inhibition of the caspase machinery does not necessarily prevent cell death indefinitely, and it seems that alternative cell death pathways are activated as a result of the inhibition of caspases (Stefanis, 2005;Volbracht *et al.*, 2001). This has revealed the existence of alternative cell death pathways which are independent of caspases (Lorenzo & Susin, 2004;Stefanis, 2005).

Apoptosis-inducing factor (AIF) is a pro-apoptotic protein which may have a key role in neuronal injury. Upon mitochondrial permeabilization, AIF translocates from its normal location at the mitochondrial inter-membrane space to the nucleus, inducing chromatin condensation and DNA degradation. In most models AIF-mediated apoptosis is independent of caspases (Cheung *et al.*, 2005;Wang *et al.*, 2004). Endonuclease G is another protein that may translocate to the nucleus to induce caspase-independent DNA degradation (Lorenzo *et al.*, 1999;Lorenzo & Susin, 2004). Thus, neurons can die by the involvement of caspase-dependent mechanisms, caspase-independent mechanisms or both, depending on the insult. In general the mitochondria are crucial for both caspase-mediated and caspase-independent pathways (Li *et al.*, 2005;Wang *et al.*, 2004). The mitochondrion may be seen as a weapons store where a cocktail of caspase-dependent and -independent pro-apoptotic proteins are dormant until a death process is triggered (Lorenzo & Susin, 2004). AIF and caspases may cooperate in the cell death cascade, and their individual contribution may depend on the specific apoptosis-inducing stimulus and the cell type (Cande *et al.*, 2002) .

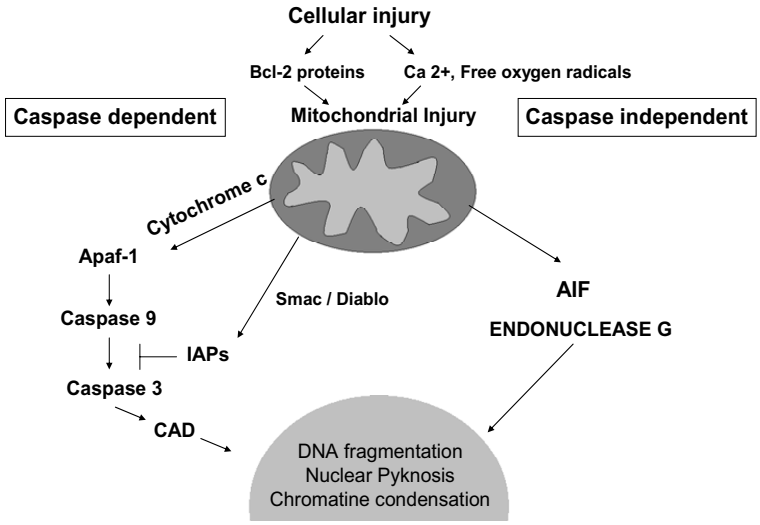


Figure 3. Release of caspase-dependent and caspase-independent pro-apoptotic factors from the mitochondria. Diverse toxic influences induce mitochondrial membrane permeabilization to cause the release of apoptotic molecules from the mitochondria. The release of pro-apoptotic factors such as Smac/Diablo may facilitate caspase activation through their inhibitory function on inhibitory apoptotic proteins (IAPs). Activated caspase-3 is required to convert iCAD into its active form, CAD (caspase-activated DNase), which enters the nucleus inducing internucleosomal DNA fragmentation and advanced chromatin condensation typically appearing as nuclear fragmentation. In response to apoptotic stimuli mitochondria may also release caspase-independent cell death effectors such as apoptosis inducing factor (AIF) or Endonuclease G. These factors translocate to the nucleus inducing high molecular weight (HMW) DNA fragmentation and less advanced chromatin condensation appearing as nuclear pycnosis (see text) (derived from Cregan *et al.*, 2004).

**4.4 Excitotoxicity**

The term excitotoxicity refers to the ability of glutamate and structurally related amino acids to damage neurons (for review see Choi, 1992). Glutamate is the major excitatory neurotransmitter in the brain, and glutamate-mediated synaptic transmission is critical for the

normal functioning and development of the nervous system (McDonald & Johnston, 1990). Glutamate acts on four different families of ionotrophic receptors; the N-methyl-D-aspartate (NMDA) receptor, the a-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor, the kainite receptor, and on metabotrophic receptors linked to G-proteins (Choi, 1992). Over-activation of the NMDA receptor by glutamate accounts for the majority of glutamate excitotoxicity, with a lesser role for non-NMDA receptor activation (Choi, 1992). Excessive extra-cellular glutamate or increased sensitivity of the NMDA receptor to glutamate may induce increased $Ca^{2+}$ influx and release of $Ca^{2+}$ from intracellular stores leading to the activation of $Ca^{2+}$-dependent enzymes and mitochondrial production of reactive oxygen species which may initiate cell death (Choi, 1992;Leist & Nicotera, 1998).

Excitotoxic cell death has formerly been reported to be mainly necrotic (Leist & Nicotera, 1998). More recent evidence has shown that cells which are exposed to excitotoxic injury, but are able to maintain ATP production for some time, die from apoptosis, while neurons which do not maintain sufficient energy production undergo necrosis (Ankarcrona *et al.*, 1995;Leist & Nicotera, 1998). While some studies have found that relatively low concentrations of glutamate may activate caspase-3 (Du *et al.*, 1997;Zhao *et al.*, 2000), it is now generally held that glutamate-induced apoptosis is independent of caspase activation in most *in vitro* model systems (Cheung *et al.*, 2005;Wang H *et al.*, 2004). The exact mechanisms by which excitotoxic injury induces caspase-independent apoptosis is incompletely understood, however, both calcium-dependent proteases called calpains (Stefanis, 2005;Polster *et al.*, 2005) and AIF are reported to be involved (Wang *et al.*, 2004).

Taken together, excitotoxic neuronal death may involve both necrotic and apoptotic cell death dependent on the magnitude of the insult and the metabolic state of the neuron. In most model systems of cell death excitotoxic cell death is independent of caspase activity *in vitro*.

### 4.5 Detection of apoptosis

There is no definition for either necrosis or apoptosis that precisely distinguishes between the different forms of cell death. It is also a matter of debate whether apoptosis and necrosis represent discrete modes of cell or death or rather a continuum, where classical apoptosis and necrosis represent two extremes (Northington *et al.*, 2005;Raffray & Cohen, 1997).

| | Apoptosis | Necrosis |
|---|---|---|
| **Occurence** | Scattered, single cells | Massive tissue injury |
| **Cytoplasm** | Shrinkage: condensed and dehydrated; normal organelles; later fragments | Swelling: Endoplasmatic reticulum, and mitochondria |
| **Nucleus** | Chromatin condenses into masses adjacent to the nuclear envelope, later pycnosis or fragmentation | Ill defined, randomly dispersed, smaller chromatin masses; later lysed |
| **Plasma and nuclear membrane** | Intense blebbing; apoptotic bodies containing normal cytoplasmic organelles and nuclear chromatin | Membrane injury/lysis, leakage of intracellular contents |
| **Tissue response** | No inflammation; phagocytosis of apoptotic bodies | Inflammation |

Table 1. Comparison of the features of apoptosis and necrosis (adapted from Allen *et al.*, 1997).

In the cytoplasm activated caspases cause breakdown of the cytoskeleton, loss of contact with neighbouring cells, cell shrinkage, and blebbing of the cytoplasm followed by disintegration into apoptotic bodies. In contrast to necrosis, the plasma membrane and cytoplasmic organelles of the apoptotic cell remain intact. *In vivo* the apoptotic cell is readily phagocytized by the organism, however, due to the absence of phagocytosis in neuronal cultures, the cell membrane of apoptotic cells also disintegrate eventually; so called secondary necrosis (from Foster JR, 2000). Parameters which denote membrane integrity, such as LDH- release to the supernatant and dye exclusion tests such as trypan blue, propidium iodide, or ethidium homodimer are more reliable in the detection of necrosis than apoptosis.

Two different apoptotic pathways are thought to mediate DNA fragmentation and condensation during apoptosis. In one pathway DNA is cleaved into high molecular weight (HMW) fragments of 50-300 kb. This pathway is accompanied by chromatin condensation at the nuclear periphery and, at least in some paradigms of cell death, pycnotic nuclear condensation. This pathway does not require activation of caspases, and other cell

death effectors such as AIF or Endonuclease G have been shown to activate this pathway (Lorenzo *et al.*, 1999;Lorenzo & Susin, 2004). The other pathway is caspase-dependent and involves activated caspase-3, which in turn converts iCAD into its active form CAD (caspase-activated DNase). CAD enters the nucleus inducing internucleosomal DNA fragmentation and advanced chromatin condensation, often appearing as nuclear fragmentation (Susin *et al.*, 2000;Walker *et al.*, 1999).

It is now generally held that only HMW DNA fragmentation is essential for apoptosis (Susin *et al.*, 2000;Walker *et al.*, 1999). This implies that the final morphological and biochemical features of caspase-dependent apoptosis such as oligonucleosomal DNA fragmentation (the DNA ladder) and morphological nuclear fragmentation, may not always be present in apoptotic cells. In cerebellar granule cells glutamate induces typical signs of apoptosis such as pycnotic nuclear chromatin condensation and HMW DNA degradation, while exposure to a different apoptotic stimulus such as staurosporine induces a different apoptotic phenotype with nuclear fragmentation and typical DNA laddering (Nicotera *et al.*, 1999).

With emphasis on the *in vitro* situation, detection of apoptosis may include; 1) typical apoptotic morphological hallmarks of the cell body, like condensation and fragmentation which are detectable by light microscopy or electron microscopy, 2) nuclear morphological hallmarks such as nuclear condensation and fragmentation visible after the staining with dyes such as Hoechst 33342, 3) direct measurements of the activity of executioners of the apoptotic process such as activated caspases, members of the Bcl-2 family or cytochrome c, 4) biochemical hallmarks, occurring as a result of the apoptotic process, such as PARP cleavage, oligonucleosomal DNA fragmentation, and systems that permit the localisation of DNA breaks, like in situ end labelling (ISEL) or the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL), and 5) the ability of caspase inhibitors or other inhibitors of the apoptotic process (PARP inhibitors, AIF inhibitors) to prevent apoptotic cell death (or morphologies) (Foster JR, 2000;Joashi *et al.*, 1999).

### 4.6 Bilirubin and apoptosis

While evidence of apoptosis is scarce in newborn infants and intact animals exposed to bilirubin, *in vitro* studies strongly suggest that apoptosis is a prominent feature in bilirubin-mediated neuronal injury. Evidence includes studies on primary cultures of cortical neurons from fetal rodents (Grojean *et al.*, 2000;Grojean *et al.*, 2001;Rodrigues *et al.*, 2000;Rodrigues

*et al.*, 2002a;Rodrigues *et al.*, 2002b;Rodrigues *et al.*, 2002c;Silva *et al.*, 2001b;Silva *et al.*, 2002), on primary cultures of astrocytes from fetal rodents (Rodrigues *et al.*, 2000;Rodrigues *et al.*, 2002b;Rodrigues *et al.*, 2002c;Silva *et al.*, 2001b;Silva *et al.*, 2002), on murine hepatoma cells (Seubert *et al.*, 2002), on cultured bovine endothelial cells (Akin *et al.*, 2002), and on colon cancer cells (Keshavan *et al.*, 2004). Evidence of apoptosis has come forth through the use of apoptotic markers such as characteristic nuclear morphological changes (Grojean *et al.*, 2000;Grojean *et al.*, 2001;Rodrigues *et al.*, 2000;Rodrigues *et al.*, 2002a;Rodrigues *et al.*, 2002b;Rodrigues *et al.*, 2002c;Seubert *et al.*, 2002;Silva *et al.*, 2001b;Keshavan *et al.*, 2004), mitochondrial cytochrome c release in intact cells (Rodrigues *et al.*, 2002a;Seubert *et al.*, 2002;Keshavan *et al.*, 2004), or from isolated mitochondria after direct exposure to bilirubin (Rodrigues *et al.*, 2000;Rodrigues *et al.*, 2002c), caspase-3 activation (Rodrigues *et al.*, 2002a;Seubert *et al.*, 2002;Keshavan *et al.*, 2004), caspase-8 activation (Seubert *et al.*, 2002;Keshavan *et al.*, 2004), TUNEL-staining (Silva *et al.*, 2001b;Silva *et al.*, 2002), oligonucleosomal DNA fragmentation (Akin *et al.*, 2002), and PARP cleavage (Akin *et al.*, 2002;Rodrigues *et al.*, 2002a).

Recent investigations have shown that bilirubin causes the release of inflammatory mediators such as IL-6 and TNF-α in cultured astrocytes (Fernandes *et al.*, 2004;Fernandes *et al.*, 2006). MAP kinases are involved in the regulation of this release, and treatment with specific inhibitors of MAP kinases such as p38, JNK and ERK prevented the release of these cytokines and subsequent apoptosis (Fernandes *et al.*, 2007).

### 4.7 Bilirubin and excitotoxicity

Several studies both *in vivo* and *in vitro* suggest that bilirubin may exert its toxic effects at least in part over the ionotropic NMDA receptor. In piglets bilirubin increased the affinity of the NMDA receptor for MK-801 in the brain, possibly due to conformational changes of the ion channel complex (Hoffman *et al.*, 1996). In the brain glutamate uptake by astrocytes normally prevents excitotoxic glutamate elevations in the extra cellular space  Exposure of nerve cells to bilirubin enhances the extra cellular concentration of glutamate either by decreased uptake (Silva *et al.*, 1999;Wennberg *et al.*, 1994), or by enhanced release (Fernandes *et al.*, 2004).

Gunn rats with substantial hyperbilirubinemia were consistently more susceptible to brain injury after injections of NMDA into the brain than non-jaundiced controls. Concurrent treatment with the NMDA receptor blocker MK-801 reduced cell death

(McDonald *et al.*, 1998). Two *in vitro* studies by Grojean *et al* on cultured cortical neurons from rat pups showed that apoptosis induced by a low bilirubin concentration (0.5 μM) alone or concurrent with hypoxia was completely abrogated by treatment with MK-801 (Grojean *et al.*, 2000;Grojean *et al.*, 2001). Their results indicated that bilirubin-mediated excitotoxicity was executed predominantly by caspase-dependent pathways (Grojean *et al.*, 2000).

## 5.1 SUMMARY OF BACKGROUND FOR THE THESIS

To date, no unifying, single mechanism of bilirubin toxicity has been demonstrated. The mechanisms operating at the BBB to regulate bilirubin entry into the brain are incompletely understood. Also, neither studies on bilirubin entry into the brain, nor the elimination of bilirubin from the brain have satisfactorily explained the preference for bilirubin accumulation in certain areas of the brain as seen at autopsy of kernicteric infants.

The wide variety of deleterious effects caused by bilirubin may suggest that bilirubin excerts its toxic effects on multiple sites of cellular metabolism. While apoptotic and excitotoxic mechanisms have been convincingly demonstrated in bilirubin-mediated toxicity, the relative impact of excitotoxic pathways and their relation to caspase-mediated and non caspase-mediated cell death needs further investigation. In a recent paper Shapiro stated that "the different patterns of expression of bilirubin- induced injury may relate to 1) the amount and duration of exposure to bilirubin, 2) variation in susceptibility of the nervous system, 3) the relative amount of necrosis vs. apoptosis produced, and 4) whether surviving neurons become functionally normal or are more susceptible to other stressors either at the time of hyperbilirubinemia or afterwards" (Shapiro, 2005).

Recent evidence from studies on newborn rodents suggests that the immature brain may be more susceptible to both excitotoxicity (McDonald & Johnston, 1990) (McDonald *et al.*, 1998) and apoptotic cell death (Hu *et al.*, 2000;Lesuisse & Martin, 2002) (Zhu *et al.*, 2005) than the adult brain. These findings should encourage further studies of the role of excitotoxicity and apoptosis in bilirubin-mediated injury in newborns. Also, no prior investigations have been undertaken on neuronal cells of human origin.

## 5. 2 AIMS OF THE THESIS

Our understanding of the mechanisms of bilirubin entry into the brain and the mechanisms of neuronal injury is inadequate. Bilirubin is a substrate for the membrane transport protein P-gp which has been attributed an important role in the regulation of entry into and expulsion of compounds from the brain. In the first study (paper I) we hypothesized 1) that treatment with drugs known to inhibit P-gp function would modify bilirubin entry into the brain of young adult rats and 2) that such drugs might affect the regional distribution pattern of bilirubin in the brain.

In the remaining studies (papers II, III and IV) we used human NT2-N neurons to determine the modes of cell death and its temporal evolution after exposure to various concentrations of bilirubin in a model of continuous exposure to bilirubin for up to 96 h and in a model of short term exposure (6 h) to bilirubin. To elucidate the roles of caspases and excitotoxicity, we investigated the effects of treatments with two different caspase inhibitors and/or an NMDA receptor antagonist in both continuous and short term exposure (paper III and IV). In additional experiments we evaluated the added impact of serum deprivation in our model of long term bilirubin exposure (96 h) (paper IV).

## 6. SUMMARY OF RESULTS

### Paper I

We hypothesized that pre-treatment with drugs known to inhibit P-gp function administered in clinically relevant doses in the newborn would alter the uptake of bilirubin in the brain of 32-36 days old Spraque Dawley rats. In the first arm of the study, the animals received pre-treatment with an iv infusion of the respective drug (or saline in controls) 10 min before an iv bolus of 50 mg/kg bilirubin given over 5 min. The animals were sacrificed 15 min after the initiation of the bilirubin infusion and brain bilirubin was determined by acid chloroform extraction. Due to significant differences in serum bilirubin values (rats receiving verapamil) a brain-to-serum bilirubin ratio was calculated. Control animals (n = 12) had a brain/serum bilirubin ratio of 3.2 ± 0.8 (x $10^{-3}$). Rats receiving pre-treatment all showed enhanced brain/serum bilirubin ratios (all x $10^{-3}$); propanolol 4.7 ± 1.6 (n = 7), ceftriaxone 7.6 ± 2.5 (n = 7), rifampin 5.3 ± 1.4 (n = 6), verapamil 4.6 ± 1.5 (n = 11), and erythromycin 4.0 ± 0.7 (n = 6). The increase in brain-to-serum bilirubin ratios was significant for all treatments ($p < 0.05$, two tailed t-test with Welch correction) except for erythromycin. Ceftriaxone is also a displacer of bilirubin at the albumin binding site, which may have contributed to the increased brain bilirubin in animals treated with this drug.

In the second study arm we hypothesized that pre-treatment with two of the drugs shown to increase total brain bilirubin, ceftriaxone and rifampin, would alter the regional distribution of bilirubin in the brain. With the exception that radioactive bilirubin was used (20 μCi, for each animal), the procedure was identical to the one described above. Analysis of seven different brain regions (cortex, hippocampus, striatum, hypothalamus, midbrain, cerebellum and medulla) by scintillation counting showed that the concentrations differed significantly among brain regions in all three study groups ($p < 0.001$). The pattern of distribution was similar in controls and ceftriaxone-treated rats in that both groups had significantly higher bilirubin concentrations in the cerebellum. In the rifampin-treated rats the distribution pattern was different in that the bilirubin concentration was significantly lower in the cerebral cortex than in the cerebellum and the medulla. However, these changes in distribution were not in accordance with a kernicteric staining pattern.

**Paper II**

NT2-N neurons were exposed to different concentrations of bilirubin and bovine serum albumin (BSA) at molar ratios (MR) of 1.5 and 3. Bilirubin induced a dose dependent (0.66 - 250 μM/L bilirubin) decrease in neuronal viability as measured with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assays for both MRs at 24 h. Bilirubin/BSA MR 3 was significantly more potent than MR 1.5 in reducing neuronal viability ($p < 0.0001$). All further experiments were undertaken with bilirubin/BSA MR 1.5, and multiple, independent measures of cell damage were used to evaluate neuronal injury after 6, 24, and 48 hours.

2 and 5 μM bilirubin caused necrotic cell death in a small subpopulation within 24 h. 10 and 25 μM caused substantial early necrotic cell death at 6 h, further increasing up to 24 h, but not thereafter. High concentrations of bilirubin (100 μM) caused almost complete necrotic cell death at 6 h accompanied by severe early impairment of MTT reduction. Apoptosis was the predominant mode of cell death after exposure to bilirubin concentrations ≤ 10 μM. Apoptosis was associated with maintained neuronal metabolism as indicated by MTT reduction. We defined apoptosis basically according to morphological criteria to consist of nuclear condensation or nuclear fragmentation. These two entities displayed differences in temporal evolution, as nuclear condensation appeared earlier and did not increase beyond 24 h (a maximum value of 25 ± 8 % in neurons exposed to 25 μM), while nuclear fragmentation first occurred at 24 h and continued to increase up to 48 h (a maximum value of 37 ± 11 % in neurons exposed to 10 μM). PARP cleavage and oligonucleosomal DNA fragmentation (DNA laddering) was found in cells treated with 5 μM bilirubin, but not with higher bilirubin concentrations. 2 μM staurosporine (used as a positive control for apoptosis) caused predominantly apoptotic cell death with a predominance of fragmented nuclei (61 % fragmentation at 48 h) and prominent PARP cleavage and DNA laddering.

To conclude, bilirubin induced both apoptosis and necrosis in neurons. Apoptosis was the predominant mode of cell death after exposure to low and moderate bilirubin concentrations.

**Paper III**

To investigate the role of caspases and excitotoxicity in bilirubin-induced cell death, we exposed NT2-N neurons to 5 μM bilirubin (a concentration known to induce apoptosis) or 2 μM staurosporine (positive apoptosis control), and investigated the effects of treatments with the specific caspase-3 inhibitor, zDEVD.FMK (20 and 100 μM), or the general caspase inhibitor, zVAD.FMK (20 and 100 μM), and/or the NMDA receptor antagonist MK-801 (10 μM) during a 24 h or 48 h exposure.

Bilirubin increased caspase-3 activity 2.3-fold after six hours. Despite this, treatment with zDEVD.FMK did not significantly reduce cell death. Treatment with the general caspase inhibitor zVAD.FMK significantly enhanced neuronal survival, however, this effect was not reflected in the MTT assays. General caspase inhibition reduced nuclear fragmentation (at morphological evaluation) and oligonuclesomal DNA fragmentation (the DNA ladder) more potently than caspase-3 inhibition. Caspase inhibition did not affect necrotic cell death (condensed nuclei with disrupted membrane). In cells exposed to staurosporine caspase-3 activity increased 7.8-fold after a six hour exposure, and both caspase inhibitors highly significantly reduced neuronal death at 24 and 48 h. Caspase inhibition markedly changed the pattern of nuclear morphology and DNA fragmentation in cells exposed to staurosporine in a similar way as seen in bilirubin-mediated injury.

Treatment with MK-801 reduced bilirubin-induced cell death after 48 h (40 ± 22 % vs 21 ± 17 % undamaged nuclei, $p = 0.03$), but significant protective effects were not found in the MTT assays. Treatment with MK-801 reduced apoptotic nuclear condensation, but did not influence the number of cells displaying nuclear fragmentation. Treatment with MK-801 had no effect on the proportion of necrotic nuclei. Co-treatment with MK-801 and zVAD.FMK (100 μM) reduced apoptotic morphologies after 24 h (88 ± 6 % vs 58 ± 16 % undamaged nuclei, $p = 0.001$) and 48 h (59 ± 21 % vs 21 ± 17 % undamaged nuclei, $p = 0.0002$). MTT assays showed similar effects of combined treatment. Combined treatment also showed significant effects versus treatment with MK-801 alone after 24 h (88 ± 6 % versus 71 ± 17 % undamaged nuclei, $p < 0.05$), but was not significant after 48 h ($p = 0.051$). Taken together, bilirubin induces apoptosis in human NT2-N neurons by excitotoxic mechanisms and by caspase-mediated pathways. Apparently these pathways are, at least in part, distinct in this model. Concomitant inhibition of both these pathways synergistically prevented cell death.

**Paper IV**

We used human NT2-N neurons to investigate delayed effects of short-term exposure to unconjugated bilirubin (UCB). Cell viability was evaluated with MTT reduction assays and nuclear morphology. A 6 h exposure to 1, 5, or 25 μM UCB and serum deprivation significantly diminished MTT reduction (74 ± 11%, 67 ± 15%, and 59 ± 6% of control values, $p = 0.01$, 1 vs. 25 μM). 96 h after rescue of neurons with removal of UCB and re-incubation in the original serum-containing medium, delayed effects were evident as recovery (1 μM UCB), intermediate cell death (5 μM UCB), or near complete cell death (25 μM UCB). In this model, treatment with the specific caspase-3 inhibitor, zDEVD.FMK (100 μM), or the pancaspase inhibitor zVAD.FMK (100 μM) did not improve viability in rescued neurons exposed to 5 μM UCB. On the other hand, treatment with the NMDA receptor antagonist MK-801 (10 μM) enhanced the number of undamaged nuclei versus controls (86 ± 14% vs. 50 ± 12%, $p = 0.001$), but had no impact on MTT reduction. In a different model with 102 h neuronal exposure to UCB and serum deprivation, we found an additional toxic impact of serum deprivation. Separate experiments suggested that this was a result of late caspase-mediated toxicity. The impact of a 6 h serum deprivation alone appeared to be modest in rescued neurons. We conclude that UCB-mediated effects are reversible in this model. Blockade of excitotoxic mechanisms, but not caspase activity, may prevent delayed cell death.

# 7. METHODOLOGICAL CONSIDERATIONS

The methods are stated in "Methods" in the respective papers. The following section is concerned with important methodological considerations of particular importance for the reliability of the studies.

### 7.1 Rat models and membrane transport proteins

The Gunn rat develops hyperbilirubinemia postnatally due to a congenital deficiency in the activity of glucuronyl transferase (McDonald *et al.*, 1998), and has been much used in the study of bilirubin-induced encephalopathy. The Gunn rat expresses P-gp (Jon Watchko personal communication). However, due in part to the high cost of Gunn rats we choose Sprague Dawley rats for our experiments. This rat strain also expresses P-gp (King *et al.*, 2001), and has been much used in the study of bilirubin entry into and elimination from the brain (Hansen & Cashore, 1995;Hansen, 1995;Hansen, 1996;Hansen & Allen, 1996b). In these rats hyperbilirubinemia is created by injection of solubilized bilirubin. A problem in the use of newborn rats for experiments is that iv infusions and adequate blood sampling is technically extremely difficult due to their small size. Thus, nearly all studies have used young adult animals at the age of approximately five weeks (Hansen & Cashore, 1995;Hansen, 1995;Hansen, 1996;Hansen & Allen, 1996b). This raises doubt about their relevance to the question of bilirubin toxicity in the newborn period. To some extent this may be counteracted by the fact that rat brain development continues at least until three months of age (Altman, 1969). The ontogeny of membrane transport protein and their functionality in newborn rats is, however, not well known.

In the Sprague Dawley rat expression of P-gp has been shown in olfactory neurons from embryonic day 14 (KulkarniNarla *et al.*, 1997). In Wistar rats, brain P-gp was not detected until postnatal day 7 and adult levels were reached at postnatal day 28, mainly in the brain capillary endothelial cells in the cerebellum, the hippocampus and the frontal cortex (Matsuoka *et al.*, 1999). In mice, brain P-gp was expressed from embryonic day 16, and adult levels were reached by postnatal day 21 (Tsai *et al.*, 2002). This evolution seems to parallel the expression of P-gp in the human brain (Tsai *et al.*, 2000). Organic anion transport protein (OATp) is expressed in the brain of adult rats both at the BBB and in the choroid plexus (Gao *et al.*, 1999;Gao *et al.*, 2000). However, information on the expression and function of OATp and MRP in the newborn is, to our knowledge virtually non-existing.

### 7.2 NT2-N neurons in the study of perinatal brain injury

The human teratocarcinoma-derived cell line Ntera2/clone D 1 has the ability to differentiate into post-mitotic neurons when treated with retinoic acid (RA) (Pleasure *et al.*, 1992). RA, RA-binding proteins and RA receptors are detected in the embryonic brain, and RA is thought to play an important role in the differentiation of neurons during development (Horton & Maden, 1995;Rossant *et al.*, 1991). According to our protocol Ntera2/clone D 1 cells were treated with RA for 4 weeks. At this stage of development, 5 % of the cells are neurons as judged by the presence of neuron-specific markers (Pleasure *et al.*, 1992). Neuronal cells were then mechanically dislodged from the bottom non-neuronal layer. Thereafter, treatment with mitotic inhibitors for another four weeks eliminates almost all the remaining non-neuronal cells, but has no apparent effect on NT2-N neurons (Pleasure *et al.*, 1992). After two weeks of treatment with mitotic inhibitors, 95 % of the cells are differentiated neurons with a wide network of processes which can be identified as dendrites and axons (Pleasure *et al.*, 1992).

In non-differentiated cells (prior to RA-treatment) apoptosis is a rare event. However, during RA treatment apoptosis increases three fold (Zigova *et al.*, 2001). Glutamate-evoked currents are detected in all cells after 6 weeks (Younkin *et al.*, 1993), and a gradual sensitivity to glutamate develops in parallel to the increased expression of NMDA receptors (Munir *et al.*, 1995), and non-NMDA receptors (Hardy *et al.*, 1994). At the time of the experiments the density of NMDA receptors in the NT2-N neurons is 1/10 of that found in mature rat hippocampal membranes, but is similar to that found in human neurons (Munir *et al.*, 1996). NT2-N neurons also express functional GABA receptors, predominantly of the same subtype as found at early stages in the rat brain (Matsuoka *et al.*, 1997;Neelands *et al.*, 1998), and functional calcium channels (Neelands *et al.*, 2000). A recent study showed that apoptotic functional genes of the extrinsic apoptotic pathways were significantly up-regulated after exposure to ethanol in NT2-N neurons (Chen *et al.*, 2005)

Human NT2-N neurons are not derived from the central nervous system. However, upon treatment with RA these cells develop characteristics of neurons which are reflected in their morphological appearance, expression of protein markers and receptors and the acquired post-mitotic state. There are strong arguments that these cells may function in a neuron-like manner. Transplantation of NT2-N cells into the striatum after focal cerebral ischemia in rats has been shown to improve motor function (Borlongan *et al.*, 1998). Furthermore, in human stroke patients the transplantation of terminally differentiated NT2-N neurons survived for 27 months without reverting to a neoplastic state (Nelson *et al.*, 2002).

Human NT2-N neurons have proven a useful model in the study of HI cell death (Almaas *et al.*, 2000;Almaas *et al.*, 2002;Rootwelt *et al.*, 1998) and to elucidate apoptotic mechanisms (Cardoso *et al.*, 2004a;Cardoso *et al.*, 2004b;Walker *et al.*, 1999). Caspase-3 activation has been shown after exposure to serum deprivation in NT2-N neurons (Walker *et al.*, 1999). Furthermore, calpain inhibitors reduced hypoxic injury in this cell line (Almaas *et al.*, 2003). No investigations have addressed the role of AIF or endonuclease G in cell death in NT2-N neurons.

### 7.3 Solubility and toxicity of bilirubin in culture medium

In studies of cultured cells the culture medium is the equivalent of the interstitial fluid of the brain. Very little information exists on bilirubin concentrations in the interstitial fluid that may be relevant to the induction of bilirubin-mediated injury *in vivo*. In one study concentrations of 5 μmol/l bilirubin were found in the cerebrospinal fluid of infants with a mean bilirubin concentration of 222 μmol/l (Meisel *et al.*, 1981). This value corresponds well with the bilirubin concentrations in brain tissue of jaundiced animals (Bratlid, 1990). As stated previously, the solubility of bilirubin has been estimated to be as low as 7 nmol/L in aqueous solutions (Brodersen, 1979). Once aggregates are formed, changes in unbound bilirubin concentrations occur, giving rise to experimental variability. In aqueous solutions the bilirubin molecule aggregates at pH < 8.5, and more so the more acidic the environment (Boiadjiev *et al.*, 2004). Most experimental systems add albumin to bind bilirubin, as albumin confers stability to bilirubin in solution. Hence, bilirubin toxicity in a system of cultured neurons is dependent on variables such as the bilirubin concentration, its binding to albumin and other constituents in the culture medium, pH, and temperature (Amit *et al.*, 1990). Lowering the bilirubin/albumin molar ratios < 1 not only prevents aggregation of bilirubin, but also largely prevents bilirubin toxicity in cell cultures (Cowger, 1971;Lie & Bratlid, 1970;Schiff *et al.*, 1985).

Hayward *et al* showed that in incubation medium containing 12.5 - 150 μM bilirubin with bilirubin/human serum albumin (HSA) molar ratio (MR) of 1.5 and 3, loss of bilirubin due to aggregation was < 10 % after 24 h. Cellular uptake of bilirubin in a human neuroblastoma cell line was “considerable” after 90 min, and the authors concluded that the preferred bilirubin/albumin ratios for *in vitro* toxicity studies were between 1.5 and 3 (Hayward *et al.*, 1987). A later study by the same group showed that the uptake of bilirubin in neuroblastoma cells was dose dependent and linear at a bilirubin/HSA MRs of 1.5 and 3,

where uptake was defined as the total amount of bilirubin associated with the cells including both surface bound and (possibly) internalized bilirubin. By lowering the pH from 7.8 to 7.4 and 7.0 bilirubin uptake was enhanced 3 and 10 times respectively. 35 - 125 μM bilirubin (bilirubin/ HSA 0.8 - 1.5 molar ratio) and pH 7.4, induced toxicity dependent on the bilirubin/albumin ratio, bilirubin concentration, and the duration of exposure (Amit *et al.*, 1990).

Based on these findings, we performed initial experiments with bilirubin/BSA MRs of 1.5 and 3. We found that bilirubin at both ratios dose-dependently impaired MTT reduction assays over a wide concentrations range (0.66 - 250 μM) measured at 24 h (paper II). Differences in the toxic effects between the two MRs were significant; however, differences were smaller than anticipated, possibly due to increased aggregation of bilirubin with MR 3. We therefore chose MR 1.5 for our later experiments, and kept pH at a slightly supra-physiological level (7.59-7.52 over the first 24 h) to further limit bilirubin aggregation. Bilirubin has a lower affinity for BSA than HSA (Zucker & Goessling, 2000). On the other hand, BSA-bound bilirubin was found to have slower dissociation rates than HSA-bound bilirubin in cultured hepatoma cells (Zucker & Goessling, 2000). The exact impact of these differences is unclear, but the only study which has compared the effect of BSA vs. HSA on bilirubin toxicity did not find differences in the toxic effects of bilirubin (Cowger, 1971). In our experiments, light-microscopic evaluation showed a slight tendency to aggregation at all concentrations ≥ 5 μM (paper II). However, even in medium containing 25 μM bilirubin, the overall impression was the one of a homogenous bilirubin containing solution with only dispersed bilirubin aggregates. A slightly greater tendency for aggregation was found with MR 3:1. 0.66 μM bilirubin without albumin was highly aggregated in our experiments. It must be added that light-microscopy certainly has limitations, and it is not sensitive enough to discover micro-aggregation. Despite the limitation of not knowing the fraction of unbound bilirubin, this model proved reliable in reproducing a graded toxic response.

**7.4 Significance of nuclear morphological changes**

Changes in nuclear morphology were evaluated with a fluorescence staining method as described in “Methods” of paper II, III and IV. In general, cells remained adherent to the dish during the experiments, and detached nuclei accounted for less than 2 % of the total cell count. Pictures were taken from randomly selected areas with medium cell density. The densest areas were impossible to count, so that areas of lesser density had to be selected. A

potential problem is the possible bias from the person who selects the areas for counting. This selection bias was reduced by the mounting of the camera at an angle that did not yield the same picture as seen in the middle of the view of the microscope. In that way it was not possible to select solitary areas for photographing, but it was possible to avoid the densest aggregates.

The combination of the two fluorescent dyes, Hoechst 33342 and EH, was used to assess nuclear morphology and membrane integrity. Hoechst 33342 stains the nuclei of all living and dead cells, while nuclei of cells with damaged cell membranes, as seen in necrosis, are stained by EH. The detection of necrosis was based on the release of lactate dehydrogenase (LDH) and uptake of ethidium homodimer (EH) into condensed nuclei (paper II). These findings both suggest disintegration of the cell membrane and were seen in virtually all cells after treatment with very high bilirubin concentrations (100 μM). We defined as apoptotic those cells which after staining with the combination of were stained exclusively with Hoechst suggestive of an intact cell membrane, and *1)* developed chromatin condensation in the shape of a pycnotic rounded mass without disruption of the cell membrane or *2)* nuclear fragmentation observed as chromatin condensation into the shape of lobuli or separate fragments. These definitions have previously been used by our group (Almaas *et al.*, 2003) and others (Cregan *et al.*, 2002;Hamabe *et al.*, 2000;Nicotera *et al.*, 1999). We defined fragmentation as the occurrence of at least two distinct lobuli. In our experiments nuclear fragmentation was dependent on the action of caspases, as the general caspase inhibitor zVAD.FMK almost completely inhibited nuclear fragmentation induced by bilirubin or STS. Condensed nuclei (without disrupted membrane) occurred as the result of *1)* toxicity mediated by the NMDA receptor (as treatment with MK-801 reduced this phenotype, paper III), *2)* neurons exposed to bilirubin or STS and treated with ZVAD.FMK (paper II), or *3)* apoptosis caused by long-term serum deprivation which could be prevented by caspase inhibitors (paper IV, data not shown).

Due to the absence of phagocytosis in neuronal cultures, eventually the cell membrane of apoptotic cells also disintegrates to allow staining with EH, so called “secondary necrosis” (Kaal *et al.*, 1998). A limitation of the present studies was that this secondary necrosis of apoptotic condensed nuclei was indistinguishable from primary necrosis, and may have resulted in an overestimation of "necrotic” cell death, appearing as condensed nuclei with disrupted membrane, and a possible underestimation of apoptotic condensed nuclei. Also neurons displaying fragmented nuclei underwent secondary necrosis,

however, these cells were counted as fragmented irrespective of uptake of EH, as apoptotic nuclear fragmentation was thought to reflect the principal cell death mode.

All cells were counted by the principal investigator (Hankø). In all experiments a subset of cells were counted by an independent investigator (Almaas). A total of 2000 cells from the three studies (paper II, III, IV) were counted to an inter-observer agreement of 95-96 %. The principal investigator had a slight tendency to judge more cells as being condensed vs. both undamaged and fragmented. A worst case scenario may have resulted in an over-report of condensed nuclei, however, not of more than 5 %. Secondary necrosis may have resulted in an underestimation of primary condensed nuclei, so that this bias does not affect the results in the same direction.

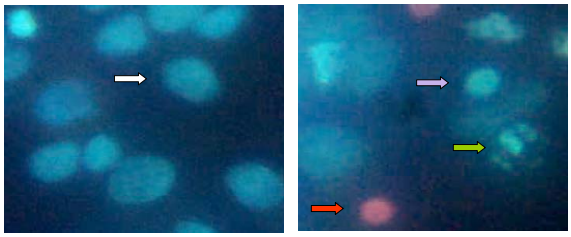


Figure 4. The combination of the two fluorescent dyes Hoechst 33342 and Ethidium Homodimer (EH) was used to assess nuclear morphology and membrane integrity respectively. (A) Predominantly healthy control cells not exposed to bilirubin. (B) Cells exposed to 5 µM bilirubin for 6 h, and photographed 96 h after removal of bilirubin. Undamaged nuclei (*white arrow*) and condensed nuclei with preserved cellular membranes (*blue arrow*) and fragmented nuclei *(green arrow)* considered apoptotic, were stained by Hoechst 33342 only. Condensed nuclei with disrupted membranes displaying uptake of EH (*red arrow*) were considered necrotic.

**7.5 MTT reduction assays**

The rate by which cells reduce MTT is a well established method to evaluate cell viability and proliferation. MTT is a tetrazolium salt which is actively transported into cells by endocytosis (Liu *et al*, 1997). A variety of sub-cellular fractions, including the cytoplasm, mitochondria,

and non-mitochondrial membranes including endosomes and lysosomes are capable of reducing MTT predominantly by an NADH-dependent mechanism. Reduced MTT is thereafter actively transported to the cell surface by exocytosis (Liu *et al.*, 1997). The crystals are solubilized with DMSO and measured by a spectrophotometer.

Impairment of all these steps may affect the MTT reduction assays, and the capacity of MTT to cross intact plasma membranes is an important experimental variable (Bernas & Dobrucki, 2000). Impaired MTT reduction is not always associated with cell death. Several studies on β-amyloid-mediated cell death have shown that impaired MTT reduction represents an early indicator of cell injury without necessarily causing cell death (Patel *et al.*, 1996;Shearman *et al.*, 1995). In astrocytes, a 4 h exposure to a moderate bilirubin concentration (17 μM) impaired endocytosis and MTT reduction without affecting the cytoskeleton, suggesting interaction of bilirubin primarily at the plasma membrane, while higher bilirubin concentrations (171 μM) also disaggregated the cytoskeleton (Silva *et al.*, 2001a). In our experiments evaluation of MTT assays and nuclear morphology at 6 h suggested that the development of apoptotic morphologies were later events than diminished MTT reduction (paper II and IV), compatible with the idea that interaction with the plasma membrane is an early event in bilirubin toxicity. In the absence of co-treatments the proportion of undamaged nuclei showed similar results as MTT assays at all time points ≥ 24 h for treatment with bilirubin, staurosporine, and serum deprivation (paper II and paper IV). These results are in good agreement with previous studies using NT2-N neurons (Almaas *et al.*, 2003;Itoh *et al.*, 1998).

*MTT assays and nuclear morphology in cells receiving anti-apoptotic treatment*

In our studies, MTT assays did not always show the same degree of protection by co-treatments as found by evaluation with nuclear morphology (paper III and IV). In neurons subjected to bilirubin and serum deprivation for 48 h (paper III), serum deprivation alone (102 h), or a 6 h bilirubin exposure with subsequent rescue and evaluation 96 h thereafter (paper IV), the protective effects of treatments found by morphological evaluation were either not reflected in the MTT assays (treatment with caspase inhibitors) or to a considerably lesser degree (treatment with MK-801). The explanation for this discrepancy is not clear. Our findings may suggest that moderate insults cause cell death in a subset of neurons which is prevented by blockade of the cell death pathways involved, but that treatment can not prevent the overall impairment on MTT metabolism in the entire neuronal population.

These results are in line with a study on rat cerebellar granule neurons exposed to potassium-deprivation where caspase inhibitors prevented apoptotic morphologies, but did not improve MTT reduction (Harada & Sugimoto, 1998). Furthermore, Lobner found that MTT assays do not always correctly quantify neuro-protective effects, and the usefulness of MTT assays in determining effects of co-treatment is dependent on the inducer of cell death (Lobner, 2000). In paper III cells subjected to staurosporine and co-treatment with caspase inhibitors, MTT reduction and the proportion of undamaged neurons showed similar results (paper III).

### 7.6 The impact of serum deprivation in the present studies

Serum deprivation is a cytotoxic environmental change that has been shown to induce cell death in various cultured cells (Deshmukh & Johnson, 1997;Kariya *et al.*, 2002). The detailed mechanisms of the execution of cell death in serum-deprived cells are uncertain, but caspase-activation is believed to play an important role in the execution of cell death in some (Batistatou *et al.*, 1993;Deshmukh & Johnson, 1997;Stefanis *et al.*, 1996), but not all cell lines (Hamabe *et al.*, 2000). A 6 h serum deprivation caused moderate impairment of the MTT assays. After subsequent neuronal rescue with washout of the serum free medium and re-addition of serum containing medium, MTT reduction did not deteriorate further and no delayed cell death was found after 96 h (paper IV). After a 102 h serum deprivation, cell death was substantial (paper IV), and concomitant exposure to UCB (1, 5 and 25 μM) caused dose-dependent bilirubin toxicity additional to the effects of serum deprivation. To elucidate the mechanisms of cell death induced by serum deprivation, we performed additional experiments on neurons exposed to serum deprivation alone. Cell death induced by serum deprivation for 102 h was reduced by co-treatment with caspase inhibitors, but not by treatment with the NMDA-receptor antagonist MK-801 (paper IV). Similar experiments after a 24 h serum deprivation (control experiments, paper III) showed treatment effects of caspase inhibitors or MK-801.

Our findings indicated that serum deprivation and bilirubin may have additive toxic effects. Serum deprivation may contribute to cell death by a late induction of caspase-dependent cell death pathways. In our model of concurrent long term serum deprivation and bilirubin, serum deprivation definitely had impact on the cell death pathways involved after a 102 h (paper IV). In retrospect, the additive effects of serum deprivation at earlier time points (24 and 48 h) cannot be ruled out (paper II and III). The impact of a 6 h serum deprivation on rescued neurons at evaluation 96 h after neuronal rescue was assumed to be minor (paper IV).

# 8. DISCUSSION OF RESULTS

## 8.1 P-gp inhibitors and bilirubin entry into the brain

Watchko *et al* showed that brain bilirubin content was almost two fold higher in knockout mice devoid of P-gp than in wild type animals after an iv injection of bilirubin (Watchko *et al.*, 1998). In paper I a similar experimental procedure was used to induce bilirubin entry into the brain ten minutes after the injections of different P-gp inhibitors. Ceftriaxone is both a P-gp inhibitor and a highly potent displacer of bilirubin. As the brain/bilirubin ratio increased more in ceftriaxone-treated animals (to 237 %) than in knock out mice devoid of P-gp (Watchko *et al.*, 1998), the bilirubin-displacing property of the drug apparently had an additive impact on bilirubin entry into brain. The increase in total brain bilirubin uptake after treatment with drugs without significant bilirubin-displacing properties varied from 24% (erythromycin not significant) to 66 % (rifampin) compared to control animals pre-treated with saline. This is similar to the increase in brain bilirubin induced by other known risk factors such as hyperosmolarity or hypercapnia, where brain bilirubin increased by 39 % and 70 % respectively (Hansen TWR *et al.*, 1989). The increased entry into the brain was, however, considerably less than the one reported for P-gp substrates with a higher affinity for P-gp than bilirubin, such as ivermectin (2.5- fold) or rhodamine (3-fold) after P-gp inhibition with Cyclosporine A (Marques-Santos *et al.*, 1999).

In experimental animal models where the permeability of the BBB has been increased by inducing hyperosmolarity or hypercapnia, the regional distribution of brain bilirubin has shown a poor correlation with the one seen in kernicteric brains of human neonates (Hansen TWR *et al.*, 1989;Hansen, 1996;Levine *et al.*, 1982). In our experiments the regional distribution patterns in control rats and ceftriaxone-treated rats were similar in that the cerebellum showed enhanced uptake compared to most other brain regions. However, other brain areas which are severely affected in kernicteric infants such as the hippocampus or the basal ganglia did not show altered bilirubin uptake compared to other brain regions. In rats pre-treated with rifampin, an agent without known bilirubin-displacing properties, the bilirubin content was significantly lower in the cortex compared to the cerebellum and the medulla. If P-gp-mediated bilirubin efflux at the BBB made an important contribution to the regional distribution pattern of bilirubin in the brain, it is likely that treatment with P-gp inhibitors would provide an increased entry into the areas of the brain which are protected from bilirubin by P-gp; e.g. treatment with P-gp inhibitors would more potently enhance the entry of bilirubin into the frontal cortex than into the cerebellum. Taken together, treatment

with P-gp inhibitors had limited impact on the regional distribution pattern of brain bilirubin in this model. The observed changes did not suggest that membrane transport proteins like P-gp are responsible for the regional distribution pattern of brain bilirubin found in kernicterus.

Our results are compatible with two studies by Drion *et al* where pre-treatment with the second generation P-gp blocker PSC or verapamil increased the distribution volume of colchicines (a cytotoxic drug) by 8- and 4- fold respectively in eight grey areas of the brain (Drion *et al.*, 1996;Drion *et al.*, 1997). The entry of colchicine was similarly enhanced in all areas of the brain. A role for P-gp in determining the distribution pattern can not be completely ruled out from the present studies. The full potential of P-gp inhibition may not have been reached in the present model, as it is unknown whether P-gp was fully saturated with P-gp inhibitor in all areas of the brain.

Up-regulation of P-gp in humans is similar to the one found in rodents (as discussed previously). In the human brain P-gp is expressed in capillary endothelial cells, astrocytes, and choroid plexus epithelium by 23 weeks of gestation (Tsai *et al.*, 2000). At term the distribution pattern is similar to the one found in the adult brain, however, with markedly less density (Tsai *et al.*, 2000). Hence, the ontogeny of P-gp suggests that the effects of P-gp inhibitors are less in newborn rats than in young adult animals as used in the present study. Information on the ontogeny of OATp and MRP in newborn animal species or humans is to our knowledge virtually non-existing. Due to the limited knowledge on the ontogeny of membrane transport proteins, it is difficult to extrapolate our findings to the jaundiced newborn human. It would be worthwhile to verify our findings in newborn animals, however due to their small size studies requiring iv infusions or blood sampling in newborn rodents is technically extremely difficult. This suggests the use of different animal models in future studies.

MRP is found at the BBB in humans and rodents (Liang & Aszalos, 2006;Tamai & Tsuji, 2000), and OATp is expressed in rat brain both at the BBB and in the choroid plexus (Gao *et al.*, 1999;Gao *et al.*, 2000). Unconjugated bilirubin is a substrate for MRP (Pascolo *et al.*, 2001) and organic anion transport protein (OATp) (Cui *et al.*, 2001). As many P-gp inhibitors, among them verapamil and rifampin are also MRP inhibitors (Courtois *et al.*, 1999;Cullen *et al.*, 2001), it can not be claimed that the drugs used in the present study exerted their function exclusively at the P-gp inhibitor. Drug inhibition of OATp by the drugs used in our experiments is to our knowledge not studied.

A possible contribution of membrane transport proteins in the pathogenesis of kernicterus may have important implications. Based on our findings Hascoet proposed that

since genetic polymorphism in the human P-gp gene has been demonstrated, this could explain the susceptibility of some newborns to bilirubin toxicity. It is possible that specific genetic markers may predict susceptibility to bilirubin toxicity similar to the role of genetic markers as means to improve anti-leucemic or anti-retroviral therapy (Hascoet, 2003).

To conclude, we have shown that treatment with several drugs known to inhibit P-gp may cause increased entry of bilirubin into the brain of young adult rats. The effect of treatment on the distribution pattern of brain bilirubin was modest and the relevance with respect to the bilirubin distribution pattern as seen in kernicterus is uncertain. We speculate that drugs inhibiting P-gp or other membrane transport proteins may increase the risk of bilirubin encephalopathy in the hyperbilirubinemic infant. However, the limitations of our study, such as the limited knowledge on the ontogeny of membrane transport proteins, the overlap in substrate specificities of the different transport proteins, and the use of inhibitors that are not entirely specific for P-gp, highlight the need for robust models that allow studies of the specific roles of membrane transport proteins in the entry and elimination of bilirubin in the brain of newborns.

**8.2 Short-term exposure to bilirubin results in recovery of function or delayed cell death**

Some studies have suggested that the risk of significant neurological abnormalities correlates with the duration of hyperbilirubinemia (Devries *et al.*, 1985;Ozmert *et al.*, 1996). Also, clinical studies suggest that early stages of bilirubin encephalopathy are reversible (Paludetto *et al.*, 2002;Shapiro, 1993), but reversibility of bilirubin toxicity has never been shown in intact neuronal cultured cells. Reversibility of an apoptosis-inducing stimulus has been extensively studied in neuronal growth factor (NGF)-deprived neurons. Sympathetic neurons deprived of NGF are capable of resuming neuronal growth and metabolism when NGF is added back into the cultures (Deshmukh *et al.*, 1996;Deshmukh *et al.*, 2000). We found that removal of bilirubin and re-incubation in serum-containing medium provided recovery as measured with MTT assays and nuclear morphology in neurons exposed to 1 μM bilirubin for 6 hours (paper IV). Long-term exposure to 1 μM bilirubin, however, significantly increased cell death (compared to serum deprivation alone), showing that this bilirubin concentration may induce permanent cell injury if the exposure time is prolonged.

As discussed elsewhere, interference with several different steps in MTT metabolism may impair MTT reduction. The capacity of MTT to cross intact plasma cell membranes is an important experimental variable (Bernas & Dobrucki, 2000). In astrocytes, a

4 h exposure to moderate bilirubin concentrations (17 μmol/l) impaired endocytosis and MTT reduction without affecting the cytoskeleton, suggesting interaction with MTT at the plasma membrane (Silva *et al.*, 2001a). Evidence suggests that injury to the cell membrane is a key factor in bilirubin toxicity (Eriksen *et al.*, 1981;Vazquez *et al.*, 1988). After the initial formation of a bilirubin anion-phospholipid complex at the cell membrane, bilirubin has a tendency to take up $H^+$ ions and form bilirubin acid leading to cell membrane destruction (Eriksen *et al.*, 1981;Vazquez *et al.*, 1988). As our experiments were conducted at slightly alkaline pH (pH 7.5 -7.6) the tendency to form membrane-aggressive bilirubin acid may have been modest. After the bilirubin washout and addition of the original serum-containing medium (paper IV) bilirubin may have been removed from the cell membrane by its binding to serum constituents consistent with previous studies (Brito & Brites, 2003;Cowger, 1971;Vazquez *et al.*, 1988).

In cultured hepatoblastoma cells bilirubin crosses the cell membrane by a mechanism involving spontaneous diffusion (Zucker & Goessling, 2000). While such a phenomenon has not been firmly established in neurons, one *in* vi*vo* study strongly suggested that bilirubin crosses plasma membranes (Hansen *et al.*, 2001). An additional mechanism of possible importance is that bilirubin may be metabolized in the mitochondria by a process of oxidation (Hansen & Allen, 1996a;Hansen & Tommarello, 1998). However, the possible clinical impact of such a mechanism in the brain is not known. Furthermore, it has not been shown in cultured cells.

*In vitro* studies have shown reversibility of certain bilirubin-mediated effects like phosphorylation of synapsin in synaptosomes from rat brain (Hansen *et al.*, 1988), and glutamate uptake in cultured astrocytes (Silva *et al.*, 1999). Amit and co-workers reported that incubation with 25–100 μM bilirubin (bilirubin:HSA molar ratio 1.5) for 1-2 h and rescue with serum free medium in a human neuroblastoma cell line caused delayed and near complete cell death after 24 h, leading to the conclusion that bilirubin toxicity was a "progressive and irreversible process" (Amit *et al.*, 1989b). The main reason for the different conclusions from our study is likely to be the high bilirubin concentration used. Results obtained with 25 μM bilirubin were similar in both studies. In a different model the addition of albumin to a 1.1:1 molar ratio (BSA:bilirubin) in L-929 cells exposed to 25 μM bilirubin for 1-30 min resulted in the extraction of a significant proportion of the bilirubin from the cells, but it did not prevent subsequent loss of cell viability (Cowger, 1971). A combination of different factors may have resulted in reversal of bilirubin toxicity in our model. These factors may include 1) the use of a low bilirubin concentration (1 μM), 2) the conduct of the

experiments at slightly alkaline pH, 3) the use of the original serum-containing medium after the 6 h bilirubin-exposure, which may have resulted in *i*) an extraction of bilirubin from the neuronal surface due to binding of bilirubin to serum constituents, *ii*) optimal conditions for cellular metabolic recovery post- exposure, and 4) long recovery time after bilirubin exposure (96 h).

To conclude, short term exposure to bilirubin in human NT2-N neurons may be followed by recovery of function or delayed neuronal death. These effects are dependent on the bilirubin concentrations and the duration of exposure. Our findings are compatible with clinical studies demonstrating that permanent neurological sequel may be related to the magnitude and duration of bilirubin exposure, and that the effects of bilirubin on the brain may be reversible. Our model may be suitable to study reversible effects of bilirubin toxicity *in vitro.*

**8.3 Bilirubin induces apoptosis and necrosis in human NT2-N neurons**

Several studies have found that bilirubin induces concentration- and time-dependent injury in cultured cells (Akin *et al.*, 2002;Amit *et al.*, 1989a;Amit *et al.*, 1989b;Cowger, 1971;Grojean *et al.*, 2000;Silberberg *et al.*, 1970;Silva *et al.*, 2001b). Many of these studies were either conducted on non-neuronal cells or predate the recognition of apoptosis. Our studies showed that bilirubin induced a concentration-dependent injury as measured with MTT assays (paper II and IV) and nuclear morphology (paper IV). Bilirubin concentrations ≤ 25 μM caused apoptosis, and in neurons exposed to ≤ 10 μM bilirubin, apoptosis was the major form of cell death. Very high bilirubin concentration (100 μM) induced a rapid decline in MTT reduction and complete necrotic cell death within 24 h, however, even low and moderate insults caused necrosis within 24 h in a subpopulation of neurons (paper II).

Our studies are the first to investigate the relation between bilirubin-induced apoptosis and necrosis in a human neuronal cell line. Species differences have been shown to play a role in the response to cell death inducing stimuli (Nitsch *et al.*, 2000), and studies on stillborn infants have suggested that the fraction of apoptotic cells may be higher in human tissues than suggested from animal experiments (Edwards *et al.*, 1997). Silva et al found that a short bilirubin exposure (4 h) to 17 μM bilirubin (bilirubin:HSA molar ratio 3:1) resulted in similar degrees of apoptosis and necrosis in cultured astrocytes from fetal rats. Necrosis predominated over apoptosis at higher bilirubin concentrations (86 μM) and longer exposure times (22 h). In neurons, evaluated at 4 h only, the proportions of necrosis and apoptosis were

similar (Silva *et al.*, 2001b). Another study by the same group showed that a 4 h exposure to 86 μM bilirubin (bilirubin/HSA = 3:1) induced apoptosis in 25 % of cultured neurons from fetal rat brain cortex. The proportion of apoptotic cells decreased with lower bilirubin/HSA ratios, indicating that the prevalence of apoptosis increased with higher concentrations of unbound bilirubin (Rodrigues *et al.*, 2002a). After exposure to low bilirubin concentrations (0.5 μM without albumin vehicle) for 96 h, apoptosis was slightly more prevalent than necrosis (16 and 12 % respectively) in primary cultured neurons from embryonic rat forebrain (Grojean *et al.*, 2000). Seubert *et al* investigated bilirubin-induced cell death (1-100 μM bilirubin and a fixed concentration of 40 μM BSA in 10 % serum containing medium) in three different murine hepatoblastoma cell lines after a 24 h exposure. They found that both apoptotic and necrotic cell death increased in a concentration-dependent fashion, with a predominance for necrotic cell death at higher bilirubin concentrations (Seubert *et al.*, 2002).

Our findings are in agreement with previous studies on non-human cells showing that apoptosis and necrosis may co-exist after bilirubin-mediated injury. However, our results may suggest that cells of human origin differ somewhat from primary cell cultures from rodents. In human NT2-N neurons apoptosis was much more prevalent than necrosis after exposure to low bilirubin concentrations. Furthermore, apoptosis was a late event and occurred more than 6 h after the onset of bilirubin exposure. Our findings are well in accordance with the classical paradigm that toxic stimuli may induce necrosis or apoptosis depending on the intensity of the insult. Typically, necrotic cell death is an early event in a subpopulation of cells while apoptosis is a slower process in a different subset of neurons (Bonfoco *et al.*, 1995). This has been confirmed in studies caused by different insults such as glutamate (Ankarcrona *et al.*, 1995), acidosis (Ding *et al.*, 2000) or methyl mercury (Castoldi *et al.*, 2000) *in vitro*, and hypoxia-ischemia (Beilharz *et al.*, 1995;Martin *et al.*, 1998) in animal models *in vivo*. Furthermore, the temporal evolution of apoptosis and necrosis in our studies was consistent with the ones found after HI injury (Northington *et al.*, 2001b) and excitotoxic injury (PorteraCailliau *et al.*, 1997) in neonatal rodents *in vivo*.

The execution of apoptosis is an energy-requiring process. In neurons treated with ≤10 μM bilirubin, i.e. bilirubin concentrations inducing cell death predominantly as apoptosis, metabolic activity was maintained as measured with MTT reduction throughout the observation periode (paper II). This finding was compatible with preserved energy levels in cells undergoing apoptosis. It has been claimed that MTT reduction at least in part reflects mitochondrial activity and the production of ATP (Kaneko *et al.*, 1995), however, a good

correlation between MTT assays and the energy status of the cell has not been firmly established (Harada & Sugimoto, 1998;Kaneko *et al.*, 1995).

Apoptotic nuclei could be divided into two distinct groups by their morphological appearance. Nuclear condensation was an early event and the proportion of condensed nuclei did not increase after 24 h, while nuclear fragmentation first occurred at 24 h and continued to increase for at least up to 48 h (paper II). Nuclear condensation was (at least in part) the result of excitotoxic cell death, as it could be reduced by the NMDA receptor blocker MK-801 (paper III), and seemed to be independent of caspases. Nuclear fragmentation was mediated by caspases, and was nearly completely prevented by high dose caspase inhibitors (paper III). Our findings suggest that bilirubin may induce both caspase-dependent and caspase–independent apoptosis in NT2-N neurons, and that these two pathways may co-exist in cultured neurons, as will be discussed in the following chapters.

To conclude, our results are in line with the literature showing that apoptosis and necrosis may co-exist after bilirubin-induced injury both *in vivo*, and in neuronal cultures *in vitro*. Our results suggest that cell death induced by low and moderate bilirubin-concentrations is predominantly apoptotic in NT2-N neurons. Both caspase independent- and caspase dependent apoptotic pathways appear to be involved.

### 8.4 Caspases and caspase inhibition in bilirubin-induced cell death

The execution of caspase-mediated apoptosis follows a cascade of events resulting in the activation of the executioner caspases -3, -6, or -7, where caspase-3 has been identified as a major executioner caspase (Nicholson *et al.*, 1995;Racke *et al.*, 2002). Our results (paper II, III and IV) provide evidence for a role of caspase-activation in bilirubin-induced death in NT2-N neurons. Indirect measures of caspase-3 activation like PARP cleavage and oligonucleosomal DNA fragmentation occurred after exposure to 5 μM bilirubin, but not after exposure to higher bilirubin concentrations (paper II). Direct measurement of activated caspase-3 showed a moderate increase after exposure to 5 μM bilirubin at 6 h (230 % compared to base-line values), but had returned to base-line values at 24 h (paper III), while 25 μM bilirubin did not activate caspase-3 (results not shown in paper III). In two different studies on primary cell cultures from rodents bilirubin induced a concentration-dependent caspase-3 activation where caspase-3 activation was maximal after exposure to high bilirubin concentrations (86 μM and 50 μM bilirubin respectively) (Rodrigues *et al.*, 2002a;Seubert *et al.*, 2002). Hence, in our studies caspase-3 activation appeared to be less prominent at higher

bilirubin concentrations than previously reported from studies on primary cell cultures from rodents. This finding was consistent with the concentration-dependent effects of bilirubin on apoptotic morphologies as discussed in the previous chapter.

Substrate preferences of the different caspases have been exploited for the development of caspase inhibitors. Caspase inhibitors are peptides that compete for caspase binding with the substrates of activated caspases (Degterev *et al.*, 2003). Despite effects on nuclear morphology, treatment with caspase inhibitors enhanced neuronal survival only to a very limited extent, and this effect was not reflected in the MTT assays (paper III and IV). Experiments with long term bilirubin exposure combined with serum deprivation (96 h) showed effects of both caspase inhibitors, but our results suggest that long term serum deprivation affected these results (paper IV). Only one previous study has investigated the effects of caspase inhibition in bilirubin-induced injury. Grojean *et al* found that both caspase-1 and caspase-3 inhibitors completely abolished both apoptosis and necrosis after treatment with 0.5 μM bilirubin for 96 h in cultured cortical rodents from fetal rats (Grojean *et al.*, 2000). This study was undertaken under serum free conditions, however, serum was replaced by growth factors and hormones to eliminate the impact of serum deprivation. Both species differences and the low bilirubin concentrations used in their study may explain the differences between their results and ours. Our data do not exclude the possibility that caspase inhibitors may prevent cell death caused by very low bilirubin concentrations.

Evidence from *in vitro* studies suggests that caspase inhibitors may induce only transient protection in most, if not all cell lines (for reviews see Kroemer & Martin, 2005;Stefanis, 2005). Several investigations have shown that nuclear fragmentation is prevented when caspases are inhibited, however, the cells die from caspase-independent apoptosis (Cregan *et al.*, 2004;Daugas *et al.*, 2000). Alternative caspase-independent pathways are activated when caspases are inhibited or a parallel pathway becomes dominant in the presence of caspase inhibitors (for review see Stefanis, 2005). AIF may play a role in the execution of this cell death as caspase inhibitors may block caspase activity, but not AIF translocation from the mithochondrion to the nucleus (Cregan *et al.*, 2002). Caspase inhibition may also cause a switch from apoptosis to necrosis (Green & Kroemer, 1998;Hirsch *et al.*, 1997), however, caspase inhibition had no effect on the proportion of necrotic cells in our assays (paper III).

It is generally held that the intrinsic pathway is the principal caspase-dependent apoptotic pathway in neurons (Stefanis, 2005). Our data could not determine whether bilirubin causes caspase dependent apoptosis by the intrinsic or extrinsic apoptotic pathway.

The possible contribution of the extrinsic apoptotic pathway is interesting in light of data suggesting that bilirubin may exert its principal effect on the cellular plasma membrane. However, several authors report that bilirubin induces caspase-mediated apoptosis through the intrinsic apoptotic pathway (Rodrigues *et al.*, 2000;Rodrigues *et al.*, 2002a;Seubert *et al.*, 2002). In colon cancer cells cells bilirubin induces apoptosis by activating caspase-3, caspase-9 and cytochrome c without activating caspase-8, strongly suggesting that apoptosis was mediated by the intrinsic pathway (Keshavan *et al.*, 2004).

To summarize, our results demonstrate that caspases are activated and play a role in the execution of bilirubin-mediated cell death in a neuronal cell line of human origin. Caspase-3 is activated after exposure to low bilirubin concentrations, but treatment with caspase inhibitors had only a very modest apoptosis reducing effect (paper III). Caspase inhibition did, however, have an impact on apoptotic nuclear morphologies and DNA-degeneration patterns.

**8.5 Excitotoxicity in bilirubin-induced cell death**

Treatment with MK-801 significantly enhanced neuronal viability as measured by the proportion of undamaged nuclei in a model of continuous exposure to bilirubin and serum deprivation after 48 (paper III) and 102 h (paper IV). In a different model, a 6 h exposure to 5 μM bilirubin followed by removal of bilirubin, MK-801 significantly increased the proportion of undamaged nuclei 96 h thereafter (paper IV). In the MTT assays significant protective effects were, however, only found after continuous bilirubin exposure for 102 h (paper IV). Our results indicate that treatment with MK-801 may reduce cell death, but the lack of effect on neuronal function as suggested by the MTT assays raises doubt as to whether MK-801 restores all aspects of neuronal function. An alternative understanding is that neurons may recover (or die) after the observation period. Experiments on serum-deprived neurons have showed that functional recovery after an insult may take up to two weeks (Deshmukh *et al.*, 1996).

Our findings confirm that bilirubin exerts its toxicity by excitotoxic mechanisms as previously found both *in vivo* (Hoffman *et al.*, 1996;McDonald *et al.*, 1998) and *in vitro* (Grojean et al., 2000). MK-801 reduced cell death more potently than caspase inhibitors, and this effect was not biased by the impact of serum deprivation (paper IV). Based on the complete protection by treatments with caspase-1 inhibitors, or caspase-3 inhibitors, or MK-801 on necrotic and apoptotic cell death Grojean et al concluded that excitotoxicity was

mediated by caspase-dependent pathways. Our findings strongly suggest that NMDA receptor-mediated apoptosis was not mediated by caspases. First, the most prominent effect of treatment with MK-801 was the reduction of apoptotic condensed nuclei without affecting the proportion of fragmented nuclei or oligonucleosomal DNA-laddering. Second, treatment with MK-801 concomitantly with bilirubin exposure had no influence on caspase-3 activation, and, third, combined treatment with MK-801 and a pancaspase inhibitor synergistically reduced apoptosis, suggesting that these agents exerted their effects on separate apoptotic pathways (paper III).

The current understanding is that in most cases excitotoxicity is not mediated by caspases (Cheung *et al.*, 2005;Lankiewicz *et al.*, 2000;Wang H *et al.*, 2004). If caspases are not responsible for mediating excitotoxicity, what is? Increased calcium concentrations may activate a group of proteases called calpains which seem to act upstream of the mitochondria. Calpains cleave multiple substrates involved in cell death (Stefanis, 2005), and may execute cell death at least in part by an AIF-mediated mechanism (Polster *et al.*, 2005).
Also, studies on NMDA receptor mediated cell death both *in vivo* and *in vitro,* have shown that AIF may translocate from the mitochondria to the nucleus (Wang H *et al.*, 2004). In several models of cell death AIF has been shown to induce apoptosis as nuclear condensation (Cheung *et al.*, 2005;Cregan *et al.*, 2002;Wang H *et al.*, 2004). As this apoptotic phenotype was reduced by the NMDA receptor antagonist MK-801 in our experiments, our data are compatible with a role for AIF in bilirubin-induced apoptosis. However, a contribution of AIF in bilirubin-mediated apoptosis has never been addressed. The possible involvement of cell death effectors such as AIF or Endonuclease G in bilirubin toxicity needs further clarification.

Several *in vivo* investigation in adult rodents have shown synergistic effects of treatment with caspase inhibitors and NMDA receptor antagonists in models of hypoxic ischemic encephalopathy (HIE) (Ma *et al.*, 1998;Schulz *et al.*, 1998) and trauma (Liang *et al.*, 2003). In these investigations synergistic treatment effects were attributed to the inhibition of separate cell death pathways by caspase inhibitors and NMDA receptor antagonists. This may be different in immature rats where NMDA receptor-mediated toxicity may induce caspase-dependent apoptosis. Injections of NMDA into the striatum of 7 day-old rat pups induced neuronal loss accompanied by DNA laddering and caspase-3 activation (Nath *et al.*, 2000). Furthermore, in a neonatal rat model of HIE caspase-3 activation was reduced by treatment with MK-801 (Puka-Sundvall *et al.*, 2000).

To conclude, we found that excitotoxic mechanisms are involved in bilirubin-mediated injury in NT2-N neurons in line with prior *in vivo* and *in vitro* investigations. Excitotoxic cell death pathways were, at least in part, distinct from caspase-mediated pathways (paper III), and concurrent inhibition of both pathways provided synergistic protection. Furthermore, treatment with MK-801 was more promising than caspase inhibition in reducing delayed cell death after a short term exposure to bilirubin.

**8.6 Can treatment with anti-apoptotic agents prevent bilirubin-mediated injury?**

The immature brain may be more susceptible to both excitotoxicity and cell death from apoptosis than the adult brain. In the immature brain key elements of apoptosis are constitutively up-regulated (Blomgren *et al.*, 2001;Johnston MV *et al.*, 2002). This up-regulation seems to involve both caspase-3 mediated pathways (Blomgren *et al.*, 2001;Zhu *et al.*, 2000), caspase-8 activation compatible with a role for the extrinsic apoptotic pathway (Northington *et al.*, 2001a), and AIF (Zhu *et al.*, 2005;Zhu *et al.*, 2007). Also, excitotoxicity seems to play a more prominent role in the neonate than in the adult (Blomgren *et al.*, 2001;Johnston *et al.*, 2002;McDonald *et al.*, 1988).

Extrapolating from human NT2-N neurons to jaundiced newborns is tenuous. To date, evidence for bilirubin-induced apoptosis *in vivo* is confined to light microscopy in the Gunn rat animal model of neonatal jaundice and kernicterus (McDonald *et al.*, 1998). However, the idea that anti-apoptotic therapies may keep neurons alive for a time sufficient to reduce bilirubin-mediated stress and to establish a tissue milieu compatible with long term survival and function is worth pursuing. Most of our knowledge on the effects of anti-apoptotic treatments in the newborn is derived from neonatal models of HIE in rodents. Biochemical and neuro-pathological data suggest efficacy of caspase inhibition in reducing the neuronal cell death in different neonatal models of HIE in rodents for up to 22 days (for review see Northington FJ *et al.*, 2005). However, to date no studies on the effects of caspase-inhibition in neonatal animal models of HIE have examined the neurological outcome. Also, there are contradictory reports with respect to whether or not the inhibition of caspases actually improves long term functional outcome in adult models of HIE (Gillardon *et al.*, 1999;Xu *et al.*, 1999).

Treatments with the present generation of caspase inhibitors or NMDA receptor antagonists in the jaundiced newborn may not be without problems. Caspase inhibitors and MK-801 exhibit low penetration into the brain after iv administration and are mostly

administered by intra-cerebroventricular injections in research animals (Loetscher *et al.*, 2001). Both caspases and NMDA receptors have important physiological properties, and to interrupt these functions may in itself be harmful (Ikonomidou & Turski, 2002;Kroemer & Martin, 2005). Studies with agents combating targets such as MAPK pathways, AIF, PARP, and BCL-2 family proteins are upcoming (for reviews see Mehta *et al.*, 2006;Kroemer & Martin, 2005).

Two recent studies with minocycline, a second generation tetracycline with good penetration into the brain, deserve special attention. Minocycline prevented bilirubin-induced cerebellar hypoplasia in the Gunn rat, and it reduced cell death in cerebellar granule neurons (Lin *et al.*, 2005). Furthermore, treatment with minocyclin 15 min prior to recordings improved brainstem auditory evoked potentials in jaundiced Gunn rats exposed to sulfisoxazole (Geiger *et al.*, 2006). Inhibition of MAPK phosphorylation by bilirubin was associated with the neuroprotective effects of minocyclin (Lin *et al.*, 2003). In addition it has been shown that minocyclin mediates neuroprotection by blocking the release of apoptogenic factors such as cytochrome c and AIF (Lin *et al.*, 2003).

## 9. CONCLUDING REMARKS

The recognition of apoptosis has resulted in an explosive increase in research on cell death mechanisms during the last decade. In this period surprisingly little research has been undertaken on the mechanisms of bilirubin-induced cell death compared to for example HIE. This may be due to the assumption that bilirubin-induced injury has become rare in the industrialized world. However, the apparent resurgence of kernicterus (Ebbesen, 2000;Johnson *et al.*, 2002), the recent indications of permanent but subtle sequel even in patients with moderate hyperbilirubinemia (Grimmer *et al.*, 1999;Soorani-Lunsing *et al.*, 2001), and concern about whether apparent reversible bilirubin-mediated effects are indeed harmless (Maisels *et al.*, 2004), should urge increased efforts to understand the mechanism of bilirubin-induced injury. Furthermore, while treatments to reduce elevated bilirubin levels, such as phototherapy, have proven effective in reducing elevated TSB levels, evidence is lacking that such therapies actually reduce subsequent bilirubin-mediated brain injury (Maisels *et al.*, 2004).

In this thesis we have shown that drugs known to inhibit P-gp may increase bilirubin entry into the brain to an extent that may have clinical consequences. Transport of bilirubin by membrane transport proteins may be an important mechanism of bilirubin-mediated injury, and well designed studies to elucidate these mechanisms are warranted. Furthermore, it is of vital importance to substantiate that apoptotic cell death *in vitro* is a phenomenon that also occurs *in vivo*. It is reasonable to believe that new insights into the mechanisms of brain injury obtained from basic science investigations will provide new treatments for neurological disorders in the future. For an otherwise healthy infant admitted to hospital with extremely high TSB levels, a time window for treatment with neuroprotective agents may exist prior to the onset of exchange transfusions. However, before new therapies may be put into clinical practice it must be determined whether treatments promote more benefit than harm.

## 10. CONCLUSIONS

1.Administration of drugs known to inhibit P-gp increases the entry of bilirubin into the brain at a magnitude of possible clinical significance in young adult rats. Effects on the distribution pattern of bilirubin in the brain were modest and not in accordance with a kernicteric staining pattern.

2.Short-term exposure to bilirubin may be followed by recovery of function or delayed neuronal death in human NT2-N neurons, depending on the magnitude of the insult and the duration of exposure.

3.Apoptosis and necrosis may co-exist after bilirubin-induced injury in NT2-N neurons. Apoptosis is the predominant cell death mode after exposure to low and moderate bilirubin concentrations.

4.Caspases are activated in bilirubin-induced apoptosis in NT2-N neurons. Treatment with caspase inhibitors had a modest or no impact on cell death, however, treatment with caspase inhibitors had impact on nuclear morphology and DNA-degradation patterns.

5.Low and moderate bilirubin concentrations induced excitotoxic cell death in NT2-N neurons primarily as caspase-independent apoptosis. Inhibition of excitotoxicity with the NMDA receptor antagonist MK-801 was more promising than caspase inhibitors in preventing cell death.

6.Bilirubin-mediated apoptosis in NT2-N neurons is executed by excitotoxic and caspase-mediated pathways which are, at least in part, distinct. Concurrent inhibition of both pathways resulted in synergistic protection.

7.Serum deprivation concomitantly with bilirubin exposure may influence the cell death pathways involved. This should be taken into consideration when studying bilirubin-mediated injury *in vitro*.

## ERRATA

In paper II, page 179 in ”Abbreviations”; “MTT, 3-4[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide”, should read “ MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide”.

In paper II, page 182 in Figure 5, in the histogram “Undamaged nuclei” and “Control” the asterix † is erroneously placed above the column at 6 h, but should in stead be placed over the column at 24 h.

In paper II, page 184 in the first column; “ In a study on cortical neurons from newborn rats by Grojean et al (5), 0.4 μM UCB (without added albumin) induced predominantly apoptotic cell death (16 % apoptotic versus 8 % necrotic cells at 96 h) “, should read “…..Grojean et al (5), 0.5 μM UCB (without added albumin) induced predominantly apoptotic cell death (16 % apoptotic versus 12 % necrotic cells at 96 h)”.

In paper III in “Abbreviations” on page…, and under “Materials” on the same page “MTT, 3-4[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide”, should read “ MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide.

In paper III errors in the data handling were discovered after publication. The Editors of *Pediatric Research* are informed, and an erratum has been published (Pediatric Research 2007 62(3):318). In this thesis the erratum and the corrected figures appear in “Publications” after paper III.

# REFERENCES


Ahdab-Barmada M. (2000). The Neuropathology of Kernicterus: Definitions and Debate. pp. 75-88.

Ahlfors, C. E. (2000). Measurement of plasma unbound unconjugated bilirubin. *Analytical Biochemistry* 279, 130-135.

Ahmed H, Yukubu AM, & Hendrickse RG (1995). Neonatal jaundice in Zaria, Nigeria - a second prospective study. *West Afr J Med* 14(1), 15-23.

Akin, E., Clower, B., Tibbs, R., Tang, J., & Zhang, J. (2002). Bilirubin produces apoptosis in cultured bovine brain endothelial cells. *Brain Research* 931, 168-175.

Allen, R. T., Hunter, W. J., & Agrawal, D. K. (1997). Morphological and biochemical characterization and analysis of apoptosis. *Journal of Pharmacological and Toxicological Methods* 37, 215-228.

Almaas, R., Pytte, M., Lindstad, J. K., Wright, M., Saugstad, O. D., Pleasure, D., & Rootwelt, T. (2003). Acidosis has opposite effects on neuronal survival during hypoxia and reoxygenation. *Journal of Neurochemistry* 84, 1018-1027.

Almaas, R., Saugstad, O. D., Pleasure, D., & Rootwelt, T. (2000). Effect of barbiturates on hydroxyl radicals, lipid peroxidation, and hypoxic cell death in human NT2-N neurons. *Anesthesiology* 92, 764-774.

Almaas, R., Saugstad, O. D., Pleasure, D., & Rootwelt, T. (2002). Neuronal formation of free radicals plays a minor role in hypoxic cell death in human NT2-N neurons. *Pediatric Research* 51, 136-143.

Altman, J. (1969). Autoradiographic and Histological Studies of Postnatal Neurogenesis .3. Dating Time of Production and Onset of Differentiation of Cerebellar Microneurons in Rats. *Journal of Comparative Neurology* 136, 269-&.

Amit, Y., Chan, G., Fedunec, S., Poznansky, M. J., & Schiff, D. (1989a). Bilirubin Toxicity in A Neuro-Blastoma Cell-Line N-115 .1. Effects on Na+K+ Atpase, [H-3]-Thymidine Uptake, L-[S-35]-Methionine Incorporation, and Mitochondrial-Function. *Pediatric Research* 25, 364-368.

Amit, Y., Fedunec, S., Thomas, P. D., Poznansky, M. J., & Schiff, D. (1990). Bilirubin-Neural Cell-Interaction - Characterization of Initial Cell-Surface Binding Leading to Toxicity in the Neuroblastoma Cell-Line N-115. *Biochimica et Biophysica Acta* 1055, 36-42.

Amit, Y., Poznansky, M. J., & Schiff, D. (1989b). Bilirubin Toxicity in A Neuro-Blastoma Cell-Line N-115 .2. Delayed-Effects and Recovery. *Pediatric Research* 25, 369-372.

Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., & Nicotera, P. (1995). Glutamate-Induced Neuronal Death - A Succession of Necrosis Or Apoptosis Depending on Mitochondrial-Function. *Neuron* 15, 961-973.

Atkinson, L. R., Escobar, G. J., Takayama, J. I., & Newman, T. B. (2003). Phototherapy use in jaundiced newborns in a large managed care organization: Do clinicians adhere to the guideline? *Pediatrics* 111, E555-E561.

Baranano, D. E., Rao, M., Ferris, C. D., & Snyder, S. H. (2002). Biliverdin reductase: A major physiologic cytoprotectant. *Proceedings of the National Academy of Sciences of the United States of America* 99, 16093-16098.

Bart J., Groen H.J.M., Hendrickse N.H., van der Graf W.T.A., Vaalburg W., & de Vries E.G.E. (2000). The blood-brain barrier and oncology:new insights into function and modulation. *Cancer Treat Rev* 26(6), 449-462.

Batistatou, A., Merry, D. E., Korsmeyer, S. J., & Greene, L. A. (1993). Bcl-2 Affects Survival But Not Neuronal Differentiation of Pc12 Cells. *Journal of Neuroscience* 13, 4422-4428.

Beaulieu, E., Demeule, M., Ghitescu, L., & Beliveau, R. (1997). P-glycoprotein is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain. *Biochemical Journal* 326, 539-544.

Beilharz, E. J., Williams, C. E., Dragunow, M., Sirimanne, E. S., & Gluckman, P. D. (1995). Mechanisms of Delayed Cell-Death Following Hypoxic-Ischemic Injury in the Immature Rat - Evidence for Apoptosis During Selective Neuronal Loss. *Molecular Brain Research* 29, 1-14.

Bellamy, W. T., Dalton, W. S., Kailey, J. M., Gleason, M. C., Mccloskey, T. M., Dorr, R. T., & Alberts, D. S. (1988). Verapamil Reversal of Doxorubicin Resistance in Multidrug-Resistant Human Myeloma Cells and Association with Drug Accumulation and Dna Damage. *Cancer Research* 48, 6365-6370.

Beneke R. (1907). Ueber den Kernikterus der neugeborenen. Muenchener Medizinische Wochenschrift 54, 2023-2027.

Bengtsson & Vernehol (1974). Follow-Up Study of Hyperbilirubinemia in Healthy, Full-Term Infants Without Isoimmunization. *Acta Paediatrica Scandinavica* 63, 70-80.

Bernas, T. & Dobrucki, J. W. (2000). The role of plasma membrane in bioreduction of two tetrazolium salts, MTT, and CTC. *Archives of Biochemistry and Biophysics* 380, 108-116.

Bhutani, V. K. (2001). Neonatal hyperbilirubinemia and the potential risk of subtle neurological dysfunction. *Pediatric Research* 50, 679-680.

Bhutani, V. K., Johnson, L. H., Maisles M.J, Newmann T.B., Phibbs C., Stark A.R., & Yeargin-Allsopp M. (2004). Kernicterus: Epidemiological strategies for its prevention through system-based approaches. *J Perinat* 24, 650-662.

Blomgren, K., Zhu, C. L., Wang, X. Y., Karlsson, J. O., Leverin, A. L., Bahr, B. A., Mallard, C., & Hagberg, H. (2001). Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia - A mechanism of "pathological apoptosis"? *Journal of Biological Chemistry* 276, 10191-10198.

Boiadjiev, S. E., Watters, K., Wolf, S., Lai, B. N., Welch, W. H., McDonagh, A. F., & Lightner, D. A. (2004). pK(a) and aggregation of bilirubin: Titrimetric and ultracentrifugation studies on water-soluble pegylated conjugates of bilirubin and fatty acids. *Biochemistry* 43, 15617-15632.

Bonfoco, E., Krainc, D., Ankarcrona, M., Nicotera, P., & Lipton, S. A. (1995). Apoptosis and Necrosis - 2 Distinct Events Induced, Respectively, by Mild and Intense Insults with N-Methyl-D-Aspartate Or Nitric-Oxide Superoxide in Cortical Cell-Cultures. *Proceedings of the National Academy of Sciences of the United States of America* 92, 7162-7166.

Borlongan, C. V., Tajima, Y., Trojanowski, J. Q., Lee, V. M. Y., & Sanberg, P. R. (1998). Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2N cells) promotes functional recovery in ischemic rats. *Experimental Neurology* 149, 310-321.

Bratlid, D. (1990). How Bilirubin Gets Into the Brain. *Clinics in Perinatology* 17, 449-465.

Bratlid, D., Cashore, W. J., & Oh, W. (1984). Effect of Acidosis on Bilirubin Deposition in Rat-Brain. *Pediatrics* 73, 431-434.

Brito, M. A. & Brites, D. (2003). Effect of acidosis on bilirubin-induced toxicity to human erythrocytes. *Molecular and Cellular Biochemistry* 247, 155-162.

Brodersen & Bartels, P. (1969). Enzymatic Oxidation of Bilirubin. *European Journal of Biochemistry* 10, 468-&.

Brodersen, R. (1979). Bilirubin - Solubility and Interaction with Albumin and Phospholipid. *Journal of Biological Chemistry* 254, 2364-2369.

Brown AK & Johnson L (1996). Loss of concern about jaundice and the re-emergence of kernicterus in full-term infants in the era of managed care. In The year book of neonatal and perinatal medicine. Mosby Year Book, eds. Fanaroff A & Klaus M, pp. 17-28. Mosby, St. Louis.

Cande, C., Cohen, I., Daugas, E., Ravagnan, L., Larochette, N., Zamzami, N., & Kroemer, G. (2002). Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. *Biochimie* 84, 215-222.

Cardoso, S. A., Rego, A. C., Penacho, N., & Oliveira, C. R. (2004a). Apoptotic cell death induced by hydrogen peroxide in NT2 parental and mitochondrial DNA depleted cells. *Neurochemistry International* 45, 693-698.

Cardoso, S. M., Santana, I., Swerdlow, R. H., & Oliveira, C. R. (2004b). Mitochondrial dysfunction of Alzheimer's disease hybrids enhances A beta toxicity. *Journal of Neurochemistry* 89, 1417-1426.

Cashore, W. J. (1980). Free Bilirubin Concentrations and Bilirubin-Binding Affinity in Term and Preterm Infants. *Journal of Pediatrics* 96, 521-527.

Castoldi, A. F., Barni, S., Turin, I., Gandini, C., & Manzo, L. (2000). Early acute necrosis, delayed apoptosis and cytoskeletal breakdown in cultured cerebellar granule neurons exposed to methylmercury. *Journal of Neuroscience Research* 59, 775-787.

Chang, H. Y. & Yang, X. L. (2000). Proteases for cell suicide: Functions and regulation of caspases. *Microbiology and Molecular Biology Reviews* 64, 821- 829.

Chen, W., Tang, Z. H., Fortina, P., Patel, P., Addya, S., Surrey, S., Acheampong, E. A., Mukhtar, M., & Pomerantz, R. J. (2005). Ethanol potentiates HIV-1 gp120-induced apoptosis in human neurons via both the death receptor and NMDA receptor pathways. *Virology* 334, 59-73.

Cheung, E. C. C., Melanson-Drapeau, L., Cregan, S. P., Vanderluit, J. L., Ferguson, K. L., McIntosh, W. C., Park, D. S., Bennett, S. A. L., & Slack, R. S. (2005). Apoptosis-inducing factor is a key factor in neuronal cell death propagated by BAX-dependent and BAX-independent mechanisms. *Journal of Neuroscience* 25, 1324-1334.

Choi, D. W. (1992). Excitotoxic Cell-Death. *Journal of Neurobiology* 23, 1261-1276.

Conlee, J. W. & Shapiro, S. M. (1991). Morphological-Changes in the Cochlear Nucleus and Nucleus of the Trapezoid Body in Gunn Rat Pups. *Hearing Research* 57, 23-30.

Courtois, A., Payen, L., Vernhet, L., de Vries, E. G. E., Guillouzo, A., & Fardel, O. (1999). Inhibition of multidrug resistance-associated protein (MRP) activity by rifampicin in human multidrug-resistant lung tumor cells. *Cancer Letters* 139, 97-104.

Cowger, M. L. (1971). Mechanism of Bilirubin Toxicity on Tissue Culture Cells - Factors That Affect Toxicity, Reversibility by Albumin, and Comparison with Other Respiratory Poisons and Surfactants. *Biochemical Medicine* 5, 1- 16.

Cregan, S. P., Dawson, V. L., & Slack, R. S. (2004). Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene* 23, 2785-2796.

Cregan, S. P., Fortin, A., MacLaurin, J. G., Callaghan, S. M., Cecconi, F., Yu, S. W., Dawson, T. M., Dawson, V. L., Park, D. S., Kroemer, G., & Slack, R. S. (2002). Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *Journal of Cell Biology* 158, 507-517.

Cremer RJ, Perryman PW, & Richard DH. (1958). Influence of light on the hyperbilirubinemia of infants. Lancet 1, 1094-1117.

Cui, Y., Konig, J., Leier, I., Buchholz, U., & Keppler, D. (2001). Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6. *Journal of Biological Chemistry* 276, 9626-9630.

Cullen, K. V., Davey, R. A., & Davey, M. W. (2001). Verapamil-stimulated glutathione transport by the multidrug resistance-associated protein (MRP1) in leukaemia cells. *Biochemical Pharmacology* 62, 417-424.

Daugas, E., Susin, S. A., Zamzami, N., Ferri, K. F., Irinopoulou, T., Larochette, N., Prevost, M. C., Leber, B., Andrews, D., Penninger, J., & Kroemer, G. (2000). Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *Faseb Journal* 14, 729-739.

Day, R. & Haines, M. S. (1954). Intelligence Quotients of Children Recovered from Erythroblastosis Fetalis Since the Introduction of Exchange Transfusion. *Pediatrics* 13, 333-338.

Day, R. L. (1954). Inhibition of Brain Respiration Invitro by Bilirubin - Reversal of Inhibition by Various Means. *Proceedings of the Society for Experimental Biology and Medicine* 85, 261-264.

Degterev, A., Boyce, M., & Yuan, J. Y. (2003). A decade of caspases. *Oncogene* 22, 8543-8567.

Deshmukh, M. & Johnson, E. M. (1997). Programmed cell death in neurons: Focus on the pathway of nerve growth factor deprivation-induced death of sympathetic neurons. *Molecular Pharmacology* 51, 897-906.

Deshmukh, M., Kuida, K., & Johnson, E. M. (2000). Caspase inhibition extends the commitment to neuronal death beyond cytochrome c release to the point of mitochondrial depolarization. *Journal of Cell Biology* 150, 131-143.

Deshmukh, M., Vasilakos, J., Deckwerth, T. L., Lampe, P. A., & Johnson, E. M. (1996). Genetic and metabolic status of NGF-deprived sympathetic neurons saved by an inhibitor of ICE family proteases. *Journal of Cell Biology* 135, 1341-1354.

Devries, L. S., Lary, S., & Dubowitz, L. M. S. (1985). Relationship of Serum Bilirubin Levels to Ototoxicity and Deafness in High-Risk Low-Birth-Weight Infants. *Pediatrics* 76, 351-354.

Diamond, I. & Schmid, R. (1967). Oxidative Phosphorylation in Experimental Bilirubin Encephalopathy. *Science* 155, 1288-&.

Ding, D., Moskowitz, S. I., Li, R., Lee, S. B., Esteban, M., Tomaselli, K., Chan, J., & Bergold, P. J. (2000). Acidosis induces necrosis and apoptosis of cultured hippocampal neurons. *Experimental Neurology* 162, 1-12.

Dore, S., Takahashi, M., Ferris, C. D., Hester, L. D., Guastella, D., & Snyder, S. H. (1999). Bilirubin, formed by activation of heme oxygenase-2, protects neurons against oxidative stress injury. *Proceedings of the National Academy of Sciences of the United States of America* 96, 2445-2450.

Drion, N., Lemaire, M., Lefauconnier, J. M., & Scherrmann, J. M. (1996). Role of P-glycoprotein in the blood-brain transport of colchicine and vinblastine. *Journal of Neurochemistry* 67, 1688-1693.

Drion, N., Risede, P., Cholet, N., Chanez, C., & Scherrmann, J. M. (1997). Role of P-170 glycoprotein in colchicine brain uptake. *Journal of Neuroscience Research* 49, 80-88.

Du, Y. S., Bales, K. R., Dodel, R. C., HamiltonByrd, E., Horn, J. W., Czilli, D. L., Simmons, L. K., Ni, B. H., & Paul, S. M. (1997). Activation of a caspase 3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurons. *Proceedings of the National Academy of Sciences of the United States of America* 94, 11657-11662.

Earnshaw, W. C., Martins, L. M., & Kaufmann, S. H. (1999). Mammalian caspases: Structure, activation, substrates, and functions during apoptosis. *Annual Review of Biochemistry* 68, 383-424.

Ebbesen, F. (2000). Recurrence of kernicterus in term and near-term infants in Denmark. *Acta Paediatrica* 89, 1213-1217.

Edwards, A. D., Cox, P., Hope, P. L., Azzopardi, D. V., Squier, M. V., & Mehmet, H. (1997). Apoptosis in the brains of infants suffering intrauterine cerebral injury. *Pediatric Research* 42, 684-689.

Egdell HG & Stanfield JP (1972). Paediatric neurology in Africa: a Ugandan report. *Br Med J* 1, 548-552.

Eriksen, E. F., Danielsen, H., & Brodersen, R. (1981). Bilirubin-Liposome Interaction - Binding of Bilirubin Dianion, Protonization, and Aggregation of Bilirubin Acid. *Journal of Biological Chemistry* 256, 4269-4274.

Falcao, A. S., Bellarosa, C., Fernandes, A., Brito, M. A., Silva, R. F. M., Tiribelli, C., & Brites, D. (2007). Role of multidrug resistance-associated protein 1 expression in the in vitro susceptibility of rat nerve cell to unconjugated bilirubin. *Neuroscience* 144, 878-888.

Fernandes, A., Falcao, A. S., Silva, R. F. M., Brito, M. A., & Brites, D. (2007). MAPKs are key players in mediating cytokine release and cell death induced by unconjugated bilirubin in cultured rat cortical astrocytes. *European Journal of Neuroscience* 25, 1058-1068.

Fernandes, A., Falcao, A. S., Silva, R. F. M., Gordo, A. C., Gama, M. J., Brito, M. A., & Brites, D. (2006). Inflammatory signalling pathways involved in astroglial activation by unconjugated bilirubin. *Journal of Neurochemistry* 96, 1667-1679.

Fernandes, A., Silva, R. F. M., Falcao, A. S., Brito, M. A., & Brites, D. (2004). Cytokine production, glutamate release and cell death in rat cultured astrocytes treated with unconjugated bilirubin and LPS. *Journal of Neuroimmunology* 153, 64-75.

Foster JR (2000). Detection and Biomarkers of Apoptosis. In *Apoptosis in Toxicology*, ed. Roberts R, pp. 213-232. Taylor and Francis Limited, London.

Fricker, G. & Miller, D. S. (2004). Modulation of drug transporters at the blood-brain barrier. *Pharmacology* 70, 169-176.

Gao, B., Hagenbuch, B., Kullak-Ublick, G. A., Benke, D., Aguzzi, A., & Meier, P. J. (2000). Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. *Journal of Pharmacology and Experimental Therapeutics* 294, 73-79.

Gao, B., Hagenbuch, B., & Meier, P. J. (1999). Opioid peptides are substrates of the OATP gene family. *Hepatology* 30, 465A.

Gartner LM (1994). Neonatal Jaundice. *Pediatrics in Review* 15, 422-432.

Geiger, A. S., Rice, A. C., & Shapiro, S. M. (2006). Minocycline is neuroprotective against acute bilirubin toxicity in jaundiced gunn rat pups. *Annals of Neurology* 60, S115.

Gennuso, F., Fernetti, C., Tirolo, C., Testa, N., L'Episcopo, F., Caniglia, S., Morale, M. C., Ostrow, J. D., Pascolo, L., Tiribelli, C., & Marchetti, B. (2004). Bilirubin protects astrocytes from its own toxicity by inducing up-regulation and translocation of multidrug resistance-associated protein 1 (Mrp1). *Proceedings of the National Academy of Sciences of the United States of America* 101, 2470-2475.

Gillardon, F., Kiprianova, I., Sandkuhler, J., Hossmann, K. A., & Spranger, M. (1999). Inhibition of caspases prevents cell death of hippocampal CA1 neurons, but not impairment of hippocampal long-term potentiation following global ischemia. *Neuroscience* 93, 1219-1222.

Green, D. & Kroemer, G. (1998). The central executioners of apoptosis: caspases or mitochondria? *Trends in Cell Biology* 8, 267-271.

Grimmer, I., Berger-Jones, K., Buhrer, C., Brandl, U., & Obladen, M. (1999). Late neurological sequelae of non-hemolytic hyperbilirubinemia of healthy term neonates. *Acta Paediatrica* 88, 661-663.

Groenendaal, F., van der Grond, J., & de Vries, L. S. (2004). Cerebral metabolism in severe neonatal hyperbilirubinemia. *Pediatrics* 114, 291-294.

Grojean, S., Koziel, V., Vert, P., & Daval, J. L. (2000). Bilirubin induces apoptosis via activation of NMDA receptors in developing rat brain neurons. *Experimental Neurology* 166, 334-341.

Grojean, S., Lievre, V., Koziel, V., Vert, P., & Daval, J. L. (2001). Bilirubin exerts additional toxic effects in hypoxic cultured neurons from the developing rat brain by the recruitment of glutamate neurotoxicity. *Pediatric Research* 49, 507-513.

Hahm, J. S., Ostrow, J. D., Mukerjee, P., & Celic, L. (1992). Ionization and Self-Association of Unconjugated Bilirubin, Determined by Rapid Solvent Partition from Chloroform, with Further-Studies of Bilirubin Solubility. *Journal of Lipid Research* 33, 1123-1137.

Hamabe, W., Fukushima, N., Yoshida, A., & Ueda, H. (2000). Serum-free induced neuronal apoptosis-like cell death is independent of caspase activity. *Molecular Brain Research* 78, 186-191.

Hampton, M. B. & Orrenius, S. (1998). Redox regulation of apoptotic cell death. *Biofactors* 8, 1-5.

Hansen T.W.R, Øyasaeter S, Stiris T, & Bratlid D (1989). Effects of sulfisoxazole, hypercarbia, and hyperosmolality on entry of bilirubin and albumin into brain regions of young rats. *Biology of the Neonate* 56, 22-30.

Hansen, T. W. R. (1995). Acute Entry of Bilirubin Into Rat-Brain Regions. *Biology of the Neonate* 67, 203-207.

Hansen, T. W. R. (1996). Bilirubin entry into and clearance from rat brain during hypercarbia and hyperosmolality. *Pediatric Research* 39, 72-76.

Hansen, T. W. R. (2000a). Bilirubin oxidation in brain. *Molecular Genetics and Metabolism* 71, 411-417.

Hansen, T. W. R. (2000b). Kernicterus in term and near-term infants - the specter walks again. *Acta Paediatrica* 89, 1155-1157.

Hansen, T. W. R. & Allen, J. W. (1996a). Bilirubin-oxidizing activity in rat brain. *Biology of the Neonate* 70, 289-295.

Hansen, T. W. R. & Allen, J. W. (1996b). Hemolytic anemia does not increase entry into, nor alter rate of clearance of bilirubin from rat brain. *Biology of the Neonate* 69, 268-274.

Hansen, T. W. R., Bratlid, D., & Walaas, S. I. (1988). Bilirubin Decreases Phosphorylation of Synapsin-I, A Synaptic Vesicle-Associated Neuronal Phosphoprotein, in Intact Synaptosomes from Rat Cerebral-Cortex. *Pediatric Research* 23, 219-223.

Hansen, T. W. R. & Cashore, W. J. (1995). Rates of Bilirubin Clearance from Rat-Brain Regions. *Biology of the Neonate* 68, 135-140.

Hansen, T. W. R., Mathiesen, S. B. W., & Walaas, S. I. (1996). Bilirubin has widespread inhibitory effects on protein phosphorylation. *Pediatric Research* 39, 1072-1077.

Hansen, T. W. R. & Tommarello, S. (1998). Effect of phenobarbital on bilirubin metabolism in rat brain. *Biology of the Neonate* 73, 106-111.

Hansen, T. W. R., Tommarello, S., & Allen, J. W. (2001). Subcellular localization of bilirubin in rat brain after in vivo i.v. administration of [H-3]bilirubin. *Pediatric Research* 49, 203-207.

Harada, J. & Sugimoto, M. (1998). Inhibitors of interleukin-1 beta-converting enzyme-family proteases (caspases) prevent apoptosis without affecting decreased cellular ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in cerebellar granule neurons. *Brain Research* 793, 231-243.

Hardy, M., Younkin, D., Tang, C. M., Pleasure, J., Shi, Q. Y., Williams, M., & Pleasure, D. (1994). Expression of Non-Nmda Glutamate-Receptor Channel Genes by Clonal Human Neurons. *Journal of Neurochemistry* 63, 482-489.

Harris, R. C., Lucey, J. F., & Maclean, J. R. (1958). Kernicterus in Premature Infants Associated with Low Concentrations of Bilirubin in the Plasma. *Pediatrics* 21, 875-884.

Hayward, D., Amit, Y., Chan, G., Fedunec, S., & Schiff, D. (1987). Solubility and Stability of Bilirubin(Br) in Tissue-Culture Incubates. *Clinical Research* 35, A234.

Hayward, D., Schiff, D., Fedunec, S., Chan, G., Davis, P. J., & Poznansky, M. J. (1986). Bilirubin Diffusion Through Lipid-Membranes. *Biochimica et Biophysica Acta* 860, 149-153.

Higgins, C. F. & Gottesman, M. M. (1992). Is the Multidrug Transporter A Flippase. *Trends in Biochemical Sciences* 17, 18-21.

Hintz, S. R., Gaylord, T. D., Oh, W., Fanaroff, A. A., Mele, L., & Stevenson, D. K. (2001). Serum bilirubin levels at 72 hours by selected characteristics in breastfed and formula-fed term infants delivered by cesarean section. *Acta Paediatrica* 90, 776-781.

Hirsch, T., Marchetti, P., Susin, S. A., Dallaporta, B., Zamzami, N., Marzo, I., Geuskens, M., & Kroemer, G. (1997). The apoptosis-necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. *Oncogene* 15, 1573-1581.

Hoffman, D. J., Zanelli, S. A., Kubin, J., Mishra, O. P., & DelivoriaPapadopoulos, M. (1996). The in vivo effect of bilirubin on the N-methyl-D-aspartate receptor/ion channel complex in the brains of newborn piglets. *Pediatric Research* 40, 804-808.

Horton, C. & Maden, M. (1995). Endogenous Distribution of Retinoids During Normal Development and Teratogenesis in the Mouse Embryo. *Developmental Dynamics* 202, 312-323.

Hsia, D. Y. Y., Allen, F. H., Diamond, L. K., & Gellis, S. S. (1953). Serum Bilirubin Levels in the Newborn Infant. *Journal of Pediatrics* 42, 277-285.

Hsia, D. Y. Y., Allen, F. H., Gellis, S. S., & Diamond, L. K. (1952). Erythroblastosis Fetalis .8. Studies of Serum Bilirubin in Relation to Kernicterus. *New England Journal of Medicine* 247, 668-671.

Hu, B. R., Liu, C. L., Ouyang, Y., Blomgren, K., & Siesjo, B. K. (2000). Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. *Journal of Cerebral Blood Flow and Metabolism* 20, 1294-1300.

Ikonomidou, C. & Turski, L. (2002). Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurology* 1, 383-386.

Ip, S., Chung, M., Kulig, J., O'Brien, R., Sege, R., Glicken, S., Maisels, M. J., & Lau, J. (2004a). An evidence-based review of important issues concerning neonatal hyperbilirubinemia. *Pediatrics* 114, E130-E153.

Ip, S., Lau, J., Chung, M., Kulig, J., Sege, R., Glicken, S., & O'Brien, R. (2004b). Hyperbilirubinemia and kernicterus: 50 years later. *Pediatrics* 114, 263-264.

Itoh, T., Itoh, A., Horiuchi, K., & Pleasure, D. (1998). AMPA receptor-mediated excitotoxicity in human NT2-N neurons results from loss of intracellular Ca2+ homeostasis following marked elevation of intracellular Na+. *Journal of Neurochemistry* 71, 112-124.

Ives, N. K., Bolas, N. M., & Gardiner, R. M. (1989). The Effects of Bilirubin on Brain Energy-Metabolism During Hyperosmolar Opening of the Blood-Brain-Barrier - An Invivo Study Using P-31 Nuclear Magnetic-Resonance Spectroscopy. *Pediatric Research* 26, 356-361.

Jette, L., Murphy, G. F., Leclerc, J. M., & Beliveau, R. (1995). Interaction of Drugs with P-Glycoprotein in Brain Capillaries. *Biochemical Pharmacology* 50, 1701-1709.

Joashi, U. C., Greenwood, K., Taylor, D. L., Kozma, M., Mazarakis, N. D., Edwards, A. D., & Mehmet, H. (1999). Poly(ADP ribose) polymerase cleavage precedes neuronal death in the hippocampus and cerebellum following injury to the developing rat forebrain. *European Journal of Neuroscience* 11, 91-100.

Johnson, L. H., Bhutani, V. K., & Brown, A. K. (2002). System-based approach to management of neonatal jaundice and prevention of kernicterus. *Journal of Pediatrics* 140, 396-403.

Johnston MV, Nakajima W, & Hagberg H (2002). Mechanisms of hypoxic neurodegeneration in the developing brain. *The Neuroscientist* 8(3), 212-220.

Kaal, E. C. A., Veldman, H., Sodaar, P., Joosten, E. A. J., & Bar, P. R. D. (1998). Oxidant treatment causes a dose-dependent phenotype of apoptosis in cultured motoneurons. *Journal of Neuroscience Research* 54, 778-786.

Kaneko, I., Yamada, N., Sakuraba, Y., Kamenosono, M., & Tutumi, S. (1995). Suppression of Mitochondrial Succinate-Dehydrogenase, A Primary Target of Beta-Amyloid, and Its Derivative Racemized at Ser Residue. *Journal of Neurochemistry* 65, 2585-2593.

Kariya, S., Takahashi, N., Ooba, N., Kawahara, M., Nakayama, H., & Ueno, S. (2002). Humanin inhibits cell death of serum-deprived PCI2h cells. *Neuroreport* 13, 903-907.

Karp, W. B. (1979). Biochemical-Alterations in Neonatal Hyper-Bilitrubinemia and Bilirubin Encephalopathy - Review. *Pediatrics* 64, 361-368.

Katoh, R., Kashiwamata, S., & Niwa, F. (1975). Studies on Cellular Toxicity of Bilirubin - Effect on Carbohydrate-Metabolism in Young Rat-Brain. *Brain Research* 83, 81-92.

Keshavan, P., Schwemberger, S. J., Smith, D. L. H., Babcock, G. F., & Zucker, S. D. (2004). Unconjugated bilirubin induces apoptosis in colon cancer cells by triggering mitochondrial depolarization. *International Journal of Cancer* 112, 433-445.

Killander A, Michaelsson M, Muller-Eberhard U, & Sjølin S (1963). Hyperbilirubinemia in fullterm newborn infants: A follow-up study. *Acta Peadiatr Scand* 52, 481-484.

King, M., Su, W., Chang, A., Zuckerman, A., & Pasternak, G. W. (2001). Transport of opioids from the brain to the periphery by P-glycoprotein: peripheral actions of central drugs. *Nature Neuroscience* 4, 268-274.

Kroemer, G. & Martin, S. J. (2005). Caspase-independent cell death. *Nature Medicine* 11, 725-730.

KulkarniNarla, A., Getchell, T. V., & Getchell, M. L. (1997). Differential expression of manganese and copper-zinc superoxide dismutases in the olfactory and vomeronasal receptor neurons of rats during ontogeny. *Journal of Comparative Neurology* 381, 31-40.

Lankiewicz, S., Luetjens, C. M., Bui, N. T., Krohn, A. J., Poppe, M., Cole, G. M., Saido, T. C., & Prehn, J. H. M. (2000). Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. *Journal of Biological Chemistry* 275, 17064-17071.

Lee T.C. & Hsia D.Y. (1959). Experimental studies on blood-spinal fluid barrier for bilirubin. *J Lab Clin Med* 54, 512-524.

Leist, M. & Nicotera, P. (1997). The shape of cell death. *Biochemical and Biophysical Research Communications* 236, 1-9.

Leist, M. & Nicotera, P. (1998). Apoptosis, excitotoxicity, and neuropathology. *Experimental Cell Research* 239, 183-201.

Leist, M., Single, B., Castoldi, A. F., Kuhnle, S., & Nicotera, P. (1997). Intracellular adenosine triphosphate (ATP) concentration: A switch in the decision between apoptosis and necrosis. *Journal of Experimental Medicine* 185, 1481-1486.

Lesuisse, C. & Martin, L. J. (2002). Immature and mature cortical neurons engage different apoptotic mechanisms involving caspase-3 and the mitogen-activated protein kinase pathway. *Journal of Cerebral Blood Flow and Metabolism* 22, 935-950.

Levine, R. L., Fredericks, W. R., & Rapoport, S. I. (1982). Entry of Bilirubin Into the Brain Due to Opening of the Blood-Brain-Barrier. *Pediatrics* 69, 255-259.

Levine, R. L., Fredericks, W. R., & Rapoport, S. I. (1985). Clearance of Bilirubin from Rat-Brain After Reversible Osmotic Opening of the Blood-Brain-Barrier. *Pediatric Research* 19, 1040-1043.

Li, J., Spletter, M. L., Johnson, D. A., Wright, L. S., Svendsen, C. N., & Johnson, J. A. (2005). Rotenone-induced caspase 9/3-independent and -dependent cell death in undifferentiated and differentiated human neural stem cells. *Journal of Neurochemistry* 92, 462-476.

Liang, C. L., Yang, L. C., Lu, K., Hsu, H. C., Cho, C. L., Chen, S. D., Huang, H. Y., & Chen, H. J. (2003). Neuroprotective synergy of N-methyl-(D)-aspartate receptor antagonist (MK801) and protein synthesis inhibitor (cycloheximide) on spinal cord ischemia-reperfusion injury in rats. *Journal of Neurotrauma* 20, 195-206.

Liang, X. J. & Aszalos, A. (2006). Multidrug transporters as drug targets. *Current Drug Targets* 7, 911-921.

Lie, S. O. & Bratlid, D. (1970). Protective Effect of Albumin on Bilirubin Toxicity on Human Fibroblasts. *Scandinavian Journal of Clinical & Laboratory Investigation* 26, 37-&.

Lightner, D. A. & McDonagh, A. F. (2001). Structure and metabolism of natural and synthetic bilirubins. *J Perinatol.* Suppl 1, 13-16.

Lightner, D. A., Reisinger, M., & Landen, G. L. (1986). On the Structure of Albumin-Bound Bilirubin - Selective Binding of Intramolecularly Hydrogen-Bonded Conformational Enantiomers. *Journal of Biological Chemistry* 261, 6034-6038.

Lin, J. H. & Yamazaki, M. (2003). Role of P-glycoprotein in pharmacokinetics - Clinical implications. *Clinical Pharmacokinetics* 42, 59-98.

Lin, S. Z., Wei, X., Bales, K. R., Paul, A. B. C., Ma, Z. Z., Yan, G. M., Paul, S. M., & Du, Y. S. (2005). Minocycline blocks bilirubin neurotoxicity and prevents hyperbilirubinemia-induced cerebellar hypoplasia in the Gunn rat. *European Journal of Neuroscience* 22, 21-27.

Lin, S. Z., Yan, C., Wei, X., Paul, S. M., & Du, Y. S. (2003). p38 MAP kinase mediates bilirubin-induced neuronal death of cultured rat cerebellar granule neurons. *Neuroscience Letters* 353, 209-212.

Liu, Y. B., Peterson, D. A., Kimura, H., & Schubert, D. (1997). Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *Journal of Neurochemistry* 69, 581-593.

Lobner, D. (2000). Comparison of the LDH and MTT assays for quantifying cell death: validity for neuronal apoptosis? *Journal of Neuroscience Methods* 96, 147-152.

Loetscher, H., Niederhauser, O., Kemp, J., & Gill, R. (2001). Is caspase-3 inhibition a valid therapeutic strategy in cerebral ischemia? *Drug Discovery Today* 6, 671-680.

Lorenzo, H. K. & Susin, S. A. (2004). Mitochondrial effectors in caspase-independent cell death. *Febs Letters* 557, 14-20.

Lorenzo, H. K., Susin, S. A., Penninger, J., & Kroemer, G. (1999). Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. *Cell Death and Differentiation* 6, 516-524.

Lucey, J. F., Hibbard, E., Windle, W. F., Behrman, R. E., & Degallar (1964). Kernicterus in Asphyxiated Newborn Rhesus Monkeys. *Experimental Neurology* 9, 43-&.

Ma, J. Y., Endres, M., & Moskowitz, M. A. (1998). Synergistic effects of caspase inhibitors and MK-801 in brain injury after transient focal cerebral ischaemia in mice. *British Journal of Pharmacology* 124, 756-762.

Maisels MJ (1999). Jaundice. In *Neonatology, Pathophysiology & Management of the Newborn*, eds. Avery GB, Fletcher MA, & MacDonald MG, pp. 765-820. Lippincott Williams & Wilkins, Philadelphia.

Maisels, M. J. (2006). What's in a name? Physiologic and pathologic jaundice: The conundrum of defining normal bilirubin levels in the newborn. *Pediatrics* 118, 805-807.

Maisels, M. J., Baltz, R. D., Bhutani, V. K., Newman, T. B., Palmer, H., Rosenfeld, W., Stevenson, D. K., & Weinblatt, H. B. (2004). Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. *Pediatrics* 114, 297-316.

Maisels, M. J. & Newman, T. B. (2001). Bilirubin and neurological dysfunction - Do we need to change what we are doing? *Pediatric Research* 50, 677-678.

Marques-Santos, L. F., Bernardo, R. R., de Paula, E. F., & Rumjanek, V. M. (1999). Cyclosporin A and trifluoperazine, two resistance-modulating agents, increase ivermectin neurotoxicity in mice. *Pharmacology & Toxicology* 84, 125-129.

Martin, L. J., Al Abdulla, N. A., Brambrink, A. M., Kirsch, J. R., Sieber, F. E., & Portera-Cailliau, C. (1998). Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: A perspective on the contributions of apoptosis and necrosis. *Brain Research Bulletin* 46, 281-309.

Matsuoka, T., Kondoh, T., Tamaki, N., & Nishizaki, T. (1997). The GABA(A) receptor is expressed in human neurons derived from a teratocarcinoma cell line. *Biochemical and Biophysical Research Communications* 237, 719-723.

Matsuoka, Y., Okazaki, M., Kitamura, Y., & Taniguchi, T. (1999). Developmental expression of P-glycoprotein (multidrug resistance gene product) in the rat brain. *Journal of Neurobiology* 39, 383-392.

McDonald, J. W. & Johnston, M. V. (1990). Physiological and Pathophysiological Roles of Excitatory Amino-Acids During Central-Nervous-System Development. *Brain Research Reviews* 15, 41-70.

McDonald, J. W., Shapiro, S. M., Silverstein, F. S., & Johnston, M. V. (1998). Role of glutamate receptor-mediated excitotoxicity in bilirubin-induced brain injury in the Gunn rat model. *Experimental Neurology* 150, 21-29.

McDonald, J. W., Silverstein, F. S., & Johnston, M. V. (1988). Neurotoxicity of N-Methyl-D-Aspartate Is Markedly Enhanced in Developing Rat Central Nervous-System. *Brain Research* 459, 200-203.

Mehta SL, Manhas N, & Raghubir R. (2006). Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Research Reviews , 1-33.

Meisel P, Jahrig D, & Jahrig K (1981). Bilirubin im Liquor cerebrospinalis Neugebrener: I. Vergleichende Untersuchungen des Liquor Cerebrospinalis. *Kinderarztliche Praxis* 49, 633-642.

Morphis, L., Constantopoulos, A., Matsaniotis, N., & Papaphilis, A. (1982). Bilirubin-Induced Modulation of Cerebral Protein-Phosphorylation in Neonate Rabbits in vivo. *Science* 218, 156-158.

Munir, M., Lu, L. H., Wang, Y. H., Luo, J. H., Wolfe, B. B., & McGonigle, P. (1996). Pharmacological and immunological characterization of N-methyl-D-aspartate receptors in human NT2-N neurons. *Journal of Pharmacology and Experimental Therapeutics* 276, 819-828.

Munir, M., Lu, L. S., & McGonigle, P. (1995). Excitotoxic cell death and delayed rescue in human neurons derived from NT2 cells. *Journal of Neuroscience* 15, 7847-7860.

Nath, R., Scott, R., Nadimpalli, R., Gupta, R., & Wang, K. K. W. (2000). Activation of apoptosis-linked caspase(s) in NMDA-injured brains in neonatal rats. *Neurochemistry International* 36, 119-126.

Neelands, T. R., Greenfield, L. J., Zhang, J., Turner, R. S., & Macdonald, R. L. (1998). GABA(A) receptor pharmacology and subtype mRNA expression in human neuronal NT2-N cells. *Journal of Neuroscience* 18, 4993-5007.

Neelands, T. R., King, A. P. J., & Macdonald, R. L. (2000). Functional expression of L-, N-, P/Q-, and R-type calcium channels in the human NT2-N cell line. *Journal of Neurophysiology* 84, 2933-2944.

Nelson, P. T., Kondziolka, D., Wechsler, L., Goldstein, S., Gebel, J., DeCesare, S., Elder, E. M., Zhang, P. J., Jacobs, A., McGrogan, M., Lee, V. M. Y., & Trojanowski, J. Q. (2002).

Clonal human (hNT) neuron grafts for stroke therapy - Neuropathology in a patient 27 months after implantation. *American Journal of Pathology* 160, 1201-1206.

Newman, T. B. & Klebanoff, M. A. (1993). Neonatal Hyperbilirubinemia and Long-Term Outcome - Another Look at the Collaborative Perinatal Project. *Pediatrics* 92, 651-657.

Newman, T. B., Liljestrand, P., Jeremy, R. J., Ferriero, D. M., Wu, Y. W., Hudes, E. S., & Escobar, G. J. (2006). Outcomes among newborns with total serum bilirubin levels of 25 mg per deciliter or more. *New England Journal of Medicine* 354, 1889-1900.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. T., Yu, V. L., & Miller, D. K. (1995). Identification and Inhibition of the Ice/Ced-3 Protease Necessary for Mammalian Apoptosis. *Nature* 376, 37-43.

Nicotera, P. & Leist, M. (1997). Energy supply and the shape of death in neurons and lymphoid cells. *Cell Death and Differentiation* 4, 435-442.

Nicotera, P., Leist, M., Fava, E., Berliocchi, L., & Volbracht, C. (2000). Energy requirement for caspase activation and neuronal cell death. *Brain Pathology* 10, 276-282.

Nicotera, P., Leist, M., & Ferrando-May, E. (1998). Intracellular ATP, a switch in the decision between apoptosis and necrosis. *Toxicology Letters* 103, 139-142.

Nicotera, P., Leist, M., & Manzo, L. (1999). Neuronal cell death: a demise with different shapes. *Trends in Pharmacological Sciences* 20, 46-51.

Nilsen, S. T., Finne, P. H., Bergsjo, P., & Stamnes, O. (1984). Males with Neonatal Hyperbilirubinemia Examined at 18 Years of Age. *Acta Paediatrica Scandinavica* 73, 176-180.

Nitsch, R., Bechmann, I., Deisz, R. A., Haas, D., Lehmann, T. N., Wendling, U., & Zipp, F. (2000). Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). *Lancet* 356, 827-828.

Northington, F. J., Ferriero, D. M., Flock, D. L., & Martin, L. J. (2001a). Delayed neurodegeneration in neonatal rat thalamus after hypoxia-ischemia is apoptosis. *Journal of Neuroscience* 21, 1931-1938.

Northington, F. J., Ferriero, D. M., Graham, E. M., Traystman, R. J., & Martin, L. J. (2001b). Early neurodegeneration after hypoxia-ischemia in neonatal rat is necrosis while delayed neuronal death is apoptosis. *Neurobiology of Disease* 8, 207-219.

Northington FJ, Graham EM, & Martin LJ. (2005). Apoptosis in perinatal hypoxic-ishemic brain injury: How important is it and should it be inhibited? Brain Research Reviews 50, 244-257.

Nwaesei, C. G., Vanaerde, J., Boyden, M., & Perlman, M. (1984). Changes in Auditory Brain-Stem Responses in Hyperbilirubinemic Infants Before and After Exchange-Transfusion. *Pediatrics* 74, 800-803.

Ostrow, J. D., Mukerjee, P., & Tiribelli, C. (1994). Structure and Binding of Unconjugated Bilirubin - Relevance for Physiological and Pathophysiological Function. *Journal of Lipid Research* 35, 1715-1737.

Ozmert, E., Erdem, G., Topcu, M., Yurdakok, M., Tekinalp, G., Genc, D., & Renda, Y. (1996). Long-term follow-up of indirect hyperbilirabinemia in full-term Turkish infants. *Acta Paediatrica* 85, 1440-1444.

Paludetto, R., Mansi, G., Raimondi, F., Romano, A., Crivaro, V., Bussi, M., & D'Ambrosio, G. (2002). Moderate hyperbilirubinemia induces a transient alteration of neonatal behavior. *Pediatrics* 110, 1 - 5..

Pardridge, W. M. (1998). CNS drug design based on principles of blood-brain barrier transport. *Journal of Neurochemistry* 70, 1781-1792.

Pascolo, L., Fernetti, C., Garcia-Mediavilla, M. V., Ostrow, J. D., & Tiribelli, C. (2001). Mechanisms for the transport of unconjugated bilirubin in human trophoblastic BeWo cells. *Febs Letters* 495, 94-99.

Patel, A. J., Gunasekera, S., Jen, A., & deSilva, H. A. R. (1996). Beta-amyloid-mediated inhibition of redox activity (MTT reduction) is not an indicator of astroglial degeneration. *Neuroreport* 7, 2026-2030.

Pearlman, M. A., Gartner, L. M., Lee, K. S., Eidelman, A. I., Morecki, R., & Horoupian, D. S. (1980). Association of Kernicterus with Bacterial-Infection in the Newborn. *Pediatrics* 65, 26-29.

Pleasure, S. J., Page, C., & Lee, V. M. Y. (1992). Pure, Postmitotic, Polarized Human Neurons Derived from Ntera-2 Cells Provide A System for Expressing Exogenous Proteins in Terminally Differentiated Neurons. *Journal of Neuroscience* 12, 1802-1815.

Polster, B. M., Basanez, G., Etxebarria, A., Hardwick, J. M., & Nicholls, D. G. (2005). Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. *Journal of Biological Chemistry* 280, 6447-6454.

Polster, B. M. & Fiskum, G. (2004). Mitochondrial mechanisms of neural cell apoptosis. *Journal of Neurochemistry* 90, 1281-1289.

PorteraCailliau, C., Price, D. L., & Martin, L. J. (1997). Excitotoxic neuronal death in the immature brain is an apoptosis-necrosis morphological continuum. *Journal of Comparative Neurology* 378, 70-87.

Puka-Sundvall, M., Hallin, U., Zhu, C. L., Wang, X. Y., Karlsson, J. O., Blomgren, K., & Hagberg, H. (2000). NMDA blockade attenuates caspase-3 activation and DNA fragmentation after neonatal hypoxia-ischemia. *Neuroreport* 11, 2833-2836.

Racke, M. M., Mosior, M., Kovacevic, S., Chang, C. H. S., Glasebrook, A. L., Roehm, N. W., & Na, S. Q. (2002). Activation of caspase-3 alone is insufficient for apoptotic morphological changes in human neuroblastoma cells. *Journal of Neurochemistry* 80, 1039-1048.

Raffray, M. & Cohen, G. M. (1997). Apoptosis and necrosis in toxicology: A continuum or distinct modes of cell death? *Pharmacology & Therapeutics* 75, 153-177.

Rodrigues, C. M. P., Sola, S., & Brites, D. (2002a). Bilirubin induces apoptosis via the mitochondrial pathway in developing rat brain neurons. *Hepatology* 35, 1186-1195.

Rodrigues, C. M. P., Sola, S., Castro, R. E., Laires, P. A., Brites, D., & Moura, J. J. G. (2002b). Perturbation of membrane dynamics in nerve cells is an early event during bilirubin-induced apoptosis. *Journal of Lipid Research* 43, 885-894.

Rodrigues, C. M. P., Sola, S., Silva, R., & Brites, D. (2000). Bilirubin and amyloid-beta peptide induce cytochrome c release through mitochondrial membrane permeabilization. *Molecular Medicine* 6, 936-946.

Rodrigues, C. M. P., Sola, S., Silva, R. F. M., & Brites, D. (2002c). Aging confers different sensitivity to the neurotoxic properties of unconjugated bilirubin. *Pediatric Research* 51, 112-118.

Rootwelt, T., Dunn, M., Yudkoff, M., Itoh, T., Almaas, R., & Pleasure, D. (1998). Hypoxic cell death in human NT2-N neurons: Involvement of NMDA and non-NMDA glutamate receptors. *Journal of Neurochemistry* 71, 1544-1553.

Rossant, J., Zirngibl, R., Cado, D., Shago, M., & Giguere, V. (1991). Expression of A Retinoic Acid Response Element-Hsplacz Transgene Defines Specific Domains of Transcriptional Activity During Mouse Embryogenesis. *Genes & Development* 5, 1333-1344.

Ryan, C. A. & Salvesen, G. S. (2003). Caspases and neuronal development. *Biological Chemistry* 384, 855-861.

Sano, K., Nakamura, H., & Matsuo, T. (1985). Mode of Inhibitory-Action of Bilirubin on Protein Kinase-C. *Pediatric Research* 19, 587-590.

Schiff, D., Chan, G., & Poznansky, M. J. (1985). Bilirubin Toxicity in Neural Cell-Lines N115 and Nbr10A. *Pediatric Research* 19, 908-911.

Schinkel, A. H. & Jonker, J. W. (2003). Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Advanced Drug Delivery Reviews* 55, 3-29.

Schinkel, A. H., Wagenaar, E., Vandeemter, L., Mol, C. A. A. M., & Borst, P. (1995). Absence of the Mdr1A P-Glycoprotein in Mice Affects Tissue Distribution and Pharmacokinetics of Dexamethasone, Digoxin, and Cyclosporine-A. *Journal of Clinical Investigation* 96, 1698-1705.

Schmorl G. (1904) .Zur Kenntnis der Ikterus Neonatorum. *Verh Dtsch Pathol Ges* 6, 109-115.

Schulz, J. B., Weller, M., Matthews, R. T., Heneka, M. T., Groscurth, P., Martinou, J. C., Lommatzsch, J., von Coelln, R., Wullner, U., Loschmann, P. A., Beal, M. F., Dichgans, J., & Klockgether, T. (1998). Extended therapeutic window for caspase inhibition and synergy with MK-801 in the treatment of cerebral histotoxic hypoxia. *Cell Death and Differentiation* 5, 847-857.

Sedlak, T. W. & Snyder, S. H. (2004). Bilirubin benefits: Cellular protection by a biliverdin reductase antioxidant cycle. *Pediatrics* 113, 1776-1782.

Seubert, J. M., Darmon, A. J., El Kadi, A. O. S., D'Souza, S. J. A., & Bend, J. R. (2002). Apoptosis in murine hepatoma Hepa 1c1c7 wild-type, C12, and C4 cells mediated by bilirubin. *Molecular Pharmacology* 62, 257-264.

Sgro, M., Campbell, D., & Shah, V. (2006). Incidence and causes of severe neonatal hyperbilirubinemia in Canada. *Canadian Medical Association Journal* 175, 587-590.

Shapiro SM (2005). Definition of the clinical spectrum of kernicterus and bilirubin-induced neurologic dysfunction. *J Perinatol* 25, 54-59.

Shapiro, S. M. (1988). Acute Brain-Stem Auditory Evoked-Potential Abnormalities in Jaundiced Gunn-Rats Given Sulfonamide. *Pediatric Research* 23, 306-310.

Shapiro, S. M. (1993). Reversible Brain-Stem Auditory-Evoked Potential Abnormalities in Jaundiced Gunn-Rats Given Sulfonamide. *Pediatric Research* 34, 629-633.

Shapiro, S. M. (2003). Bilirubin toxicity in the developing nervous system. *Pediatric Neurology* 29, 410-421.

Shapiro, S. M. & Conlee, J. W. (1991). Brain-Stem Auditory Evoked-Potentials Correlate with Morphological-Changes in Gunn Rat Pups. *Hearing Research* 57, 16-22.

Shearman, M. S., Hawtin, S. R., & Tailor, V. J. (1995). The Intracellular Component of Cellular 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (Mtt) Reduction Is Specifically Inhibited by Beta-Amyloid Peptides. *Journal of Neurochemistry* 65, 218-227.

Silberberg D.H., Johnson, L., & Ritter, L. (1970). Factors Influencing Toxicity of Bilirubin in Cerebellum Tissue Culture. *Journal of Pediatrics* 77, 386-&.

Silva, R., Mata, L. R., Gulbenkian, S., Brito, M. A., Tiribelli, C., & Brites, D. (1999). Inhibition of glutamate uptake by unconjugated bilirubin in cultured cortical rat astrocytes: Role of concentration and pH. *Biochemical and Biophysical Research Communications* 265, 67-72.

Silva, R. F. M., Mata, L. M., Gulbenkian, S., & Brites, D. (2001a). Endocytosis in rat cultured astrocytes is inhibited by unconjugated bilirubin. *Neurochemical Research* 26, 793-800.

Silva, R. F. M., Rodrigues, C. M. P., & Brites, D. (2001b). Bilirubin-induced apoptosis in cultured rat neural cells is aggravated by chenodeoxycholic acid but prevented by ursodeoxycholic acids. *Journal of Hepatology* 34, 402-408.

Silva, R. F. M., Rodrigues, C. M. P., & Brites, D. (2002). Rat cultured neuronal and glial cells respond differently to toxicity of unconjugated bilirubin. *Pediatric Research* 51, 535-541.

Silvermann W.A., Andersen D.H., Blanc W.A., & Crozier D.N. (1956). A difference in mortality rate and incidence of kernicterus among premature infants allotted to two prophylactic antibacterial regimens. *Pediatrics* 18, 614-625.

Slater, L. M., Sweet, P., Stupecky, M., & Gupta, S. (1986). Cyclosporine-A Reverses Vincristine and Daunorubicin Resistance in Acute Lymphatic-Leukemia Invitro. *Journal of Clinical Investigation* 77, 1405-1408.

Soorani-Lunsing, I., Woltil, H. A., & Hadders-Algra, M. (2001). Are moderate degrees of hyperbilirubinemia in healthy term neonates really safe for the brain? *Pediatric Research* 50, 701-705.

Stefanis, L. (2005). Caspase-dependent and -independent neuronal death: Two distinct pathways to neuronal injury. *Neuroscientist* 11, 50-62.

Stefanis, L., Park, D. S., Yan, C. Y. I., Farinelli, S. E., Troy, C. M., Shelanski, M. L., & Greene, L. A. (1996). Induction of CPP32-like activity in PC12 cells by withdrawal of trophic support - Dissociation from apoptosis. *Journal of Biological Chemistry* 271, 30663-30671.

Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K. F., Irinopoulou, T., Prevost, M. C., Brothers, G., Mak, T. W., Penninger, J., Earnshaw, W. C., & Kroemer, G. (2000). Two distinct pathways leading to nuclear apoptosis. *Journal of Experimental Medicine* 192, 571-579.

Tamai, I. & Tsuji, A. (2000). Transporter-mediated permeation of drugs across the blood-brain barrier. *Journal of Pharmaceutical Sciences* 89, 1371-1388.

Tiker, F., Gulcan, H., Kilicdag, H., Tarcan, A., & Gurakan, B. (2006). Extreme hyperbilirubinemia in newborn infants. *Clinical Pediatrics* 45, 257-261.

Tsai, C., Ahdab-Barmada, M., Daood, M. J., & Watchko, J. F. (2000). P-glycoprotein expression in the developing human central nervous system: Cellular and tissue localization. *Pediatric Research* 47, 436A.

Tsai, C. E., Daood, M. J., Lane, R. H., Hansen, T. W. R., Gruetzmacher, E. M., & Watchko, J. F. (2002). P-glycoprotein expression in mouse brain increases with maturation. *Biology of the Neonate* 81, 58-64.

Vazquez, J., Garciacalvo, M., Valdivieso, F., Mayor, F., & Mayor, F. (1988). Interaction of Bilirubin with the Synaptosomal Plasma-Membrane. *Journal of Biological Chemistry* 263, 1255-1265.

Volbracht, C., Leist, M., Kolb, S. A., & Nicotera, P. (2001). Apoptosis in caspase-inhibited neurons. *Molecular Medicine* 7, 36-48.

Volpe JJ (2003). Bilirubin and Brain Injury. In *Neurology of the Newborn* pp. 521-546. W.B.Saunders Company, Philadelphia.

Walaas, S. I. & Greengard, P. (1991). Protein-Phosphorylation and Neuronal Function. *Pharmacological Reviews* 43, 299-349.

Walker, P. R., Leblanc, J., Carson, C., Ribecco, M., & Sikorska, M. (1999). Neither caspase-3 nor DNA fragmentation factor is required for high molecular weight DNA degradation in apoptosis. *Mechanisms of Cell Death* 887, 48-59.

Wallerstein H. (1946).Treatment of severe erythtroblastosisby simultaneous removal and replacement of the blood of the newborn infant. Science 103, 583-584.

Wang H, Yu S-W, Koh DK, Lew J, Coombs C, Bowers W, Federoff HJ, Poirier GG, Dawson TD, & Dawson VL. (2004).Apoptosis-inducing factor substitutes for caspase executioners in NMDA-triggered excitotoxic neuronal death. J Neuroscience 24, 10963-10973.

Watchko, J. F. (2005a). Bilirubin induced apoptosis in vitro: Insights for kernicterus. *Pediatric Research* 57, 177-178.

Watchko, J. F. (2005b). Vigintiphobia revisited. *Pediatrics* 115, 1747-1753.

Watchko, J. F., Daood, M. J., & Hansen, T. W. R. (1998). Brain bilirubin content is increased in P-glycoprotein-deficient transgenic null mutant mice. *Pediatric Research* 44, 763-766.

Wennberg, R. P. (1988). The Importance of Free Bilirubin Acid Salt in Bilirubin Uptake by Erythrocytes and Mitochondria. *Pediatric Research* 23, 443-447.

Wennberg, R. P. (2000). The blood-brain barrier and bilirubin encephalopathy. *Cellular and Molecular Neurobiology* 20, 97-109.

Wennberg, R. P., Gospe, S. M., Rhine, W. D., Seyal, M., Saeed, D., & Sosa, G. (1993). Brain-Stem Bilirubin Toxicity in the Newborn Primate May be Promoted and Reversed by Modulating Pco2. *Pediatric Research* 34, 6-9.

Wennberg, R. P., Johansson, B. B., Folbergrova, J., & Siesjo, B. K. (1991). Bilirubin-Induced Changes in Brain Energy-Metabolism After Osmotic Opening of the Blood-Brain-Barrier. *Pediatric Research* 30, 473-478.

Wennberg, R. P., Yin, J., & Enan, E. (1994). Bilirubin Inhibits Dopamine and Glutamate Uptake by Synaptosomes. *Pediatric Research* 35, A389.

Widlak, P. & Garrard, W. T. (2005). Discovery, regulation, and action of the major apoptotic nucleases DFF40/CAD and endonuclease G. *Journal of Cellular Biochemistry* 94, 1078-1087.

Wyllie, A. H. (1987). Apoptosis - Cell-Death Under Homeostatic Control. *Archives of Toxicology* 3-10.

Xu, D. G., Bureau, Y., McIntyre, D. C., Nicholson, D. W., Liston, P., Zhu, Y. X., Fong, W. G., Crocker, S. J., Korneluk, R. G., & Robertson, G. S. (1999). Attenuation of ischemia-induced cellular and behavioral deficits by X chromosome-linked inhibitor of apoptosis protein overexpression in the rat hippocampus. *Journal of Neuroscience* 19, 5026-5033.

Younkin, D. P., Tang, C. M., Hardy, M., Reddy, U. R., Shi, Q. Y., Pleasure, S. J., Lee, V. M. Y., & Pleasure, D. (1993). Inducible Expression of Neuronal Glutamate Receptor Channels in the Nt2 Human Cell-Line. *Proceedings of the National Academy of Sciences of the United States of America* 90, 2174-2178.

Zamzami, N., Brenner, C., Marzo, I., Susin, S. A., & Kroemer, G. (1998). Subcellular and submitochondrial mode of action of Bcl-2-like oncoproteins. *Oncogene* 16, 2265-2282.

Zhao, X. R., Newcomb, J. K., Pike, B. R., Wang, K. K. W., d'Avella, D., & Hayes, R. L. (2000). Novel characteristics of glutamate-induced cell death in primary septohippocampal cultures: Relationship to calpain and caspase-3 protease activation. *Journal of Cerebral Blood Flow and Metabolism* 20, 550-562.

Zhu, C., Wang, X., Huang, Z., Qiu, L., Xu, F., Vahsen, N., Nilsson, M., Eriksson, P. S., Hagberg, H., Culmsee, C., Plesnila, N., Kroemer, G., & Blomgren, K. (2007). Apoptosis-inducing factor is a major contributor to neuronal loss induced by neonatal cerebral hypoxia-ischemia. *Cell Death and Differentiation* 14, 775-784.

Zhu, C., Wang, X., Xu, F., Bahr, B. A., Shibata, M., Uchiyama, Y., Hagberg, H., & Blomgren, K. (2005). The influence of age on apoptotic and other mechanisms of cell death after cerebral hypoxia-ischemia. *Cell Death and Differentiation* 12, 162-176.

Zhu, C. L., Wang, X. Y., Hagberg, H., & Blomgren, K. (2000). Correlation between caspase-3 activation and three different markers of DNA damage in neonatal cerebral hypoxia-ischemia. *Journal of Neurochemistry* 75, 819-829.

Zigova, T., Willing, A. E., Saporta, S., Daadi, M. M., McGrogan, M. P., Randall, T. S., Freeman, T. B., Sanchez-Ramos, J., & Sanberg, P. R. (2001). Apoptosis in cultured hNT neurons. *Developmental Brain Research* 127, 63-70.

Zou, H., Henzel, W. J., Liu, X. S., Lutschg, A., & Wang, X. D. (1997). Apaf-1, a human protein homologous to C-elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90, 405-413.

Zucker, S. D. & Goessling, W. (2000). Mechanism of hepatocellular uptake of albumin-bound bilirubin. *Biochimica et Biophysica Acta-Biomembranes* 1463, 197-208.

Zucker, S. D., Goessling, W., & Hoppin, A. G. (1999). Unconjugated bilirubin exhibits spontaneous diffusion through model lipid bilayers and native hepatocyte membranes. *Journal of Biological Chemistry* 274, 10852-10862.