Review Article

Bilirubin neurotoxicity: a Narrative Review on long lasting, insidious, and dangerous effects

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Background and Objective: Elevated levels of UCB due to overproduction and/or defective clearance can severely impact the CNS leading to fatal encephalopathy or kernicterus spectrum disorders (KSD) associated with motor and auditory impairments. Still unknown is the preferential distribution of UCB in specific CNS regions and the long-lasting disabilities derived from severe neonatal hyperbilirubinemia. One of the aspects that remains uncertain is how unconjugated hyperbilirubinemia determines neural cell sequelae that may predispose to the development of neurodevelopmental, psychiatric, and neurodegenerative disorders. How UCB damages neurons and glial cells, and the injuries that can occur in more susceptible brain areas, thus potentially leading to permanent CNS dysfunction, is far from being clear. In this review, we summarize the neuropathological effects of unconjugated bilirubin (UCB) and its free species (Bf) with a focus on the dysregulation of the central nervous system (CNS) cell homeostasis and subsequent toxic paracrine signaling effects. Direct or indirect actions of glial cells on UCB-induced neurodegeneration are also critically reviewed.

Methods: An exhaustive electronic search of the literature was performed with PubMed and Google Scholar on bilirubin neurotoxicity-related topics to identify relevant articles from 1947 to 2021. Languages other than English, German and French were excluded.

Key Content and Findings: We specifically focused on the neurotoxic species of UCB and provided neuro- and gliocentric views in the context of neurodevelopmental alterations. Potential novel neuroprotective and regenerative strategies, including the use of extracellular vesicles (EVs) and their loading with medicines or microRNAs, were also addressed. Our perspectives on the future application of human advanced models and EVs to investigate UCB-induced neurotoxicity/KSD and subsequent pathological insults in early-life and lasting outcomes are outlined.

Conclusions: We believe that this information could provide the next step for newborn screening using promising noninvasive biomarkers in the era of precision medicine to develop new and combinatorial therapeutic approaches at the forefront of translation.

Keywords: Advanced BIND models and therapeutic opportunities; bilirubin-induced neuroinflammation and targets; intercellular (mis) communication by bilirubin; toxic bilirubin and neurodegeneration; UCB-induced glial aberrancies

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Introduction

The features of bilirubin deposition in the brain were initially described by Orth (1) and later designated as "kernicterus" by Schmorl (2) in the last quarter of the 19th century. Today, more than a century later, and despite the extensive research, multiple management recommendations and guidelines (3-8), cases of acute bilirubin encephalopathy (ABE) are still being described during the early neonatal period (9,10), particularly in low- and/or middle-income countries (11-14). Recent reviews recapitulate the spectrum of disorders associated with bilirubin neurotoxicity and kernicterus, highlighting the toxic role of elevated free bilirubin (Bf) levels (15), i.e., unconjugated bilirubin not bound to its main blood transporter, albumin. They also emphasize several risk factors and co-morbidities that can lead to increased concentrations of serum albumin-bound unconjugated bilirubin (UCB), accounting for elevated Bf levels and subsequent neurotoxicities (*Figure 1*). Moreover, the available preventive and treatment options, as well as the recommendations are identified to manage ABE and kernicterus spectrum disorders (KSD) (16-20).

With regards to the neurotoxic actions of UCB, four main factors, acting alone or in combination, are implicated as illustrated in *Figure 1*: increased bilirubin production, impaired hepatic uptake, reduced bilirubin conjugation, and defective liver clearance (21). The excessive production of UCB in the first days of life primarily derives from the relative polycythemia and breakdown of hemoglobin, as well as from the increased red blood cell (RBC) turnover in neonates, with a rate of 6 to 8 mg/kg/day, more than twice the production as adults (22). Bilirubin is generated from heme degradation, catalyzed by heme-oxygenase (HO) to form biliverdin, which is then metabolized by biliverdin reductase (BVR) to Bf or UCB (if bound to albumin) (17). Other risk factors are implicated in UCB overproduction. This is the case for hemolytic diseases, e.g., glucose-6-phosphate dehydrogenase (G6PD) deficiency with increased erythrocyte fragility and hemolysis (23,24). The relative prevalence of G6PD deficiency (25), associated with neonatal hyperbilirubinemia (26) and prematurity (27), makes both conditions significant risk factors. Notably, UCB can bind to RBCs (28,29) causing shape alterations and increased fragility that culminate in increased hemolysis, further enhancing UCB and Bf production (*Figure 1*) (30-33).

UCB dissociates from albumin before entering the liver and may be impacted by decreased delivery or by inefficient hepatocyte uptake due to sinusoidal protein polymorphisms (21,34). Low hepatic gene expression of the bilirubin uridine diphospho-glucuronosyltransferase 1A1 (UGT1A1), as well as UGT1A1 enzyme deficiency in Gilbert's disease (partial) and Crigler-Najjar types I (total, CN1) and II (almost total, CN2) syndromes, impairs bilirubin conjugation with (mostly) glucuronic acid (35-37), thus leading to increased levels of UCB and Bf in circulation. Enzyme polymorphisms may also play a role (35,38). Of note, CN1 syndrome leads to fatal outcomes with kernicteric features, unless liver transplantation is performed (39). Finally, excretion of conjugated bilirubin into the bile, mainly mediated by multidrug resistance-associated protein 2 (MRP2), is a key player for conjugated bilirubin elimination from the liver (40) and stool output (41). MRP2 deficiencies may cause the re-uptake of conjugated bilirubin into circulation (Dubin-Johnson syndrome) and the presence of cholestasis may lead to its elimination in urine (42).

Other main risk factors for bilirubin-induced neurological damage (BIND) during neonatal hyperbilirubinemia are: (I) prematurity that affects all the UCB clearance mechanisms and increase neural cell susceptibilities to its harmful effects (10,43-47); and (II) hypoxia-ischemia (48), sepsis (49), hypoalbuminemia (50) and acidosis (31,51,52) that contribute to increase Bf concentrations and its entrance in the central nervous system (CNS) after crossing the blood-brain barrier (BBB) (*Figure 1*), causing neuronal damage and glial activation.

Breastfeeding has also been associated with an increased incidence of hyperbilirubinemia, but the causes for "breast milk jaundice" or "breastfeeding failure jaundice" are not completely clear (53,54). However, an association with intestinal flora colonization status has been recently described (55,56). This may be important since it has been reported that the lack of microbiota in jaundiced babies may lead to the reabsorption of non-polar UCB in the intestine and may contribute to the development of BIND (57).

A less considered risk factor for UCB neurotoxicity in neonates is the apparent lack of societal awareness for this condition (58), together with early discharge policies practiced by birthing centers and maternity services that impair early detection and timely therapeutics, which are crucial to prevent UCB encephalopathies (59).

In summary, newborn infants overproduce UCB and have a decreased ability to eliminate UCB, thus increasing their susceptibility for UCB-induced neurodegeneration, oligodendrocyte dysfunction, astrocyte reactivity, and microglia activation in specific brain regions, which can lead

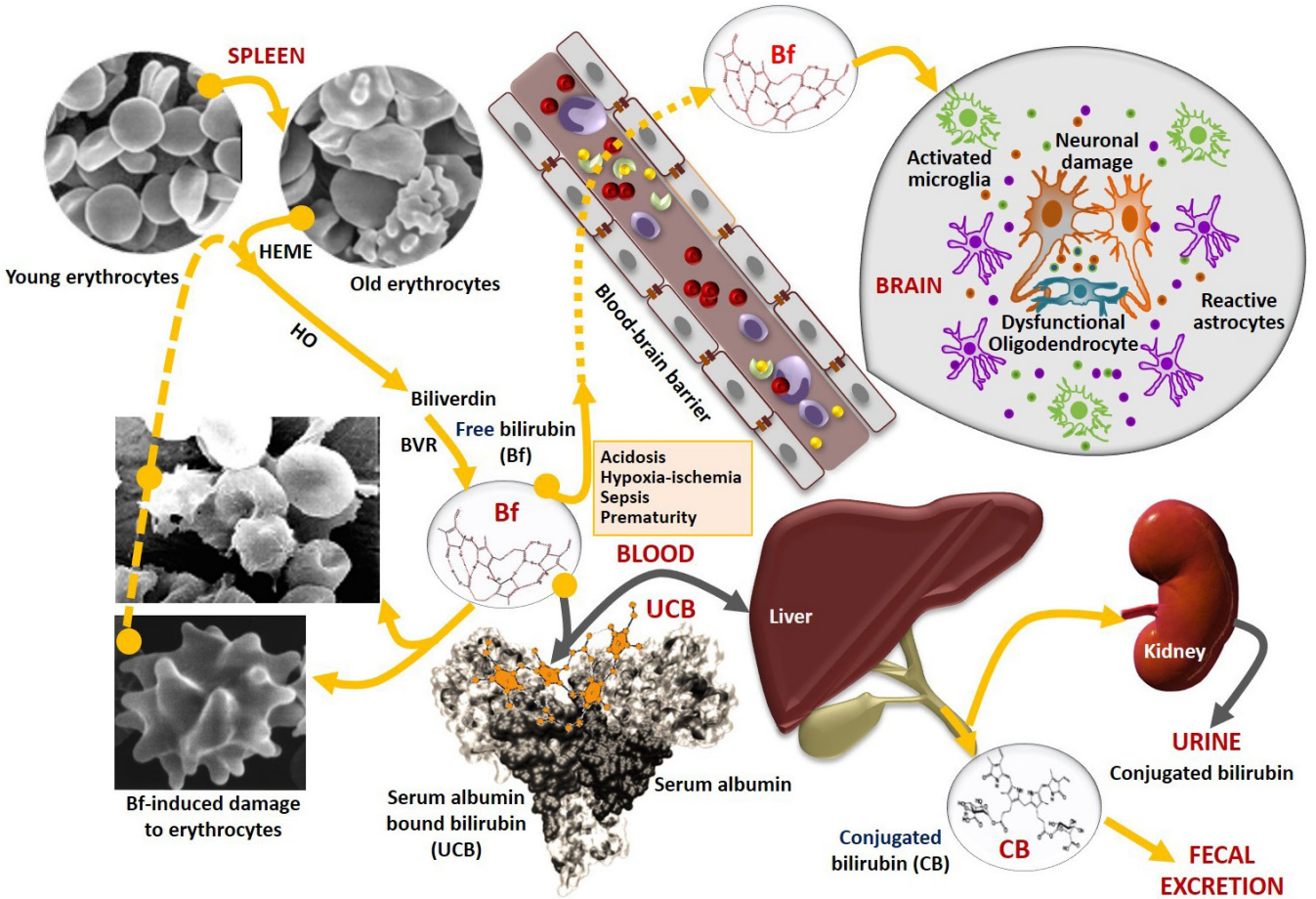

Figure 1 Schematic representation of bilirubin production, transport, conjugation, and clearance, highlighting the distribution of the circulating species, risk factors and passage across the blood-brain barrier into the brain. Bilirubin is mainly produced by the degradation of hemoglobin prosthetic group, Heme from erythrocytes reaching its lifespan (old), by the enzymatic action of heme oxygenase (HO), orignates biliverdin, which is then immediately converted to bilirubin by biliverdin reductase (BVR). Bilirubin not bound to albumin (free, Bf) is in equilibrium with those bound to albumin (UCB), which is transported from the blood circulation into the liver for conjugation with the glucuronic acid mediated by the bilirubin uridine diphosphoglucuronosyltransferase 1A1 (UGT1A1), to form conjugated bilirubin (CB). CB is then excreted into bile and its final degradation products eliminated in feces. In cholestatic conditions, CB may return into the circulation and be excreted in urine. When UCB is overproduced and exceeds the albumin binding capacity, Bf concentration raises, binds to erythrocytes and causes hemolysis, as well as crosses the blood-brain barrier, mainly in the presence of risk factors like acidosis, hypoxia-ischemia, sepsis, and hypoalbuminemia. In the brain, Bf interacts with neurons and glial cells (astrocytes, microglia, and oligodendrocytes) causing several neuropathological sequelae.

to neurological sequelae with different severe long-term morbidities.

This review summarizes the concepts associated with UCB, Bf, and erythrocyte-linked neurotoxic species, using descriptive neuro- and gliocentric views, and addressing the key role of intercellular paracrine dysregulation to homeostatic imbalance and BIND. Future research using human advanced models and extracellular vesicles (EVs) to clarify pathological mechanisms associated with BIND and long-term sequelae are outlined, and the relevance of their use as new therapeutic tools in personalized medicine are also addressed.

We present the following article in accordance with the Narrative Review reporting checklist (available at https://pm.amegroups.com/article/view/10.21037/pm-21-37/rc).

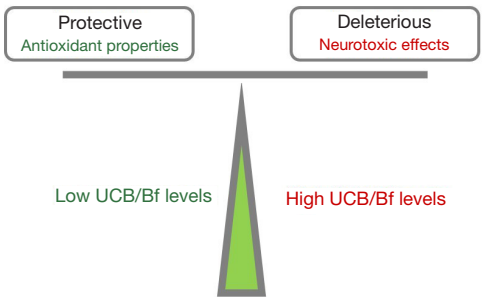

Figure 2 The beneficial and harmful double-edge sword effects of unconjugated bilirubin (UCB) in oxidative and neuroinflammatory conditions accordingly to its low and high concentrations. When at physiological or slightly elevated levels, UCB and its free species (Bf) not bound to human serum albumin exert antioxidant and anti-inflammatory protective mechanisms. In contrast, their increased concentrations are deleterious to the brain, involving neurodegeneration and glial activation or even causing death or permanent severe outcomes.

Methods

We conducted an exhaustive literature search on the electronic databases including PubMed, MEDLINE and Google Scholar, from January 1947 to April 2021, to identify all relevant studies mentioning serum bilirubin in relation to neonatal hyperbilirubinemia, bilirubin encephalopathy and kernicterus. A combination of the search terms included UCB-induced neurodegeneration, unbound or free bilirubin, bilirubin binding to albumin, bilirubin binding capacity of albumin, bilirubin-induced neurotoxicity, red cell binding of bilirubin, erythrocyte-bound bilirubin, neonatal jaundice, bilirubin metabolism in infants, bilirubin uptake, conjugation and clearance by the liver, bilirubin encephalopathy, kernicterus, oligodendrocytes, astrocytes, neurons and microglia. Languages other than English, German and French were excluded. Selection included clinical, animal and cellular studies. The selection process was conducted by both Authors, following agreement on search criteria, and selected references shared in a common database. The reference list also included studies identified manually, and studies referenced for other purposes.

Pathological implications of neurotoxic bilirubin species

Before addressing the neuropathological effects of the increased concentrations of UCB and Bf, it is perhaps worthwhile to describe the controversies regarding the beneficial and harmful effects of UCB, which directly depend on its "physiological" (slightly elevated) or markedly increased concentrations. The pleiotropic role of UCB at low levels as an antioxidant (60-62), though still controversial (63,64), and its anti-inflammatory effects (65-67), have contributed to a poor understanding, and sometimes even a dismissive attitude towards the harmful consequences of high UCB levels, either in neonatal life or as a consequence of inherited unconjugated hyperbilirubinemias, such as Gilbert, CN1 and CN2 syndromes. As an example, a shift between antioxidant and pro-oxidant actions may occur between intracellular UCB values of 7 ng/mg protein and those above 25 ng/mg protein, respectively (68). The dual effects of UCB (*Figure 2*) are even more difficult to understand when several therapeutic approaches have used low concentrations of UCB to treat several pathologies based on its antioxidant and anti-inflammatory properties (66,69-74). However, the harmful effects of UCB at high concentrations and the severe neurological consequences that unfortunately still occur should not be disregarded (75).

For that reason, we will focus on neuropathological issues, brain lesions and sequelae resulting from ABE and chronic kernicterus most often associated with bilirubin levels of 19 mg/dL or higher (16), which have been mostly addressed (53). In contrast, the life-long consequences of moderate levels of total or UCB, such as those surpassing 5 mg/dL in the first 2 to 4 days and up to values below 18 mg/dL (76,77) on the CNS are still unknown. One consequence is the association between neonatal hyperbilirubinemia and autism spectrum disorder (78), which has been only suspected before or even denied (53,79). Auditory brainstem function is also impaired in neonates with hyperbilirubinemia (80), and hearing screening tests have shown a relevant association between bilirubin levels and abnormal auditory activity in jaundiced newborns (81) that have been associated with KSD (5,16,82,83). As would be expected, the risk for auditory damage is increased in preterm infants, where bilirubin levels considered "safe" for term babies can lead to irreversible lesions (84). Alterations of the sensorimotor system due to elevated UCB concentrations assessed at several developmental ages have been reviewed by Lunsing (85). Abnormalities in the visuocortical function were observed at 3 months of age in children who had total bilirubin levels between 10 and 25 mg/dL at postnatal (PN) day 3 (86). Delayed

neurodevelopmental outcomes at 6 months (87) and 1 year of age (88) were also found in term newborns, even with moderate hyperbilirubinemia accordingly to recent prospective cohort studies performed in India.

Though usually not associated with cognitive abnormalities, the literature is divided on this issue (89-93). Neurobehavioral disabilities with lower rates of school completion and full-time employment, as well as reading difficulties, were related to the existence of hyperbilirubinemia (94). Interestingly, a recent study using hippocampal neurons and animal models revealed that UCB induces the deposition of the amyloid-β (Aβ) peptide and tau hyperphosphorylation, establishing a link between an early exposure to bilirubin and Alzheimer's disease (AD) features in later life (95), thus reinforcing its long-term effects. In a later study, high UCB levels, together with decreased serum concentrations of albumin, were found in dementia patients with Aβ and intravenous administration of albumin produced beneficial effects on daily function and dementia severity in AD patients (96).

In conclusion, neonatal-associated UCB and BIND may contribute to auditory and motor deficits (97), but also be associated with developmental delay, cognitive impairment, behavioral problems as well as poor executive function, and psychiatric disorders (98,99).

Free and erythrocyte-bound bilirubin

Bf was first designated as the fraction of bilirubin that was not conjugated and bound to albumin, distinct from the conjugated species. This concept was introduced in 1958 with an Italian publication (100), followed by a French one (101) (and many others) until 1969 to 1972, when the low concentration of the non-protein-bound bilirubin species started to be estimated and was determined to be around 10^{-10} or 10^{-9} mol/L (102,103). The authors, at that time, designated this fraction as Bf or unbound and suggested that it could increase under some conditions to 10^{-6} or 10^{-5} mol/L. First determinations used the Sephadex G-25 elution technique for the separation of Bf and albumin-bound bilirubin (104,105) and the enzymatic oxidation with hydrogen peroxide and horseradish peroxidase (106). The former method was even commercialized and recognized as a valuable aid to neonatologists in preventing bilirubin encephalopathy (107,108). Then, when the two processes were compared, the peroxidase method was found to require less volume of serum and to be more sensitive for the assessment of Bf concentration (109). All these studies were fundamental to finally separate two species of UCB, the one bound to albumin and the free species, which is the most toxic fraction (15,110,111). Determination of Bf and the estimation of reserve albumin binding capacity was then complemented by the erythrocyte-bound bilirubin (29,112). All these methods were thereafter reviewed (113). Now, some studies have assessed the modifications caused by the binding of bilirubin to erythrocytes, either for morphological changes or induced hemolysis, and consequences that it could have in aggravating the risk of BIND (*Figure 1*) (30,114). Another important contribution to the relevance of the toxic levels of Bf was the understanding about the bilirubin displacement from albumin by competitive binding of endogenous compounds and several drugs that promoted an increase of its levels (106,115-118).

Neurocentric view of BIND

The notion that UCB reaches the brain by crossing the BBB was probably concluded from studies performed in the mid-1960s using various animal models of experimental bilirubin encephalopathy (119). Such studies also suggested that neurologic damage was related to UCB concentration in the brain. Furthermore, UCB seemed not to be a passive player in BBB dynamic properties, but it possibly could trigger several damaging mechanisms that impair the barrier function at the level of brain microvascular endothelial cells (120) in a time-dependent manner (121). These effects were observed both *in vitro* and in *post-mortem* brain sections of infants with kernicterus (122).

Once in the brain, UCB interacts with neurons and may cause irreversible damage. Initial studies in experimental kernicterus already proposed that UCB diffuses through the neuroplasm, interacting with the Golgi complex, neurotubules, and endoplasmic reticulum (ER) of neurons, diffusing into the axoplasm and causing axonal destruction (123). Another long-recognized target for UCB is the mitochondria, where UCB damages respiration, uncouples oxidative phosphorylation, and induces brain mitochondrial swelling, even at low micromolar concentrations (124,125). We found that UCB also impairs the mechanisms associated with mitochondrial fusion-fission dynamics as depicted in *Figure 3* (unpublished data), which are associated with the maintenance of cellular quality (126). Elevated mitochondrial fusion, here assessed by mitofusin 2 protein immunostaining, favors the generation of interconnected mitochondria to increase cell bioenergetics efficiency when facing an insult

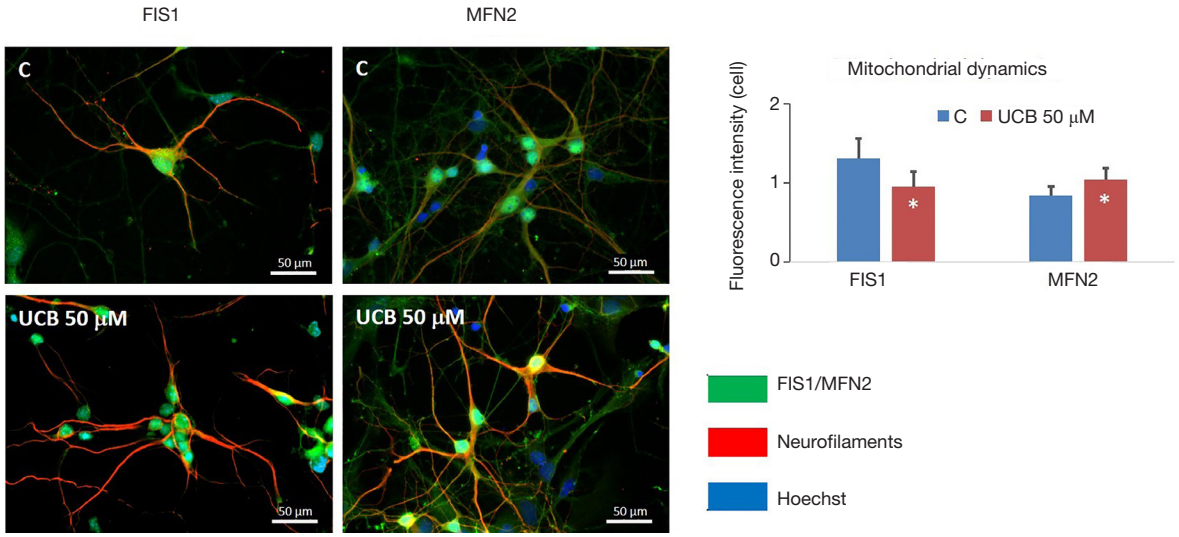

Figure 3 Alterations in the mitochondrial mechanisms of fusion-fission dynamics induced by unconjugated bilirubin (UCB) in rat cortical neurons. Neurons were incubated for 4 h at 37 ℃ with UCB at 50 µM plus 100-µM human serum albumin (HSA), and data compared with controls (cells with albumin, but no UCB added). Bars represent the mean fluorescence values (± SEM) from at least four different microphotographs and normalized by the number of cells in each photograph for fission 1 protein (FIS1) and mitofusin 2 protein (MFN2). *, P<0.05 *vs.* control (C). Unpublished data obtained by RFM Silva at the D Brites laboratory.

as a cell survival mechanism (127). In contrast, fission that we determined through the expression of the mitochondrial fission 1 protein (FIS1) is associated with numerous mitochondrial fragments and its decrease may lead to reduced mitochondria motility (128). UCB also triggers mitochondrial membrane permeabilization, with the release of cytochrome *c*, and activation of caspases 3 and 9, that culminate in neuronal apoptosis as described previously (129,130) and us as well (131).

Although the exact toxic mechanisms are still not clear, it is becoming apparent that UCB impairs neuronal cells by a plethora of effects that eventually culminate in cell death by necrosis- and apoptosis-like mechanisms (132,133), which may involve glutamate excitotoxicity (134). In fact, several reviews describe multiple neurotoxic mechanisms for UCB, like inhibition of neurite outgrowth and ramification (135), alteration of neuronal membrane microfluidity, impairment of axonal arborization, and increased nitrosative stress (136) that, together with glutamate, seem to mediate arborization impairment (137) and to alter synaptic transmission (138). Not surprisingly, immature cells appear to be more sensitive to UCB neurotoxicity (139,140), correlating with the proposed age-related window of susceptibility to UCB neurological damage (141). It is important to note that most of these results were obtained in experimental conditions that mimic the true pathophysiological conditions, i.e., with clinically relevant molar ratios of UCB compared with human serum albumin (HSA), avoiding excessive aggregation and precipitation (142).

Studies using live calcium imaging reinforce the role of ER stress in UCB neurotoxicity to hippocampal neurons previously described as one of the most UCB-susceptible neuronal subpopulations (143,144), with a disruption of calcium homeostasis in neuronal cells, but not in astrocytes. This agrees with the previous report of Qaisiya *et al.* showing the involvement of ER stress in neuroinflammation and apoptosis in the SH-SY5Y differentiated neuronal cells (145). Interestingly, a similar mechanism involving ER stress and calpain (an intracellular Ca^{2+}-dependent cysteine protease) was shown to interfere with oligodendrocyte maturation by UCB-induced demise of oligodendrocyte precursor cells (*Figure 4*) (146), impacting axonal myelination (147) and thus, potentially disturbing axonal conduction and, consequently, neuronal communication.

Other novel findings highlight the potential of UCB to disrupt neuronal communication, such as the inhibition of lipid raft-dependent functions at the specific level of the nerve cell adhesion molecule 1 (L1) that is involved in neuronal signaling (148). Using patch-clamp techniques, Shi *et al.* found that UCB increased the spontaneous firing rates of neonatal neurons in brainstem slices in a calcium-

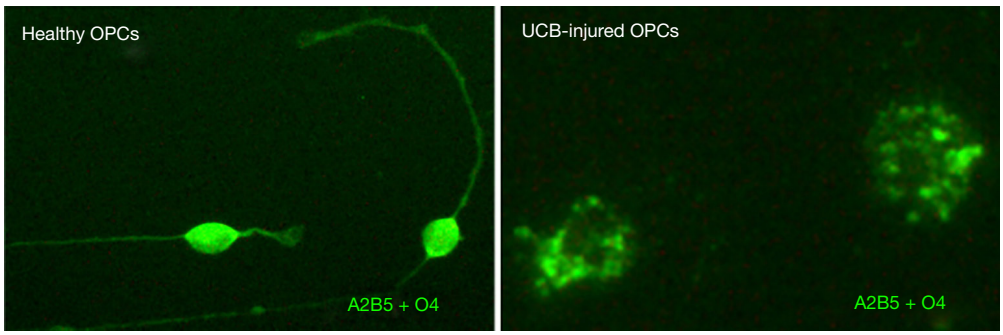

Figure 4 Unconjugated bilirubin (UCB) is harmful to oligodendrocyte progenitor cells (OPCs). Isolated OPCs were incubated for 8 h at 37 ℃ in the absence (healthy) or in the presence of UCB at 50 µM plus 100-µM human serum albumin (HSA) (injured). Cells were immunolabelled with specific antibodies, A2B5 for OPCs and O4 that stains the transition from OPCs to differentiated oligodendrocytes. Representative pictures are shown. Magnification: 630×. Unpublished data obtained by A Barateiro at the D Brites laboratory.

dependent manner, upregulating the voltage-gated sodium channels by promoting their recruitment to the neuronal membrane (149). Furthermore, Albanna *et al.* showed that moderate UCB levels were able to modify the function of voltage gated Cav2.3 calcium channels, impairing neurotransmission in retinal neurons (150). Moreover, using a mouse model of neonatal hyperbilirubinemia, it was observed that UCB-induced oxidative stress may damage cerebellar DNA (151), which may then contribute to neuronal cell death.

Finally, a link between maternal micronutrients, the nutritional status of the newborn (152) and the deficient enzymatic antioxidant defenses implicated in neuronal damage by UCB (153), may additionally constitute targets for therapeutic interventions in the management of BIND, by potentially exerting protective and regenerative effects.

Gliocentric view of BIND

Besides neurons, it is now recognized that glial cells are relevant players in most neurodegenerative diseases, contributing to the initiation and/or propagation of neuropathological cascades, either by gain or loss of function (154). Actually, glial cells, once considered as the glue between neurons, are presently acknowledged as key players in the brain immune system and in multiple physiological processes linked to synaptic plasticity, energy metabolism, learning and memory formation, among others (155). The intricate balance of homeostatic and inflammatory functions influences the onset and the progression of neurodegenerative diseases (156). Moreover, neurological disorders usually involve feedback loops that disseminate and perpetuate the disease (157), mostly mediated by the cell-secreted soluble factors and release of small (exosomes) and large EVs (158,159), already observed in the cerebrospinal fluid (CSF) of patients with ABE (160). We propose that neuronal selectivity in BIND converges with non-cell autonomous mechanisms involving signaling mechanisms and non-neuronal cell types, thus requiring a better understanding. In this section, we will address data on glial sensitivity to UCB, i.e., the view of a more integrated "gliocentric brain" (161), providing further information on targets to unravel and prevent UCB brain lesions and their sequela, and then assist in the insult recovery.

Myelin damage

The myelinating cells of the CNS, the oligodendrocytes, are generated from bipolar oligodendrocyte progenitor cells (OPCs) that arise between 10 and 18 weeks of gestation in humans (162,163). Maturation of oligodendrocytes start at 28 to 40 weeks of gestation and proceeds during the early postnatal period (141). Oligodendrocytes constitute 5% to 8% of total glial cells (163). The first report on ultrastructural changes in the Gunn rat with bilirubin encephalopathy identified the presence of myelin debris in the cytoplasm of neurons, which also presented mitochondrial alterations and glycogen-filled vacuoles (164). UCB was shown to bind to myelin and was suggested to be associated with its retention in the brain (165,166). However, UCB also caused cerebellar myelin fragmentation in *in vitro* cultures (167), and myelin loss was observed in biopsy samples from a kernicteric preterm infant (168). Lesions in the myelin sheath of

spiral ganglion cells were observed in neonatal guinea pigs exposed to hyperbilirubinemia. Neuroimaging studies in infants at risk for kernicterus identified white matter abnormalities (169). When assessed for *in vitro* effects, UCB was shown to impair OPCs (*Figure 4*) (146) and oligodendrocytes (170), as well as to disturb the differentiation of OPCs into myelinating oligodendrocytes (147). Further studies, using rat organotypic cerebellar slices demonstrated that treatment with 20-nM Bf led to a reduction in the number of myelinated fibers, together with the gene expression of the myelin basic protein (171). The data validate that concentrations mimicking neonatal unconjugated hyperbilirubinemia impair myelination. Using a new kernicterus mouse model with *Ugt1a1* gene deletion, it was possible to confirm the presence of cerebellum atrophy by the elevated UCB concentrations, together with axonal loss and decreased myelination, which was similarly noticed in the medulla oblongata and pons, but not in the corpus callosum (172). In summary, deficits in myelination should be considered as targets when developing new therapeutic strategies for BIND.

Microglia polarization

Microglia are the resident macrophages of the CNS that are derived from the yolk sac and travel to the brain during early development (173). Microglia represent 5% of total glial cells in the human cortical brain (174) and show phenotypical heterogeneity, regional diversity, and are highly complex and dynamic and with interchangeable phenotypes (175) (*Figure 5*). Microglia release interleukin (IL)-1β and tumor necrosis factor-alpha (TNF-α), among other cytokines (176), which regulate homeostasis or are involved in neuroinflammation and pathology. Besides phagocytic and pruning functions, microglia regulate myelin uptake, neurogenesis, and cerebral angiogenesis (175). The first data on possible lipid droplet-accumulating microglia were obtained in the Gunn rat cerebellum in 1986 (177), a model of CN1. In conditions leading to HO-1 induction, producing biliverdin from heme (*Figure 1*), this enzyme was found to be mainly localized in microglia and involved in their activation, but it is unclear whether this might lead to beneficial or harmful effects (178-180). A pioneer study showed that UCB activates microglia leading to the release of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, as well as glutamate, while also inducing cell death by apoptosis and necrosis (181), suggesting that these cells may have an important role in BIND and, consequently, are promising targets to modulate excessive neuroinflammation. Certain UCB photoproducts also produce neuro-inflammatory effects that may even surpass those of 140-nM Bf (182). Therefore, it is not surprising that microglia are activated after intracerebral hemorrhage and can be associated with bilirubin production in the CNS and its oxidation products (183), while also facilitating early inflammation by neutrophil brain infiltration (184).

Interaction of UCB with microglia first impacts on protective mechanisms associated with the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF-κB), together with increased phagocytosis, and later release of pro-inflammatory cytokines (185). In early responses to UCB, microglia may then have a protective intervention (185,186). However, in chronic or long-lasting hyperbilirubinemia, such benefits may no longer be supported (187-189). Therefore, the good may turn bad with the release of excessive inflammatory mediators. Actually, microglia are known by their dual neuroprotective and neuroinflammatory roles among the kaleidoscope of polarized phenotypes (190) (*Figure 5*). In the steady-state, microglia have a ramified morphology with highly motile processes constantly surveying the neighboring environment. Changes in brain homeostasis leads to alterations in microglia shape and process motility. The acquired amoeboid morphology is associated with phagocytic ability and mild inflammation, the rod-shape with activation by mild neurodegeneration, and the hypertrophic with excessive immune reaction. When damaged by chronic insults or senescence, microglia become dystrophic and are ineffective in supporting neural cell homeostasis (191-199). Using transcriptional single-cell sorting, it was possible to identify several immune-related classes and disease-associated microglia (DAM) phenotypes, based on a specific set of genes found in AD models and patients (200-202).

Activation of microglia was observed in the hippocampus and cerebellum of mice with hyperbilirubinemia (172,203,204) and in rat cerebellar slice cultures treated with UCB (171), where the induction of excitotoxic and neurodegenerative processes were identified. However, we still need to better understand microglial population diversity, in which each member may perform unique functions in a disease-context-dependent fashion (205). As already mentioned, senescent microglia associated with aging and AD (192,206,207) show loss of function and chronic release of pro-inflammatory mediators. Caldeira

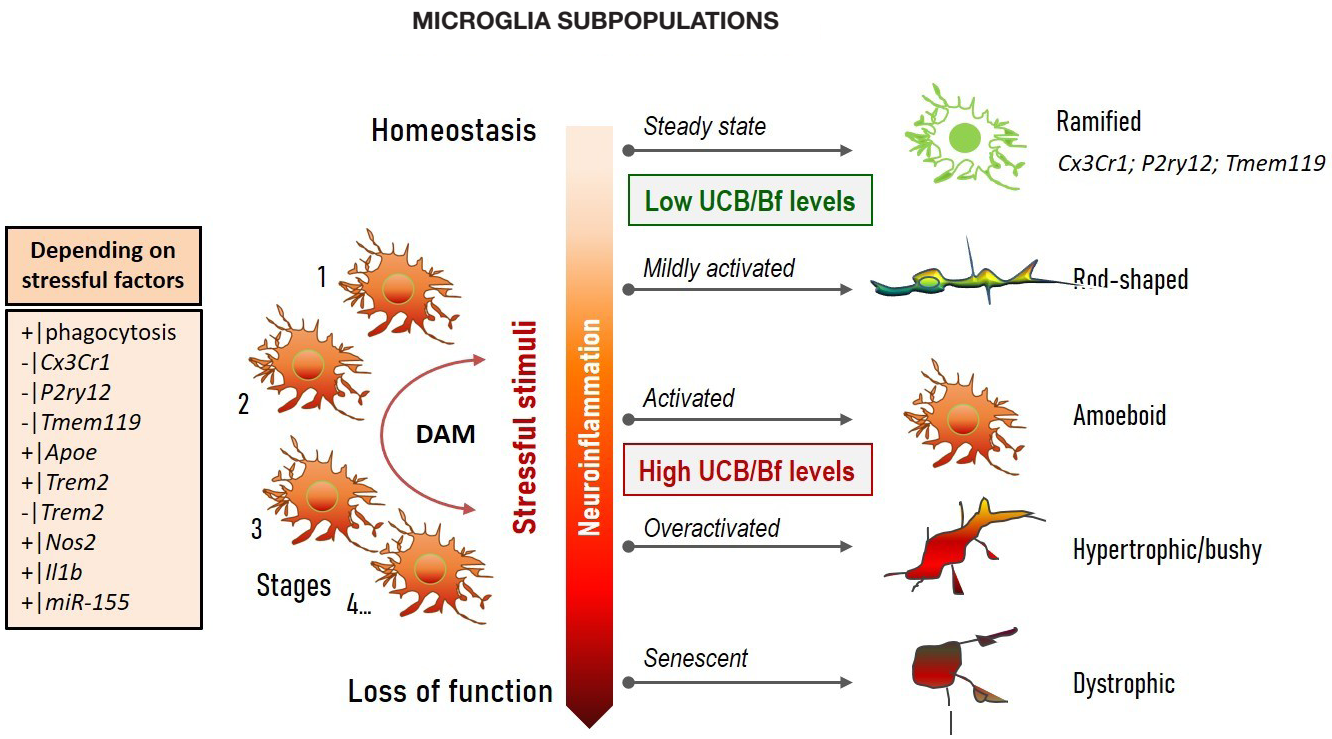

Figure 5 Simplified microglial phenotypic categorization in homeostatic and in inflammatory conditions, accordingly to intensity, type, and duration of unconjugated bilirubin (UCB) and/or free bilirubin (Bf) treatment. Homeostatic microglia are known for their immune surveillance and regulation of neural cell networks, with a ramified morphology and motile processes. The bipolarized or rod-shaped microglia are highly proliferative, express both pro- and anti-inflammatory markers, and are associated to mild neurodegeneration and repair. Phagocytic and activated microglia reveal an amoeboid shape with retracted processes. Hypertrophic and "bushy" microglia have short and poorly ramified processes and are associated with cell activation/overactivation. While early microglia activation is important to restore brain homeostasis, if chronically activated, they continuously release pro-inflammatory molecules that further increase tissue damage. Dystrophic microglia relate to a less responsive or ineffective supportive cell showing loss of processes, cytoplasmic fragmentation, and spheroid morphology. Evidence demonstrated that microglia may co-exist in different phenotypes (reparative, inflammatory, and senescent-like). Diverse activation stages occur after the transition from the steady state into a disease-associated microglia (DAM) population. Transcriptional signatures may vary in a context-dependent fashion and in the case of UCB/Bf stimulation, with the brain region, jaundice severity, and presence of co-morbidities.

et al. developed an *in vitro* microglia model able to mimic "young/responsive" and "old/senescent" microglial features (194). These authors using *in vitro* aging microglia were able to discriminate age-dependent responses by Aβ (195). When such a model was used to assess Bf-induced responses in each of the conditions, increased sickness prevailed in the younger microglia, as compared with the older cells (208), and included enhanced amoeboid morphology, NO release, and elevated high mobility group box protein 1 (HMGB1), TNF-α, and IL-6 gene expression levels. Among the vast number of small non-coding short RNAs (miRNAs) controlling post-transcriptional expression of target genes, some were recognized as inflammatory associated miRNAs (inflamma-miRNAs), and were accepted as key players in microglia function/dysfunction, polarization, and restoration (209). Among those, upregulation of miRNA(miR)-155, miR-125b, miR-21, and miR-146a by Bf was only observed in the "young" microglia, pushing the cell phenotype to an immune-polarized state, and indicating their propensity to be stimulated by Bf. However, Bf seemed to also induce a sort of microglia activation, independent of the age of the cells, based on an induced increased of CD11b staining (associated with a proinflammatory status) and on the elevation of

inducible nitric oxide synthase (iNOS) gene expression at a 100-nM concentration. On the contrary, cells behaved differently with early apoptosis exclusively noticed in "younger" microglia, and late apoptosis/necrosis only in "older" cells. Data have shown that microglia reveal age-dependent performance when stimulated by bilirubin, with beneficial and pathological properties that may vary with co-morbidities, CNS region, neurodevelopmental stage, cell maturation, jaundice duration, and hyperbilirubinemia intensity.

Astrocyte aberrancies

Astrocytes comprise nearly 35% of the total CNS population, and like microglia, they may be found in all CNS regions. Astrocytes participate in neuroinflammatory responses and show diverse subtypes that are disorder- and context-specific (210,211). Some of the biomarkers more often used in their characterization are glial fibrillary acidic protein (GFAP), S100B, glutamine synthetase, or the glutamate transporters, GLT1 and GLAST (210,212). One of the first studies using mixed fetal rat glial cells, in which 80% to 95% of cells were astrocytes, identified morphological and cytotoxic alterations, as well as age-in-culture-dependent sensitivity, when working with UCB/HSA ratios of 2, i.e., recapitulating severe hyperbilirubinemia in neonates (213). The study called attention for the higher susceptibility of immature neural cells to UCB harmful effects. The idea was later reinforced by several studies in primary cultures of astrocytes, as well as in neurons and microglia (46,47,140,208,214,215). By using the MTT assay for cell viability and mitochondrial activity assessments, Chuniaud *et al.* attributed the cytotoxicity of UCB to mitochondria failure (216). Astrocytes have a wide range of relevant functions in the brain that contribute to maintain extracellular homeostasis (217), such as their ability to uptake glutamate, thus preventing its accumulation at synapses and resulting excitotoxicity (218). UCB inhibited glutamate uptake and cell endocytosis, when rat cortical astrocytes were used (219,220). Interestingly, while the inhibition of glutamate was higher in astrocytes than in neurons, cell death and changes in redox stress were prominent in neurons (133,221), suggesting cell pathological susceptibilities. Astrocyte increased resistance may derive from the elevated expression of the multidrug resistance-associated protein 1 (MRP1) that was shown to be promoted by UCB (222).

Astrocytes detect infection and injury by neurons, microglia, oligodendrocytes, and endothelial cells, with the secretion of cytokines and growth factors that may act as immune regulators, following the activation of NF-κB. They are accepted as initiators and responders to inflammation, namely to mediators released by the activated microglia, such as IL-1β, TNF-α, and interferon gamma (IFN-γ), and they react to lipopolysaccharide (LPS) (223-226). Interestingly, UCB at 50-μM (UCB/HSA=0.5) caused apoptotic cell death and TNF-α secretion from rat cortical astrocytes, in a similar way to that of 10 ng/mL of LPS. They reacted strongly to UCB, then to LPS, in terms of necrosis, as well as to the secretion of IL-1β and glutamate, and less to the release of IL-6 (227). The cytotoxicity of UCB in astrocytes was also observed with serum from infants with unconjugated hyperbilirubinemia (228). As expected, UCB activated astrocyte signaling pathways associated with MAPKs, i.e., p38, Jun N-terminal kinase (JNK)1/2 and extracellular signal-regulated kinase (ERK)1/2 pathways, as well as the key player NF-κB (229), which further increased in hypoxia and oxygen-glucose deprivation preconditioning conditions (48). Translocation of NF-κB from the cytoplasm to the nucleus in the cortical astrocytes treated with UCB at 50 μM *plus* 100-μM HSA was shown to peak at 2 to 4 h of interaction (230) (*Figure 6*), much later than in microglia, where the peak was observed 30 min after exposure (185), evidencing the early activation of microglia by UCB. We also noticed an induced release of IL-1β and TNF-α from UCB-treated cortical astrocytes with increased expression of the TNF-α receptor (TNFR)1 and IL-1β receptor (IL-1R)1 (231). Moreover, UCB reduced the cytokine pro-forms while activated their converting enzymes, ICE and TACE, respectively (231,232). ICE or caspase-1 activation that leads to pyroptotic cell death (233), pro-inflammatory processes (234), and inflammasome activation, including the regulator of innate immunity NLR family pyrin domain containing 3 (NLRP3) (235), was demonstrated in cultured rat cortical astrocytes after exposure to UCB (236). Astrocytes, acquire phenotypic aberrancies in neurodegenerative diseases and are activated by neuroinflammation and stressful factors, such as UCB, contributing to pathophysiological paracrine signaling events, mainly mediated by EVs containing miRNAs (237-240). In such a way, dysfunctional astrocytes actively contribute to cell homeostatic imbalance in the brain, as will be further explained in the next section.

Dysregulated neuron-glia interplay: the gearbox?

Brain function depends on coordinated interactions and

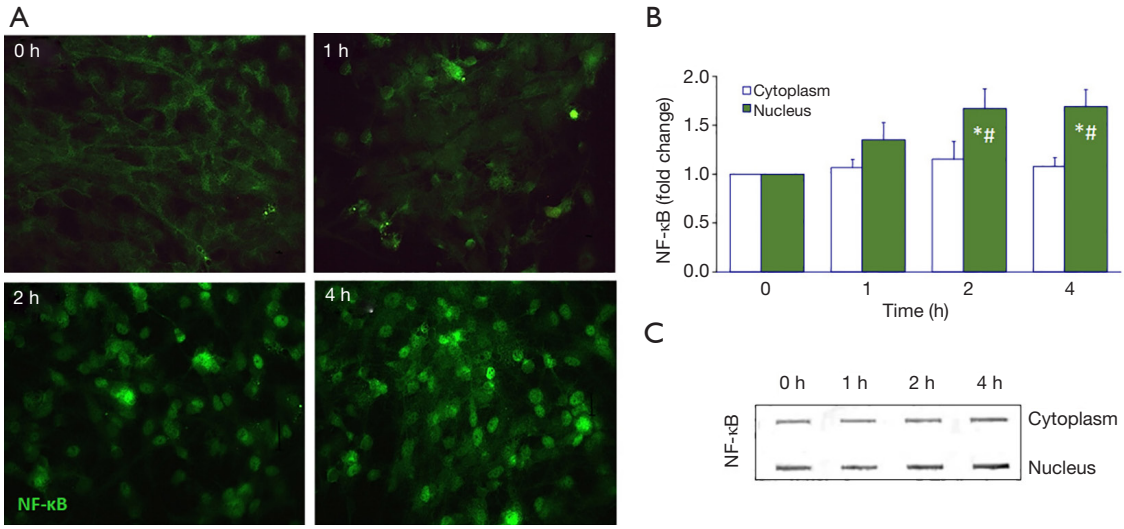

Figure 6 Unconjugated bilirubin (UCB) induces the translocation of nuclear factor kappa B (NF-κB) from the cytoplasm to the nucleus of cortical astrocytes. Astrocytes were incubated for 1, 2, and 4 h at 37 ℃ with UCB at 50 μM plus 100-μM human serum albumin (HSA), and data compared with controls (cells with no UCB added, time =0 h). (A) Representative images of NF-κB immunoreactivity using an anti-NF-κB primary antibody. Magnification: ×400. (B) Bars represent the NF-κB-fold change values (±SD) from at least three independent experiments. (C) Cytosolic and nuclear protein extracts were processed for Slot blot analysis of p65 NF-kB expression. *, P<0.05 *vs.* controls at time point 0 h; #, P<0.05 *vs.* cytosolic extracts. Unpublished data obtained by A Fernandes at the D Brites laboratory.

intercellular signals between neurons and glial cells that sustain cell homeostatic balance (241,242). In disease, secretion of pathological signaling molecules and EVs from donor cells determine autocrine and paracrine signaling dysregulation (243,244). Changes in neuro-immune homeostasis and neuroinflammation may start at the neurovascular unit composed by the BBB elements: (I) on one side, the endothelial cells, pericytes, and the astrocyte end foots; and (II) on the other side the glial cells, neurons, and the extracellular matrix. Disruption of BBB by UCB is well documented (120,245-247) and facilitate the entrance of elevated Bf levels into the brain, its interaction with neuronal cells and the emergence of BIND (*Figure 1*). Disruption of the BBB by bilirubin and hypercarbia/hyperosmolarity (52,248) may also allow the passage of albumin-bound bilirubin (249), though the permeability is higher for the Bf species (250). Within the brain, the binding of Bf to cells is facilitated by acidosis (251), increasing the risk of BIND. Pathological synapse loss and dysfunction depends on the maturation of neuronal circuits, proper function of glial cells, and synaptic refinement (252). By using co-culture systems of dorsal root ganglia neurons from rat embryos with OPCs, it was possible to establish that UCB toxicity on pre-myelinating oligodendrocytes interferes with their maturation and leads to incomplete myelination (147). Considering that oligodendrocytes modulate synaptic transmission through the release of brain-derived neurotrophic factor (BDNF) in the developing brain (253), the delay caused by bilirubin in the maturation of OPCs may then be critical to brain development and BIND disorder.

The concerted activity of neuron-astrocyte communication by neuromodulators, neurotransmitters, glutamate, and calcium (254) is determinant in neurodevelopment and associated diseases (255). It may differ by brain region and with stimulus duration, as observed after injection of bilirubin into the brain (256). Protective astrocyte pathways involve the release of neurotrophic factors, neuropoietic cytokines and a plethora of protective mediators (257), which modulate the propensity of the cells to injury. This was what we observed when the addition of 50-μM UCB plus 100-μM HSA to neuron-astrocyte cocultures did not produce immediate neurotoxic effects (258). Cell recognition triggers pro-survival effects that should have protected cells from UCB injury. Instead, when neuron-astrocyte homeostasis was established for 24 h prior to the addition of the same UCB/HSA molar ratio, then increased neuronal cell death (apoptosis and necrosis), reduced

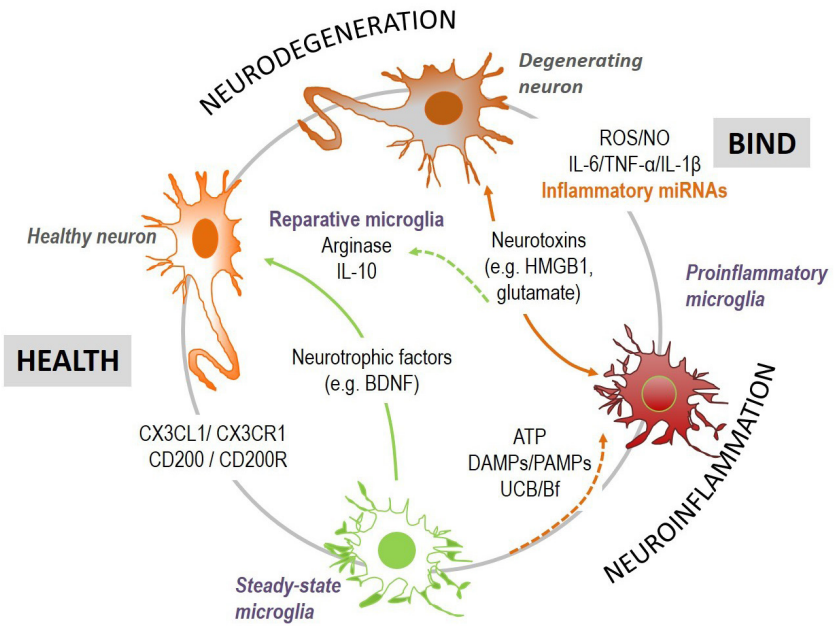

Figure 7 Neuron-microglia interactions in bilirubin-induced neurological damage (BIND) associated to neuroinflammation and neurodegeneration. Microglia-neuronal signaling involves several secretome-associated mediators, including cytokines, chemokines, growth factors, and extracellular vesicles (EVs), among others, that sustain cell homeostasis. Stimulation of neuroinflammation by injury, as that caused by unconjugated bilirubin (UCB)/free bilirubin (Bf) and by infection, leads to neuronal degeneration and microglial activation, which may then switch to reparative microglia or be overactivated. DAMPs, damage-associated molecular patterns; HMGB1, high mobility group box protein 1; IL, interleukin; NO, nitric oxide; PAMPS, pathogen-associated molecular patterns; TNF, tumor necrosis factor; ROS, reactive oxygen species.

neurite extension and ramification, together with S100B and nitric oxide (NO) release, were then revealed (259).

Microglia also modulate CNS homeostasis by: (I) releasing BDNF (that controls neuronal network excitability) (260), secretome functional signaling associated factors and EVs; and (II) bidirectional communication that involves CD200 and fractalkine (CX3CL1) in neurons and the target receptors CD200R and CX3CR1 in microglia (261) (*Figure* 7). During neuroinflammation by damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and ATP, microglia release pro-inflammatory cytokines (e.g., IL-6, TNF-α, IL-1β), inflamma-miRNAs, reactive oxygen species (ROS) and NO, which may either account to injury repair (262), or to neurodegeneration if excessive (263-265). In such dysregulated homeostasis, increased extracellular neurotoxins, such as HMGB1 and glutamate, further contribute to neuronal dysfunction and microglia overactivation (266). In contrast, reparative/restorative microglia from the integration of pro- and anti-inflammatory mediators, expressing IL-10 and arginase (267,268), intervenes in balancing health and disease. Thus, good or bad cellular environmental conditions may affect the cell response to the UCB stimulus. Indeed, astrocytes may either reduce microglia inflammatory reaction (269), or become more reactive when receiving specific inflammatory mediators from the activated microglia (224), aggravating neuroinflammation, and neurodegeneration. Secretome from UCB-treated astrocytes or neurons, added to UCB-treated microglia modulated IL-1β secretion and enhanced phagocytosis (269), highlighting the benefits of homeostatic cell drivers over the UCB immediate microglia cytotoxicity. Thus, cells establish a very strong communication until attaining homeostasis; but also, their crosstalk after UCB insult may act in a synergistic way to cause a neurotoxic environment. In hippocampal organotypic cultures from Wistar rats at 2 and 8 PN days, harmful effects at PN8 were higher than at PN2 after treatment with 140-nM Bf (144), as well as after 14 days in culture relatively to that observed after only 7 days (270). However, we cannot dismiss the

fact that cells may become more susceptible with time in culture. When the effects of microglia in the hippocampus response to UCB was assessed by using microglia-depleted and non-depleted organotypic cultures (PN7-10 plus 72-h slice functional recovery), release of glutamate and NO, as well as cell demise, were higher in the presence of microglia after treatment with 50-μM UCB (at a UCB/HSA =0.5), indicating the joint action of neurons and glial cells in overall nerve cell toxicity surplus (137).

Lately, EVs were shown to be key players in cell-to-cell signaling (209,242,271). These vesicles, besides lipids, proteins, and genetic material, include miRNAs (243) and may release their contents into the extracellular space or into a neighboring cell after fusion or uptake (271), thus sustaining homeostasis or propagating the disease. The small EVs/exosomes produced by neural cells easily cross the BBB and allow reciprocal communication between the CNS and the peripheral circulation, being considered promising biomarkers (272). Profiling of exosomal proteins or miRNAs in serum and CSF showed promise as relevant markers in several brain disorders (273-276). In a recent study, quantitative proteomic characterization of EVs from the CSF of infants with ABE identified the involvement of four proteins associated to immune-inflammation and signaling pathways (160).

In conclusion, neurons and glial cells establish concerted actions that preserve brain function from injury, but also work in a synergistic fashion when the cell homeostatic balance is severely damaged, accounting for the time-dependent aggravating effects of a sustained hyperbilirubinemia. EVs are important players in supporting health or in contributing to disease, and may turn out to be important tools to identify infants at risk of BIND.

Perspectives

Bilirubin encephalopathy and associated KSD have been neglected conditions with limited funding and unsatisfactory health interventions due to insufficient knowledge of the underlying pathological mechanisms. Current available treatment options and potential therapies were recently reviewed (18). Some of the tested interventions include ursodeoxycholic acid (UDCA) or its glycoconjugate (GUDCA) (129,130,139,232,247,277-279), minocycline (280,281), bioactive compounds (282-284), and small-molecule activators (285,286).

Though a few of the strategies were assessed for efficacy in clinical trials (278,279), most were tested in pre-clinical models, from cell cultures to organotypic systems and animal models. Translation of such data to the clinic is a critical challenge that frequently disappoints due to biological discrepancies and different response mechanisms to perturbations among species (287). Reprogramming of dysfunctional neural cells toward pro-regenerative functions, as suggested for microglia (288), may also provide new therapeutic opportunities to prevent excessive UCB-induced neuroinflammation and neurodegeneration. However, strategies able to produce cell revival with modulatory medicines, such as the incorporation of medicines in exosomes, miRNA-based therapies, or cell replacement strategies are innovative approaches that are yet far from being developed or tested in the field of hyperbilirubinemia. The use of fibroblasts from jaundiced infants that can be differentiated into neural cells according to brain regions by reprogramming techniques (induced pluripotent stem cells or iPSCs), or by direct conversion, may also bring new opportunities. These advanced models will be important tools for drug testing, or use in regenerative strategies (e.g., autologous transplantation). Of note, induced hepatocytes from iPSCs transplanted into Gunn rats produced a decline of 30% to 60% of UCB and biliary excretion of bilirubin glucuronides, ameliorating hyperbilirubinemia and showing promise in the treatment of unconjugated inherited liver diseases (289), namely in CN1.

Given the complexity and the multiple factors associated to the risk of BIND or KSD in severe neonatal hyperbilirubinemia, combination of therapeutic strategies might be considered. Circulating exosomes may not only then be used as noninvasive novel biomarkers to help in the clinic, but also for personalized medicine. Engineered exosomes (290), biomimetic exosomes (291), and miRNA-enriched EVs (292), could also contribute to a better cell survival and revival, when used together with the traditional therapeutic interventions, hopefully facilitating neuro-regeneration in infants with bilirubin-associated brain injury.

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Footnote

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