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Neurotoxicity of Unconjugated Bilirubin in Mature and Immature Rat Organotypic Hippocampal Slice Cultures

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Keywords
Bilirubin · Neurotoxicity · Albumin · Neuroprotection · Brain · Organotypic slice

Abstract
Background: The physiopathology of bilirubin-induced neurological disorders is not completely understood. ***Objectives:*** The aim of our study was to assess the effect on bilirubin neurotoxicity of the maturity or immaturity of exposed cells, the influence of different unconjugated bilirubin (UCB) and human serum albumin (HSA) concentrations, and time of UCB exposure. ***Methods:*** Organotypic hippocampal slices were exposed for 48 h to different UCB and HSA concentrations after 14 (mature) or 7 (immature) days of in vitro culture. Immature slices were also exposed to UCB and HSA for 72 h. The different effects of exposure time to UCB on neurons and astrocytes were evaluated. ***Results:*** We found that 48 h of UCB exposure was neurotoxic for mature rat organotypic hippocampal slices while 72 h of exposure was neurotoxic for immature slices. Forty-eight-hour UCB exposure was toxic for astrocytes but not for neurons, while 72-h exposure was toxic for both astrocytes and neurons. HSA prevented UCB toxicity when the UCB:HSA molar ratio was ≤1 in both mature and immature slices. ***Conclusions:*** We confirmed UCB neurotoxicity in mature and immature rat hippocampal slices, although immature ones were more resistant. HSA was effective in preventing UCB neurotoxicity in both mature and immature rat hippocampal slices.

Introduction

Hyperbilirubinemia is the most frequent clinical problem that neonatologists must deal with during the newborn period because under certain circumstances high levels of bilirubin may be potentially toxic to the central nervous system, both in term and preterm infants. In fact, sufficiently elevated levels of bilirubin can lead to acute bilirubin encephalopathy (ABE), bilirubin-induced neurological disorders (BINDs), and subsequently kernicterus, with devastating, permanent neurodevelopmental handicaps or death [1]. Therefore, recommendations

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have been developed to prevent ABE and BINDs by establishing levels of total serum bilirubin (TSB) to indicate risk of neurological damage at which to start treatment with phototherapy, the first-line treatment for neonatal hyperbilirubinemia and, eventually, with intravenous immunoglobulin or exchange transfusions [2].

Jaundice is considered physiologic when TSB level ranges from 5–6 to 7–17 mg/dL in term infants, whereas when TSB is >18 mg/dL, jaundice is considered frankly pathologic [3]. A TSB of 20 mg/dL should not be exceeded in the first 96 h of life in term newborns. After this period, a TSB of 25 mg/dL should be avoided since the efficacy of the blood-brain barrier increases with postnatal age [2]. The safe bilirubin level is lower for preterm infants: 12 mg/dL for infants with a gestational age of ≤30 weeks and 15 mg/dL for infants with a gestational age of 31–36 weeks [4, 5]. However, to establish individual safe TSB levels remains a big challenge and reported numbers have limited evidence base.

The pathophysiology of ABE and BINDs has been investigated in several studies. At autopsy of kernicteric infants, bilirubin is macroscopically visible within the globus pallidus, hippocampus, lateral ventricular walls, cerebellum, and subthalamic nuclei, reflecting its preferential deposition in specific brain areas [6, 7]. However, other evidence has shown that bilirubin can also accumulate in other brain areas, such as the cortex, striatum, midbrain, hypothalamus, cerebellum, and medulla [8]. Bilirubin neurotoxicity occurs by multiple mechanisms including cell membrane perturbation, DNA damage, changes in synaptic transmission, increase in cytokine release, inhibition of neurotrophin-mediated protective signals, and induction of cell apoptosis and necrosis [8].

However, some potential determinants of bilirubin neurotoxicity are particularly interesting from a clinical point of view, such as the maturity or immaturity of exposed cells, the influence of different unconjugated bilirubin (UCB) and human serum albumin (HSA) concentrations, and time of UCB exposure. Thus, we hypothesized that: mature cells might be less susceptible to bilirubin neurotoxicity than immature cells; increasing HSA concentration might decrease bilirubin neurotoxicity; and prolonged time of UCB exposure might increase bilirubin neurotoxicity. To assess these hypotheses, we carried out this in vitro study in rat organotypic hippocampal slice cultures, since brain slice models offer unique advantages over other in vitro platforms in that interactions between neurons or between neurons and glial cells are fundamentally preserved replicating many aspects of the in vivo context [7].

Table 1. Concentrations of unconjugated bilirubin (UCB) and human serum albumin (HSA) in culture media of organotypic hippocampal slices used for experiments (expressed as μM/L, molar ratio, mg/dL) and related calculated unbound bilirubin (nM/L)

UCB/HSA, μM/L/μM/L	UCB/HSA ratio	UCB/HSA, mg/dL/mg/dL	Calculated unbound bilirubin, nM/L
10/100	0.1	0.5850/0.665	6
100/10	10	5.850/0.067	1,140
100/100	1	5.850/0.066	215
100/200	0.5	5.850/1.329	160
200/100	2	11.700/0.665	10,074
200/200	1	11.700/1.329	288

Methods

Organotypic hippocampal slice cultures from the brains of 7- to 9-day-old Wistar rat pups (Harlan, Milan, Italy) were prepared as previously reported [9]. All experiments were performed in primary slice cultures obtained from different litters. Before the experiments, all slices were screened for viability by incubating them for 30 min with propidium iodide (PI: 5 μg/mL); slices displaying signs of neurodegeneration were discarded from the study. All the experiments with UCB were performed under light protection to avoid photodegradation.

We decided to study mature and immature organotypic hippocampal slices to mimic what may occur in term and preterm infants. According to recent studies, the first postnatal week in the rat corresponds to 19–28 weeks of gestation in humans [10]. Thus, we used hippocampal slices after 14 days of culture in vitro, considering them as mature slices, and hippocampal slices after 7 days of culture in vitro considering them as immature slices.

The first set of experiments regarded mature cells. Rat organotypic hippocampal slices were exposed for 48 h to 10–200 μM (0.585–11.700 mg/dL) of UCB (Sigma, St. Louis, MO, USA) and 10–200 μM of HSA (0.067–1.329 g/dL) without changing the medium to assess its possible neuroprotective effect (Table 1).

Under our experimental conditions, the theoretically calculated unbound fraction of UCB concentrations ranged from 6 (UCB:HSA = 0.1) to 1,140 nM (UCB:HSA = 10.0), and was 215 and 288 nM, respectively, at 100 and 200 equimolar concentrations of UCB and HSA, according to the model: $K'_f = B_t - B_f / B_f$ (HSA – $B_t + B_f$), where K'_f is the binding constant of albumin; B_t is the total bilirubin concentration; and B_f is the free bilirubin concentration, as proposed by Weisiger et al. [11] and Ostrow et al. [12]. Thus, our model generally reproduced concentrations of unbound bilirubin which are associated with the development of kernicterus in preterm (15–34 nM) and in term (>68 nM) infants [13].

The effects of glutamate receptor activation as mediators of bilirubin toxicity were evaluated by exposing mature hippocampal slices to 100 μM of UCB alone or plus HSA (100 μM) or AMPA antagonist NBQX (10 μM) or NMDA antagonist MK-801 (10 μM) or mGluR5 antagonist MPEP (30 μM) for 48 h; they were then assessed for neuronal injury using PI fluorescence.

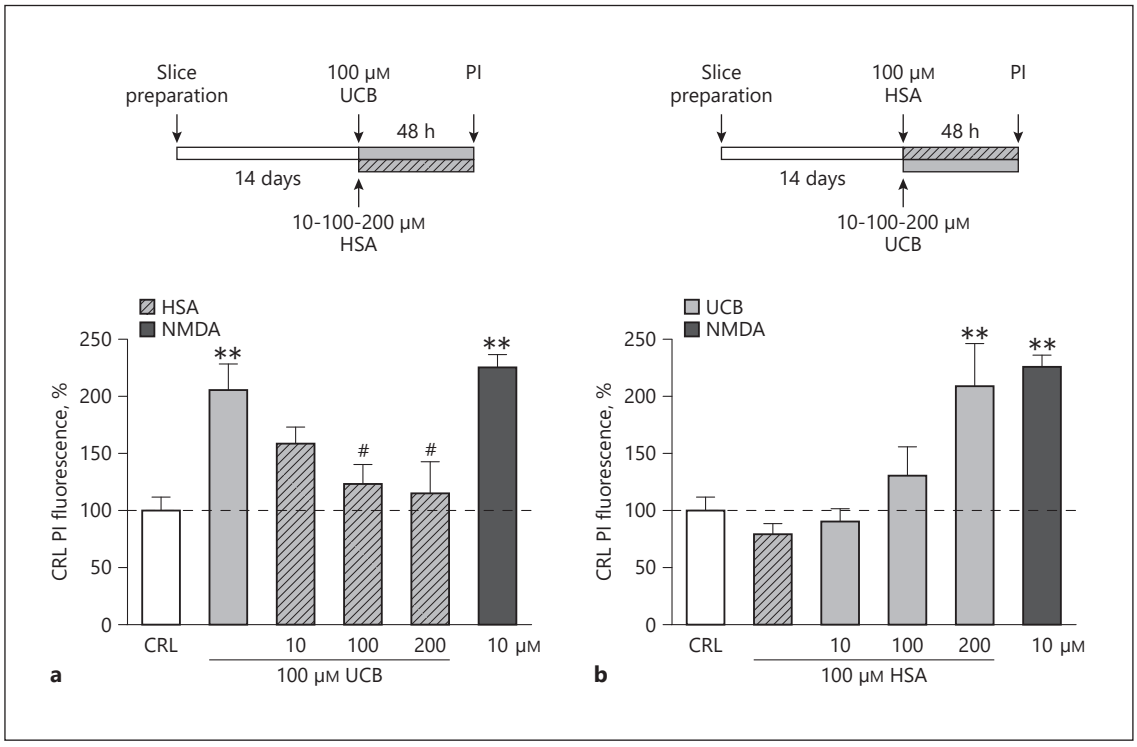

Fig. 1. Neuroprotective effects of human serum albumin (HSA) on unconjugated bilirubin (UCB)-induced toxicity in mature organotypic hippocampal slices. **a** Experimental protocol showing hippocampal slices cultured for 14 days (mature) and then exposed to 100 μM of UCB alone or plus HSA (10–200 μM) for 48 h. Quantitative analysis shows that HSA is neuroprotective against UCB toxicity at equimolar concentration. Quantitative analysis is expressed as percentage of control (CRL) PI fluorescence. Bars represent the mean ± SEM of at least 7 experiments (about ≥28 slices for each experimental point). ** $p < 0.01$ versus CRL, # $p < 0.05$ versus 100 μM UCB (ANOVA + Tukey's *w* test). **b** Experimental protocol showing hippocampal slices cultured for 14 days (mature) in vitro and then exposed to 100 μM of HSA alone or plus UCB (10–200 μM) for 48 h. Quantitative analysis shows that UCB is neurotoxic when present in concentration higher than HSA. Quantitative analysis is expressed as percentage of CRL PI fluorescence. Bars represent the mean ± SEM of at least 5 experiments (about ≥20 slices for each experimental point). ** $p < 0.01$ versus CRL (ANOVA + Tukey's *w* test).

The second set of experiments regarded immature cells. Rat organotypic hippocampal slices were exposed for 48 h to 1–450 μM of UCB after 7 days of in vitro culture without changing the medium. Neuroprotective effects of HSA and effects of glutamate receptor activation were not measured since UCB did not injure immature cell slices. However, to explore other less evident injuries, further experiments were performed only in hippocampal slices of immature cells. Thus, we investigated effects of UCB exposure in promoting cell apoptosis by incubating hippocampal slices for 48 h with 1–300 μM of UCB to measure expression of BAD, cleaved PARP-1, and AIF apoptotic proteins by Western blot analysis. Possible different effects of UCB on neurons and astrocytes were evaluated in CA1 pyramidal stratum in immature organotypic hippocampal slices exposed to 100 μM UCB for 48 h using immunostaining methods and confocal microscopy for quantitative analysis of regions of interest. Electron microscopic evidence for intraneuronal effects of UCB were assessed in CA1 pyramidal cells after incubation for 48 h with 100 μM of UCB. Moreover, further experiments assessed the effect on neurotoxicity of chronic (72 h) exposure to UCB of immature cells to better mimic what occurs in clinical practice. In these experiments, slices were exposed for 72 h to 100 μM UCB and the possible protective effect of 100 μM HSA was evaluated, with or without daily change of culture medium. Effects of chronic exposure to UCB on neurons and astrocytes was evaluated in CA1 pyramidal stratum in immature organotypic hippocampal slices using immunostaining methods and confocal microscopy for quantitative analysis of regions of interest. Slices were exposed for 72 h to 100 μM UCB with or without daily change of culture medium, alone or plus HSA (100 μM). The possible role of glutamate transporters in chronic UCB neurotoxicity was evaluated measuring mRNA expression of glutamate transporter GLAST, Glt1, EAAC1, vGluT1, and vGluT2 genes using the real-time polymerase chain reaction (PCR) and specific primers (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000494101).

Neuronal cell viability injury was evaluated in CA1 pyramidal cells using the PI method (5 μg/mL; Sigma, St Louis, MO, USA).

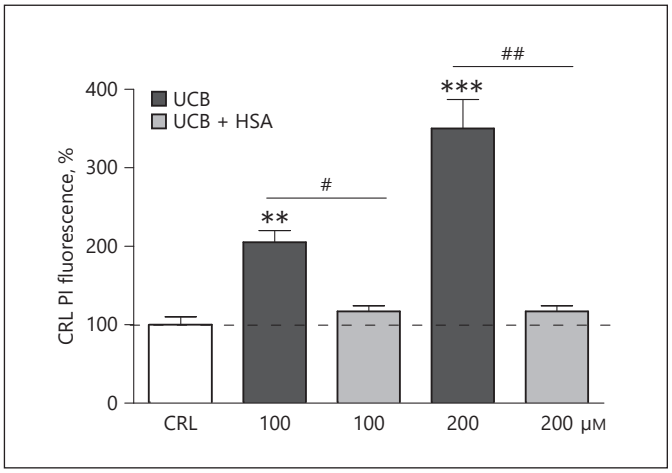

Fig. 2. Human serum albumin (HSA) is neuroprotective against unconjugated bilirubin (UCB)-induced toxicity at equimolar concentration. Mature slices were exposed to equimolar concentration (200 μM) of UCB plus HSA for 48 h and then incubated with propidium iodide (PI) to assess neuronal injury. Quantitative analysis shows that HSA is neuroprotective against UCB toxicity when present at equimolar concentration. Quantitative analysis is expressed as a percentage of CRL PI fluorescence. Bars represent the mean ± SEM of at least 6 experiments (about ≥24 slices for each experimental point). ** $p < 0.01$ versus CRL, *** $p < 0.001$ versus CRL, # $p < 0.05$ versus 100 μM UCB, ## $p < 0.01$ versus 200 μM UCB (ANOVA + Tukey's *w* test).

Statistical Analysis

Data are presented as means ± SEM of *n* experiments. In toxicity, Western blot, and immunofluorescent experiments, each experimental point consisted of 8 hippocampal slices. Statistical significance of differences between PI fluorescence intensities or Western blot optical densities was evaluated by performing one-way analysis of variance (ANOVA) followed by Dunnett's and Tukey's *w* test for multiple comparisons. Statistical significance of differences between immunofluorescence was evaluated by performing ANOVA and Newman-Keuls multiple comparison test. All statistical calculations were performed using GraphPad Prism v.5 for Windows (GraphPad Software, San Diego, CA, USA). A probability value (p) of <0.05 was considered statistically significant.

Results

Mature organotypic hippocampal slices were exposed to increasing concentrations of UCB (10–200 μM) and HSA (10–200 μM) for 48 h to resemble clinical TSB and unbound bilirubin in term and preterm infants (Table 1). We observed that UCB induces a selective CA1 pyramidal cell injury when the UCB/HSA ratio is at >1:1 (UCB/HSA 200/100 μM), whilst it is not neurotoxic when UCB and HSA were equimolar both at 100 (Fig. 1) and 200 μM (Fig. 2). Among glutamate receptor antagonists, we found that NBQX (10 μM) and MPEP (30 μM), but not MK-801 (10 μM), are neuroprotective against 100 μM UCB. This protective effect was similar to that induced by 100 μM HSA (see online suppl. Fig. S1).

Immature organotypic hippocampal slices were exposed to increasing concentrations of UCB (1–450 μM) for 48 h. Toxic effects were not found with the PI method (Fig. 3) nor with immunofluorescence. Moreover, Western blotting experiments demonstrated that UCB (100 μM for 48 h) does not affect the expression levels of the apoptotic proteins BAD, cleaved PARP-1, and AIF (see online suppl. Fig. S2), suggesting that UCB activated neither the necrotic nor apoptotic pathways in neurons. Immunostaining methods and confocal microscopy confirmed that UCB (100 μM, 48 h) is not toxic for immature neurons but evidenced a toxic effect on astrocytes (Fig. 4). However, electron microscopic evidence for intraneuronal effects of UCB reveals that CA1 pyramidal cells exposed to 100 μM UCB for 48 h displayed abnormal swollen mitochondria, smaller Golgi apparatus, swollen dendrites, and reduction of synapses, some of which are depleted of synaptic vesicles, in comparison to healthy CA1 pyramidal cells (see online suppl. Fig. S3). The chronic exposure (72 h) to 100 μM UCB of immature organotypic hippocampal slices was found neurotoxic when a change of culture medium and UCB addition were performed every day. This detrimental effect was prevented by an equimolar amount of HSA (100 μM) (Fig. 5). Chronic exposure to UCB was toxic for both neurons and astrocytes (Fig. 6). Indeed, we performed real-time PCR by measuring the levels of mRNA expression of glutamate transporter GLAST, Glt1, EAAC1, vGluT1, and vGluT2 and we found that 100 μM UCB chronic exposure induces a significant decrease in some important genes involved in gliotransmission (see online suppl. Fig. S4) confirming the detrimental effect of UCB on astroglial cells.

Discussion

In this study, we evaluated for the first time the susceptibility of mature and immature rat organotypic hippocampal slices to UCB neurotoxicity, the possible neuroprotective effects against UCB of HSA, and the effect of UCB exposure duration on its neurotoxicity in immature slices. We found that 48-h exposure to 100–200 μM UCB is neurotoxic for mature rat organotypic hippocampal slices when the UCB:HSA molar ratio is >1, while HSA is

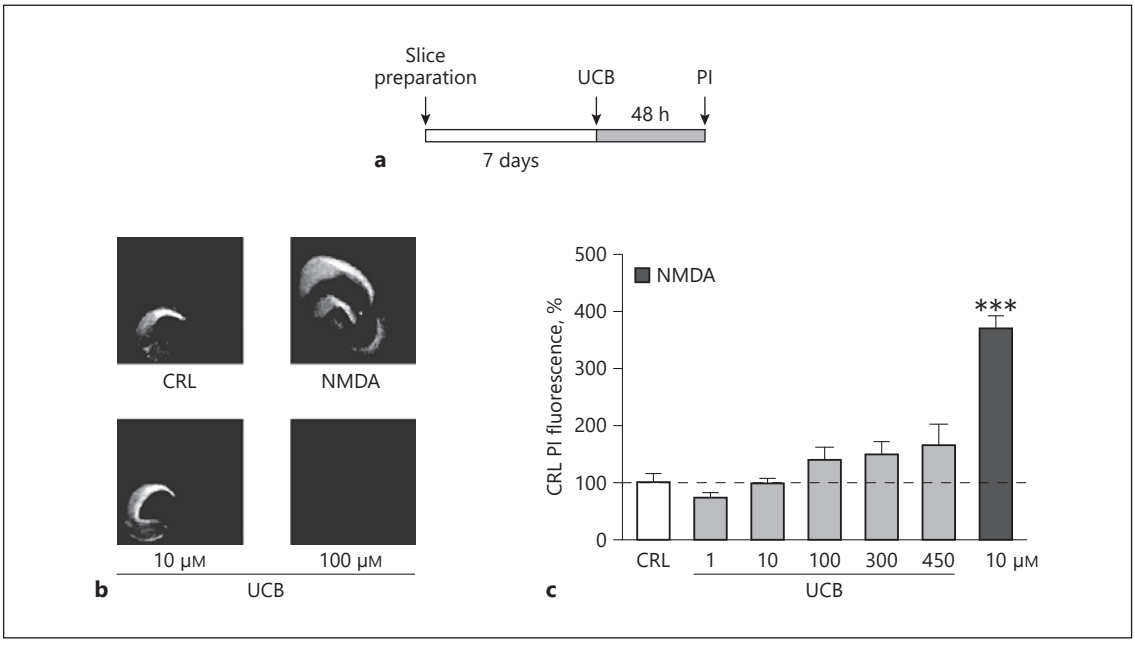

Fig. 3. Unconjugated bilirubin (UCB) does not induce damage in immature organotypic hippocampal slices. **a** Experimental protocol showing hippocampal slices cultured for 7 days (immature) and then exposed to 1–450 μM of UCB for 48 h. At the end of this period, the fluorescent dye propidium iodide (PI) was added to the medium to assess neuronal injury. **b** Qualitative analysis shows that UCB does not induce toxicity. **c** Quantitative analysis is expressed as a percentage of control (CRL) PI fluorescence. Bars represent the mean ± SEM of at least 5 experiments (about ≥20 slices for each experimental point). *** $p < 0.001$ versus CRL (ANOVA + Tukey's *w* test).

protective when the UCB:HSA molar ratio is ≤1. This toxicity was abolished by some glutamate receptor antagonists, such as NBQX and MPEP. We then demonstrated that 48-h exposure to 1–450 μM UCB does not induce necrosis or apoptosis in immature rat hippocampal slices. We observed that 48-h exposure to 100 μM UCB is not toxic for neurons but is toxic for astrocytes, although electron microscopic analysis showed that 48-h exposure to 100 μM UCB induced pathologic changes in CA1 pyramidal cells. However, a chronic exposure (72 h) to 100 μM UCB was toxic for both neurons and astrocytes and this toxicity is abolished by an equimolar amount of HSA (100 μM). We found that UCB chronic toxicity involves glutamate transporter GLAST, Glt1, EAAC1, vGluT1, and vGluT2, since their mRNA expression decreased.

Although the better potential of organotypic hippocampal slices compared to isolated cell cultures in investigating UCB-induced injuries has been well recognized [6], few studies were performed using this method [14–16], being mainly focused on UCB-induced modification of synaptic transmission. These studies are heterogeneous: donor rats were 6–8 days old [14], or 7–10 days old [15], or 2 and 8 days old [16]; rat hippocampal slices were maintained in vitro for 3 [14, 15] or 6 [16] days prior to use (to allow tissues to recover from experimental trauma caused by the isolation procedure) and, according to Bayer et al. [10], represent models of immature tissues; slices were exposed to 1–10 μM UCB plus 2–20 μM HSA (molar ratio 0.5) for 24 or 48 h [14], or 50 μM UCB plus 100 μM HSA (molar ratio 0.5) for 24 h [15], or to 70, 140, or 300 nM of unbound bilirubin for 24 h [16]. Therefore, our findings on the neurotoxic effect of UCB in mature slices when the UCB:HSA molar ratio is >1 cannot be compared to previous studies [14–16], because these had a different design and, mainly, were carried out in immature slices [10]. However, in one study, UCB did not affect cell viability [14], while in others it induced neuronal necrosis [15, 16].

We found that glutamate non-NMDA and metabotropic receptor antagonists, such as NBQX and MPEP, abolished UCB neurotoxicity in mature slices, and this result confirms the reported pivotal role of glutamate in the pathogenesis of bilirubin-induced cell damage [7, 8]. However, this did not occur with MK-801 glutamate NMDA receptor antagonist. This finding is in agreement

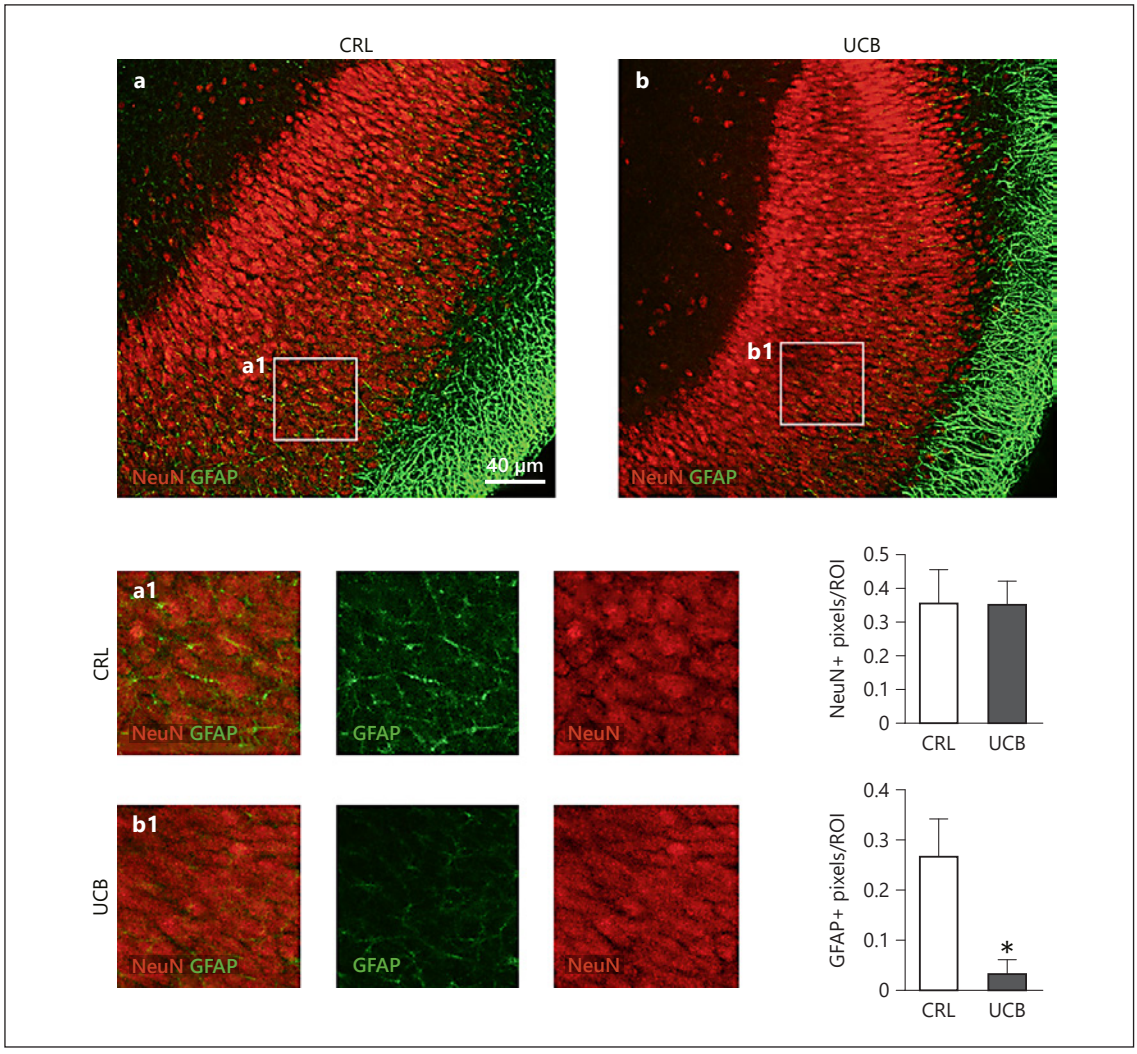

Fig. 4. Effects of unconjugated bilirubin (UCB) on neurons and astrocytes in immature organotypic hippocampal slices immunostaining of neurons (NeuN, red), and astrocytes (GFAP, green) in CA1 stratum pyramidale of immature organotypic hippocampal slices after exposure to bilirubin (100 µM, 48 h). Representative confocal microscopy images showing CA1 NeuN- and GFAP-positive cells in the pyramidal cell layer of the three experimental groups. Scale bar: 40 µm. Quantitative analysis of NeuN-positive neurons (upper panel) and GFAP-positive astrocytes (lower panel) of control (CRL) (white column, $n = 12$), and bilirubin (black column, $n = 12$) slices (mean ± SEM; * $p < 0.01$ vs. CRL; one-way ANOVA and Newman-Keuls multiple comparison test).

with Shapiro et al. [17] who found in hippocampal neurons and in Gunn rat pups that bilirubin neurotoxicity is not mediated through NMDA receptor activation. On the other hand, our results are in disagreement with Hankø et al. [18] who demonstrated in NT2-N cells, a teratocarcinoma-derived cell line, that MK-801 can only delay UCB-induced cell death; and with Grojean et al. [19] who found in primary forebrain neuronal cultures that MK-801 is protective against bilirubin-induced apoptosis and necrosis. It is difficult to explain these different results, but one key difference between our study and that of Shapiro et al. [17] and those demonstrating neuroprotection with MK-801 [18, 19] is that the former were performed in neuronal slices or cultures from hippocampi, whereas the latter were performed in teratocarcinoma-derived cell line [18] or embryonic cortical neurons [19]. In fact, in human and rat studies of bilirubin encephalopathy, the hippocampus has been shown to be affected, along with brainstem and the basal ganglia, while no injury has been demonstrated in the cortex [6].

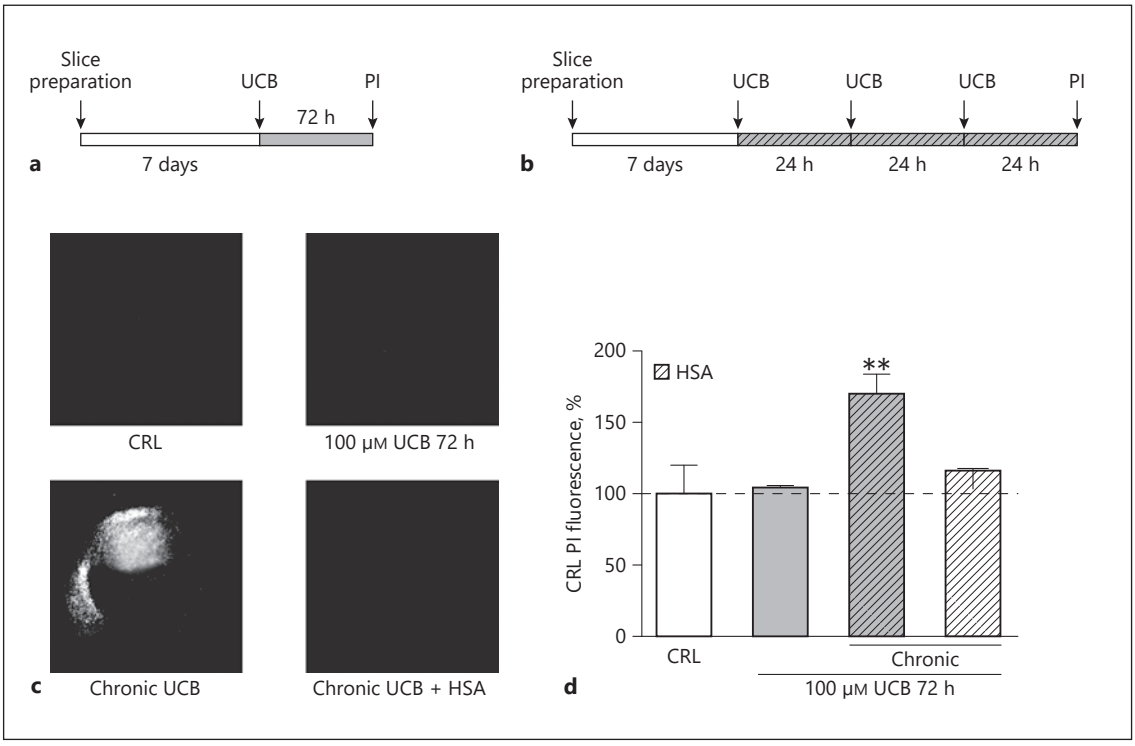

Fig. 5. The chronic application of UCB is neurotoxic in immature organotypic hippocampal slices. Experimental protocol showing immature organotypic hippocampal slices exposed to 100 μM UCB once (**a**) or exposed to a chronic application of UCB (72 h), changing the medium once a day with daily addition of 100 μM UCB, alone or plus human serum albumin (HSA: 100 μM) (**b**), and then incubated with propidium iodide (PI). **c** Qualitative analysis with observation under fluorescence optics to detect neuronal injury shows that chronic UCB exposure induces toxicity. **d** Quantitative analysis confirms UCB toxicity and shows HSA protective effect. Quantitative analysis is expressed as a percentage of control (CRL) PI fluorescence. Bars represent the mean ± SEM of at least 5 experiments (about ≥20 slices for each experimental point). ** $p < 0.01$ versus CRL (white bar), single UCB exposure (gray bar), and plus HSA (white hutched bar) (ANOVA + Tukey's *w* test).

In our study, immature organotypic hippocampal slices were more resistant to UCB neurotoxicity than mature ones, as evidenced by the longer exposure to UCB (72 vs. 48 h) needed to induce cellular injuries. These results disagree with previous studies in cell cultures of neurons and astrocytes [20, 21], which report a higher vulnerability of immature than mature cells. However, previous studies used rat cortical astrocyte and neuron cultures, which were exposed to 50–100 μM of UCB for 4 h [20, 21], while we used organotypic hippocampal slices, which were exposed to 10–200 (mature slices) or 1–450 μM (immature) of UCB for 48 h. Thus, opposing results might depend on these different experimental conditions. On the other hand, some considerations support the result reliability of our model: brain lesions in kernicterus are usually in the hippocampus but not in cortex areas [6] since the hippocampus is the most vulnerable brain region to UCB injuries [22]; moreover, brain slice models represents a better in vitro model than cell cultures because they maintain interactions between neurons and glial cells [6] and mainly with microglia whose role in the physiopathology of UCB-induced neurotoxicity has been elucidated [15]. Moreover, our findings are in agreement with a recent study demonstrating a lower vulnerability of immature than mature hippocampal slices to noxious stimuli other than UCB (i.e., alcohol) [23]. However, it is important to note that an equimolar amount of HSA abolished UCB toxicity both in mature slices exposed for 48 h and in immature slices chronically exposed to UCB confirming the role of unbound bilirubin in the pathogenesis of kernicterus [13]. Electron microscopic analysis disclosed abnormal neuronal changes of mitochondria, Golgi apparatus, dendrites, and synapses after 48 h of UCB exposure of immature slices. We speculate that these anomalies probably prelude cellular death, as reported by many studies detailing neurotoxic effects of UCB [7, 8].

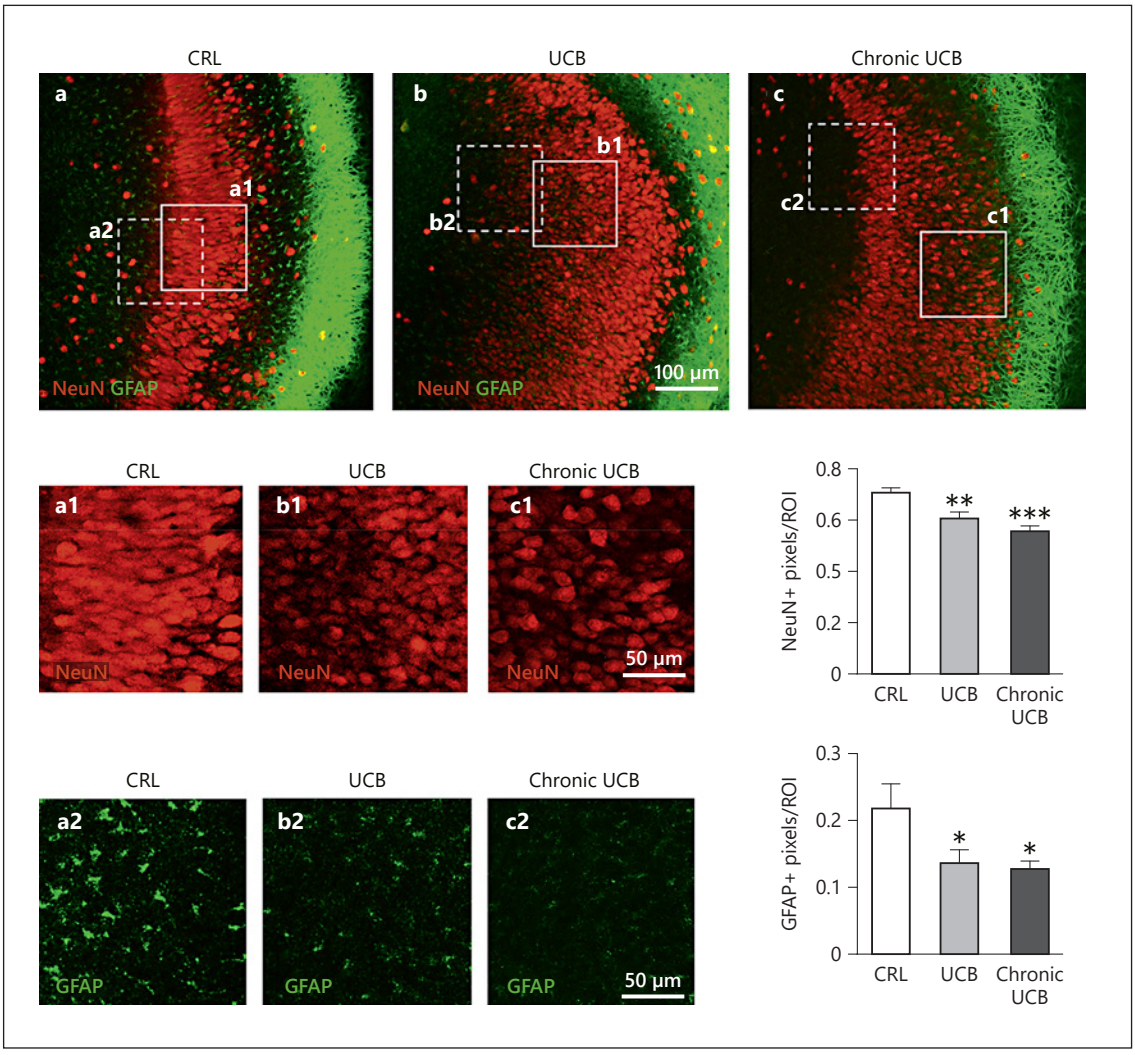

Fig. 6. Effects of chronic UCB exposure on neurons and astrocytes in immature organotypic hippocampal slices. Immunostaining of neurons (NeuN, red), and astrocytes (GFAP, green) in CA1 stratum pyramidale of immature organotypic hippocampal slices after exposure to UCB (100 µM for 72 h). Representative confocal microscopy images showing CA1 NeuN- and GFAP-positive cells in the pyramidal cell layer of the three experimental groups. Scale bar: 40 µm. Quantitative analysis of NeuN-positive neurons (upper panel) and GFAP positive astrocytes (lower panel) of CRL (white column, $n = 12$), bilirubin (one exposition, gray column, n = 12) and chronic bilirubin (black column, $n = 12$) slices (mean ± SEM; * $p < 0.05$ vs. CRL, ** $p < 0.01$ vs. CRL slices, *** $p < 0.001$ vs. CRL; one-way ANOVA and Newman-Keuls multiple comparison test).

We observed that in immature hippocampal slices, 48 h of UCB exposure injured astrocytes but not neurons, while 72 h of UCB exposure injured both neurons and astrocytes. This observation supports previous studies in neuron and astrocyte cultures reporting that astrocytes were more vulnerable than neurons to UCB toxicity [20]. On the other hand, UCB exposure reduced mRNA expression of glutamate transporters in our immature hippocampal slices and this might favor cellular death by excitotoxicity prolonging cellular exposure to glutamate. This finding confirms previous studies demonstrating that UCB exposure induces excitotoxic cellular death in rat primary cortical neurons and astrocyte cultures [21, 24] and in 7-day-old Gunn rat pups [25].

In conclusion, we found that 48 h of UCB exposure was neurotoxic for mature rat organotypic hippocampal slices, while 72 h of UCB exposure was neurotoxic for immature rat hippocampal slices. However, HSA was able to prevent UCB toxicity when the UCB:HSA molar ratio was ≤1 in both mature and immature slices.

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Disclosure Statement

The authors declare no conflicts of interest.

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