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Mapping of the consequences of bilirubin exposure in the immature rat: local cerebral metabolic rates for glucose during moderate and severe hyperbilirubinemia

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Abstract

The regional cerebral metabolic consequences of bilirubin intoxication are not well known. With the quantitative autoradiographic [^{14}C]2-deoxyglucose (2DG), we studied the effect of moderate or severe bilirubin infusion on local cerebral metabolic rates for glucose utilization (LCMRglcs) in 10 (P10) and 21 day-old (P21) rats. After an 80 or 160 mg/kg loading dose of bilirubin administered over 15 min, the speed of bilirubin infusion was reduced to 32 or 64 mg/kg/h for the following 105 min, for moderate or severe intoxication, respectively. This infusion protocol led to plasma bilirubin concentrations of 100–200 nmol/ml (moderate intoxication) or 200–300 nmol/ml (severe intoxication). Cerebral bilirubin concentration was 10 nmol/g at P10 and undetectable at P21 in moderate hyperbilirubinemia while it reached 22–34 nmol/g at both ages during severe hyperbilirubinemia. At P10, bilirubin infusion, moderate or severe, induced significant decreases in LCMRglcs in 17 and 15 brain regions of the 24 studied, respectively. At P21, moderate hyperbilirubinemia induced a decrease in LCMRglcs in only 2 regions, auditory cortex and auditory nerve. Conversely, at that age, severe bilirubin intoxication led to significant decreases in LCMRglcs in all regions studied. These results demonstrate that metabolic changes induced by bilirubin are directly correlated to its entry into the brain which occurs without any alteration in the blood-brain barrier. Indeed, the effects of the dye are quite discrete during moderate hyperbilirubinemia at P21 when no bilirubin is detectable in the brain while they are massive during severe hyperbilirubinemia at P21 and at both levels of intoxication at P10 when bilirubin has entered the brain in measurable amounts.

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1. Introduction

Hyperbilirubinemia, usually defined as physiologic jaundice of the newborn, is a common occurrence during the neonatal period and concerns 26–57% of all newborn infants [1]. However, in some infants, bilirubin concentrations reach levels high enough to induce a pathologic jaundice. In that case, the anion is able to cross the blood-brain barrier and to be deposited into the brain where it is responsible for the yellow staining of brain nuclei and the neurologic dysfunction characteristic of bilirubin encephalopathy. In this respect, small premature infants are a population at risk in which bilirubin levels that are considered to be in the normal and so called 'safe' range for the mature newborn infant may induce bilirubin encephalopathy [2,3].

In vitro, high doses of bilirubin are able to inhibit cellular respiration, glycolysis, oxidative phosphorylation, protein and lipid metabolism in adult animals [4]. Data in vivo appear more controversial. Indeed, several investigations have failed to demonstrate changes in cerebral energy metabolism after bilirubin infusion either in adult guinea pigs [5] or in newborn piglets [6]. In 15 day-old Gunn rats, bilirubin was found to inhibit glycolysis without affecting oxidative phosphorylation [7,8] while the anion induced severe mitochondrial dysfunction in adult rats after opening of the blood-brain barrier [9] or uncoupling of oxidative phosphorylation in kernicteric adult Gunn rats [10]. The cerebellum appears more vulnerable than the cerebral cortex [10,11] and neurons are more sensitive to bilirubin than glial cells [12,13]. Within neurons, some cellular types such as cerebellar Purkinje cells, hippocampal pyramidal and granular cells look affected by the dye while the other neuronal types are unaffected [10,12].

Since the consequences of bilirubin exposure on regional cerebral metabolic levels have not been explored in detail, the purpose of the present study was to map the regional sensitivity to the dye with cerebral maturation. The effects of a moderate or severe bilirubin intoxication on local cerebral metabolic rates for glucose (LCMRglc) in rats at postnatal age 10 (P10) and 21 days (P21) were explored with the quantitative autoradiographic [^{14}C]2-deoxyglucose technique of Sokoloff et al. [14] adapted to the immature rat [15]. The age of the rats has been chosen to represent the human neonatal period. Indeed, in terms of cerebral maturation, a 10 day-old rat is considered as equivalent to a human full term newborn while a 21 day-old rat corresponds to a 9 month-old infant [16,17].

2. Materials and methods

2.1. Animals and treatment

Adult Sprague–Dawley rats (Iffa-Credo Breeding Laboratories, L'Arbresle, France), 1 male and 2 females by cage, were mated together for 5 days under stan-

dard laboratory conditions on a 12:12 h light/dark cycle (lights on at 6:00 h). After delivery, litter sizes were reduced to 10 pups for homogeneity. The experiments were performed on groups of 6–8 rats at P10 and P21 (day of birth was considered as day 0). All experimentation was conducted with the highest standards of animal care.

A femoral artery and two femoral veins were catheterized with PE10 polyethylene catheters (Clay Adams, Parsippany, USA, I.D. 0.28 mm, E.D. 0.61 mm) under light halothane anesthesia, as previously described [15]. On the following day, rats were transferred to a rectangular plexiglass box to prevent free ambulation of the pups, especially at P21. A bilirubin solution (32 mg/ml) in 0.5 M NaOH, 0.055 M phosphate buffer and 5% beef serum albumin at pH 7.4 (10:20:70) was perfused through one femoral vein. The perfusion of the bilirubin solution (final concentration 3.2 mg/ml) was performed in two steps. First, a loading dose of 80 or 160 mg/kg for moderate or severe hyperbilirubinemia, respectively, was administered over 15 min. The speed of the perfusion was then reduced to 32 or 64 mg/kg/h for moderate or severe hyperbilirubinemia, respectively and the perfusion was continued for 105 min. Control animals received the same volume of saline plus phosphate buffer administered over the same time. Control animals did not receive albumin which was only given in this protocol to bind bilirubin molecules. Moreover, because of the high photosensitivity of bilirubin, the whole experiment was performed in low intensity red light.

2.2. Measurement of local cerebral glucose utilization

LCMRglcs were measured by the [^{14}C]2-deoxyglucose (2DG) method described by Sokoloff et al. [14] and adapted to the developing rat by Nehlig et al. [15]. The [^{14}C]2-deoxyglucose (4.625 MBq/kg; sp. act. 1.65–2.04 GBq/mol; Commissariat à l'Energie Atomique, Saclay, France) was injected as an i.v. pulse (125 μl/100 g) at 75 min after the onset of bilirubin or saline infusion which were continued throughout the whole 2DG procedure. During the following 45 min 9–12 timed arterial blood samples were drawn in glass capillary tubes. The blood samples were immediately centrifuged and the plasma concentration of 2DG and glucose was determined, as previously described [13].

At approximately 45 min after the pulse of 2DG, the animals were killed by decapitation. Brains were rapidly removed and frozen in isopentane chilled to −30°C, coated with chilled embedding medium (carboxymethylcellulose 4% in water), and stored at −80°C in plastic bags until sectioned and autoradiographed. The brains were then cut into 20-μm coronal sections at −18°C in a cryostat. Sections were autoradiographed on Kodak SB5 film along with calibrated [^{14}C]methylmethacrylate standards calibrated for their ^{14}C concentration in brain sections [14]. Adjacent sections were fixed and stained with thionin for histological identification of specific nuclei.

The autoradiographs were analyzed by quantitative densitometry with a computerized image processing system (Biocom 200, Les Ulis, France). Optical density measurements for each structure anatomically defined according to the developing rat brain atlas of Sherwood and Timiras [18] were made bilaterally in a minimum of 4 brain sections. All densitometry was conducted without knowledge of the treatment of the animal. Tissue ^{14}C concentrations were determined from the optical

densities of the autoradiographic representations of the tissues and a calibration curve obtained from the autoradiographs of the calibrated standards. LCMRglcs were calculated from local tissue concentration of ^{14}C, time courses of the plasma 2DG and glucose concentrations, and appropriate constants according to the operational equation of the method [14]. They were also corrected for the time lag in sampling blood through arterial catheters in small animals, as previously described [15].

2.3. Statistical analysis

LCMRglcs were determined in 24 structures in 6 groups of 6–8 animals each, 2 groups of saline- and 4 groups of bilirubin-exposed rats. The values of LCMRglc obtained for each structure underwent first a two-way analysis of variance to test the effects of age, of the nature of the treatment and the possible interaction between the age and the nature of the treatment. Then, LCMRglcs in each group of either bilirubin-exposed animals were compared with those in control animals by means of a Student's *t*-test.

3. Results

3.1. Plasma and brain bilirubin and albumin concentrations

Moderate bilirubin infusion led to plasma bilirubin concentrations of 176 ± 9 and 110 ± 15 nmol/ml at P10 and P21, respectively, while brain levels were 13 ± 5 nmol/g at P10 and undetectable at P21. After severe bilirubin infusion, plasma levels

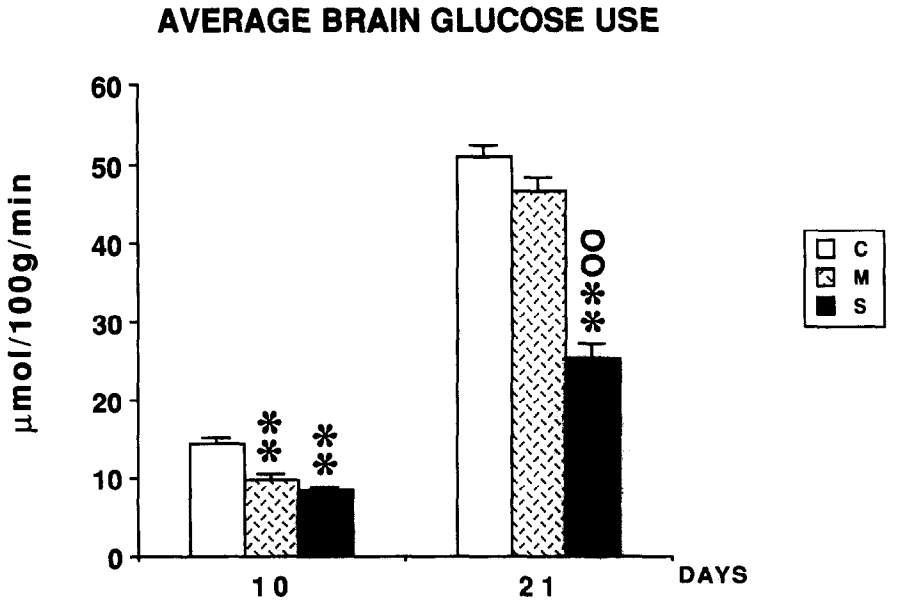

Fig. 1. Effects of moderate (M) and severe (S) hyperbilirubinemia on the average rate of cerebral glucose utilization, expressed as µmol/100 g/min and compared to control (C) values. $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant differences from control.

were 230 ± 15 and 250 ± 13 nmol/ml and cerebral bilirubin concentrations were 19 ± 5 and 27 ± 6 nmol/g at P10 and P21, respectively. Plasma albumin concentrations reached 360 ± 35 and 397 ± 21 nmol/ml during moderate hyperbilirubinemia and 421 ± 45 and 295 ± 11 nmol/ml during severe hyperbilirubinemia, at P10 and P21, respectively. Thus in both hyperbilirubinemic conditions, the albumin:bilirubin

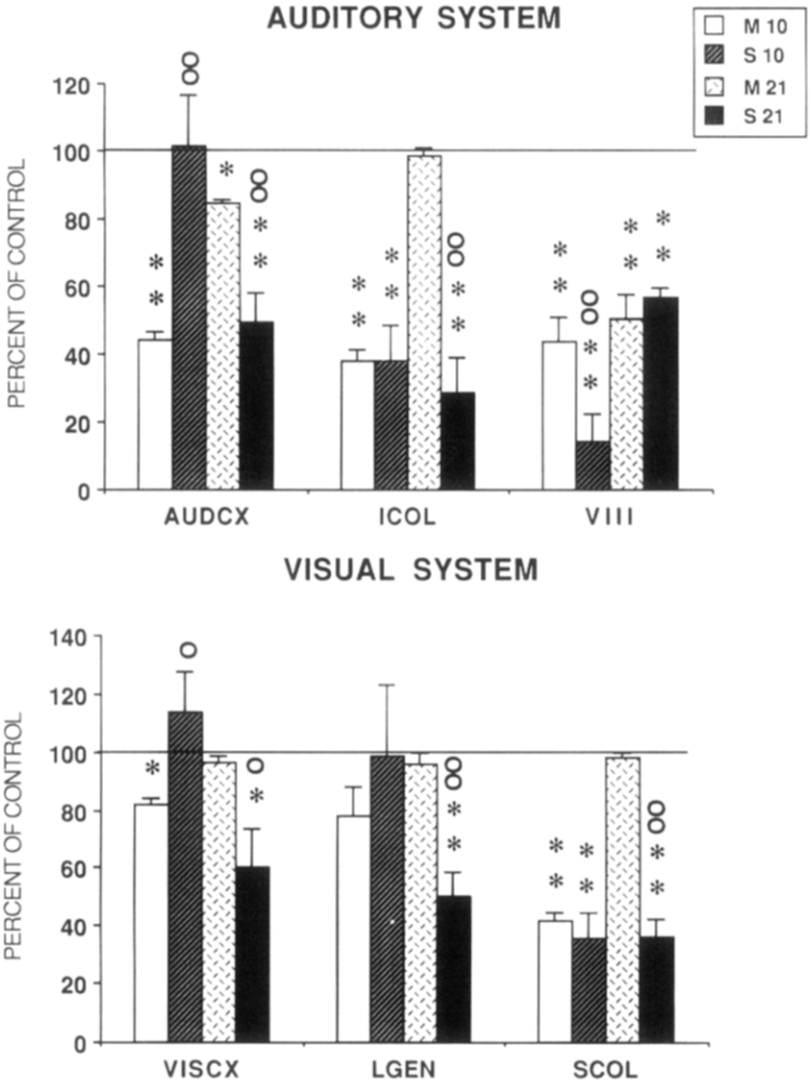

Fig. 2. Effects of moderate (M) and severe (S) hyperbilirubinemia on LCMRglcs in sensory regions of P10 and P21 rats, expressed as percent of control values. Abbreviations: AUDCX, auditory cortex; ICOL, inferior colliculus; VIII, auditory nerve; VISCX, visual cortex; LGEN, lateral geniculate nucleus; SCOL, superior colliculus. $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant differences from control. $^{o}P < 0.05$, $^{oo}P < 0.01$, statistically significant differences between severe and moderate hyperbilirubinemia.

ratio was always higher than 1, showing that bilirubin was bound to albumin in the present experimental conditions.

3.2. *Effects of bilirubin intoxication on average cerebral glucose utilization*

In P10 rats, bilirubin infusion significantly decreased average brain glucose utilization in a dose-dependent way (Fig. 1), resulting in a 32 and 42% decrease in moderate

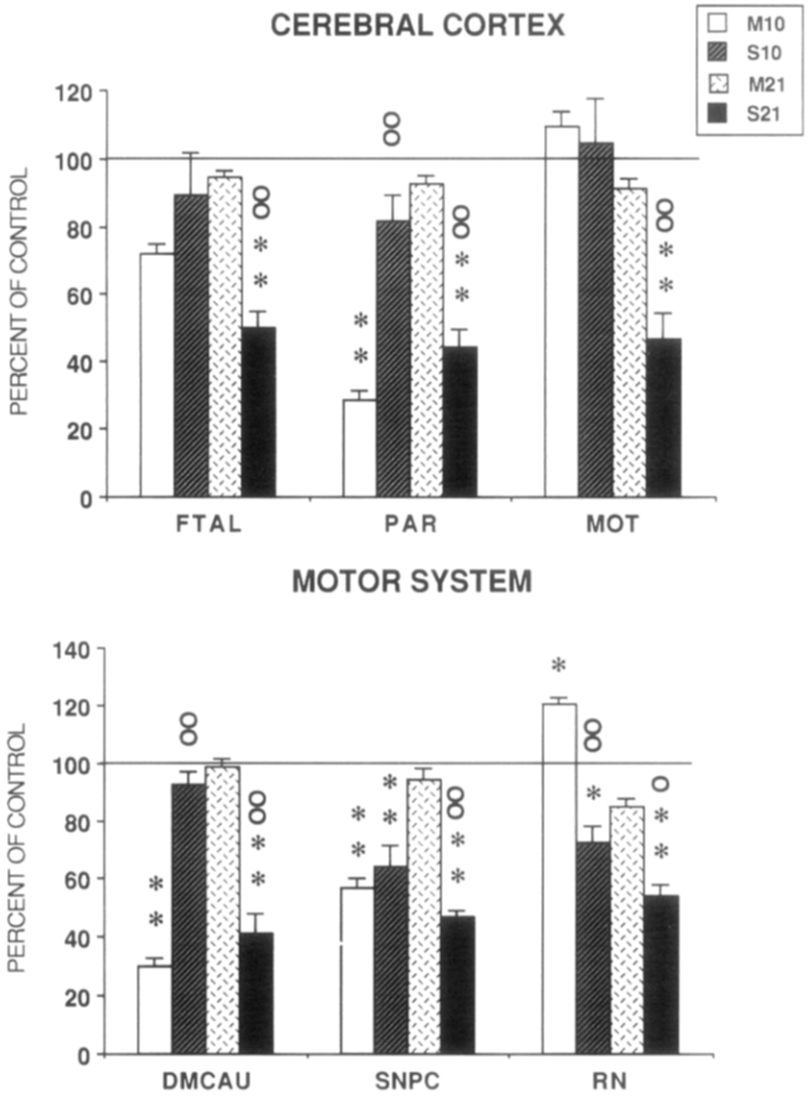

Fig. 3. Effects of moderate (M) and severe (S) hyperbilirubinemia on LCMRglcs in cerebral cortex and motor system of P10 and P21 rats, expressed as percent of control values. Abbreviations: FTAL, frontal cortex; PAR, parietal cortex; MOT, motor cortex; DMCAU, dorsomedian caudate nucleus; SNPC, substantia nigra pars compacta; RN, red nucleus. $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant differences from control. $^{o}P < 0.05$, $^{oo}P < 0.01$, statistically significant differences between severe and moderate hyperbilirubinemia.

or severe bilirubin intoxication, respectively. At P21, moderate intoxication had no effect while severe hyperbilirubinemia decreased average brain glucose utilization by 51%.

3.3. *Effects of moderate bilirubin intoxication on local cerebral metabolic rates for glucose*

Values of LCMRglcs in control P10 and P21 rats (data not shown) were in the

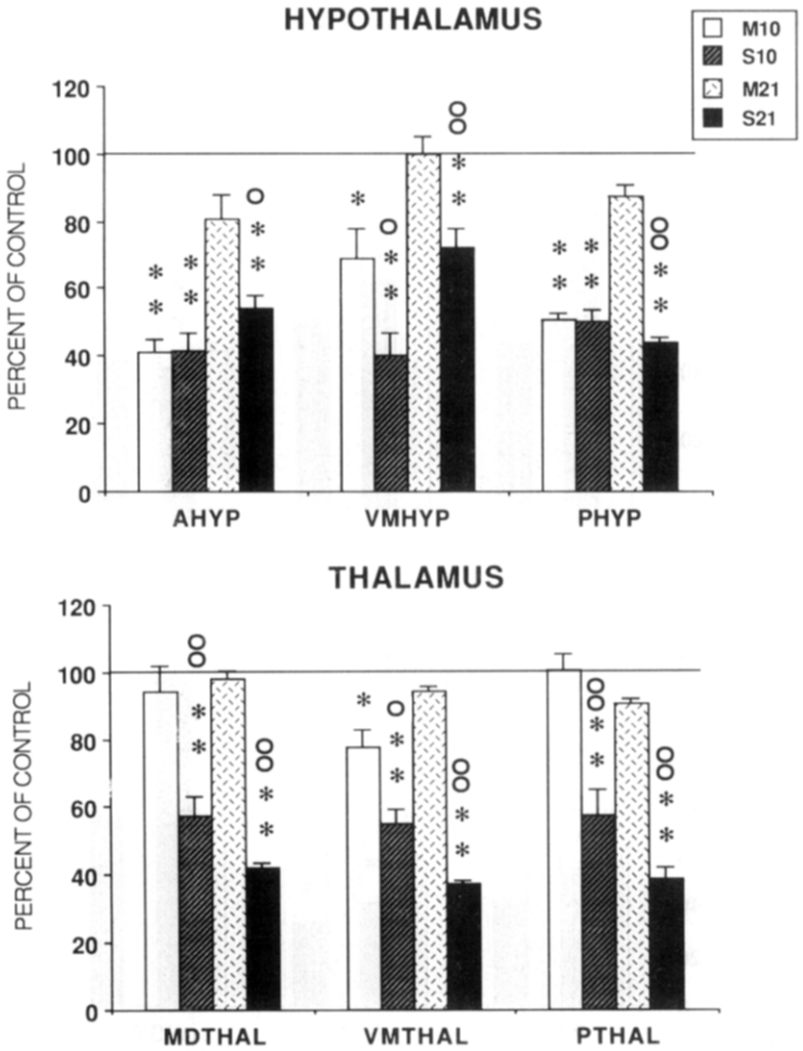

Fig. 4. Effects of moderate (M) and severe (S) hyperbilirubinemia on LCMRglcs in hypothalamus and thalamus of P10 and P21 rats, expressed as percent of control values. Abbreviations: AHYP, anterior hypothalamus; VMHYP, ventromedian hypothalamus; PHYP, posterior hypothalamus; MDTHAL, mediodorsal thalamus; VMTHAL, ventromedian thalamus; PTHAL, posterior thalamus. $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant differences from control. $^{o}P < 0.05$, $^{oo}P < 0.01$, statistically significant differences between severe and moderate hyperbilirubinemia.

range of previous data from our laboratory [15,19]. Moderate bilirubin intoxication led to a significant decrease in LCMRglcs in 17 out of the 24 structures studied at P10 (Figs. 2–5) and to an increase over control levels in 1 area, red nucleus (Fig. 3). Conversely, at P21, LCMRglcs were only significantly decreased in 2 auditory areas, auditory cortex and nerve (Fig. 2). Most marked decreases (>50%) compared to control values were recorded at P10 in the 3 auditory regions, superior colliculus (Fig. 2), parietal cortex and caudate nucleus (Fig. 3), anterior hypothalamus (Fig.

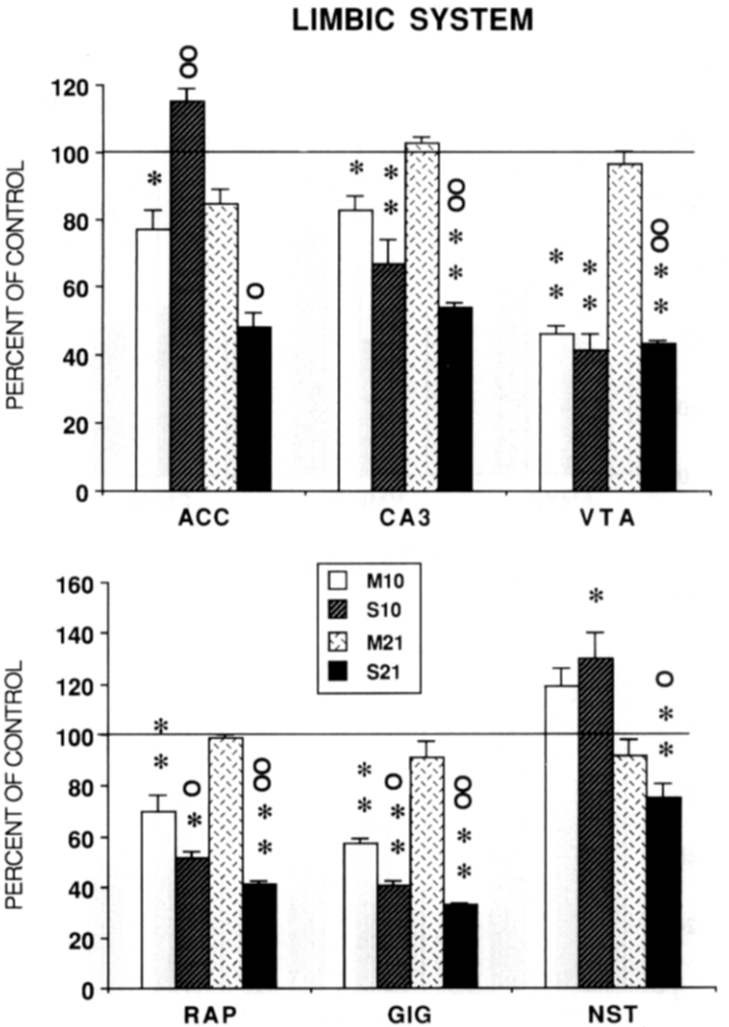

Fig. 5. Effects of moderate (M) and severe (S) hyperbilirubinemia on LCMRglcs in the limbic system of P10 and P21 rats, expressed as percent of control values. Abbreviations: ACC, accumbens nucleus; CA3, hippocampus, CA3 area; VTA, ventral tegmental area; RAP, medial raphe nucleus; GIG, gigantocellularis nucleus; NST, nucleus of the solitary tract. $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant differences from control. $^{o}P < 0.05$, $^{oo}P < 0.01$, statistically significant differences between severe and moderate hyperbilirubinemia.

4) and ventral tegmental area (Fig. 5). At P21, LCMRglc was deeply depressed in auditory nerve (–49%).

3.4. Effects of severe bilirubin intoxication on local cerebral metabolic rates for glucose

At P10, severe bilirubin intoxication significantly decreased LCMRglcs in 15 regions and increased LCMRglc in 1 area, the nucleus of the solitary tract. These decreases were especially marked (> 50%) in inferior and superior colliculi, auditory nerve (Fig. 2), hypothalamus (Fig. 4), ventral tegmental area and gigantocellularis nucleus (Fig. 5). At P10, decreases in LCMRglcs were significantly more marked in severe than in moderate hyperbilirubinemia in 8 regions, i.e. auditory nerve (Fig. 2), red nucleus (Fig. 3), ventromedian hypothalamus, the 3 thalamic nuclei (Fig. 4), medial raphe and gigantocellularis nucleus (Fig. 5). Conversely, metabolic decreases were less marked in severe than in moderate hyperbilirubinemia in auditory, visual (Fig. 2), and parietal cortex as well as in caudate nucleus (Fig. 5).

At P21, severe hyperbilirubinemia decreased LCMRglcs in all regions studied (Figs. 2–5). Metabolic depressions ranged from 43–71% compared to control levels, except in ventromedian hypothalamus (Fig. 4) and nucleus of the solitary tract (Fig. 5) where metabolic decreases reached only 25–28%. Decreases in LCMRglc were more pronounced in severe than in moderate hyperbilirubinemia in all regions, except in auditory nerve (Fig. 2).

4. Discussion

The data of the present study show that in the very immature rat, i.e. P10, bilirubin enters the brain at both levels of infusion while, at P21, the dye is only measurable after severe intoxication. Consequences of bilirubin on cerebral energy metabolism are directly correlated to its entry into the brain with marked effects at P10 at the two hyperbilirubinemia levels while, at P21, metabolic consequences are only significant after severe intoxication leading to high cerebral bilirubin levels. Moreover, all these metabolic changes occur at both ages without any alteration of the blood-brain barrier, as previously shown with the blue Evans method in severe hyperbilirubinemia of longer duration [19].

Metabolic depressions recorded in the present study are in good accordance with previous studies showing the inhibitory effects of bilirubin on cellular respiration, glycolysis [4,7,8], oxidative phosphorylation [9,10] and enzymes of the tricarboxylic acid cycle [20–22]. Decreases in LCMRglcs recorded in the present study are especially high at P10 in auditory regions, even after a moderate bilirubin intoxication. These results are in good accordance with clinical and experimental observations. Indeed, bilirubin disturbs auditory evoked potentials in both human neonates [23] and rats [24], increases the duration of sound conduction latencies, diminishes the amplitude of sound conduction waves [24,25] and induces a long-term decrease of the auditory response eventually leading to auditory loss in infants with bilirubin encephalopathy [26,27]. In Gunn rat pups, frequencies of auditory evoked potentials are abnormal and may lead to neuropathologic lesions of auditory pathways located at the level of mesencephalic afferences, particularly those related to the cochlear nucleus [28,29]. The specific vulnerability of the auditory regions to bilirubin is confirmed by the metabolic decrease specifically recorded in those areas after a

moderate hyperbilirubinemia at P21 although only traces of the anion are susceptible to be present in the brain at that age and that level of intoxication.

During moderate hyperbilirubinemia at P10, LCMRglcs were slightly increased in motor cortex, significantly increased in red nucleus and deeply depressed in caudate nucleus (Fig. 3). These metabolic changes reflect the loss of motor coordination, the moving difficulties and the lethargy of P10 rats exposed to bilirubin compared to controls, and correlate with motor disturbances reported in kernicteric human newborns [30] and ataxia and lethargy observed in newborn piglets and rabbits exposed to bilirubin [5,31]. As in motor cortex and red nucleus, hyperbilirubinemia, either moderate or severe, leads to a metabolic increase in the nucleus of the solitary tract (Fig. 5). This increase could reflect the key role of this nucleus in the control of vital functions. Indeed, this nucleus receives afferences from the heart, the stomach, the receptors of peripheral arterial blood pressure and controls cardiovascular regulation, respiration, endocrine functions and alertness [32]. The increase in LCMRglc in the nucleus of the solitary tract during hyperbilirubinemia is obviously the reflection of a protective mechanism to vital functions. However, if one could expect bilirubin-induced metabolic depressions as a result of the known depressant effect of the anion on cerebral energy metabolism [4,7–10,21,22], the scattered metabolic increases recorded in the present study raise the question of the direct or indirect effect of bilirubin on cerebral metabolic levels.

In the 10-day-old rat brain in which bilirubin enters even at a moderate level of intoxication, the metabolic response to the dye varies with the structures. In some regions (the auditory nerve, red nucleus, ventromedian hypothalamus, thalamic regions, hippocampus, medial raphe and gigantocellularis nucleus), the metabolic depression recorded is proportional to the level of bilirubin intoxication. Other regions, such as sensory relay nuclei, substantia nigra, hypothalamus and ventral tegmental area, react to bilirubin by an all or none response with a maximal depression already at the low level of intoxication. Finally, a last group of structures, i.e. cerebral cortices and caudate nucleus, exhibit a depressed metabolic level during moderate hyperbilirubinemia and go back to control levels in severe hyperbilirubinemia. The apparently paradoxical metabolic response of the latter regions to bilirubin is related to their very specific response to severe hyperbilirubinemia. Indeed, in the latter situation, metabolic levels are heterogeneous, distributed as alternate dark and light columns in cerebral cortex and dark and light dots in caudate nucleus [19]. Thus, in the same region, bilirubin may induce both metabolic increases and decreases whose mean is close to the control value and whose functional meaning remains to be elucidated.

In conclusion, there appears to be a good correlation between the entry of bilirubin at the cerebral level and the metabolic disturbances induced by the anion. Moreover, the very immature rat brain, at P10, appears to be more permeable and vulnerable to bilirubin, even at moderate levels, than the rat brain at weaning time, P21 whose metabolism is only strongly depressed by severe hyperbilirubinemia.

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