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Cortical Consequences of *In Vivo* Blockade of Monocarboxylate Transport During Brain Development in Mice

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ABSTRACT: In addition to glucose, monocarboxylates including lactate represent a major source of energy for the developing brain and appears to be crucial in the pathogenesis and recovery after brain damage. We hypothesized a role of monocarboxylates transport in the energy supply of neurons of the immature cerebral cortex. The effects of the blockade of monocarboxylates transport *in vivo* on the cortical development was investigated in neonatal mice using alpha-cyano-4-hydroxycinnamate (CIN) diluted either in DMSO (CD) or in ethanol (CE) administered intraperitoneally over postnatal day (P) P1 to P3. Injection of CIN induced a cytoarchitectonic disorganization in the parietal cortex likely due to a combination of slight disturbance of cortical neuronal migration and an increased neuronal cell death observed in CE ($p < 0.05$) but not in CD group. An increased number of activated GFAP-positive astroglia was observed in the neocortex in groups treated with CIN (CD and CE) on P10. These data: 1) Provide first evidence of deleterious effects observed *in vivo* after blockade of monocarboxylates transport in the developing brain; 2) emphasize the role of lactate during neuronal migration as a major source of energy; and 3) suggest the synergistic effect of ethanol-induced hypoglycemia in cortical brain damage induced by CIN. **(*Pediatr Res* 61: 54–60, 2007)**

Cerebral activity requires delivery of an adequate amount of energy substrates to neurons. In addition to glucose, the main source of energy for the brain, monocarboxylates including lactate play a significant role as alternative energy suppliers (1). Delivery of energy substrates from the blood to the brain requires specific transporters across the endothelial cells involved in blood-brain barrier and cellular membranes of the neurons and glia (2). Therefore, monocarboxylate transporters (MCTs) were supposed to play a key role in astrocyte/neuronal shuttle as lactate release by astrocytes is likely to be a major energy substrate coupled with neuronal activity as suggested in adults (3). Moreover, monocarboxylate including lactate play a significant role as alternative energy suppliers in developing brain (4) and under challenged conditions including excitotoxic insult or inadequate glucose supply (5–7).

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Little is known about the energy trafficking during CNS development (4). We previously emphasized the presence of monocarboxylate transporters (MCTs) in the different cell populations of the developing rat cerebral cortex and in the human visual cortex at midgestation including radial glia, mature astrocytes and ependimocytes (8,9). These studies demonstrate that lactate could be used as energy substrate in perinatal period, but physiologic relevance of its trafficking in developing brain have never been studied *in vivo*.

Alpha-cyano-4-hydroxycinnamate (CIN) is a competitive inhibitor of monocarboxylate transporters which is blood brain barrier permeable and appears to block lactate utilization by cells that import monocarboxylates (10). *In vitro*, using this transporter blocker, lactate has been reported to be a crucial energy substrate for neurons under activated conditions or during the reperfusion phase following hypoxic-ischemic insult (7,10). Similarly, blockade of monocarboxylate transport *in vivo* have been shown to exacerbate delayed ischemic neuronal damage in the adult (11). CIN could be diluted in DMSO (DMSO) but also in ethanol according to manufacturer's instructions (Sigma Chemical Co., St. Louis, MO). Ethanol is known to induce disturbances of gliogenesis and neuronogenesis (13), apoptosis and may interact with energetic metabolism inducing transient hypoglycemia (14,15); thus, this solvent might be able to potentiate any metabolic effect of CIN.

No study is available on the effect of MCT inhibitor *in vivo* under basal conditions during brain development. Here, we have explored the effect of inhibition of monocarboxylate transport on cortical development in mice using CIN. We investigated both the last steps of neuronal migration, astrocytic maturation and developmental apoptosis following neonatal CIN administration.

MATERIALS AND METHODS

Animals and experimental design. Swiss mouse pups (Iffa Credo, L'Abresle, France) were housed in our animal unit and maintained according to the guidelines issued by the Institut National de la Santé et de la Recherche Médicale (INSERM). All experimental protocols were approved by our ethics

Abbreviations: CIN, alpha-cyano-4-hydroxycinnamate; **GFAP,** glial fibrillary acidic protein; **MAP,** microtubule associated protein; **MCT,** monocarboxylates transporter

review committee. All animals were submitted to photoperiodicity (12:12h light-dark cycle) and were given free access to food and water.

The manufacturer's instructions (Sigma Chemical Co.) recommend dissolving CIN in methanol (50 mg/mL) as it is nearly insoluble in aqueous solutions. CIN is also readily soluble into DMSO. Pups received 5 intraperitoneal (IP) injections of CIN in an alcoholic (ethanol) solution (95%) or in DMSO (100%) at the dose of 80 mg/kg/d from P1 to P3, killed at P4 or P10. Controls received similar volume of ethanol or DMSO. Ethanol administration corresponded to 1 mL/kg/injection of 95% ethanol.

Animals received the five IP injections at 12-h intervals of the following drugs, in a volume of 5 μL: DMSO (control), 95% ethanol (E), CIN diluted in DMSO (CD), CIN diluted in 95% Ethanol (CE).

Weight and mortality were recorded before the injections period (P1–P3) then on P4 and P10.

Standard histologic procedures. Pups under deep anesthesia received a transcardiac infusion of phosphate buffer (PB) (pH 7.4, 0.12 M) containing 4% paraformaldehyde. The dissected brains were postfixed in the same fixative for 4h at 4°C. After rinses in 10% sucrose in PB, the cryoprotected brains were frozen in liquid nitrogen-cooled isopentane at –50°C and stored at –80°C until cryostat cutting. The brains were cut into serial 10-μm thick cryostat sections.

Full autopsy was performed, and samples of kidney, liver and skeletal muscles were fixed in formalin and snap-freezed on pups of the different groups sacrified at P5. Paraffin sections were processed using Hematoxylin and Eosin, Perls and reticulin staining was added for kidney and liver. Transverse frozen muscle sections were stained using hematoxylin and eosin, Gomori's Trichrome, NADH and cytochrome oxydase (Cox) activity (16).

Immunohistochemistry. Brain sections from frozen samples were processed for immunohistochemical analysis. The primary antibodies were directed against various antigens specific of cell types (Table 1). The primary antibodies were visualized after incubations with the appropriate species-specific biotinylated secondary antibody and the streptavidin-biotin-peroxidase complex as previously described (17).

BrdU analysis. To analyze the impact of CIN administration on neuronal migration in the cortical plate, BrdU was injected IP to Swiss pregnant mice at E15, at a dose of 50 mg/kg, twice 8 h apart. Then, pups were killed at P10 when neuronal migration is known to be completed. The perfused brains were postfixed in 70% ethanol overnight at 4°C, before embedding and cutting. First, slices were treated with 0.2% trypsine in 0.1M Tris pH 7.5 for 15 min at 37°C. Sections were incubated in 2N HCl at room temperature for 30 min and blocked with 0.1M lysine in PBS pH = 6 for 30 min and 0.2% gelatin. Sections were further processed according to similar protocol than described above. For cell counts, to avoid bias related to regional variations, the same anatomical level was examined in each group. Images of the parietal cortex area PAR1 (18) from 4–6 animals (3–4 slides per animal) were digitized using a CDD camera (Apogee Instrument Inc., Boston, MA) to allow accurate measurements.

TUNEL staining. Cell death in white matter was detected using TUNEL staining using ApopTag™ (Q-Biogen, Morgan Irvine, CA) as previously described (19). On each section, labeled nuclei were counted throughout the hemispheric cortical plate (at least 3 hemispheres per animal) at P4 in at least four animals in each group.

Microscopy. Bright field illumination was used to examine sections in the parietal cortex PAR1 (18). A CDD camera was used to digitize the region of interest to improve the accuracy of the labeled-cell counts. In each group, three to five animals at each developmental stage were studied; at least three nonadjacent fields from either right or left hemisphere in a section included within a square-grid reticule were examined. Sections were observed at magnification ×20 (0.25 mm^2) to get the most reliable cell counts according to the cellular morphology of each marker considered.

Statistical analysis. Results were expressed as means ± SEM. Statistical analysis of the histologic data were performed using one-way ANOVA with a Dunnett comparison posttest (GraphPad Prism version 3.03 for Windows, GraphPad Software).

Table 1. *Primary antibodies used for immunohistochemistry*

Markers	Labeled structures	Manufacturer	Dilution
GFAP	Astrocytes	Sigma Biosciences, St. Louis, MO	1/1000
NeuN	Neurons	Chemicon, Temecula, CA	1/1000
MAP2	Neurons	Chemicon, Temecula, CA	1/500
BrdU	DNA	Novocastra, Newcastle, UK	1/200
Cleaved-Caspase 3	Cell death	Cell Signaling, Beverly, MA	1/200

RESULTS

Phenotype following* in vivo *administration of CIN. Mortality rates were 0% in the DMSO control group, 15% in the ethanol (E) group, 10% in the CIN-DMSO (CD) group, and 50% in the CIN-ethanol (CE) group. Most of deaths occurred after animals exhibited lethargy that lasted more than 3 h and may be ascribable to energetic failure following CIN injections. CIN administration using either DMSO or ethanol as solvent was not associated with reductions in body weights of the pups on P4 (Fig. 1*A*). In contrast, a dramatic increase in lactate concentrations in serum was observed one hour following CIN administration (Fig. 1*B*). As expected, 95% ethanol alone (E) or CIN diluted in 95% ethanol (CE) induced a 42% transient decrease in glucose concentrations in serum one hour after injection, likely due to ethanol peak at the same time points (Fig. 1*C–D*). Three hours after injections, difference in both glucose levels and ethanol concentrations were no longer statistically significant between treated and control animals.

Because CIN was reported to induce mitochondrial dysfunction, mitochondrial markers and enzymatic functions following injections to mouse pups was investigated. Microscopical examination of Hematoxylin and Eosin, Perls and reticulin staining of kidney and liver and Gomori's Trichrome, NADH and cytochrome oxydase activity on skeletal muscle failed to observe any difference in mitochondrial functions and morphology between animals having received CIN and controls (Fig. 2) as suggested in previous reports (11,20)

Disturbance of neuronal migration following CIN administration. Administration of CE induced to slight alteration in the cortical architecture of the parietal cortex observed in cresyl violet staining on P5 pups, including an increased apoptotic feature of the nucleus and a cytoarchitectonic disorganization in the parietal cortex (Fig. 3*A–B*) in layer IV, in CE group compared with control. Regarding these alterations

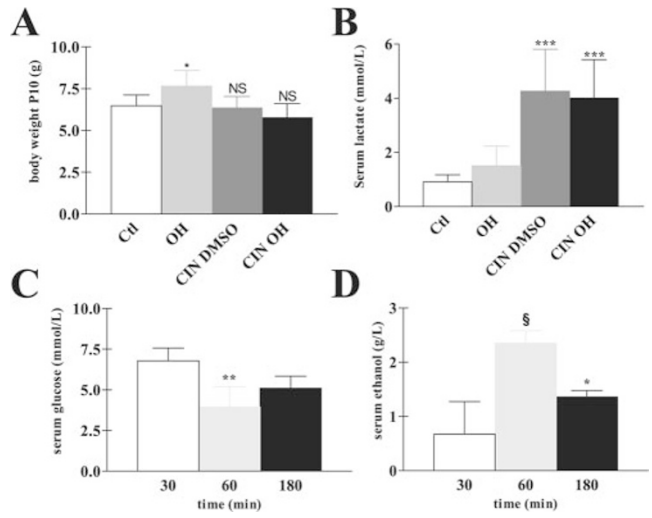

Figure 1. Clinical and biologic phenotype of CIN injected mouse pups. (*A*) Body weight of mouse pups at P10 after injection of either 95% ethanol (OH), or CIN diluted into DMSO (CIN-DMSO) or 95% ethanol (CIN-OH) (n = 6–15). (*B*) Serum lactate concentrations 1 h after the injection of either 95% ethanol, or CIN diluted into DMSO or 95% ethanol (n = 6–10). (*C–D*) Serum glucose and ethanol concentrations after injection of CIN diluted into 95% ethanol (n = 6–9). *$p < 0.05$; **$p < 0.01$; §$p < 0.001$.

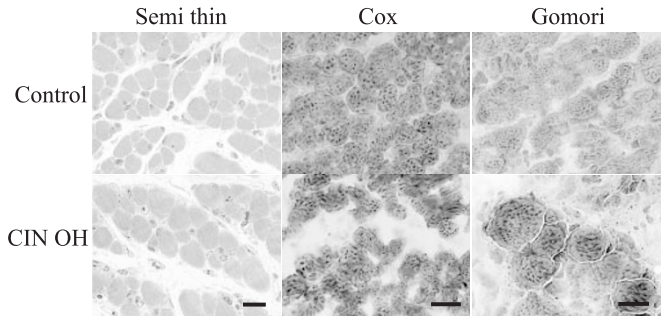

Figure 2. Mitochondria and CIN administration: Representative microphotographs on semi-thin section, and after Cox and Gomori staining in skeletal muscle of control pups (upper panel) and animal treated with CIN-ethanol (CIN OH) (lower panel). Bar = 100 μm.

and the potential ability of CIN to block lactate transfer from radial glial cells to neurons during the neonatal period in mouse, we next ask the question whether CIN could interact with neuronal migration in the neocortex. BrdU injections were performed at a developmental stage when most of neurons subsequently migrating into layers II and III were dividing in the germinal zone (E15). Labeling with anti-BrdU antibody at the end of neuronal migration (P10) showed a 45% decrease in the number of labeled neurons in the cortical plate at P10 in mouse who received CE or CD compared with the other groups ($p < 0.05$) (Figs. 3*C, D*). In contrast, neither DMSO nor ethanol alone was found to be able to decrease the density of labeled neurons in the cortical plate. Double labeling using neuronal marker NeuN demonstrated that most of BrdU-labeled cells are neurons (Fig. 4).

Synergistic effect of CIN and ethanol administration-induced apoptotic cell death in developing cortical plate. The decreased number of neurons in the developing cortical plate following CIN administration may be related to an increased neuronal cell death. To test this hypothesis, two cell death markers were used: TUNEL labeling and cleaved-caspase 3 at P4, at a time point corresponding to the physiologic apoptotic cell death occurring in the neocortex (21). When cortical column was considered to count cell death in the cortical plate, ethanol administration alone induced a not-significant 1.5-fold increase in the number of cleaved-caspase 3 + cells (Fig. 5*A,C*) or TUNEL+ cells (Fig. 5*B, D*). In contrast, apoptotic cell death was significantly three-fold higher in pups who received CE compared with control group ($p < 0.05$) whereas CD had no effect on cell death detected using both TUNEL and anti-cleaved caspase 3 immunohistochemistry. Semi fine sections of 1 μm were processed from the same groups to visualize nuclear feature of apoptosis (Fig. 5*E*). Both nuclear fragmentation and condensation was observed in dead or dying neurons in the cortical plate. In contrast, we failed to demonstrate any difference between CD and CE using Fluoro-Jade B/C suggesting that CIN-induced cell death is likely to be mostly apoptotic (data not shown). Apoptotic cells have shown to be neurons using double labeling with cleaved caspase 3 and microtubule associated protein 2 (MAP2) markers (Fig. 6).

CIN administration and astrogliosis in the developing cortical plate. To study the impact of CIN on astroglia, glial

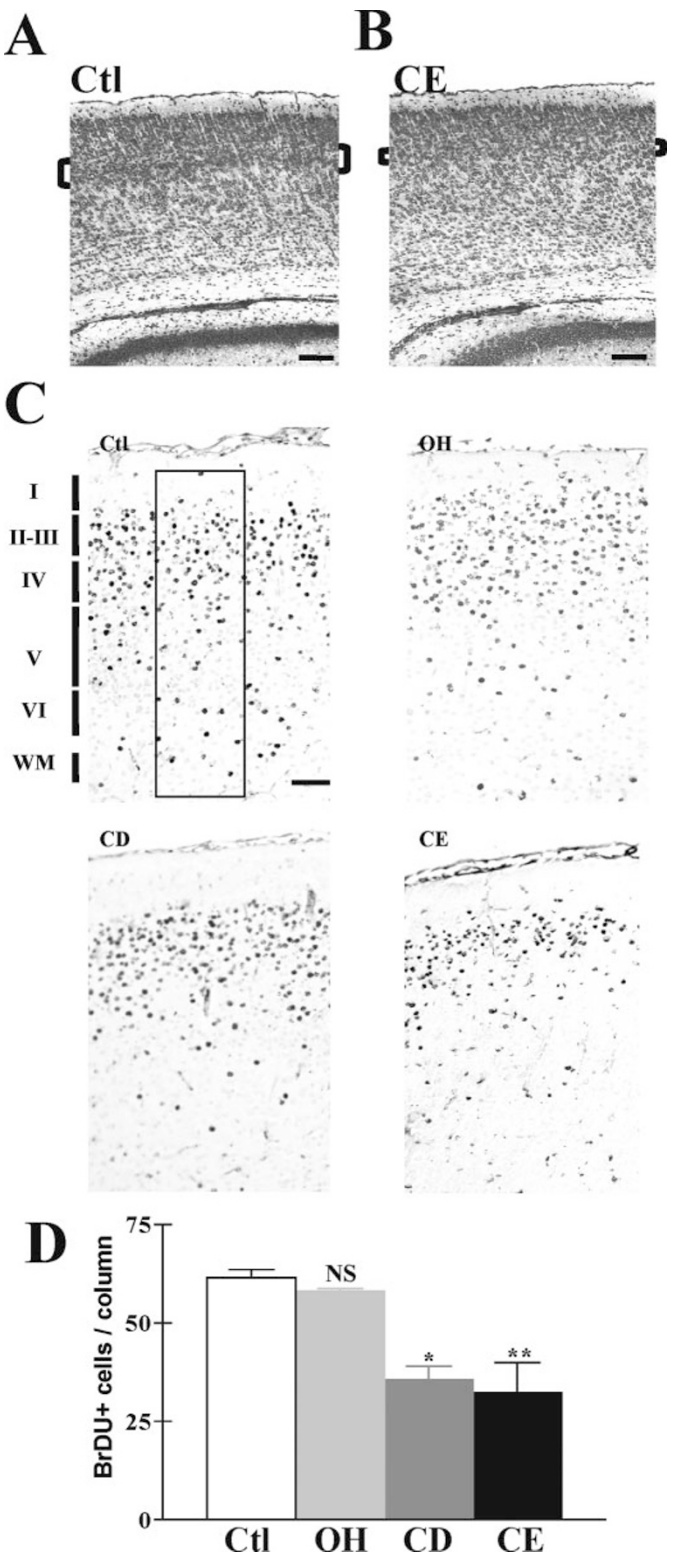

Figure 3. Disturbance of neuronal migration after injection of CIN. (*A,B*) Disruption of thin organization of the layer IV (brackets) in P5-pups treated with CIN-Ethanol (*B*) compared with controls (*A*). Bars = 300 μm. (*C*) Representative microphotographs showing BrdU + cells having migrated into cortical layers on P10 in control (Ctl) and pups treated with ethanol (OH) CIN-DMSO (CD) and CIN-OH (CE). WM, white matter. Bars = 150 μm. (*D*) Quantitative analysis of BrdU + cells in the cortical plate at P10 (column of interest are represented in *C*) ($n = 4$). *$p < 0.05$; **$p < 0.01$.

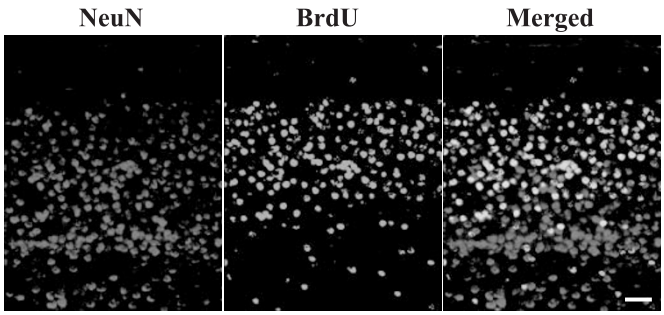

Figure 4. Double labeling of BrdU-labeled cells. Most of BrdU-labeled cells in layers 1–4 co-localized with the neuronal marker NeuN. Bar = 50 μm.

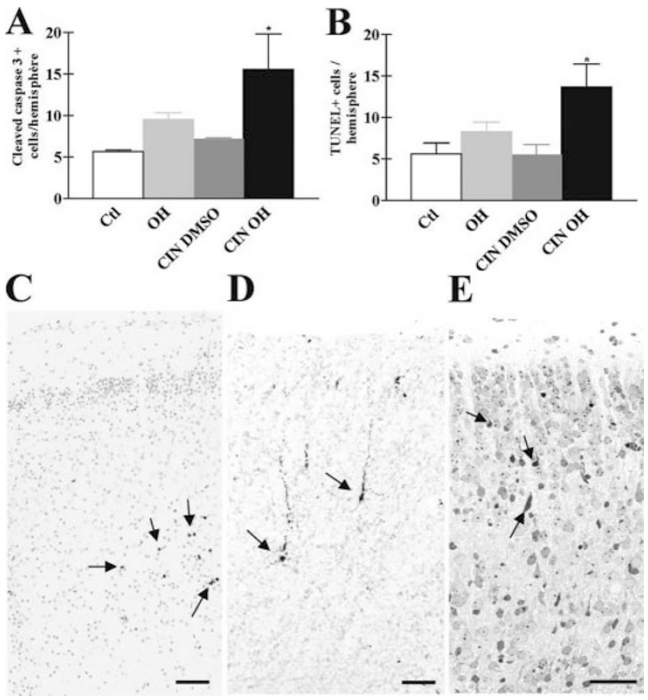

Figure 5. Apoptotic cell death and CIN: (*A, B*) Quantitative analysis of cleaved-caspase 3 + cells (*A*) and TUNEL + cells (*B*) in the cortical plates in the different experimental groups on P4 (n = 4–5). *$p < 0.05$; using one-way ANOVA with a Dunnett comparison post-test. (*C, D, E*) Representative microphotographs of TUNEL + cells (*C*) or cleaved-caspase 3 + cells (*D*), and semi-thin section (*E*) in the cortical plate (n = 4–5). Arrows show dying cells in the cortical plate. Bars = 250 μm.

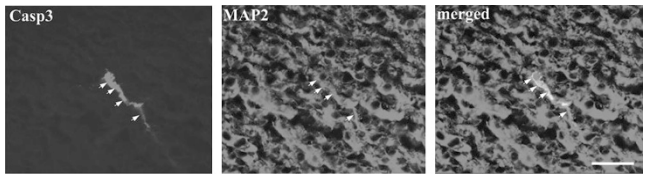

Figure 6. Double labeling of cleaved-caspase 3 + cells using MAP2 neuronal marker. Arrows show both cell body and axon of a dying neuron. Bar = 50 μm.

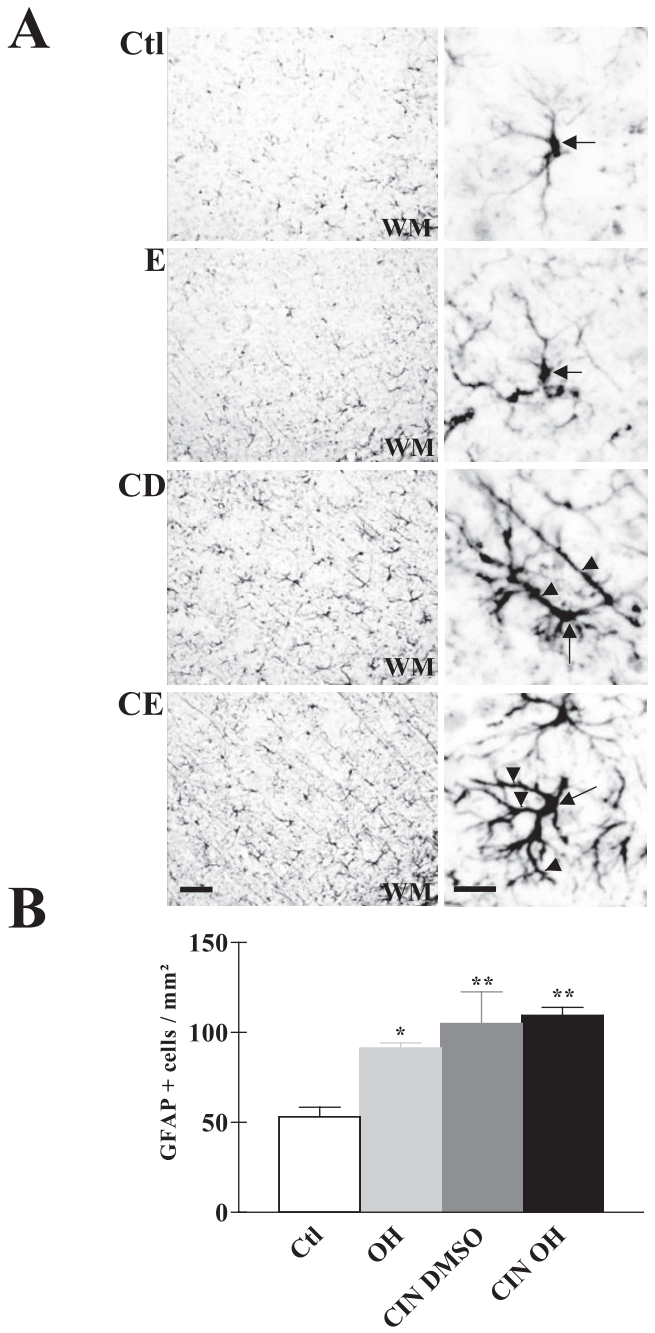

Figure 7. Astrogliosis and CIN: (*A*) GFAP immunocytochemistry on P10 in parietal cortex (PAR1) in control (Ctl), 95% ethanol-injected (E) or CIN-injected animals (CD for CIN diluted in DMSO and CE for CIN diluted in 95% ethanol). Bars = 250 μm. Note the increase in GFAP + cell density after injection of CIN and alterations in cell morphology observed at higher magnification (right part, arrows point cell body and arrowheads point processes). WM, white matter. Bars = 25 μm. (*B*) Quantitative analysis of GFAP + cells density in parietal cortex in the same experimental groups (n = 3–5). *$p < 0.05$; **$p < 0.01$.

fibrillary acidic protein (GFAP) staining was investigated as a marker for radial glia differentiation into mature astrocytes. The density of GFAP positive cells were significantly higher in parietal cortex in groups receiving CIN and ethanol alone compared with controls on P10 ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 7*A–B*). Similar increase was observed in hippocampus. Mature astrocytes displayed an activated aspect with an increased number of processes and larger cell bodies in the CIN-treated groups (Fig. 7*A*, high magnification panel). To further confirm the increased expression of GFAP in the cortical plate of animals subjected to CIN during neonatal period, a western blotting analysis compared P21 control animals and P21 animals having received CIN together with

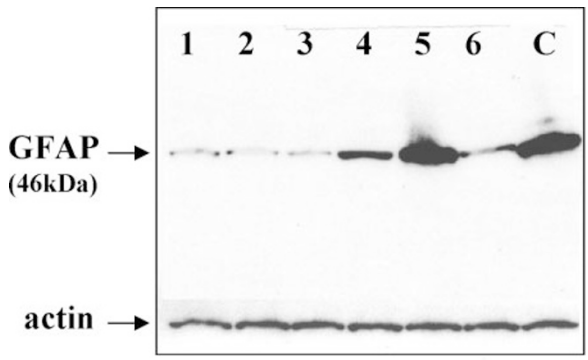

Figure 8. Western blotting analysis comparing GFAP expression in P21 control animals (lines 1–3) and P21 animals injected with CIN-OH during the neonatal period (lines 4–6). C, positive control of GFAP expression in adult mice.

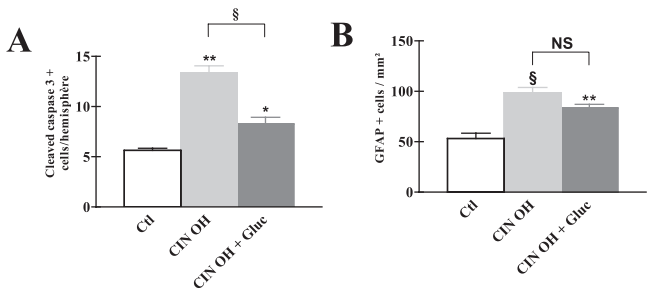

Figure 9. Effect of glucose administration on CIN-OH-induced cell death and astrogliosis in the developing brain. (*A*) Quantitative analysis of cleaved-caspase 3 + cells in the cortical plate in the different experimental groups on P4 ($n = 4$). (*B*) Quantitative analysis of GFAP + cells density in parietal cortex in the same experimental groups ($n = 4$). §$p < 0.001$; **$p < 0.01$; *$p < 0.05$; NS, not significant.

ethanol. A dramatic increased in GFAP signal was observed (Fig. 8).

CIN administration and impact of ethanol-induced hypoglycemia The synergistic effect between CIN and ethanol on neuronal cell death in the developing cortical plate was hypothesized to be related to the deprivation of neuronal energy supply by combination of the ethanol-induced hypoglycemia and the CIN-induced blockage of lactate shuttle. To test this hypothesis, we ask the question whether administration of glucose could abrogate this synergistic effect. Pups were injected with a similar volume of CIN diluted into ethanol and 15% glucose (5 μL). Serum glucose levels were found stable one to 6 h after ip injection (data not shown). Neuronal cell death and astrogliosis were investigated on P4 and P10, respectively. A significant reduction of cell death was observed in CIN-OH + glucose animals compared with animals injected with CIN-OH alone ($p < 0.001$) (Fig. 9*A*). In contrast, despite a slight reduction, glucose was not able to significantly abrogate CIN-induced astrogliosis (Fig. 9*B*).

DISCUSSION

Our data show the first *in vivo* evidence of the deleterious effect of MCTs blockade on cortical development in mice. We demonstrated that CIN administration was associated with disturbance of neuronal migration in the parietal cortex and an increased astrogliosis. In addition, CIN-induced cell death was found to be synergistic with ethanol toxicity used as solvent in this study.

Metabolic changes following CIN administration. During gestation and postnatal life, energy demands for cerebral development uses substantial amounts of monocarboxylate and/or ketone bodies followed later on by progressive cerebral glucose utilization according to the maturation of the blood brain-barrier (4,22). In parallel, the pattern of expression MCTs in the cerebral cortex varied at different stages of development (8,23). In particular, during early postnatal life, vessel walls expressed preferentially MCT1 whereas mature astrocytes expressed preferentially MCT2 (8,24). In this study, CIN acts as a monocarboxylic acid uptake inhibitor in a competitive manner with MCTs (7,10). Increased lactate concentration in serum following CIN administration was detected suggesting a reduced transport from vessels to astrocytes through endothelial cells. This increase in serum lactate concentrations was associated with ethanol-induced hypoglycemia leading to a high rate of mortality, and lethargy among those animals that survived but did not affect growth.

Blockade of monocarboxylate trafficking and disturbance of neuronal migration. The cerebral cortex in animals who received CIN just after birth displayed slight abnormalities in cortical architecture but significant disturbed neurons migration processes. Neuronal migration is an active process requiring high levels of energy demand along radial glial cells containing large amounts of glycogen (25) and maybe the presence of MCT1 (8). Most of neuronal migration is prenatal and CIN administration to pregnant mice just before birth, induced dramatic death for the embryos associated with critical cerebral dystrophy as neuronal ectopies (unpublished data). For this study, the disturbed postnatal neuronal migration processes following neonatal CIN administration could be correlated to the blockade of the trafficking of lactate to the neurons by MCT1 in the radial glial and/or of MCT2 in the astrocytes according to the astrocyte/neuronal shuttling theory (1). Even if ethanol has also been associated with subtle neuronal migration disturbances, the reduction in BrdU-labeled cells in CIN groups was also likely due to an increased cell death triggered by ethanol-induced hypoglycemia and not only to a disruption in neuronal migration.

Blockade of monocarboxylate trafficking, ethanol and cell death in the cerebral cortex. Disturbance of neuronal migration following CIN administration in our model also may account for an excess of cell death. Interestingly, a significant increase of cell death using TUNEL and active caspase-3 staining was only observed in the brain following treatment of CIN diluted in ethanol. We also observed a slight nonsignificant increase of cell death in pups who received ethanol alone consistently with previous report (15), that led us to verify the impact of a ethanol-induced hypoglycemia. Massive cell death occurs in deep hypoglycemia lasting for more than 3 h (26), which could

not explain solely the cell death in our animals as we observed only a slight transient (<1–2 h) hypoglycemia. Moreover, we did not find the typical pattern of cell death reported during hypoglycemia in CA1 and dentate gyrus regions known to be the most vulnerable during the hypoglycaemic shock (26). Indeed, we did not observe any significant difference in number of cell death in CA1 region compared with CA2–CA3 of hippocampus among experimental groups (data not shown).

Synergic effects of CIN with ethanol toxicity. The high mortality rate and massive cell death detected in pups following treatment of CIN diluted in ethanol compared with pups following treatment of CIN diluted in DMSO animals strongly supports the hypothesis of a double hit insult-related cell death. Ethanol is known to induce disturbances of gliogenesis and neuronogenesis in the developing murine brain (13) and widespread apoptosis but its mechanism of toxicity is still not well understood and may interact with the energetic metabolism by several ways (14,15). However, ethanol alone does not seem to be responsible for the apoptotic cell death following treatment of CIN diluted in ethanol as we did not observe a significant cell death in animals treated with ethanol alone. Moreover, serum ethanol concentration should be maintained at or above 200 mg/dL for 4 consecutive hours to trigger extensive neurodegeneration (15) which was not observed in our ethanol-treated mice. These observations support the hypothesis of the synergistic effect of CIN and ethanol leading to an increased neuronal cell death occurring through an apoptotic but not necrotic pathway. One explanation would be the addition of a slight ethanol-induced hypoglycemia to the blockage of lactate shuttle by CIN which deeply deprives the neuronal energetic resources. By administrating glucose together with CIN-OH, neuronal cell death but not astrogliosis was significantly reduced compared with CIN-OH alone, suggesting that ethanol-induced hypoglycemia play a key role in the CIN-OH-induced cell death. Alternatively, ethanol may interfere by multiple other neuronal pathways. For example, ethanol has both NMDA antagonist and GABAmimetic properties (27). The NMDA/glutamate antagonist properties may reduce the rate of glycolysis and subsequently lactate in astrocytes. NMDA has also anti-apoptotic (protective) effects (28). A single intoxication episode induced by any of these agents is sufficient to cause widespread neurodegeneration throughout many brain regions (14,15). Changes in gene expression can also occur rapidly after ethanol exposure and persist for long periods of time (29).

Blockade of monocarboxylate trafficking and subsequent astrogliosis. Finally, we report here a protracted astrogliosis induced by CIN injection during the neonatal period in mouse. However, in contrast to the CIN-induced consequences observed on neuronal cell death, no synergistic effect was detected between CIN and ethanol. The coupling between synaptic activity and glucose utilization is a central physiologic principle of brain function. Astrocytes play a central role in neurometabolic coupling, and the basic mechanism involves glutamate-stimulated aerobic glycolysis; the sodium-coupled re-uptake of glutamate by astrocytes triggers glucose uptake and processing *via* glycolysis, resulting in the release of lactate from astrocytes. Particulate glycogen likely to be intracellularly metabolised into alternative substrates in radial glia and mature astrocytes accounts for this phenomenon (12). Thus, inhibitors such as 4-CIN may have deleterious effects on this neurometabolic coupling regardless of neuronal cell death.

We speculate that CIN may have effects on astrocytes directly by activating glycolysis to compensate lactate deprivation of axons, and decreasing the oxidative metabolism of pyruvate derived from both glucose and lactate in mitochondria.

We also speculate that the metabolic effects of these two drugs were not found synergistic on astroglial activation because: astrogliosis is a non specific maker of neuronal cell death, and astrocytes are less vulnerable to a metabolic challenge based on activation of PFK2 (phosphofructo-2-kinase) (30).

In summary, blockade of lactate shuttle induces disturbance of neuronal migration and an apoptotic pattern of neurodegeneration when CIN is associated with ethanol. Our data emphasize the crucial role of monocarboxylates to supply energetic demand during both neuronal migration, neuronal cell death and astrocytic maturation. Characterization of the physiologic importance of monocarboxylate transport in the developing brain is a relevant question for the understanding of cerebral metabolisms in the neonate and in clinical disorders occurring during hypoxia-ischemia insults or energy deprivation.

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