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Inhibition of Glutamate Uptake by Unconjugated Bilirubin in Cultured Cortical Rat Astrocytes: Role of Concentration and pH

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The molecular basis of bilirubin toxicity to nerve cell function is still unclear. Since astrocytes are the main transporters of synaptically released glutamate and impaired glutamate uptake results in neuronal death, we investigated the effect of unconjugated bilirubin (UCB) on [^{3}H]glutamate uptake in cultured rat astrocytes and the role of bilirubin ionization on toxicity. Astrocytes were incubated for 5–15 min, with UCB concentrations from 17 to 342 μM and UCB/albumin molar ratios of 0.2–3.0, at pH 7.0, 7.4, and 8.0. Exposure of astrocytes for 15 min to 85.5 μM UCB and 28.5 μM albumin resulted in a 63.1% decrease of glutamate uptake ($p < 0.01$). Interestingly, the effect demonstrated to be correlated with the UCB/albumin molar ratio ($r = -0.986$, $p < 0.01$) and a significant decrease was observed for a UCB/albumin molar ratio as low as 0.8. Inhibition of glutamate transport was also pH-dependent as it occurred at 7.4 ($p < 0.05$) and 8.0 ($p < 0.01$), but not at 7.0, suggesting that the monoanionic species of UCB accounted for the inhibition. These findings indicate that UCB, and more precisely the monoanionic species, impairs a crucial function of astrocytes such as glutamate transport and support a potential role of astrocyte function in the pathogenesis of UCB-related brain damage (kernicterus).

Key Words: **bilirubin cytotoxicity; bilirubin encephalopathy; glial cells; neurotransmitter uptake.**

Abbreviations used: UCB, unconjugated bilirubin; DMEM, Dulbecco's modified Eagle's medium; HSA, human serum albumin; GFAP, glial fibrillary acidic protein.

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Deposition of unconjugated bilirubin (UCB) in the central nervous system is the major factor causing bilirubin encephalopathy during severe neonatal hyperbilirubinemia (1). Neurological manifestations of UCB toxicity range from transient or definitive auditory (2–4) and visual (5) impairment to death, as a consequence of kernicterus (6–8). Distribution of unbound UCB among body fluids and tissues differs for its three ionic species, whose proportions are determined by pH (9). At pH 7.4 the fully protonated UCB (diacid) is the dominant species (83%) and the monoanion constitutes 16%, but there is less than 1.5% dianion. As pH increases from 7.0 to 8.0, the proportion of UCB diacid declines as the proportion of monoanion increases steeply; the proportion of dianion, however, shows little increase until the pH is above 7.5 and it still constitutes only 17% of unbound UCB at pH 8.0 (9).

UCB cytotoxicity has been demonstrated in various cell types (10–13), including astrocytes (14) and neurons (15, 16). UCB may affect viability by interfering with the metabolism, depolarization, and transmitter functions of neurons (15, 17–21). In electron microscopic and autoradiographic studies on experimental kernicterus, astroglia has been indicated as the main transporter of UCB from blood to neurons (22). Although once thought to be merely passive supporting elements, astrocytes have recently been recognized as one of the most functional cells in the brain (23, 24) where they regulate energy homeostasis (25), maintain the blood-brain barrier and have phagocytic, immune and detoxification functions (26). *In vitro* (27, 28) and *in vivo* (29) studies have also established a key role for astrocytes in the post-synaptic removal of the excitatory neurotransmitters, such as aspartate and glutamate. Glutamate is considered to be the most important excitatory amino acid neurotransmitter in the

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brain, being released by a great number of synapses (30). Both astrocytes and neurons present glutamate transporters in their plasma membranes, but astrocytes, have a higher uptake capacity, which can recapture all glutamate released by neurons (28, 31). Impairment of glutamate uptake into astrocytes causes an increase of its extracellular concentration, resulting in overstimulation of neurotransmitter receptors and excitotoxic neuronal death (32).

The present study examines whether astrocytes, the main transporters of synaptically-released glutamate, are involved in UCB induced excitotoxicity. We report here that UCB has a time- and concentration-dependent inhibitory effect on glutamate uptake by cultured cortical rat astrocytes. The increase in toxicity from pH 7.0 to 8.0 suggests that the monoanion is the offending UCB species.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium, (DMEM) and fetal calf serum were purchased from GIBCO BRL (Life Technologies Inc., Grand Island, U.S.A.). Antibiotic antimycotic solution (20×), human serum albumin fraction V, fatty acid free (HSA), rabbit antibody anti-glial fibrillary acidic protein (GFAP) and goat antibody anti-rabbit-fluorescein isothiocyanate were from Sigma (St. Louis, MO). Unconjugated bilirubin (UCB), also from Sigma, was purified according to the method of McDonagh and Assisi (33). [^{3}H]Glutamate (specific activity 49 Ci/mmol) was obtained from Amersham Life Science (Buckinghamshire, UK). For uptake studies, 25 nCi of [^{3}H]glutamate, plus unlabeled glutamate to a final concentration of 50 μM (hereafter cited as labeled glutamate), were added per milliliter of culture medium. Other chemicals, purchased from Merck (Darmstadt, Germany) were of analytical grade.

Cell culture. Astrocyte primary cultures were prepared by an adaptation of the method of Blondeau *et al.* (34), using 2-day-old Wistar rats. Briefly, the brain was collected after decapitation in DMEM containing 2 g/L $NaHCO_3$, 6 g/L glucose and 1% antibiotic antimycotic solution (culture medium), and the meninges, blood vessels and white matter were removed. The cortex was homogenized by mechanical fragmentation, and the cell suspension passed sequentially through steel screens of 230 μm, 104 μm and 73.3 μm pore size. Cells were then collected by centrifugation (700*g*, 10 min) and resuspended in culture medium supplemented with 10% fetal calf serum. Finally, the cell suspension was plated (0.8 mL/well) at 2.0×10^5 cell/cm^2 in 12-well multidishes (Corning Costar Corp., Cambridge, MA) and incubated at 37°C in the presence 5% CO_2. Culture medium was replaced at days 7 and 10, and the confluent cultures used at day 11. All cell uptake and toxicity studies were performed at 37°C under 5% CO_2, in the dark.

Cell viability. Cell viability was assessed based on the trypan blue dye exclusion by viable cells, comparing cultures incubated for 15 min in the presence of 114 μM HSA, with vs without 342 μM UCB. This UCB/HSA molar ratio of 3, was the most toxic condition used in the present study.

Morphological analysis. Cells were morphologically characterized by phase contrast microscopy and by indirect immunocytochemistry for GFAP using a primary rabbit anti-GFAP antibody followed by a fluorescent-labeled secondary goat anti-rabbit antibody.

[^{3}H]Glutamate uptake. Labeled glutamate was added to each culture well (35), and cells were incubated for 2, 4, 6, 8, 14, 20, and 30 min, at pH = 7.4. At the end of each incubation period, medium was aspirated and the glutamate uptake was stopped by three washes with ice-cold isotonic saline buffer (145 mM NaCl, 5 mM sodium phosphates, pH 7.4), followed by immediate addition of 1 mL of 1 M NaOH to promote cell lysis (36). An aliquot of each cell lysate was taken for protein estimation by the method of Lowry *et al.* (37) and for radioassay. For radioassay, 0.5 mL sample was added to 10 mL of Optiphase "Hisafe 2" (Wallac, Finland), and counted in a Beckman LS 6000LL liquid scintillation spectrometer with internal quench correction.

Time-dependent effect. Purified UCB was dissolved in 0.1 N NaOH in order to prepare a "stock" solution at a concentration of 8.55 mM. The UCB "stock" solution was added to 57 μM HSA in culture medium (without fetal calf serum) to obtain a final UCB concentration of 171 μM and a UCB/HSA molar ratio of 3. The pH of 7.4 was restored using HCl. Labeled glutamate was added to the cultivated astrocytes either simultaneously with, or following a pre-incubation of 5 and 15 min with the UCB/HSA solution (pH 7.4), and glutamate uptake over 7 min was measured as described above. Results were compared with control values, obtained in the presence of HSA without UCB. Proper scintillation quench correction was checked using a cell lysate aliquot incubated with UCB but without labeled glutamate.

Effect of UCB concentration. Cultured cells were incubated in the absence (control) or in the presence of 17.1, 85.5, 171 and 342 μM UCB at a constant molar ratio of UCB/HSA = 3, for 15 min at 37°C and pH 7.4, in the dark. A further 7 min incubation with [^{3}H]glutamate and unlabeled glutamate preceded the determination of glutamate uptake. Reversibility of UCB toxicity was assessed in cells that were incubated with 85.5 and 171 μM UCB/HSA solutions for 15 min, and then washed three times (10 min each at 37°C) with a 171 μM HSA solution, a procedure able to remove UCB externally bound to cell membrane. Glutamate uptake was evaluated as described above.

Effect of unconjugated bilirubin/human serum albumin molar ratio. A 100 μM solution of HSA in culture medium was prepared and aliquots of the UCB "stock" solution were added to achieve UCB/HSA molar ratios of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2. Cells were incubated with these UCB/HSA solutions for 15 min at pH 7.4, and then glutamate uptake assessed as described above. Results were compared with those obtained for control experiments, from which UCB was excluded.

Effect of UCB ionic species. Astrocytes were incubated for 15 min with solutions containing UCB 120 μM and HSA 100 μM, prepared at pH values of 7.0, 7.4 and 8.0 in DMEM containing 10 mM Hepes. Labeled glutamate was then added and glutamate uptake over 7 min was estimated as above.

Statistical analysis. Results are expressed as mean ± SEM. Comparisons between UCB treated cells and control cells are made using the two-tailed *t* test for a two-sample population and were considered statistically significant when *p* values where lower than 0.05.

RESULTS

Effect of Excess UCB on Morphology and Viability of Astrocytes

As shown in Fig. 1, astrocytes exposed for 15 min to 342 μM UCB in the presence of 114 μM HSA showed no alterations in morphology (Fig. 1A) or in the GFAP immunoreactive pattern (Fig. 1B), compared with that described for normal protoplasmic (type 1) astrocytes (38). Trypan blue exclusion for astrocytes exposed to UCB (Fig. 1C) was virtually identical to that observed in controls (83.6 ± 1.2% vs 84.8 ± 1.8%, N.S.). Thus,

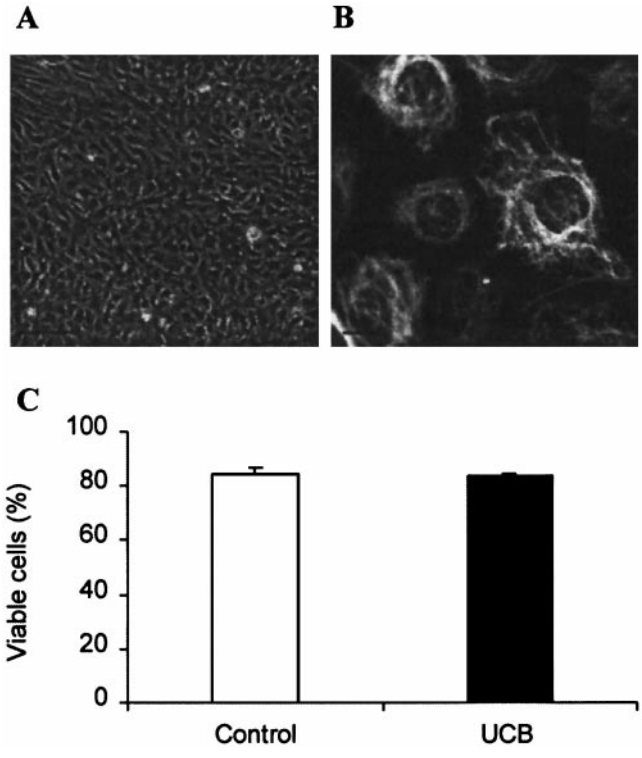

FIG. 1. Incubation of cultured astrocytes with unconjugated bilirubin (UCB) does not modify cell morphology (A), immunoreactivity for glial fibrillary acidic protein (B) and cell viability (C) of cultured astrocytes after incubation with UCB. Cells were isolated from the cortex of 2-day-old rat brains and cultured in DMEM (10% fetal calf serum) for 10 days, at 37°C, in the presence of 5% CO_2. Astrocytes were incubated with 342 μM UCB for 15 min, at a constant UCB/human serum albumin molar ratio of 3. Incubations were performed at 37°C and pH 7.4. Control was performed in the absence of UCB. The flat, polygonal type 1 astrocytes are confluent as shown by phase-contrast microscopy (A) and exhibit strong immunoreactivity for glial fibrillary acidic protein (B) by fluorescent microscopy. Bar = 10^4 μm (A) and 10^3 μm (B). Cell viability was assessed by trypan blue dye exclusion and the results are expressed as mean ± SEM (n = 6).

UCB at the highest concentration did not affect either astrocyte viability or morphology.

Time Course of [^{3}H]Glutamate Uptake by Astrocytes

As shown in Fig. 2, uptake was linear over the first 7 min and reached a plateau from 20 min onward (0.61 ± 0.10 pmol/mg protein). Accordingly the 7 min value was selected as the time interval to determine glutamate uptake in the following experiments.

Time and Concentration-Dependence of Inhibition of Glutamate Uptake by UCB

As shown in Fig. 3A, when cells were exposed to 171 μM UCB and 57 μM HSA, glutamate uptake was decreased in a time-dependent manner, to 66.1 ± 7.2% of control at 5 min ($p < 0.05$) and to 35.2 ± 6.2% after 15 min of incubation ($p < 0.01$). Accordingly, the 15 min

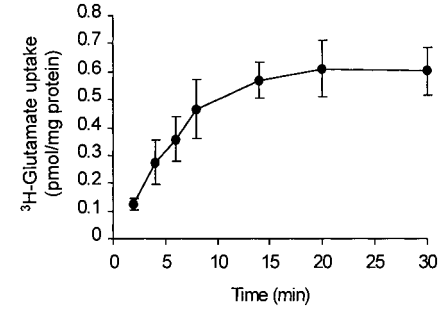

FIG. 2. Time-course of [^{3}H]glutamate uptake by astrocytes in primary culture. Cells were incubated with 25 nCi/ml [^{3}H]glutamate and 50 mM unlabeled glutamate for various time periods, ranging from 2 to 30 min, at 37°C, in the presence of 5% CO_2 (pH 7.4). Uptake was stopped by three washes with ice-cold isotonic saline phosphate buffer, followed by addition of 1 N NaOH to lyse cells and scintillations counted in cell lysates from each data point. Results are mean ± SEM (n = 4).

pre-incubation time with UCB was used in the subsequent assessment of the effects of increasing concentrations of UCB. As shown in Fig. 3B, glutamate uptake was not affected at the lowest UCB concentration tested (17.1 μM) (−11.6%, N.S.). By contrast, UCB

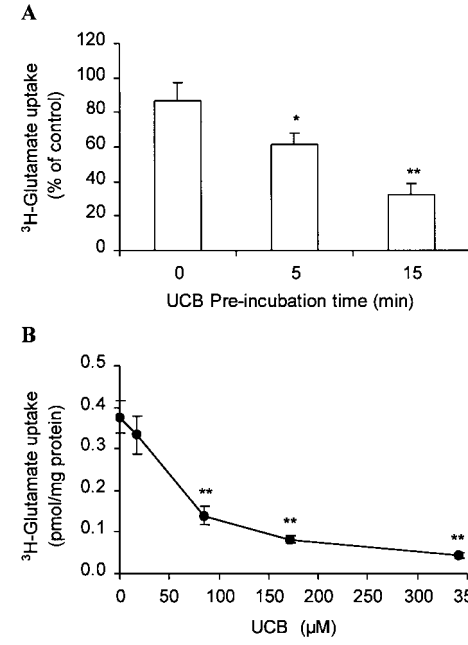

FIG. 3. Time- and concentration-dependent dependent inhibition of astrocyte glutamate uptake by unconjugated bilirubin (UCB). (A) Cells were incubated with 171 μM UCB and 57 μM human serum albumin for 0, 5 and 15 min. (B) Cells were incubated for 15 min with UCB concentrations, ranging from 17.1 to 342 μM, at a constant UCB/human serum albumin molar ratio of 3. Incubations were performed at 37°C and pH 7.4, in the presence of 5% CO_2. Glutamate uptake was measured over a 7 min time period following the experimental procedure described in the legend to Fig. 2. Controls were performed in the absence of UCB. Results are expressed as mean ± SEM (n = 8); *$p < 0.05$; **$p < 0.01$.

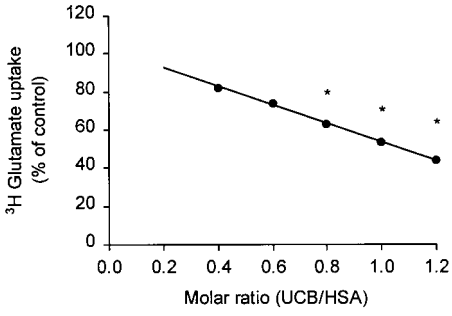

FIG. 4. Effect of unconjugated bilirubin (UCB)/human serum albumin (HSA) molar ratio on astrocyte glutamate uptake. Cells were incubated for 15 min, at 37°C and pH 7.4, with UCB and 100 μM HSA in order to achieve UCB/HSA molar ratios ranging from 0.2 to 1.2. Incubation conditions and analytical procedures as in Fig. 2. Glutamate has a negative linear relationship to UCB/HSA molar ratio ($y = -47.9x + 102$, $r = -0.99$, $p < 0.01$). Results are expressed as mean ± SEM ($n = 14$); *$p < 0.05$ vs control in the absence of UCB.

concentrations of 85.5, 171, and 342 μM, at a constant UCB/HSA molar ratio of 3, decreased the glutamate uptake significantly from control values, by 63.1, 78.3 and 88.3%, respectively ($p < 0.01$).

Glutamate uptake was measured in astrocytes exposed to UCB/HSA molar ratios varying from 0.2 to 1.2 at a constant HSA concentration of 100 μM (Fig. 4). A negative linear correlation was observed between the UCB/HSA molar ratio and the glutamate uptake as per cent of control ($y = -47.9x + 102$, $r = -0.99$, $p < 0.01$). Glutamate uptake was decreased significantly ($p > 0.05$) at molar ratios of 0.8 and higher.

Reversibility of Inhibitory Effect of UCB

Incubation of astrocytes with 85.5 μM UCB and 28.5 μM HSA for 15 min, followed by washing three times with 171 μM HSA, restored glutamate uptake to near control values (90.1 ± 16.1%). By contrast, when cells were first incubated with 171 μM UCB, washing with HSA only slightly restored the uptake of glutamate, from 21.7 ± 2.1% to 38.9 ± 7.5% of control values ($p < 0.01$ vs controls).

Effect of pH on the Inhibition of Glutamate Uptake by UCB

As shown in Fig. 5, 15 min exposure of astrocytes to 120 μM UCB in the presence of 100 μM HSA inhibited glutamate uptake similarly at pH 7.4 and 8.0, to 70.4 ± 8.4% ($p < 0.05$) and to 66.6 ± 6.3% ($p < 0.01$) of control values, respectively. By contrast, glutamate uptake was not inhibited significantly at pH 7.0 (90.6 ± 9.1% of control uptake measured in the absence of UCB). An inverse correlation was obtained between glutamate uptake pH and ($r = -0.60$, $p < 0.05$).

DISCUSSION

These results indicate that glutamate uptake by astrocytes is inhibited significantly when the cells are exposed to unbound UCB. The decrease in glutamate transport is observed at UCB/HSA molar ratios as low as 0.8 ($p < 0.05$), a value found in some neonates who develop UCB encephalopathy (39–42), albeit at much higher total UCB and HSA concentrations. Interestingly, unbound UCB concentration calculated for this molar ratio, accordingly to Pascolo *et al.* (43), is of the same magnitude (~600 nM) of that found in jaundiced newborns with 362 μM (21 mg/dL) of total UCB and 452 μM (3 g/dL) HSA.

Various toxic effects of UCB have been reported on neuronal and glial cell activities (44, 45), such as alterations on postsynaptic potentials, morphological alterations and decreased mitochondrial activity. It has been proposed (20, 46, 47) that glutamate excitotoxicity is implicated in UCB neuronal damage. Our study is the first to correlate UCB cytotoxicity in astrocytes with the, a finding of particular relevance since these cells are primarily responsible for the clearance of this neurotransmitter from the extracellular space in the brain.

The absence of morphological alterations in astrocytes, following UCB treatment, is most likely due to the short term incubation period (15 min) used in this study. In fact, such alterations were only observed after a 2-h exposure to UCB (44). This observation is consistent with the maintenance of cell viability after treatment with the highest UCB concentration used in this study (342 μM), supporting the conclusion that the inhibition of glutamate uptake was not related to cell destruction but rather to impairment of cellular function(s).

Pretreatment of astrocytes with UCB led to a significant time-dependent inhibition of glutamate uptake (5

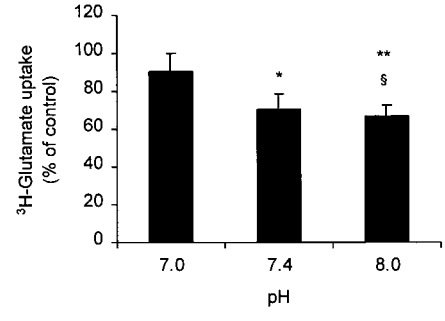

FIG. 5. pH-dependent inhibition of astrocyte glutamate uptake by unconjugated bilirubin (UCB). Cells were incubated for 15 min in the absence (control) and in the presence of 120 μM UCB and 100 μM human serum albumin at pH values of 7.0, 7.4 and 8.0. Incubation conditions and analytical procedures otherwise as in Fig. 2. Results are expressed as mean ± SEM ($n = 13$); *$p < 0.05$, **$p < 0.01$ vs control; $^{\S}p < 0.05$, pH 8.0 vs pH 7.0.

min, $p < 0.05$; 15 min, $p < 0.01$) (Fig. 3A). The progressive toxic effect observed indicates that the fast interaction of UCB with the membrane, evidenced by the slight impairment of glutamate transport, is probably followed by a complexation of the molecule with membrane phospholipids increasingly affecting glutamate uptake (11, 48, 49). This, therefore, disfavor the hypothesis that UCB and glutamate compete for a common transport mechanism. In line with previous observations in erythrocytes (12, 50) and synaptosomes (45, 47), the inhibitory effect of UCB was shown to be concentration-dependent also in astrocytes (Fig. 3B). Almost a complete inhibition of glutamate uptake was found at high UCB concentrations ($\geq$171 μM), and this inhibition was not restored following UCB removal by HSA washing, indicating that cellular damage resulting from aggregation of UCB in the membrane bilayer is irreversible.

The negative linear correlation observed between the UCB/HSA molar ratio and the glutamate uptake at a fixed HSA concentration (Fig. 4) supports the conclusion that the concentration of unbound UCB, which rises steeply at UCB concentrations exceeding the binding capacity of albumin, has an important role in UCB toxicity (51–53). Although the inhibition of glutamate uptake attained statistical significance only at UCB/HSA molar ratios $\geq$0.8, where the albumin "buffering" capacity for UCB decreases (54), glutamate uptake decreased progressively as UCB/HSA molar ratio increased even at low molar ratios, indicating that supersaturation of albumin is not necessary for this inhibitory effect. This favors the pathophysiological significance of our observations, in contrast to many other studies in which, as at our highest UCB concentrations, harmful effects of UCB were observed only when albumin binding capacity, and aqueous solubility of the unbound UCB species, were considerably exceeded (10, 11, 21, 45, 50).

As reviewed by Ostrow *et al.* (9), UCB diacid and monoanion are the two molecular species most involved in toxicity. The major decrease of glutamate uptake observed in going from pH 7.0 to 7.4 with little further inhibition at pH 8.0 (Fig. 5), indicates that the monoanion, rather than the dianion or the uncharged species, causes the reduction in glutamate uptake.

In conclusion, our studies demonstrate that UCB has a time-, concentration-, and pH-dependent inhibitory effect on glutamate uptake by cultured cortical rat astrocytes, even at concentrations below saturation of albumin. The present data support the assumption that astrocytes can no longer be considered as passive supporting cells resistant to injury (55), since damage to astrocytes can directly, or indirectly, promote neurodegeneration. These findings point to a potential role for astrocytes in contributing to the development of UCB encephalopathy during severe neonatal hyperbilirubinemia and indicate that future therapies considering astrocytes as potential targets should not be disregarded.

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