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Astrocytes as Mediators of Methylmercury Neurotoxicity: Effects on *D*-Aspartate and Serotonin Uptake

Key Words
Aspartate
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Abstract
In this study we address the effects of methylmercuric chloride (MeHgCl), a metal that is preferentially sequestered in astrocytes, on 5-HT and glutamate/aspartate uptake by rat primary astrocyte cultures. Quantitative autoradiography (ARG) combined with glial acidic fibrillary protein (GFAP) immunocytochemistry, as well as intact-cell (bulk) measurements of radiolabel uptake of these neurotransmitters were performed in 7- and 21-day-old primary astrocyte cultures. MeHg (10 μ*M* for 30 min) treatment of astrocytes (21 days in culture) significantly inhibited the Na^+-dependent and fluoxetine-sensitive [^{3}H]5-HT uptake. *D*-aspartate uptake in 7- and 21-day-old cultures was even more sensitive to MeHg, leading to >99% inhibition of *D*-aspartate uptake by astrocytes (30 min; 10 μ*M* MeHg). These results imply that the Na^+-dependent and fluoxetine-sensitive 5-HT uptake, as well as the Na^+-dependent *L*-glutamate/*D*-aspartate uptake systems in primary astrocyte cultures are sensitive to low concentrations of MeHg. Since astrocytic removal of glutamate (and aspartate) and 5-HT from the extracellular space in situ is crucial to the maintenance of chemical homeostasis, MeHg-induced uptake inhibition of 5-HT and aspartate could have cytotoxic effects on neighboring neurons.

Introduction

Recent studies [1–3] have established that rat primary astrocyte cultures of 1- to 4-day-old rats exhibit high-affinity, Na^+-dependent and fluoxetine-sensitive serotonin (5-HT) uptake with a K_m for 5-HT of 0.4 μ*M* and a K_i for fluoxetine of 23 n*M* which corresponds to the characteristics for 5-HT transport in brain. The demonstration of [^{3}H]5-HT uptake by primary cultured astrocytes in vitro suggests that astrocytes in situ may also function by actively removing 5-HT from the extracellular environment via the same high-affinity, fluoxetine- and Na^+-sensitive mechanism.

The V_{max} for the glutamate carrier in astrocytes is higher than that in synaptosomes or neurons, making astrocytes a likely major means of transmitter removal during normal neurotransmission in vivo [4, 5]. This quantitative data is derived from in vitro studies; the longer the astrocytes remain in culture, the higher the K_m and the lower the V_{max}. The glutamate carrier cotrans-

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ports Na^+ amd glutamate inward and K^+ outward. Based on its electrogenicity it was postulated that the stoichiometry was 3 Na^+ plus glutamate in and 1 K^+ out. Thus, for one transport cycle, a positive charge is transported inward, making it electrogenic. Kimbelberg et al. [6], using $^{22}Na^+$ and ^3H-glutamate, demonstrated a stoichiometry for uptake of 2–3 $^{22}Na^+$ with 1 glutamate in cultured neonatal primary astrocytes with a K_m of approximately 70 μ*M* for glutamate. It would thus appear that the carrier can be both voltage- as well as ion gradient-dependent, and disturbances of ion gradients may reduce the effectiveness of glutamate uptake. The electrogenic astrocyte carrier can also substitute aspartate for glutamate [7].

Astrocytes in the mammalian CNS are known targets for MeHg, consistent with the preferential accumulation of MeHg in astrocytes, in both human and nonhuman primate brains [8–11]. A prominent feature of prenatal MeHg poisoning is the reduction in CNS mitotic activity [12], and the interference with neuron migration [13–15]. Effects of MeHg on neuronal migration in culture reveal cessation of cell movement at 10 μ*M* MeHg. Abnormalities of neuronal migration are also prominent pathological features of MeHg-affected human brains [16]. Since postmitotic neurons normally migrate outward along radial glial processes [17], it is not surprising that damage to the astrocytes interferes with neuronal positioning and orientation. The initial site of MeHg injury appears to be the astrocytic plasma membrane with a marked shift in the distribution of anionic groups and loss of filopodial activity [18].

The biochemical mechanisms underlying MeHg neurotoxicity have not been clearly defined. Recently, it was observed that treatment with low micromolar concentrations of MeHg inhibits the uptake of the excitotoxic neurotransmitter, *L*-glutamate, into cultured mouse and rat cerebral cortical astrocytes [19, 20]. Astrocytes are a major site of glutamate inactivation [6, 21, 22] and survival of neurons exposed to glutamate is enhanced by co-culture with astrocytes [23]. It follows that astrocytic failure to control the levels of glutamate in the extracellular fluid may indirectly contribute to MeHg-induced damage to juxtaposed neurons [10, 20, 24]. To further test whether astrocytic homeostasis may be affected by MeHg, we studied its effects on *D*-aspartate uptake (a nonmetabolizable substrate for the Na^+-dependent *L*-glutamate/*L*-aspartate carriers) and a second Na^+-dependent neurotransmitter uptake, 5-HT, in neonatal rat primary astrocyte cultures. Studies were performed both by quantitative autoradiography (ARG; 21-day-old cultures) and intact-cell measurements (bulk; 7- and 21-day-old cultures) of radiolabel uptake of these neurotransmitters.

Materials and Methods

Materials

Fluoxetine hydrochloride was a gift of Eli Lilly & Co., Indianapolis, Ind., USA. Ketanserin(+) tartrate and spiperone HCl were obtained from Research Biochemicals Inc., Natick, Mass., USA [2-4-6-7-side chain-^3H]-5-HT creatinine sulfate (specific activity, 11.1 Ci/mmol) and *D*-[2,3-^3H]-aspartic acid (specific activity, 300 mCi/mg) were obtained from Amersham Life Sciences, Arlington Heights, Ill., USA. Polyclonal primary antibody against glial fibrillary acidic protein (GFAP) was obtained from Accurate Chemical and Scientific Company, Westbury, N.Y., USA. Secondary antibodies were obtained from Tago Immunochemical Company, Burlingame, Calif., USA. Autoradiography supplies were from Eastman Kodak, Rochester, N.Y., USA. All other chemicals were from Sigma Chemical Company, St. Louis, Mo., USA.

Tissue Cultures

The cerebral hemispheres of newborn rats (Sprague-Dawley) were removed, the meninges were carefully dissected off, and the tissue was dissociated using Dispase II (Boehringer-Mannheim Biochemicals, neutral protease, Dispase Grade II). Cultures were prepared as described by Frangakis and Kimelberg [25]. The cell suspension was seeded at a density of 1×10^4 cells/cm^2 onto 25-mm coverslips present in 6-well dishes and grown in Eagle's MEM supplemented with 10% heat-inactivated horse serum (HS) or 10% fetal bovine serum (FBS). The FBS was from a batch previously found to be permissive for [^3H]5-HT uptake in primary astrocyte cultures.

Autoradiographic Studies

Uptake of [^3H]5-HT and D*-Aspartate by Autoradiography.* Na^+-containing HCO_3^- buffer (see above) was used for uptake experiments. Primary astrocyte cultures were initially washed 4 times with 2 ml of this buffer at room temperature after being transferred to new 6-well dishes. Following the first wash with Na^+-containing buffer, coverslips to be tested in Na^+-free conditions were washed 3 times with Na^+-free buffer (where NaCl was replaced by 122 m*M* N-methyl-*D*-glucamine hydrochloride [NMDG·HCl], and $NaHCO_3$ was replaced by 22 m*M* triethylamine bicarbonate plus 3 m*M* $KHCO_3$). Following the wash steps, coverslips were incubated for 25 min at 37 °C in an incubator with a 5% CO_2/95% air atmosphere in 2 ml of preincubation buffer that contained 10^{-4} *M* pargyline to inhibit both monoamine oxidase-A (MAO-A) and MAO-B [26] and 10^{-5} *M* *L*-ascorbic acid to prevent the oxidation of [^3H]5-HT [27]. Next, 10^{-5} *M* ketanserin (+) tartrate to block 5-HT_{1B} in addition to 5-HT_{1C} and 5-HT_2 receptors [28, 29] and 10^{-5} *M* spiperone hydrochloride to block 5-HT_{1B} in addition to 5-HT_{1A}, 5-HT_{1C} and 5-HT_2 receptors [30] were present in the preincubation buffer in all wells for 5 min. Where indicated, 10^{-6} *M* fluoxetine hydrochloride was also present to specifically block high-affinity [^3H]5-HT uptake [31, 32]. Uptake was initiated by incubating cells with 3×10^{-7} *M* [^3H]5-HT (3.4 μCi/ml, with no added cold 5-HT) for 30 min. at 37 °C in Na^+-containing or Na^+-free HCO_3^- buffer. Uptake was terminated by rapidly washing all wells 6 times with ice-cold phosphate-buffered saline (PBS). The composition of the PBS

was 137 mM NaCl, 15 mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$ and 0.7 mM $CaCl_2$ and the pH was 7.4. Cells were then fixed in Zamboni's fixative (2% paraformaldehyde, 7.5% picric acid in 0.1 M sodium phosphate buffer pH 7.4) [33] for 30 min at 4 °C.

Uptake of [^3H]-D-Aspartate Uptake by Autoradiography. Uptake of [^{3}H]-D-aspartate was measured as described above, but without pargyline, ascorbic acid, and ketanserin (+) tartrate.

Immunocytochemical Staining. Coverslips containing the fixed cells were rinsed three times with ice-cold PBS and then permeabilized by incubation in PBS containing 250 μg/ml saponin for 30 min. All antibody incubations were performed for 45 min in PBS containing saponin and 2% normal goat serum (NGS) and were terminated by three rinses with PBS. Cells were incubated with the appropriate primary and secondary antibodies for GFAP. Cells were incubated with a 1:20 dilution of polyclonal rabbit antibody raised against bovine GFAP (Accurate Chemical & Scientific Co.), followed by incubation with a 1:40 dilution of rhodamine-conjugated goat antirabbit secondary antibody (Tago, Inc.). Controls during staining consisted of the omission of primary antibody and incubation only with the secondary antibody. In some experiments, nonimmune serum was substituted as a control for the primary GFAP antibody, followed by incubation with secondary antibody.

Autoradiographical Procedures and Grain Analysis. Coverslips were mounted cell side up on glass slides with 1:1 xylene/permount solution and air-dried for 12 h at room temperature in the dark. The mounted coverslips were then dipped in a 1:1 solution of NTB2 emulsion and MilliQ water which contained 1% glycerol to reduce stress artifacts [34], at a temperature of 40 °C. They were then dried for 2 h in a high humidity atmosphere to lessen latent image formation and thereby reduce background grain development [34, 35], air dried in normal humidity for another 2 h and then placed in a light-tight box containing desiccant for 10–12 days at 4 °C. Development was always carried out for 2 min at 4 °C since this protocol led to moderate grain densities over uptake-positive cells with very low background. Slides were developed with a 1:1 Kodak Dektol developer/MilliQ water solution, bathed for 30 s in 1% acetic acid to arrest development, exposed to Kodak fixer for 10 min an then rinsed 4 times in tap water at 5-min intervals. Following development, the experimental coverslip was covered with a second coverslip using a 25 μl PBS-glycerol drop containing 2% DABCO [1–4-diazabicyclo (2.2.2) octane] to prevent fading of fluorescence [36]. Control coverslips were processed in exactly the same way without being exposed to radioactivity [35], and control background grain levels were examined over cells as well as acellular areas.

Morphologically intact GFAP(+) astrocytes were identified initially only by visualization under rhodamine fluorescence as this helped prevent the recognition of colocalized grains, which could bias the selection of cells during data analysis. Cell morphometric measurements and grain counts (both for 5-HT and D-aspartate) were performed over the rhodamine-fluorescence video image of astrocyte cell bodies, and separately over their associated processes. However, it was often necessary to analyze intense [^{3}H]-D-aspartate uptake over cells directly as it obscured the GFAP-immunofluorescence intensity. The immunofluorescence rather than the phase-contrast video image was analyzed to ensure uniform and accurate data collection. This approach caused grains juxtaposed to the cell membrane or on it [35] to be excluded from analysis.

To avoid bias in cell selection during data analysis, coverslips were examined systematically in successive left to right sweeps and visualized only under rhodamine fluorescence so as to identify GFAP(+) cells without seeing their associated grains. Cells with sharp, intensely fluorescent and uniformly distributed GFAP(+) filaments that appeared morphologically intact were analyzed by the JAVA image analysis system (Jandel, Corte Madera, Calif., USA). The rhodamine fluorescence image of the cell was captured by a video camera and digitized by the JAVA image analysis system. Morphometric measurements including length, breadth and area of the cell and/or processes were made independently using cursor measurements. The number of grains over the rhodamine fluorescence image of the cell and/or its processes were counted by eye to ensure accuracy. A total of 25 cultured cells were analyzed for each experimental condition where [^{3}H]5-HT or [^{3}H]-D-aspartate uptake were examined.

Intact-Cell (Bulk) Measurement of Radiolabeled Uptake in 21-Day-Old Cultures

Uptake of [^3H]5-HT. HEPES buffer was used in all the uptake experiments, unless otherwise specified, and consisted of the following: 122 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM D-glucose and 25 mM HEPES (N-2-hydroxyethylpiperazine N′-2-etahesulfonic acid). Buffered solutions were maintained at pH 7.4 by the addition of 1 N NaOH and at 37 °C by a water bath. Prior to MeHg exposure, primary astrocytes grown in 12-well culture dishes with heat-inactivated HS-supplemented MEM were washed (×4) with 1 ml of buffer (37 °C). Following the wash, 1 ml of buffered MeHg solution (1 and 10 μM) was added to MeHg-treated cells. Each well was then washed (×4) with 1 ml of buffer, followed by a 5-min incubation with 10^{-4} M pargyline to inhibit both MAO-A and MAO-B [26] and 10^{-5} M L-ascorbic acid to prevent the oxidation of [^{3}H]5-HT [27]. Also, where specified as a control, 10^{-6} M fluoxetine hydrochloride was present to specifically block high-affinity [^{3}H]5-HT uptake [31, 32] during the 5-min incubation period. The contents of each well were then aspirated (not washed) and uptake of [^{3}H]5-HT was initiated. Where required, Na^+-free buffer was used as an additional control (NaCl was replaced by 122 mM N-methyl-D-glucamine hydrochloride [NMDG · HCl]). Uptake was performed by incubating cells with 3×10^{-7} M [^{3}H]5-HT (4.92 μCi/ml, with no added cold 5-HT) for 30 min at 37 °C in Na^+-containing or, where required, Na^+-free HEPES buffer. All wells received both 10^{-4} M pargyline and 10^{-5} M L-ascorbic acid during the actual 30 min [^{3}H]5-HT uptake. 10^{-6} M fluoxetine hydrochloride was added where needed as a control. Uptake was terminated by rapidly washing all wells (×4) with 1 ml mannitol wash solution (0 °C). The composition of the mannitol wash solution was 0.29 M mannitol, 10 mM Tris (hydroxymethyl) aminomethane, 0.5 M $Ca(NO_3)_2 \cdot 4H_20$ (pH 7.4). Each well was aspirated until dry and the cellular contents of each well subsequently lysed with 1 ml 1 N NaOH for 15 min. Aliquots (800 μl) were removed from each well and added to 5 ml of Ecoscint (National Diagnostics, Manville, N.J., USA) for counting in a β-scintillation counter (Beckman LS 3801, Beckman Instruments, Irvine, Calif., USA). An additional 100-μl aliquot was taken from each well for protein determination according to Pierce BCA/Elisa protein analysis according to the method of Goldschmidt and Kimelberg [37].

Uptake of [^3H]-D-Aspartate. Uptake of [^{3}H]-D-aspartate was measured as described above; however, in the absence of pargyline and ascorbic acid. Uptake was initiated by aspirating the medium and adding 1.0 ml of prewarmed buffer containing 1.0 mCi of [^{3}H]-D-aspartic acid (45 mCi/mmol) supplemented with nonlabeled D-aspartate to a final concentration of 100 μM. Uptake was allowed to proceed for 10 or 30 min, and after rapid washing with 0.29 M mannitol solu-

tion and solubilization overnight, aliquots were removed for counting and for protein determination as described above.

Statistical Analysis

Significant differences between cell groups in the quantitative ARG and bulk measurements were determined using a one-way randomized ANOVA design. Where the overall test of significance ($p < 0.05$) led to a rejection of the null hypothesis, post hoc comparisons [38] were carried out to determine the source of the effect (Scheffe's test for the ARG data and Newman-Keuls test for the bulk data). Statistical calculations were performed with CSS:Statistica software (StatSoft Inc., Tulsa, Okla., USA).

Results

Autoradiographic Studies in GFAP(+) Cells

[^{3}H]5-HT Uptake by 7- and 21-Day-Old Cultured Astrocytes by ARG. Throughout this study results for [^{3}H]5-HT uptake of ARG were calculated from 25 cultured astrocytes for each experimental condition. Background cellular grain densities were calculated from 25 control cells that were not exposed to [^{3}H]5-HT during experiments. [^{3}H]5-HT uptake in cultured astrocytes (7 and 21 days old) with varying experimental conditions is shown in figure 1. When compared to the uptake (100%) in Na^+-containing buffer, uptake of [^{3}H]5-HT in Na^+-free buffer was 17.5 ± 2.2 and 20.7 ± 1.5% for 7- and 21-day-old cultures, respectively ($p < 0.00001$ and $p < 0.00001$, respectively, by ANOVA and Fisher's LSD test). In fluoxetine (1 μ*M*)-treated astrocytes [^{3}H]5-HT uptake was 67.5 ± 7.9 and 20.3 ± 1.0% of that in Na^+-containing buffer for 7- and 21-day-old cultures, respectively ($p < 0.02$ and $p < 0.00001$, respectively). MeHg was also more potent (both at 1 and 10 μ*M*) in inhibiting [^{3}H]5-HT uptake in 21-day-old cultures. When compared to the uptake in Na^+-containing buffer, uptake of [^{3}H]5-HT in the presence of 1 μ*M* MeHg (30 min) was 87.8 ± 7.1 and 73.5 ± 5.6% for 7- and 21-day-old cultures ($p < 0.8$ and $p < 0.02$), respectively. At 10 μ*M* MeHg [^{3}H]5-HT uptake was 90 ± 7.5 and 63.7 ± 3.1% of controls for 7- and 21-day-old cultures ($p < 0.9$ and $p < 0.0006$), respectively.

Figures 2A and B are phase-contrast and brightfield photomicrographs of positive [^{3}H]5-HT uptake by 21-day-old cultured astrocytes in Na^+-containing buffer. Figures 2C and D depict uptake in the presence of 1 μ*M* fluoxetine in Na^+-containing buffer. Figures 2E and F show astrocytes in the presence of MeHg. Grain densities were lower over GFAP(+) cells treated with both 1 (fig. 2F) or 10 μ*M* MeHg (fig. 2H) compared with controls (see also fig. 1 for the graphic data derived from 25 cells).

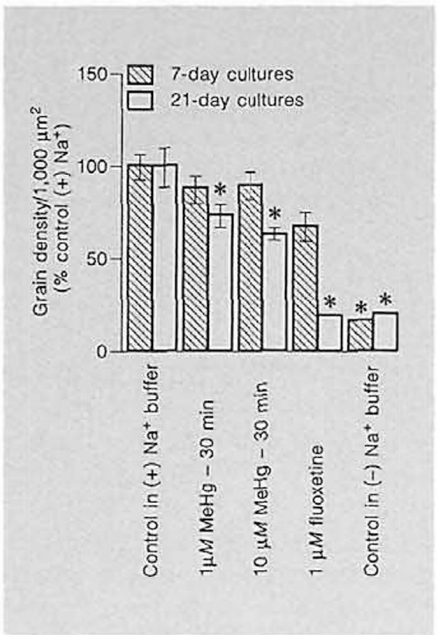

Fig. 1. Percent mean [^{3}H]5-HT uptake (controls = grain density/100 μm² cell area in (+) Na^+ buffer) measurements by ARG in 7- and 21-day-old primary astrocyte cultures showing the effects of omission of Na^+ (–Na^+), addition of 1 μ*M* fluoxetine, and exposure to MeHg (1 and 10 μ*M*). As noted in the methods, throughout this study, mean [^{3}H]5-HT uptake was calculated from 25 astrocytes for each condition. *Significantly reduced compared with control. Means ± SEM.

[^{3}H]-D-Aspartate Uptake by 7- and 21-Day-Old Cultured Astrocytes by ARG. The mean [^{3}H]-*D*-aspartate grain densities over 7- and 21-day-old cultured astrocytes are shown in figure 3. [^{3}H]-*D*-aspartate uptake in Na^+-free buffer was 1% of controls both in 7- and 21-day-old cultures ($p < 0.000001$ and $p < 0.00001$, respectively). In the presence of 1 m*M* *L*-aspartic β-hydroxamate in the buffer, [^{3}H]-*D*-aspartate uptake was 25.7 ± 4.4 and 1.8 ± 0.01% of controls for 7- and 21-day-old cultures ($p < 0.00001$ and $p < 0.00001$), respectively. Thirty minutes exposure to MeHg, both at 1 and 10 μ*M*, led to significant inhibition of [^{3}H]-*D*-aspartate uptake ($p < 0.00001$) in 7- and 21-day-old cultures.

Figure 4 illustrates the phase-contrast and brightfield photomicrographs, respectively, of [^{3}H]-*D*-aspartate uptake by astrocytes (21 days old) in a number of experimental conditions. Very intense [^{3}H]-*D*-aspartate uptake by cultured astrocytes is seen in Na^+-containing buffer (fig. 4A, B). Figures 4C and D show the inhibitory effect

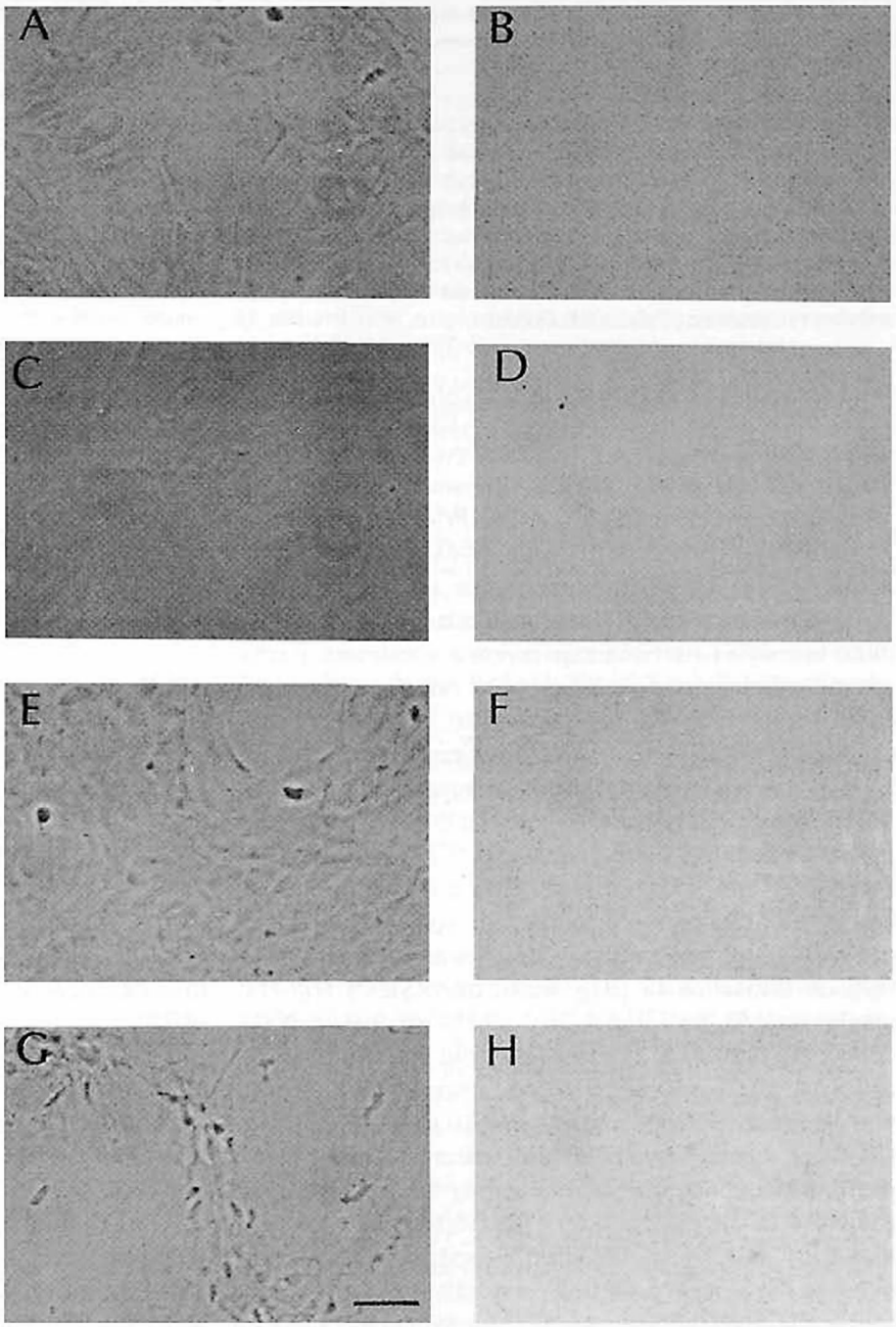

Fig. 2. Phase (**A**) and brightfield (**B**) photomicrographs of 21-day-old astrocyte cultures showing [^{3}H]5-HT uptake in Na^+-containing buffer. Astrocytes showed moderately positive uptake of [^{3}H]5-HT by ARG with grain densities of 104.3± 11.2 grains/1,000 μm^2 cell area. Scale bar = 50 μm. **C, D** Phase and brightfield photomicrographs of [^{3}H]5-HT uptake in astrocytes treated with 1 μ*M* fluoxetine. Grain densities over the astrocytes was 21.1± 1.0 grains/1,000 μm^2 showing that [^{3}H]5-HT uptake was reduced in the presence of fluoxetine in the buffer. **E–G** Phase and brightfield photomicrographs of [^{3}H]5-HT uptake in astrocytes treated with 1 μ*M* (**E, F**) and 10 μ*M* (**G, H**) MeHg. Grain densities over astrocytes was 76.7± 5.2 and 66.4± 3.3 grains/1,000 μm^2 for 1 and 10 μ*M* MeHg, respectively.

of $Na^+(-)$ buffer on [^{3}H]-*D*-aspartate uptake. Figures 4E and F illustrate the effect of 1 m*M* *L*-aspartic acid β-hydroxamate on the 30-min uptake of *D*-aspartate in 21-day-old cultures. Both removal of Na^+ from the buffer and treatment with the glutamate analog almost completely abolish the uptake of aspartate by these cultures as shown in the graphic data for 25 cells in figure 3. Aspartate uptake is also inhibited by treatment of astrocytes with MeHg. Grain densities are reduced over GFAP(+) cells treated with 1 μ*M* (fig. 4G, H); 10 μ*M* MeHg (fig. 4I, J) treated cells appear completely void of [^{3}H]-*D*-aspartate labeling (see also fig. 3 for bulk measurements).

Bulk Cell Measurements of Radiolabeled Uptake of 5-HT and D-Aspartate

[^{3}H]5-HT Uptake in 21-Day-Old Cultures. The effect of MeHg on astrocytic [^{3}H]5-HT uptake is shown in figure 5. Removal of Na^+ from the buffer led to a time-dependent decrease in uptake of [^{3}H]5-HT (60.5 ± 0.1 and 47.4 ± 2.2% of controls for 10 and 30 min uptake, respectively; $p < 0.00017$ and $p < 0.00012$, respectively). [^{3}H]5-HT uptake was reduced in a time-dependent fashion in the presence of 1 μ*M* fluoxetine to 36.1 ± 1.8 and 32.5 ± 1.8% of controls for 10 and 30 min uptake, respectively ($p < 0.00015$ and $p < 0.00016$, respectively). Exposure of the cultures to 1 μ*M* MeHg for 10 and 30 min (101.2 ± 0.1 and 99 ± 1.5% of control uptake, respectively), and 10 μ*M* MeHg for 10 min (101.8 ± 1.9% of controls) did not affect [^{3}H]5-HT uptake; a significant effect on [^{3}H]5-HT uptake was noted, however, upon exposure to 10 μ*M* MeHg for 30 min (75.6 ± 1.3% of controls; $p < 0.00015$), consistent with the data derived from the ARG studies (fig. 1).

[^{3}H]-D-Aspartate in 21-Day-Old Cultures. The effect of MeHg on the uptake of [^{3}H]-*D*-aspartate is illustrated in figure 6. Na^+-free buffer led to 50.1 ± 0.01% and 43.3 ± 4.1% of control uptake at 10 and 30 min, respectively ($p < 0.0008$ and $p < 0.00025$, respectively). Exposure of astrocytes to 1 μ*M* MeHg for 10 or 30 min did not alter [^{3}H]-*D*-aspartate uptake (101.8 and 98.3% of controls, respectively). In contrast, exposure to 10 μ*M* MeHg led to significant time-dependent inhibition of [^{3}H]-*D*-aspartate uptake to 57.9 ± 4.42 and 13.5 ± 3.3% of controls, for 10 and 30 min, respectively ($p < 0.0003$ and $p < 0.00025$, respectively).

Viability of MeHg-Treated Cells

Astrocytes exposed to MeHg for 30 min appeared morphologically intact. To ascertain whether MeHg treatment may have long-lasting effects on astrocytic viability, astrocytes exposed to 10 μ*M* MeHg for 30 min were washed and cultured for additional 1.5–2 weeks in MEM, and stained for GFAP as previously described. Unlike control cells (fig. 7a, b), MeHg-treated cells (fig. 7c, d) appeared retracted, with increased intercellular space. There was no apparent loss of GFAP fluorescence intensity in MeHg-treated cells, but the GFAP did not appear to be as filamentous as in control cells. In addition, the cytoplasm of MeHg-treated cells showed increased granularity and vacuolization compared with controls. To further test their viability, MeHg-treated cells were also treated at 2 weeks after MeHg treatment with 0.08% trypan blue. Nearly all the cells appeared intact and excluded the dye, suggesting that cell viability is not compromised by MeHg treatment.

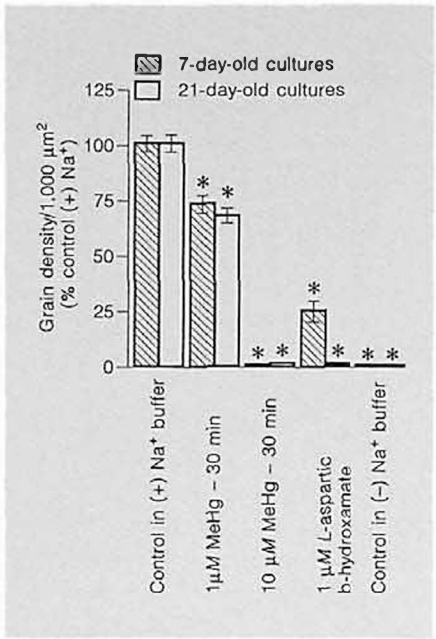

Fig. 3. Percent mean [^{3}H]-*D*-aspartate uptake (controls = grain density/100 μm² cell area in (+) Na^+ buffer) in 7- and 21-day-old primary astrocytes cultures showing the effects of omission of Na^+ ($-Na^+$), effect of 1 μ*M* fluoxetine, and MeHg (1 μ*M* and 10 μ*M* for 10 or 30 min). Throughout this study, mean [^{3}H]-*D*-aspartate uptake was calculated from 25 astrocytes for each condition. *Significantly reduced compared with control. Means ± SEM.

Discussion

The present study corroborates the earlier finding of Brookes and Kristt [19], Aschner et al. [24], and Albrecht et al. [20], both in mouse and rat astrocytes, that astrocytic glutamate (aspartate) uptake is highly sensitive to MeHg. These findings support the concept that inhibition of glutamate uptake by astrocytes may in part mediate MeHg neurotoxicity. The present study also extends the inhibitory effects of MeHg to the uptake of a second neurotransmitter: 5-HT.

Moderately high grain densities over astrocytes were observed with [^{3}H]5-HT in Na^+-containing buffer. Treatment of the astrocytes with ketanserin and spiperone ensured that this could not represent binding to 5-HT_1 or 5-HT_2 receptors subtypes. The uptake of [^{3}H]5-HT was also Na^+- and fluoxetine-sensitive. Compared to [^{3}H]5-HT uptake in age-matched astrocytic cultures, the uptake

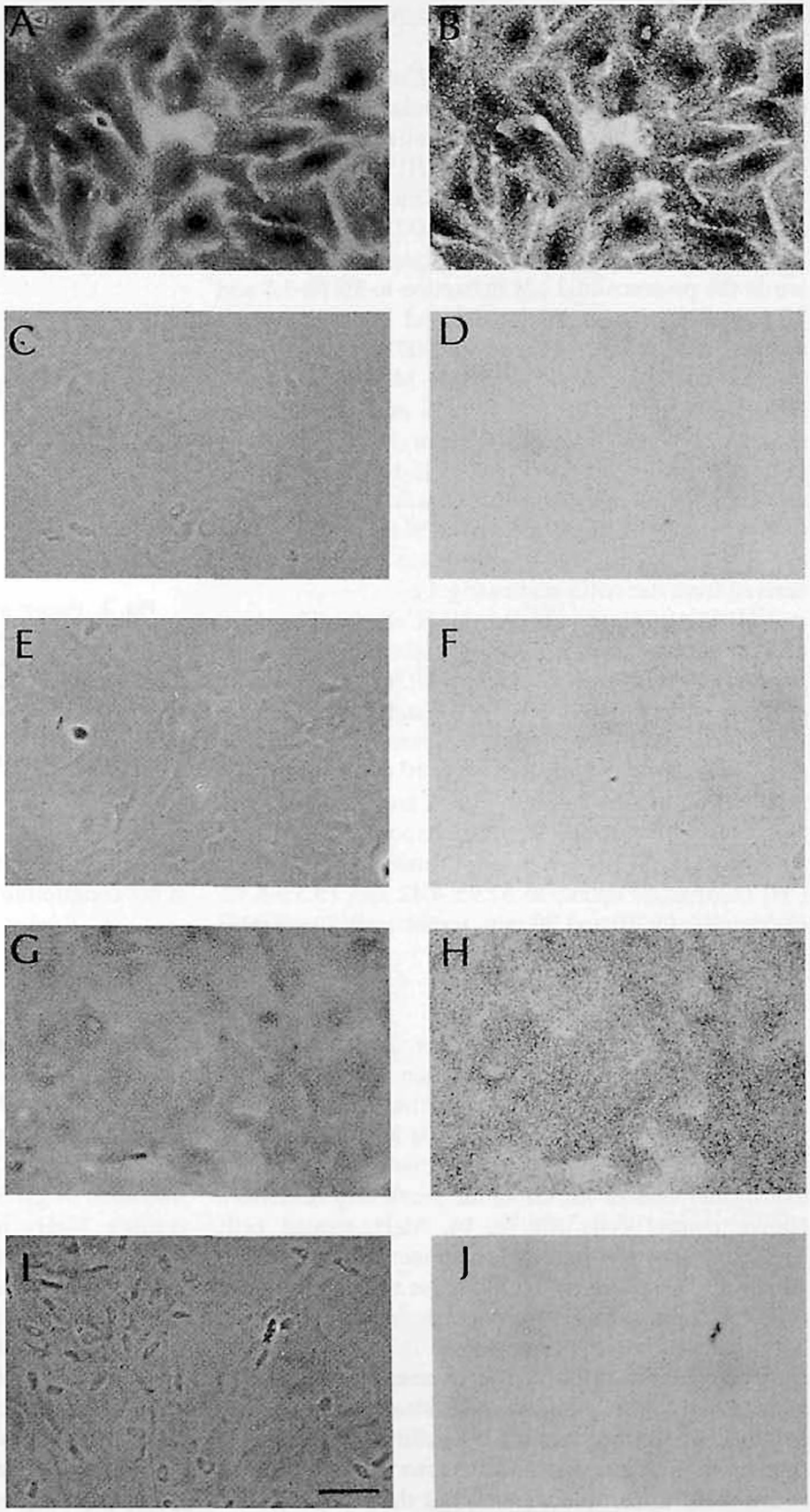

Fig. 4. Phase (**A**) and brightfield (**B**) photomicrographs of 21-day-old astrocyte cultures showing [^{3}H]-*D*-aspartate uptake in Na^+-containing buffer. Astrocytes showed intense uptake of [^{3}H]-*D*-aspartate with grain densities of 286.6± 10.5 grains/1,000 μm^2 cell area. Scale bar = 50 μm. **C, D** Phase and brightfield photomicrographs of [^{3}H]-*D*-aspartate uptake in astrocytes in Na^+ (−) buffer. Grain densities over the astrocytes was 2.5± 0.2 grains/1,000 μm^2 showing almost complete inhibition (> 99%) of [^{3}H]-*D*-aspartate uptake in the absence of Na^+ in the buffer. **E, F** Phase and brightfield photomicrographs of [^{3}H]-*D*-aspartate uptake in astrocytes treated with 1 m*M* *L*-aspartic acid β-hydroxamate, an analog of *L*-glutamate. Grain densities over the astrocytes was 5.2± 0.4 grains/1,000 μm^2, comparable to the density in Na^+ (−) buffer. **G–J** Phase and brightfield photomicrographs of [^{3}H]-*D*-aspartate uptake in astrocytes treated with 1 μ*M* (**G, H**) and 10 μ*M* (**I, J**) MeHg. Grain densities over astrocytes was 195.1± 10.1 and 4.8± 0.3 grains/1,000 μm^2 Na^+ (−) buffer for 1 and 10 μ*M* MeHg, respectively.

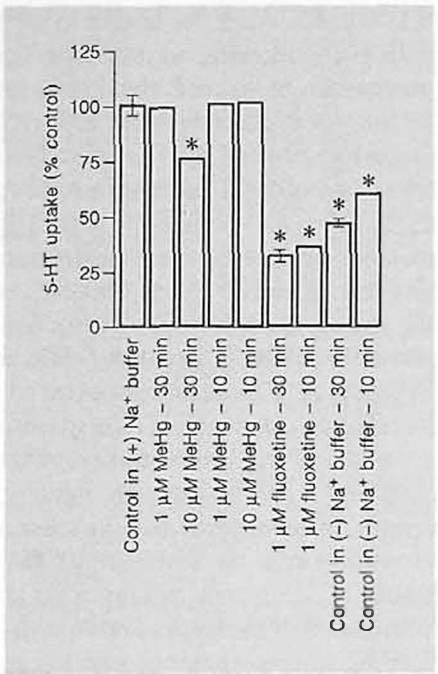

Fig. 5. Bulk measurements of radiolabeled [^{3}H]5-HT uptake in 21-day-old primary astrocyte cultures (controls = DPM/mg protein in (+) Na^+ buffer). Shown are the effects of omission of Na^+ ($-Na^+$), addition of 1 μM fluoxetine, and incubation with MeHg (1 and 10 μM). Each point represents the mean ± SEM (4 wells/experiment). For further details please refer to the text. *Significantly inhibited compared with control.

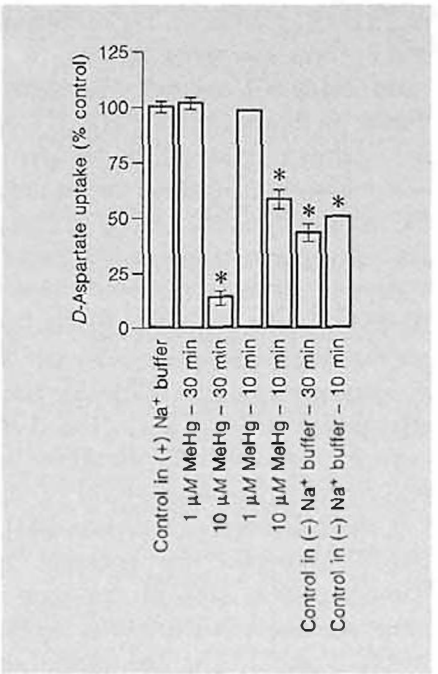

Fig. 6. Bulk measurements of radiolabeled [^{3}H]-*D*-aspartate uptake in 21-day-old primary astrocyte cultures (controls = DPM/mg protein in (+) Na^+ buffer). Shown are the effects of omission of Na^+ ($-Na^+$), and incubation with MeHg (1 and 10 μM). Each point represents the mean ± SEM (4 wells/experiment). For further details please refer to the text. *Significantly inhibited compared with control.

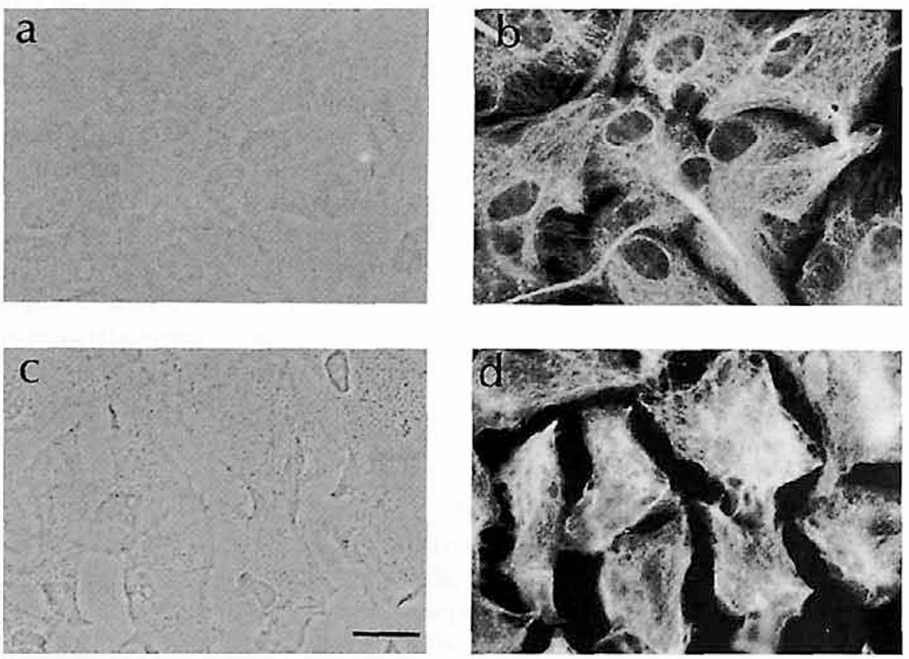

Fig. 7. Rhodamine-conjugated GFAP immunofluorescence (**a**) and brightfield (**b**) photomicrographs of 5-week-old astrocyte cultures (i.e. non-MeHg-treated controls). Rhodamine-conjugated GFAP immunofluorescence (**c**) and brightfield (**d**) photomicrographs of 21-day-old astrocyte cultures exposed to 10 μM MeHg and cultured for an additional 2 weeks in MEM. Although morphologically intact, unlike controls (**a, b**) their processes are retracted, their plasma appears more granulated and vacuolated, and they do not seem to form cell-to-cell contacts. Scale bar = 25 μm.

of [^{3}H]-*D*-aspartate in Na^+-containing buffer at both 7 and 21 days was much higher.

In 7-day-old cultured astrocytes the inhibition produced by fluoxetine on [^{3}H]5-HT uptake was less than the inhibition produced by Na^+-free buffer (fig. 1). This was a consistent finding for all the treatment groups in the autoradiographic (ARG) studies and implies that the Na^+-dependent component of the 5-HT uptake system at 7 days is not as sensitive to fluoxetine, which may be related to some difference in the fluoxetine-binding site on the 5-HT transporter. To our knowledge, an age-dependent increase in fluoxetine sensitivity has not been reported previously [32]. The 5-HT content and the capacity for total 5-HT uptake in the neonatal rat cerebral cortex is about one-third of that seen in adult rats [39, 40]. Kirksey and Slotkin [41] have reported that [^{3}H]5-HT uptake by neonatal rat cerebral cortex homogenates is 10% of that seen in adult rats, and a rapid increase in such uptake occurs between postnatal weeks 1 and 2. The age-related increase in fluoxetine sensitivity we found may also be related to these age-related differences in 5-HT levels and uptake related to some differences in fluoxetine-binding site on the 5-HT transporter. Consistent with the increased inhibition of [^{3}H]5-HT uptake in 21-day-old cultures (compared with 7-day-old cultures) produced by fluoxetine, and MeHg, *D*-aspartate uptake inhibition was also increased in the 21-day-old astrocytes by MeHg (1 μ*M*) and aspartic β-hydroxamate. Taken together, these observations also imply that the Na^+-dependent components of both 5-HT and *D*-aspartate are not fully developed in the 7-day-old cultures.

Glutamate (and its analog, aspartate) transport is an Na^+-dependent, carrier-mediated, electrogenic process, with the Na^+ gradient plus the membrane potential providing the driving force [6, 42, 43]. Therefore, inhibition of aspartate uptake by MeHg may be caused by one, or a combination, of the following events: (a) cell membrane depolarization; (b) inhibition of the Na^+/K^+-ATPase activity, which keeps intracellular [Na^+] low, and (c) direct inactivation of the carrier. At present, we are unable to directly distinguish between these three possibilities. One can argue against possibility (b), because it takes about 1 h before the inhibition of the Na^+/K^+-ATPase results in the dissipation of the Na^+ gradient across the astrocytic cell membrane [44].

The uptake system for 5-HT is thought to be the primary means of inactivation of 5-HT after it is released from the neuronal pool, because this system shows a relatively high affinity for the substrate with K_m values of 0.2–0.4 μ*M*. Also, the uptake system cotransports 5-HT with Na^+, allowing intracellular concentrations of the transmitter to exceed the extracellular concentrations because of utilization of the free energy available in the inwardly directed Na^+ electrochemical gradient. The likely fate of 5-HT taken up into astrocytes appears to be metabolism and removal – a pass-through, uptake, and metabolism system – rather than uptake and storage in vesicles for re-release, as occurs in nerve endings. Thus, the astrocyte could function to take up either excess or normally released levels of 5-HT, and convert it to its metabolites. Failure of astrocytes to perform such functions in the presence of MeHg may lead to a selective increase in 5-HT concentrations in the extracellular fluid. Similarly, MeHg-induced inhibition of *D*-aspartate uptake by astrocytes can increase its (and glutamate) conentration in the extracellular fluid. Abnormally high levels of excitatory amino acids causing exaggerated stimulation of excitatory amino acid receptors on the surface of adjacent neurons can trigger a destructive cascade of events that can damage neurons en masse.

A consistent finding in our experiments is the persistent greater inhibitory effects of MeHg, fluoxetine, and Na^+ – buffer on [^{3}H]5-HT uptake, and the greater inhibitory effects of MeHg, aspartic β-hydroxamate, and Na^+ – buffer on [^{3}H]-*D*-aspartate uptake by astrocytes in the autoradiographic studies (ARG) compared to the bulk measurements. This may be related to methodological differences between the two protocols, the autoradiographic experiments requiring additional fixation steps (see methodology). Nevertheless, in ARG and bulk studies of both [^{3}H]5-HT and [^{3}H]-*D*-aspartate uptake the same inhibitory trends are noted, although the degree of inhibition is not always concordant. MeHg concentrations of 10 μ*M* exert a significant effect on the uptake of both neurotransmitters by astrocytes.

In summary, MeHg inhibits the Na^+-dependent uptake of both aspartate and 5-HT in cultured astrocytes as shown by ARG and bulk measurements of radiolabeled 5-HT and *D*-aspartate. The occurrence of attenuated neurotransmitter uptake in MeHg-treated astrocytes supports the argument that astrocytes may mediate MeHg-induced neurotoxicity and warrants further study on MeHg-mediated effects on neurotransmitter homeostasis.

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