Na⁺-Dependent, Fluoxetine-Sensitive Serotonin Uptake by Astrocytes Tissue-printed from Rat Cerebral Cortex

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Previous studies have established that rat primary astrocyte cultures prepared from several brain regions of 1–4-d-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 nM, which correspond to the characteristics for this transport for other brain preparations. However, it is not known whether astrocytes *in situ* show such uptake. We addressed this question by performing ³H-5-HT uptake experiments on cortical astrocytes, within 4 hr of isolating them from 6- and 21-d-old rats by the tissue-print technique. Quantitative autoradiography was combined with GFAP and neurofilament (NF) immunocytochemistry to distinguish astrocytic from neuronal ³H-5-HT uptake. In composition, the tissue-printed (TP) cells and processes were 60–70% GFAP(+) and 10–15% NF(+). ³H-5-HT uptake (0.3 μ M 5-HT, 3.4 μ Ci/ml) in both tissue-printed GFAP(+) astrocytes and NF(+) structures was sensitive to 1 μ M fluoxetine and was also Na⁺ dependent. More than 90% of TP astrocytes from 6- and 21-d-old rats and 100% of NF(+) structures from 21-d-old rats showed positive ³H-5-HT uptake (defined as ≥ 31 grains/10³ μ m²). The highest level of uptake (≥ 191 grains/10³ μ m²) was never observed in TP astrocytes but was exhibited by about half of the NF(+) structures. In other experiments we found that ³H-5-HT uptake by 6-d-old TP astrocytes was comparable to uptake by postnatal age-matched primary cultured astrocytes that were grown in fetal bovine serum (FBS). However, primary cultured astrocytes grown in horse serum showed lower uptake than that observed with FBS, a finding similar to previous results in cultures where ³H-5-HT uptake was measured per milligram of cell protein. These results imply that high-affinity, Na⁺-dependent and fluoxetine-sensitive 5-HT uptake occurs in rat cortical astrocytes *in situ*.

[Key words: tissue-printed astrocytes, 5-HT uptake, rat, cerebral cortex, cultured astrocytes, fluoxetine, autoradiography, fetal bovine serum, horse serum, neurofilament]

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Primary astrocyte cultures derived from the cerebral cortex and other rat brain regions exhibit high-affinity, Na⁺-dependent and fluoxetine-sensitive serotonin (5-HT) uptake (Kimelberg and Katz, 1985, 1986; Amundson et al., 1992). In these reports, ³H-5-HT uptake was measured by transport studies in primary cultures that were >95% GFAP(+) (Katz and Kimelberg, 1985) or by the autoradiographic localization of grains on individual cells in such cultures and, depending on the fixative used, colocalized with GFAP staining (Kimelberg and Katz, 1985). These autoradiography experiments revealed heterogeneous 5-HT uptake among individual astrocytes cultured from the same brain region (Katz and Kimelberg, 1985; Kimelberg and Katz, 1985). Both autoradiography and transport studies also revealed marked differences in 5-HT uptake in astrocytes cultured from different brain regions (Amundson et al., 1992). The demonstration of ³H-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. However, definitive evidence of such uptake by astrocytes *in situ* is needed as conditions such as the type of serum used are known to influence 5-HT uptake in cultures (Kimelberg et al., 1992), and because uptake in cultures could represent either up- or down-regulated expression of the 5-HT uptake system, or even *de novo* expression. We thus decided to study the uptake of ³H-5-HT by astrocytes isolated acutely from brain tissue.

There have been a few previous studies of ³H-5-HT uptake in acutely isolated glial and astrocyte fractions prepared from brain tissue, as well as attempts at localizing uptake *in situ*. Uptake of labeled ³H-5-HT was seen in a “glial” fraction isolated from rabbit brain (Henn and Hamberger, 1971) and was localized over glial profiles in the rabbit area postrema by fluorescence histochemistry (Dow et al., 1973). Subsequently, such uptake was demonstrated over astrocyte profiles in cat medulla following ³H-5-HT perfusion by using electron microscopic autoradiography (Ruda and Gobel, 1980) and over the “all-glial” frog filum terminale by light microscope autoradiography (Ritchie et al., 1981). More recently, ³H-5-HT uptake was found to be high in brain slices obtained from the kainate-lesioned gliotic cortex and hippocampus of adult rats and, following intraventricular injection, was localized to periventricular and hippocampal GFAP(+) astrocytes by autoradiography (Anderson et al., 1992). However, pharmacological characterization of the 5-HT uptake system in this as well as in previous *in situ* studies proved difficult.

The technique of tissue-printing was recently used by Barres et al. (1990) to isolate astrocytes from the postnatal rat optic nerve for electrophysiological studies. These optic nerve tissue-

printed astrocytes appeared morphologically intact with numerous processes and had the large negative membrane potentials characteristic of astrocytes *in vivo* and *in vitro*. We modified this tissue-print procedure, primarily by omitting the enzymes used for promoting cell dissociation. Following ^3H -5-HT uptake experiments, where the Na^+ dependence and fluoxetine sensitivity were also examined, tissue-printed cells were immunocytochemically stained for GFAP and neurofilament (NF) and then processed for autoradiography as described earlier (Kimelberg and Katz, 1985). Our results demonstrate that high-affinity, Na^+ -dependent and fluoxetine-sensitive ^3H -5-HT uptake is a property exhibited by the majority of 6- and 21-d-old tissue-printed GFAP(+) rat cortical astrocytes, as well as by NF(+) axon-like profiles.

Some of the results presented here were reported previously in abstract form (Dave and Kimelberg, 1993).

Materials and Methods

Materials. Fluoxetine hydrochloride was a generous gift of Eli Lilly & Co., Indianapolis, IN. Ketanserin(+) tartrate and spiperone HCl were obtained from Research Biochemicals Inc., Natick, MA. [2-4-6-7-Side chain- ^3H -5-HT creatinine sulfate (specific activity, 11.1 Ci/mmol) was obtained from Amersham Life Sciences, Arlington Heights, IL. Polyclonal primary antibody against GFAP was obtained from Accurate Chemical & Scientific Co., Westbury, NY, and monoclonal primary antibody against neurofilament (200 kDa subunit) was from Boehringer-Mannheim Inc., Indianapolis, IN. Secondary antibodies were obtained from Tago Immunochemical Co., Burlingame, CA. Autoradiography supplies were from Eastman Kodak, Rochester, NY. Cell tracker fluorescent blue probe 4-chloromethyl-7-hydroxycoumarin (CMHC) was from Molecular Probes, Eugene, OR. All other chemicals were from Sigma Chemical Co., St. Louis, MO.

Preparation of tissue-printed astrocytes. The tissue-print method used in the present study was modified from the method used by Barres et al. (1990) for postnatal rat optic nerve. In the present study, under light CO_2 anesthesia, 6- and 21-d-old rat pups were killed and the superior surface of the brain exposed rapidly under sterile conditions. Brain cortical slices less than 2 mm thick were cut with a scalpel from the prefrontal, sensorimotor, and temporoparietal regions and placed in 60 mm tissue culture petri dishes containing isotonic bicarbonate buffer (final concentrations, in mM: Na^+ , 145; K^+ , 4.5; Mg^{2+} , 0.4; Ca^{2+} , 1.3; Cl^- , 127; SO_4^{2-} , 0.4; HCO_3^- , 25; and glucose, 10; the buffer was at room temperature and a pH of 7.35–7.4). Brain slices from different pups were pooled. Meninges and blood vessels were not dissected from the slices to save time and minimize tissue damage. A hand-held razor blade was next used to mince the slices completely into 1–2 mm pieces. Excess buffer was aspirated from the petri dish, leaving behind just enough to cover the tissue pieces. Buffer (40–70 μl) with 10–15 tissue pieces was pipetted gently onto the center of poly-D-lysine (10 $\mu\text{g}/\text{ml}$)-coated coverslips, one per well in six-well dishes. A second poly-D-lysine-coated coverslip was gently inverted for 2 min over the tissue pieces present on the first coverslip. No external pressure was applied over the sandwiched coverslips as it produced extensive tissue damage and yielded very few cells. Buffer (5–6 ml) was then added to each well to facilitate the unaided separation of coverslips, though fine forceps were occasionally required for this purpose. Cells usually printed better on the upper coverslip, which also had less debris. Astrocytes could almost always be identified by their typical flat or process-bearing morphology and phase dark appearance, and many appeared intact with well-defined processes. Groups of astrocytes frequently printed in exclusive “zones” that were free of neuronal elements. Neuronal elements were less frequently present and were also harder to identify by phase contrast microscopy. They were recognizable as meshworks containing numerous thin, elongated axon-like processes, but confirmation by immunocytochemistry was always necessary. Under phase-contrast microscopy we found that approximately half the coverslips used for tissue-printing yielded sufficient numbers of intact appearing astroglial cells suitable for experimental use. Tissue-printed cells were used within 4 hr of preparation for performing uptake experiments.

In preliminary experiments, calcium-free buffer (nominally calcium-free buffer containing 80 μM EGTA) and enzymes including 0.04%

collagenase, 1 mg/ml dispase, 4 mg/ml DNase, 1–15 U/ml papain, and 0.25% trypsin were tried separately and in different combinations, but in our hands always resulted in widespread cell damage. In order to improve cell yield, minimize tissue damage, and avoid the possible proteolytic digestion of membrane proteins, the tissue-print procedure we finally adopted entirely omitted the use of enzymes during dissociation and used buffer containing normal levels of Ca^{2+} (1.3 mM).

Primary astrocyte cultures. Primary astrocyte cultures were prepared by dissociating 1-d-old rat pup cerebral cortices according to the method of Frangakis and Kimelberg (1984). The cell suspension was seeded at a density of 1×10^4 cells/ cm^2 onto 25 mm coverslips present in six-well dishes and grown in Eagle's MEM supplemented with 10% heat inactivated horse serum (HIS) 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 (Kimelberg et al., 1992). Cultured astrocytes (whose time of plating was taken as day 1) were matched in postnatal age to tissue-printed cells for uptake experiments.

Uptake of ^3H -5-HT by autoradiography. Na^+ -containing HCO_3^- buffer (see above) was used for uptake experiments. Coverslips with tissue-printed cells were initially washed four times with 2 ml of this buffer at room temperature. Age-matched primary astrocyte cultures grown in HS- or FBS-supplemented MEM were washed similarly after being transferred to new six-well dishes. Following the first wash with Na^+ -containing buffer, coverslips to be tested in Na^+ -free conditions were washed three times with Na^+ -free buffer [where NaCl was replaced by 122 mM *N*-methyl-D-glucamine hydrochloride (NMDG-HCl), and NaHCO_3 was replaced by 22 mM triethylamine bicarbonate plus 3 mM 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 MAO-A and MAO-B (Fitzgerald et al., 1990) and 10^{-5} M L-ascorbic acid to prevent the oxidation of ^3H -5-HT (Wilson and Wilson, 1991). Next, 10^{-3} M ketanserin(+) tartrate to block 5-HT_{1B} in addition to 5-HT_{1A} and 5-HT₂ receptors (Leysen et al., 1982; Middlemiss and Hutson, 1990) and 10^{-5} M spiperone hydrochloride to block 5-HT_{1B} in addition to 5-HT_{1A}, 5-HT_{1C}, and 5-HT₂ receptors (Leysen et al., 1992) were present in the preincubation buffer in all wells for 5 min. Where indicated, 10^{-6} M fluoxetine hydrochloride was also present to block specifically high-affinity ^3H -5-HT uptake (Wong et al., 1975; Fuller et al., 1991). Uptake was performed by incubating cells with 3×10^{-7} M ^3H -5-HT (3.4 $\mu\text{Ci}/\text{ml}$, 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 six times with ice-cold phosphate-buffered saline (PBS). The composition of the PBS was 137 mM NaCl, 15 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 mM KH_2PO_4 , 2.7 mM KCl, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.7 mM CaCl_2 , and the pH was 7.35–7.4. Cells were then fixed in Zamboni's fixative (2% paraformaldehyde, 7.5% picric acid in 0.1 M sodium phosphate buffer, pH 7.4; Stefanini et al., 1967) for 30 min at 4°C .

Immunocytochemical staining. Coverslips containing the fixed cells were rinsed three times with ice-cold PBS and then permeabilized by incubation in PBS containing 250 $\mu\text{g}/\text{ml}$ 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 first with the appropriate primary and secondary antibodies for GFAP followed by the primary and secondary antibodies for neurofilament (NF). 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 anti-rabbit secondary antibody (Tago, Inc.). For NF staining, cells were incubated with a 1:10 dilution of mouse monoclonal antibody raised against the 200 kDa subunit of porcine NF, H chain (Boehringer-Mannheim), followed by incubation with a 1:40 dilution of fluorescein-conjugated goat anti-mouse secondary antibody (Tago, Inc.). Following the secondary antibody incubation for NF, cells were rinsed four times in double-distilled water. Controls during staining consisted of the omission of primary antibody and incubation only with the secondary antibody and were negative. In some experiments, nonimmune serum was substituted as a control for the primary GFAP and NF antibodies, followed by incubation with secondary antibody, and these were also negative.

Autoradiography. Following immunocytochemistry, coverslips were mounted cell side up on glass slides with 1:1 xylene/Permount solution and air dried for 12 hr at room temperature in the dark. The mounted coverslips were then dipped in a 1:1 solution of NTB2 emulsion and

MilliQ water that contained 1% glycerol to reduce stress artifacts (Waller, 1961), at a temperature of 41°C. They were then dried for 2 hr in a high-humidity atmosphere to lessen latent image formation and thereby reduce background grain development (Waller, 1961; see also Rogers, 1979), air dried in normal humidity for another 2 hr, and then placed in a light-tight box containing desiccant for 10–12 d 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 for 2 min at 4°C with a 1:1 Kodak Dektol developer:MilliQ water solution, bathed for 30 sec in 1% acetic acid to arrest development, exposed to Kodak fixer for 10 min, and then rinsed four times in tap water at 5 min intervals. Following development, the experimental coverslip was covered with a second coverslip using a 25 μ l PBS-glycerol drop containing 2% DABCO [1-4-diazabicyclo(2,2,2)octane] to prevent fading of fluorescence (Langanger et al., 1983). Control coverslips were processed in exactly the same way without being exposed to radioactivity (Rogers, 1979), and control background grain levels were examined over cells as well as acellular areas. Increased grain densities over control cells could result from a chemical interaction between the emulsion and underlying cell membrane components and/or because of molecules diffusing from cells and such an artifact is termed *positive chemography* (Rogers, 1979). Another artifact termed *negative chemography* refers to a diminution in grain density over cells, also due to some chemical reaction between the emulsion and underlying cells (Rogers, 1979). The occurrence of negative chemography was checked for by exposing two coverslips to room light after they were dipped in emulsion and dried. Negative chemography would have become apparent as localized grain-free areas over cells with the rest of the coverslip being completely blackened with grains.

Quantitative grain 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 with numerous processes were examined further under fluorescein optics to detect any NF(+) processes or unstained debris, overlying or associated with the GFAP(+) astrocyte. Only undamaged, well-stained GFAP(+) astrocytes without associated NF(+) elements or cellular debris ("clean" cells) were analyzed by morphometry using the JAVA image analysis system (Jandel, Corte Madera, CA). 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 was counted by eye to ensure accuracy. A total of 50 tissue-printed cells and 25 cultured cells were analyzed for each experimental condition where ^3H -5-HT uptake was examined.

Statistical analysis. Significant differences between cell groups in morphometric measurements were determined using a one-way randomized ANOVA design followed by Scheffé's test for post hoc comparison of means. Differences in ^3H -5-HT uptake under different conditions were analyzed for significance using a repeated-measures ANOVA design for 6 d astrocytes (cultured and tissue-printed), for 21 d astrocytes, for NF(+) groups, and for 6- and 21-d-old astrocyte processes. Reduced uptake was anticipated in Na^+ -free buffer or in the presence of 1 μM fluoxetine in comparison to Na^+ -containing buffer, and these results were therefore analyzed by Fisher's LSD (least significant difference test) for planned comparisons of means. Any statistically significant differences in grain densities over debris were determined by a repeated-measures ANOVA followed by Scheffé's test. Any association between uptake by cell processes and cell bodies was calculated as the Pearson product-moment correlation. Statistical calculations were performed with the help of CSS:STATISTICA software (StatSoft Inc., Tulsa, OK) and Bruning and Kintz (1987).

Results

The tissue-print method we used yielded 50–300 GFAP(+) astrocytes per coverslip and astrocytes constituted 60–70% of the total cells; the remainder consisted of NF(+) processes, capillaries, and unidentified cells. Trypan blue (0.08%) was excluded by essentially all tissue-printed GFAP(+) astrocytes. GFAP(+) astrocytes frequently printed in groups of 2–20 cells without any

or with only minimally associated neuronal elements. Prior to immunocytochemistry, astrocytes could usually be identified easily in tissue-prints as phase-dark cells with granular cytoplasm, dark nuclei, and elongated processes in contrast to the phase-light, thin, and extensively branched meshworks typically seen for neuronal elements. NF(+) structures usually printed as meshworks of long thin processes interspersed with occasional round or polygonal cell bodies. NF(+) structures with associated underlying GFAP(+) cells or processes were frequently seen and were classified as NF(+) _{underGFAP(+)}.

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 were performed over the rhodamine fluorescence video image of astrocyte cell bodies, and separately over their associated processes. The immunofluorescence rather than the phase-contrast video image was analyzed to ensure uniform and accurate data collection but would cause grains present close to the cell membrane or on it, or lying just extracellularly, possibly due to the tracking of β -particles from intracellular sites (Rogers, 1979) to be excluded from analysis. Development of emulsion-coated coverslips was carried out at 4°C for 2 min, as this protocol resulted in very low background grain densities but moderate to high grain densities over positive cells. There was no indication of positive or negative chemography over primary cultured astrocytes, tissue-printed astrocytes, or NF(+) structures (see Materials and Methods).

In Figure 1, *A* and *B* depict the results of morphometric measurements of the different tissue-printed and cultured astrocyte cell groups that were studied, and show that cultured astrocytes, compared to age-matched TP astrocytes, always had bigger cellular dimensions and areas (area of cell body only without processes). In Figure 1*B*, the mean area of HS21-cultured astrocytes was more than twice the area of HS6-cultured astrocytes, indicating that cultured astrocytes grown in horse serum-supplemented medium showed a progressive increase in cell size with time in culture. Although in Figure 1*A* the size difference between TP21 astrocytes and TP6 astrocytes appears small, the 21 d astrocytes were more than 1.5-fold greater in area. The large difference in cell areas between tissue-printed and cultured astrocytes made it essential to calculate and represent ^3H -5-HT uptake as the number of grains/ $10^3 \mu\text{m}^2$ of cell area.

^3H -5-HT uptake by 6 d tissue-printed and cultured astrocytes

Throughout this study results for ^3H -5-HT uptake were calculated from 50 tissue-printed and 25 cultured astrocytes for each experimental condition. Background cellular grain densities were calculated from 25 control cells that were not exposed to ^3H -5-HT during experiments. ^3H -5-HT uptake experiments were performed within 4 hr of printing cells. Figure 2*A–C* shows that ^3H -5-HT uptake occurred into 6 d tissue-printed (TP6) astrocytes in the presence of extracellular Na^+ . Figure 2*A* shows positive rhodamine-conjugated immunofluorescence staining for GFAP, Figure 2*B* is a phase photomicrograph, and Figure 2*C* is a bright-field view and shows that grains were distributed over the entire surface of nearly all the astrocytes and their processes. In Figure 2, *D–F* are corresponding photomicrographs that show that uptake by TP6 astrocytes was greatly diminished in Na^+ -free buffer. The low grain densities over cells

Figure 1. Morphometric measurements of length, breadth, and area of tissue-printed and cultured astrocytes. *A* depicts mean length (taken as the larger of two cell dimensions) and breadth of cells measured in μm using JAVA image analysis software (error bars = SEM). Cultured astrocytes had significantly bigger cell dimensions compared to age-matched TP astrocytes at both 6 and 21 d ($p < 0.0001$ for length, $p < 0.0001$ for breadth, derived by one-way randomized ANOVA and Scheffe's test). *B* shows mean cell areas (cell bodies only, excluding processes) obtained by outlining the perimeter of cells with a cursor. Cultured astrocytes had significantly larger cell areas at 6 and 21 d compared with age-matched TP astrocytes ($p < 0.0001$ at 6 d; $p < 0.001$ at 21 d). HS-cultured astrocytes showed a significant increase in area from 6 to 21 d ($p < 0.0001$). Mean length, breadth, and cell areas for each group were calculated from 100 cultured astrocytes and 175 tissue-printed astrocytes or NF(+) structures. HS, astrocytes cultured in horse-serum supplemented MEM; FBS, astrocytes cultured in fetal bovine serum supplemented MEM; TP, tissue-printed astrocytes; NF, neurofilament positive processes. Numbers after abbreviations indicate postnatal age in days for TP or cultured cells.

were not appreciably different from the background grain densities present over acellular areas. The photomicrographs in Figure 2 collectively indicate that ^3H -5-HT uptake by TP6 astrocytes showed a near total dependence on the presence of extracellular Na^+ .

The increased grain levels present over acellular areas, especially on the left half of photomicrographs *B* and *C* in Figure 2, reflect the binding of ^3H -5-HT to cellular debris. Only 14 grains would be expected to occur as background over the entire micrograph if additional binding had not occurred. Early in the study it became apparent that the high grain densities present over some "acellular" areas of experimental coverslips represented binding to cellular debris rather than high background grain densities due to improper experimental technique. Exten-

sive areas of cell debris with no recognizable cellular profiles or remnants were frequently observed in tissue-printed coverslips and typically showed weak and hazy rhodamine or fluorescein fluorescence. The high concentrations of ketanserin and spiperone ($10 \mu\text{M}$ each) present during uptake experiments ensured that binding to any cell surface 5-HT₁ receptor subtypes and 5-HT₂ receptors could not occur (Leysen et al., 1982; Middlemiss and Hutson, 1990; Leysen, 1992). In contrast to the moderate grain density present over debris on the left side of Figure 2*B*, low to absent grain density is seen over the cell debris in the upper half of Figure 2*E*, suggesting that the binding of ^3H -5-HT to debris could be Na^+ dependent. Table 1 shows that the grain densities over cell debris in both 6 and 21 d tissue-prints were indeed Na^+ dependent, but not fluoxetine sensitive. As

Table 1. Mean grain densities (grains/1000 $\mu\text{m}^2 \pm \text{SEM}$) over cellular debris, acellular areas, and control conditions

Cell type	Cellular debris				Acellular areas				Control cells
	(+)Na	(-)Na	(+)Fluox.	Control	(+)Na	(-)Na	(+)Fluox.	Control	
TP6	33.3 $\pm$ 2.7	8.6 $\pm$ 0.3*	38.4 $\pm$ 4.0	1.0 $\pm$ 0.3	0.7 $\pm$ 0.0	0.8 $\pm$ 0.1	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1	3.4 $\pm$ 0.7
TP21	39.5 $\pm$ 4.0	16.0 $\pm$ 1.3*	32.3 $\pm$ 2.9	0.5 $\pm$ 0.1	1.4 $\pm$ 0.1	0.9 $\pm$ 0.1	1.1 $\pm$ 0.1	0.6 $\pm$ 0.1	1.5 $\pm$ 0.3
HS6					1.0 $\pm$ 0.1	1.1 $\pm$ 0.1	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1	1.1 $\pm$ 0.3
FBS6					4.2 $\pm$ 0.8	1.9 $\pm$ 0.1	4.3 $\pm$ 0.3	0.5 $\pm$ 0.1	0.5 $\pm$ 0.2
HS21					0.8 $\pm$ 0.1	0.9 $\pm$ 0.1	0.7 $\pm$ 0.1	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1

Control columns describe measurements made over cells/coverslips not exposed to radioactive 5-HT during experiments. Grain densities over cellular debris and acellular areas were calculated over a 23,445 μm^2 area ($n = 5$ observations/value). Grain densities over control cells were calculated from 25 cells.

* $p < 0.001$ compared to the value in the (+)Na column, by ANOVA and Scheffe's test for post hoc comparison of means.

Figure 2. A series of photomicrographs showing the Na^+ dependence of ^3H -5-HT uptake in TP6 rat cortical astrocytes and the phenomenon of Na^+ -dependent binding by debris. *A*, Rhodamine-conjugated GFAP immunofluorescence photomicrograph of a group of astrocytes. *B*, Phase micrograph of the same field with debris recognizable in the left half of the micrograph. *C*, Bright-field micrograph showing positive uptake over astrocytes with grain densities that ranged from 36.3 to 70.2/ $10^3 \mu\text{m}^2$. In *B* and *C*, grains present on the left side of the micrograph represent binding to cellular debris (see Table 1). *D*, Rhodamine-conjugated GFAP immunofluorescence photomicrograph from a sister coverslip where uptake was performed in Na^+ -free buffer. *E* and *F* are the phase and bright-field photomicrographs and grain densities over the astrocytes ranged from 10 to 39 grains/ $10^3 \mu\text{m}^2$, indicating that ^3H -5-HT uptake was reduced in Na^+ -free buffer. Scale bar, 25 μm .

“uptake” by functional 5-HT transporters present in debris should have been fluoxetine sensitive, the absence of fluoxetine sensitivity implies the occurrence of some type of Na^+ -dependent ^3H -5-HT binding rather than “uptake” via the high-affinity fluoxetine-sensitive transport system.

In experiments with Na^+ -containing buffer, none or only oc-

casional grains were observed over some astrocyte cell bodies that were surrounded by debris that, however, had moderately high grain densities, suggesting a low occurrence of such non-specific ^3H -5-HT binding on at least some astrocytes. Even if nonspecific binding comparable to that present over debris occurred over astrocytes, it was still relatively easy to identify

Table 2. ^3H -5-HT uptake grain densities (mean $\pm$ SEM, per 1000 μm^2 cell area)

Cell type	(+)Na	(-)Na	(+)Fluox.
TP6	78.7 $\pm$ 5.2	21.2 $\pm$ 2.3****	44.1 $\pm$ 3.8****
TP21	66.4 $\pm$ 3.6	16.3 $\pm$ 1.7****	19.4 $\pm$ 2.8****
HS6	37.0 $\pm$ 5.4	3.7 $\pm$ 1.1****	15.5 $\pm$ 2.0*
FBS6	66.0 $\pm$ 7.0	18.0 $\pm$ 2.3****	30.4 $\pm$ 3.9****
HS21	35.5 $\pm$ 2.5	21.6 $\pm$ 1.6*	21.2 $\pm$ 1.0*
NF(+)	174.9 $\pm$ 41.1	21.8 $\pm$ 2.6****	16.4 $\pm$ 0.1****
NF(+) _{underGFAP(+)}	149.0 $\pm$ 15.4	22.5 $\pm$ 2.9****	22.0 $\pm$ 3.6****
TP6 processes	98.6 $\pm$ 15.1	28.1 $\pm$ 15.6*	48.4 $\pm$ 13.2
TP21 processes	84.0 $\pm$ 15.9	8.8 $\pm$ 2.6****	25.0 $\pm$ 3.6**

Number of cells analyzed for (+)Na, (-)Na or (+)Fluox. was as follows: cultured astrocytes, 25; tissue-printed astrocytes, 50; NF(+) and NF(+)_{underGFAP(+)} combined, 50.

* $p < 0.01$, ** $p < 0.001$, **** $p < 0.00001$, in a given row, compared to the value in the (+)Na column by ANOVA and Fisher's LSD test for planned comparison of means.

astrocytes that showed positive ^3H -5-HT uptake as grain densities over them were on average about twofold higher than those observed over cellular debris (e.g., see Fig. 2C) and also because binding to debris was fluoxetine insensitive (compare Tables 1 and 2).

Positive uptake in Na^+ -containing buffer by astrocytes cultured in FBS supplemented medium for 6 d (FBS6) is shown in Figure 3. Figure 4 compares the mean grain densities present over 6-d-old HS-cultured, FBS-cultured, and TP astrocytes following ^3H -5-HT uptake under different experimental conditions. The higher mean grain densities over FBS6 compared to HS6-cultured astrocytes in Na^+ -containing buffer (Fig. 4) agree with a recent report from this laboratory where the batch of FBS used in the present study also promoted 5-HT uptake by astrocyte cultures, measured as the uptake of ^3H -5-HT/mg cell protein (Kimelberg et al., 1992). Grain densities over "acellular" areas in FBS coverslips were slightly higher than in HS6 coverslips, and were 4.2 ± 0.8 grains/ $10^3 \mu\text{m}^2$ for FBS compared to 1.0 ± 0.1 for HS coverslips (Table 1). Figure 4 shows that grain densities were significantly lower in Na^+ -free buffer (-Na) compared to Na^+ -containing buffer (+Na) for HS6-cultured, FBS6-cultured, and TP6 astrocytes ($p < 0.00001$ for all three

groups by ANOVA and Fisher's LSD test). When compared to the uptake in Na^+ -containing buffer, the extent of inhibition of ^3H -5-HT uptake in Na^+ -free buffer for HS-cultured, FBS-cultured, and TP6 astrocytes was 90%, 73%, and 73%, respectively. Similarly, fluoxetine (1 μM) produced an inhibition of 58%, 54%, and 44% when uptake was compared to the uptake in Na^+ -containing buffer in the three respective astrocyte groups ($p < 0.01$, $p < 0.00001$, and $p < 0.00001$, respectively). The higher uptake in the presence of 1 μM fluoxetine in comparison with the uptake in Na^+ -free buffer in HS6, FBS6, and TP6 astrocytes was seen in three such experiments.

Figure 5 shows the distribution of grain densities for 6 d cultured HS or FBS astrocytes and TP6 astrocytes. We divided the grain densities into six ranges, with the initial range being 0–30 and other ranges representing successive increases of 40 grains/ $10^3 \mu\text{m}^2$. Uptake by HS6 astrocytes in Na^+ -containing buffer (solid bars) was distributed predominantly in the two smallest ranges, but uptake by FBS6 and TP6 astrocytes was distributed over several ranges. In contrast to HS6 and FBS6, almost no TP6 cells were represented in the lowest 0–30 range. The leftward shift in distribution observed with Na^+ -free buffer was less pronounced in the presence of fluoxetine, in agreement

Figure 3. *A* and *B* are the rhodamine-conjugated GFAP immunofluorescence and bright-field photomicrographs, respectively, of 6 d astrocytes cultured in fetal bovine serum (FBS6). ^3H -5-HT uptake was studied in Na^+ -containing buffer and from left to right in *B* these astrocytes showed moderately positive uptake with grain densities of 83.6 and 109.1 grains/ $10^3 \mu\text{m}^2$, respectively. Scale bar, 25 μm .

Figure 4. Mean ^3H -5-HT grain densities for 6 d cultured and TP astrocytes (error bars = SEM) showing the effects of omission of Na^+ ($-\text{Na}$) or the effect of $1\ \mu\text{M}$ fluoxetine. The ranking of grain densities was $\text{HS6} < \text{FBS6} < \text{TP6}$ for each experimental uptake condition. Throughout this study, mean ^3H -5-HT grain densities were calculated from 50 tissue-printed and 25 cultured astrocytes for each condition. Background grain densities (*Bkgd.*) were calculated over control cells that were processed through experiments without being exposed to radioactivity and are shown for comparison.

with the data in Figure 4. All background cells (i.e., those unexposed to ^3H -5-HT) belonged to the 0–30 range and in fact belonged to the 0–14 subrange (not depicted in Fig. 5) for all three astrocyte groups.

^3H -5-HT uptake by 21 d astrocytes

In Figure 6, *a–c* are rhodamine-conjugated GFAP immunofluorescence, phase-contrast, and bright-field photomicrographs, respectively, which show positive ^3H -5-HT uptake by TP21 astrocytes in Na^+ -containing buffer. Figure 6*d–f* depicts uptake in the presence of $1\ \mu\text{M}$ fluoxetine in Na^+ -containing buffer. Figure 6*c* shows TP21 astrocytes with good, uniformly distributed grain localization and grains were also localized to smaller GFAP(+) cell remnants at lower right. Grains were not localized over the clump of bare nuclei present below the GFAP(+) cell remnants at lower right, indicating that nonspecific ^3H -5-HT binding did not occur over these nuclei. The small, dense cluster of grains present at lower right between the GFAP(+) remnants could represent high uptake by a neuronal element or a blood platelet. The small size of the cluster made it less likely to be a positive chemography artifact where grains are often large, irregular, and sometimes clumped together (Rogers, 1979). Figure 6*d–f* shows reduced uptake over TP21 astrocytes in the presence of $1\ \mu\text{M}$ fluoxetine (see legend).

^3H -5-HT uptake by NF(+) structures

Uptake by 6 d NF(+) structures could not be studied due to the weak staining intensity observed with the 68 kDa, 150 kDa,

Figure 5. Histogram depicting the distribution of grain densities for the 6 d astrocyte groups shown in Figure 4.

Figure 6. A series of photomicrographs showing ^3H -5-HT uptake by 21 d tissue-printed astrocytes. *a-c*, Rhodamine-conjugated GFAP immunofluorescence, phase-contrast, and bright-field photomicrographs, respectively, of a group of TP21 astrocytes where uptake was performed in Na^+ -containing buffer. The grain densities over the astrocyte cell bodies ranged from 58 to 166.5 grains/ $10^3 \mu\text{m}^2$, and correspond to moderate to high levels of uptake. *d-f*, Uptake performed in the presence of $1 \mu\text{M}$ fluoxetine in Na^+ -containing buffer showing diminished uptake with grain densities of 28.4 and 36 grains/ $10^3 \mu\text{m}^2$ over the astrocyte cell bodies. Scale bar, $25 \mu\text{m}$.

and 200 kDa subunit monoclonal NF antibodies. Staining with the 200 kDa subunit NF antibody was more definite and stronger in tissue-prints from 21-d-old rats, and this antibody was used throughout for staining TP21 cells. Figure 7 shows uptake by 21 d NF(+) elements in Na^+ -containing buffer (*a-c*) and greatly reduced uptake in Na^+ -containing buffer in the presence of $1 \mu\text{M}$ fluoxetine (*d-f*). Figure 7*a* is an FITC fluorescence photomicrograph of a group of putative NF(+) axons that showed high grain densities over the two areas indicated by arrowheads

in Figure 7, *b* and *c*. These areas could represent uptake by axonal varicosities where 5-HT is stored in vesicles (Heuser and Reese, 1977; Tork, 1990). In Figure 7*d-f*, uptake was performed in the presence of $1 \mu\text{M}$ fluoxetine and fewer grains were localized to any part of the putative axons.

Mean uptake by 21-d-old astrocytes and NF(+) structures

The results shown in Figure 8 and also Table 2 were obtained from 31 microscope fields from a total of five experiments. The

Figure 7. *a–c*, ^3H -5-HT uptake in Na^+ -containing buffer by tissue-printed 21 d neurofilament(+) structures. *a*, Fluorescein-conjugated immunofluorescence photomicrograph of a bundle of putative NF(+) axons. *b* and *c*, The corresponding phase micrograph (*b*) and the bright-field micrograph (*c*) showing high grain densities of 344.3 and 417.7 grains/ $10^3 \mu\text{m}^2$ localized to the two sites indicated by arrowheads. *d–f*, Same experiment showing greatly reduced grain densities over NF(+) structures in the presence of $1 \mu\text{M}$ fluoxetine. Scale bar, $25 \mu\text{m}$.

mean grain densities over HS21-cultured astrocytes and TP21 astrocytes for uptake performed under different conditions are shown in Figure 8*A*. In contrast to 6 d preparations, uptake by HS21-cultured astrocytes and TP21 astrocytes was equally Na^+ - and fluoxetine-sensitive (for HS21 astrocytes, $p < 0.01$ for Na^+ -containing vs Na^+ -free buffer and also for Na^+ -containing buffer vs fluoxetine; similarly, $p < 0.00001$ for TP 21 astrocytes for both comparisons). The extent of inhibition produced either by

Na^+ -free buffer or by $1 \mu\text{M}$ fluoxetine was 40% for HS21-cultured astrocytes and around 70% for TP21 astrocytes. Experiments were not performed with FBS21-cultured astrocytes.

Data were collected from all NF(+) structures rather than specifically from axons with high grain densities that could then be considered serotonergic. The results thus provide an overall estimate of uptake by cortical neuronal elements and not specifically by serotonergic neurons. About half the NF(+) pro-

Figure 8. Mean ^3H -5-HT grain densities for 21 d cultured and tissue-printed astrocytes and NF(+) structures (error bars = SEM). *A* shows uptake by HS21-cultured and TP21 astrocytes. Uptake by TP21 was higher than HS21 and both were equally sensitive to the omission of Na^+ , or the presence of fluoxetine. *B*, Shows uptake by 21 d NF(+) structures, with one subgroup that stained only for NF and a second subgroup, NF(+)underGFAP(+) with associated underlying GFAP(+) processes, which would not affect uptake into NF(+) structures. NF(+) structures overlaid with GFAP(+) processes were excluded from analysis. In Na^+ -containing buffer, both NF(+) subgroups showed uptake twice as great as that seen in TP21 astrocytes.

cesses analyzed stained exclusively for NF without any associated GFAP(+) elements but the other half were associated with underlying GFAP(+) processes or cells and thus were classified, respectively, as the NF(+) and NF(+)underGFAP(+) subgroups. Data were analyzed separately for these NF(+) subgroups (Fig. 8*B*, Table 2). NF(+) elements with overlying GFAP(+) processes were excluded from analysis. Figure 8, *A* and *B*, and Table 2 indicate that grain densities in Na^+ -containing buffer for both NF(+) subgroups were at least twofold greater than those observed in TP21 astrocytes, and were equally inhibited in Na^+ -free buffer and in the presence of fluoxetine, by about 85–90% ($p < 0.00001$, for Na^+ -containing vs Na^+ -free buffer and Na^+ -containing vs fluoxetine for both NF(+) subgroups).

In tissue-prints, intimately associated GFAP(+) and NF(+) cells could reflect their anatomical relationships *in situ*, and their grain densities could provide important information about any cellular heterogeneity in ^3H -5-HT uptake *in situ*. In 21 d tissue-prints, three such intimately associated NF(+) : GFAP(+) cell pairs were identified. The NF(+) : GFAP(+) grain densities were 157:46.2, 211.9:26.1, and 339.5:78.8, so NF(+) elements in these three NF(+) : GFAP(+) cell pairs showed greater 5-HT uptake. Similarly, heterogeneity in grain densities among GFAP(+) astrocytes that printed close to each other was also consistently observed, indicating that 5-HT uptake capacities were probably differentially expressed within anatomically and functionally related groups of cortical astrocytes *in situ*.

The distributions of grain densities for HS21-cultured and TP21 astrocytes and the two NF(+) subgroups under different conditions are shown in Figures 9 and 10, respectively. In Figure 9, *A* and *B*, uptake in Na^+ -containing buffer by HS21-cultured astrocytes (represented as solid bars), was seen only in the two smallest ranges (0–30 and 31–70). By comparison, TP21 astrocytes (Fig. 9*B*) were represented predominantly in the 31–70 and 71–110 ranges. These distribution patterns closely resem-

bled the respective cell-type distributions observed for HS6 and TP6 astrocytes (Fig. 5). Uptake performed in Na^+ -free buffer or in the presence of $1 \mu\text{M}$ fluoxetine resulted in a leftward shift as more than 80% of cells were now represented in the 0–30 range. In contrast, as seen earlier in Figure 5, FBS6 and TP6 astrocytes had a much wider distribution that reflected the lesser inhibition of uptake in the presence of fluoxetine (Fig. 5).

In Figure 10, *A* and *B*, the distribution of grain densities for the NF(+) and NF(+)underGFAP(+) subgroups under different experimental conditions are shown. In Na^+ -containing buffer (represented as solid bars), 60% of cells from the NF(+) subgroup and 40% of cells from the NF(+)underGFAP(+) subgroup were represented in the ≥ 191 grains/ $10^3 \mu\text{m}^2$ range. In Na^+ -containing buffer, the NF(+)underGFAP(+) subgroup showed a more uniform representation in all ranges in contrast to the bimodal distribution observed in the NF(+) subgroup. In Figure 10, *A* and *B*, the high representation of NF(+) structures in the ≥ 191 range in Na^+ -containing buffer suggests uptake by a homogeneous group of neuronal elements such as serotonergic axons, though morphologically such NF(+) structures resembled both axons and neuronal perikarya. In Na^+ -free buffer and in the presence of $1 \mu\text{M}$ fluoxetine there was a marked leftward shift in distribution for both NF(+) subgroups.

Percentages of positive cells

In order to determine the percentages of cells that showed ^3H -5-HT uptake, we had to first determine a threshold value that reliably identified positive uptake. The grain densities over cellular debris listed in Table 1 could also represent, wholly or in part, nonspecific binding that occurred to tissue-printed astrocytes. As the binding to debris was fluoxetine insensitive, the simplest solution seemed to be to adopt a grain density of $\geq 31/10^3 \mu\text{m}^2$ as the threshold to identify positive uptake, since 32.3 ± 2.9 was the mean density observed over TP21 debris in the

Figure 9. Distribution of ^3H -5-HT grain densities in 21 d HS-cultured and TP astrocytes. **A**, Grain densities over HS21-cultured astrocytes in Na^+ -containing buffer were distributed only in the two smallest ranges and were further reduced in Na^+ -free buffer and in the presence of $1 \mu\text{M}$ fluoxetine. **B**, Grain densities over TP21 astrocytes in Na^+ -containing buffer occurred up to the 71–110 range and were equally sensitive to omission of Na^+ and to the presence of fluoxetine.

Figure 10. Distribution of ^3H -5-HT grain densities over 21 d NF(+) subgroups. **A**, Purely NF(+) structures. **B**, NF(+) under GFAP(+) subgroup (see text for description of these).

Figure 11. ^3H -5-HT grain densities over 6 and 21 d tissue-printed astrocytic processes. *B*, Percentage of processes that showed positive uptake at both ages using the ≥ 31 grains/ $10^3 \mu\text{m}^2$ criterion for positive uptake.

presence of fluoxetine. The ≥ 31 grains/ $10^3 \mu\text{m}^2$ threshold would exclude the majority of grain densities over cells that arose due to background and nonspecific binding. In Na^+ -containing buffer, 90–100% of TP6, TP21, and NF(+) structures showed positive uptake, which can also be inferred from the frequency distribution histograms shown in Figures 5, 9, and 10. Among cultured astrocytes, by contrast, only 48% of HS6, 60% of HS21, and 76% of FBS6 astrocytes showed positive uptake. In Na^+ -free buffer, less than 20% of either 6 or 21 d astrocytes showed positive uptake, and for NF(+) elements, 32 and 41% were positive. In the presence of $1 \mu\text{M}$ fluoxetine there were less than 20% positive cells in 21 d HS or TP astrocytes, but FBS6 and TP6 astrocytes had 48% and 58% positive cells. NF(+) and NF(+) _{under GFAP(+)} structures had 20% and 46% positive cells in the presence of fluoxetine, respectively.

Uptake by astrocytic processes

The results presented so far were obtained exclusively from cell bodies and uptake by processes was studied separately since they could show different patterns of ^3H -5-HT uptake. Mean grain densities over 6 and 21 d TP GFAP(+) processes under different experimental conditions are shown in Figure 11*A*. Though mean grain densities over 6 and 21 d TP astrocyte processes in Na^+ -containing buffer were comparable to those observed over the cell bodies (Figs. 4, 8*A*; Table 2), the correlation of grain densities between astrocytic processes and cell bodies was weak ($r = -0.09$ for TP6 and -0.05 for TP21 astrocytes). Uptake by the processes of TP6 and TP21 astrocytes was Na^+ dependent and fluoxetine sensitive (Fig. 11*A*, Table 2), though fluoxetine inhibited uptake to a lesser extent over 6 d processes, a finding similar to results obtained over 6 d astrocyte cell bodies. As shown in Figure 11*B*, according to the criterion of ≥ 31 grains/ $10^3 \mu\text{m}^2$ that was adopted, 100% and 67% of TP6 and TP21 processes, respectively, were uptake positive in Na^+ -containing buffer, 32% and 13% in Na^+ -free buffer, and 46% and 37% in the presence of $1 \mu\text{M}$ fluoxetine.

Discussion

Rationale for the tissue-print procedure

The results presented in this study indicate that more than 90% of tissue-printed GFAP(+) astrocytes from 6- and 21-d-old rats exhibited high-affinity ^3H -5-HT uptake that was both Na^+ dependent and fluoxetine sensitive (see Results). In a previous study from this laboratory (Anderson et al., 1992), we showed by autoradiography that GFAP(+) astrocytes *in situ* showed increased grain densities after intraventricular injection of ^3H -5-HT, but were not able to demonstrate that this uptake was fluoxetine sensitive. The present study fills this gap and demonstrates that fluoxetine-sensitive ^3H -5-HT uptake can be localized to individual GFAP(+) astrocytes isolated acutely from the CNS.

We decided to study fluoxetine-sensitive uptake in acutely isolated astrocytes from the rat brain since detailed pharmacological and quantitative studies were more likely to be successful with this preparation. A suitable method for the acute isolation of intact astrocytes from the rat optic nerve was recently described by Barres et al. (1990) for electrophysiological experiments, and we adopted this protocol exactly in preliminary experiments. However, we found that the use of $80 \mu\text{M}$ EGTA in nominally Ca^{2+} -free buffer or the dilute concentrations of enzymes such as collagenase and papain that were used by Barres et al. (1990) resulted in the death of nearly all cells obtained from the rat cerebral cortex. We developed a reproducible method for the isolation of cells from cortical slices using normal Ca^{2+} buffer and without the use of enzymes. The omission of enzymes had the added advantage of minimizing the possibility that membrane proteins, including components of the 5-HT uptake system, would be altered by enzymatic digestion.

Dimensions of acutely isolated astrocytes

We found that the mean cellular dimensions of tissue-printed astrocytes isolated from 6- and 21-d-old rats were somewhat

bigger than the 5–8 μm reported by Tse et al. (1992) for hippocampal astrocytes obtained by digesting hippocampal slices with papain. Privat and Rataboul (1986) described protoplasmic and fibrous astrocytes *in situ* from rat hippocampus, cerebral cortex, and corpus callosum and adjoining deep cortex using different staining techniques. They found that the dimensions of protoplasmic and fibrous astrocyte cell bodies were similar, and the main difference between them was the greater length of the processes of fibrous astrocytes. The length of both protoplasmic and fibrous astrocyte cell bodies was approximately 20 μm , which is similar to the results obtained by us (see Fig. 1).

Viability of cells

Tissue-printed cells often appeared morphologically intact and were usually well adorned with complete processes (Figs. 2, 6). However, an unavoidable problem was the presence of cellular debris on most coverslips, as well as areas where damaged cells were intermingled with debris. The presence of damaged cells and debris made it critically important to analyze results only from viable cells. Damaged cells could usually be identified under phase-contrast microscopy by the presence of coarsely granular cytoplasm and refractile vacuoles. It was also possible to identify damaged cells by their loss of sharp and well-defined GFAP intermediate filament fluorescence. We used 0.08% trypan blue and observed that nearly all the tissue-printed cells that appeared intact also excluded the dye. The cell tracker blue fluorescent probe 4-chloromethyl-7-hydroxycoumarin (CMHC) did not allow us to identify viable cells as the fluorescence intensity over cells was not appreciably higher than that observed over cellular debris.

Uptake and nonspecific binding

Moderately high grain densities over cellular debris were observed with ^3H -5-HT in Na^+ -containing buffer (Table 1). The use of very high concentrations of ketanserin and spiperone ensured that this could not represent binding to 5-HT₁ receptor subtypes or 5-HT₂ receptors. The binding of ^3H -5-HT to debris was also Na^+ dependent but fluoxetine insensitive. Grain densities over debris in Na^+ -containing buffer were about twofold smaller in magnitude than over cells (Tables 1, 2; Figs. 2, 3, 6, 7). Fluoxetine is a highly specific inhibitor of 5-HT uptake (Wong et al., 1975; Fuller et al., 1991) and has been shown to inhibit effectively ^3H -5-HT uptake into primary astrocyte cultures (Katz and Kimelberg, 1985). The lack of inhibition by fluoxetine of grain densities over debris was in sharp contrast to the fluoxetine sensitivity of the grain densities over cells. This finding permitted us to conclude that true ^3H -5-HT uptake into tissue-printed astrocytes and NF(+) structures had occurred. The nature of the ^3H -5-HT binding site(s) on the debris is, at present, unknown.

A grain density of $\geq 31/10^3 \mu\text{m}^2$ was adopted as the criterion to identify positive cellular uptake of ^3H -5-HT. The mean grain density present over TP21 debris in the presence of fluoxetine was 32.3 ± 2.9 ($\pm$ SEM). Since binding to “debris” was fluoxetine insensitive, this allowed us to set the threshold at ≥ 31 grains/ $10^3 \mu\text{m}^2$ since it would treat the majority of background and nonspecific binding density values present over the different cell groups studied as uptake negative. However, as only very few grains were occasionally observed over some tissue-printed astrocytes in Na^+ -containing buffer, it remains possible that the threshold of ≥ 31 grains/ $10^3 \mu\text{m}^2$ was high and resulted in the

exclusion of some astrocytes that showed weak uptake in addition to the small component due to nonspecific binding.

More than 90% of 6 and 21 d TP GFAP(+) astrocytes showed positive ^3H -5-HT uptake by the criterion of ≥ 31 grains/ $10^3 \mu\text{m}^2$. This suggests that the ability to take up 5-HT is likely to be a widespread property of cortical astrocytes *in vivo*. It can be seen from Figure 11A that processes of 6 and 21 d TP astrocytes showed mean grain densities comparable to cell bodies, though no correlation in grain densities was seen between the processes and cell bodies. Grains were typically present along the entire length of astrocytic processes. About half of the NF(+) structures examined showed grain densities in the ≥ 191 grains/ $10^3 \mu\text{m}^2$ range, which were, however, never achieved by cultured or tissue-printed astrocytes. To analyze uptake into purely GFAP(+) astrocytes it was important to do neurofilament staining since NF(+) elements in this preparation were also associated with GFAP profiles and could therefore give a false indication of astrocytic uptake.

Compared to age-matched tissue-printed astrocytes, ^3H -5-HT uptake in Na^+ -containing buffer at both 6 and 21 d was lower in cultured astrocytes (Figs. 4, 8). The percentages of HS6-, HS21-, and FBS6-cultured astrocytes that showed positive ^3H -5-HT uptake were 48%, 60%, and 76%, respectively, which were less than the 98% and 90% seen in TP6 and TP21 astrocytes. The finding that FBS-cultured astrocytes showed higher grain densities than HS-cultured astrocytes is in agreement with a previous report where total ^3H -5-HT uptake rather than autoradiographic measurements were made (Kimelberg et al., 1992). Regional differences in ^3H -5-HT uptake have been previously reported in astrocyte cultures (Amundson et al., 1992). We do not know whether such differences occur in acutely isolated cells, though it should be possible to address this question by studying uptake in astrocytes tissue-printed from different brain regions.

Developmental dependence of fluoxetine sensitivity

In 6 d FBS-cultured and TP astrocytes the inhibition produced by fluoxetine was less than the inhibition produced by Na^+ -free buffer. This was a consistent finding for TP6 or FBS-cultured astrocytes and implies that the Na^+ -dependent component of the 5-HT uptake system at 6 d is not as sensitive to fluoxetine, which may be related to some difference in the fluoxetine-binding site on the 5-HT transporter. This is of interest, but to our knowledge, an age-dependent increase in fluoxetine sensitivity has not been reported previously (Fuller et al., 1991). The 5-HT content and the capacity for total 5-HT uptake in the neonatal rat cerebral cortex are about one-third of that seen in adult rats (Bennett and Giarman, 1965; Nomura et al., 1976). Kirksey and Slotkin (1979) have reported that ^3H -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 be related to these age-related differences in 5-HT levels and uptake.

Neuronal 5-HT uptake

We noticed that the tissue-printed preparations contained several NF(+) cell processes that could correspond to axons, as well as a few NF(+) cell bodies. About half of these NF(+) profiles showed very intense uptake in the ≥ 191 grains/ $10^3 \mu\text{m}^2$ range that could represent uptake by varicosities in serotonergic projections from the dorsal and median raphe nuclei. The finding of positive uptake by 90% of NF(+) structures with very

intense uptake by 50% of them was remarkable, and fits the concept that 5-HT uptake could be a widespread property of neuronal elements. Audet et al. (1989) assessed the serotonergic innervation of adult rat cortical slices by quantifying ^3H -5-HT uptake by slices in the presence of a monoamine oxidase inhibitor. Their results suggest that each midbrain raphe cell body gives rise to at least 500,000 varicosities in the cortex, and the mean number of varicosities that make connections with individual cortical target neurons ranges from 145 to 230. This suggests that almost all cortical neurons are likely to receive some serotonergic input. Catecholamines and 5-HT are present in varicosities along neuronal axons (Tork, 1990) and we observed intense, localized ^3H -5-HT uptake along axon-like processes that could have represented uptake into serotonergic varicosities (see Fig. 7).

There are differences in the serotonergic innervation of the cortex at birth, at postnatal week 1, and by the third postnatal week. At the end of postnatal week 1, serotonergic axons arising from the dorsal and median raphe nuclei traverse through all areas of the neocortex, with dense plexuses of serotonergic axons present in layer I and VI (Wallace and Lauder, 1983; Blue and Molliver, 1985). By the third postnatal week, the pattern of serotonergic innervation closely resembles the pattern seen in adult rats (Blue and Molliver, 1985), and our results indicate that about half of 21-d-old cortical NF(+) structures showed intense ^3H -5-HT uptake. In the present study, it was not possible to analyze uptake by 6-d-old NF(+) structures due to the absence of proper immunocytochemical staining, so we do not know whether there is a progressive developmental increase in 5-HT uptake capacity in neuronal elements between the first and third postnatal weeks.

Significance and implications

The significance of astrocytic versus neuronal ^3H -5-HT uptake would depend on the magnitude of uptake exhibited by each cell type and its location relative to the architecture of the synapse. Since astrocytes outnumber neurons in the brain by about fivefold and account for up to 25% of the volume of gray matter, their overall contribution could prove very significant (Kimelberg and Norenberg, 1989). The recent cloning of the rat 5-HT transporter (Blakely et al., 1991; Hoffman et al., 1991) and the human 5-HT transporter (Lesch et al., 1993) shows that they have similar molecular weights and 92% sequence identity. There is no evidence yet for the existence of multiple 5-HT transporters in rat brain, so it seems likely that astrocytes and neurons share a common 5-HT transporter. The difference in the magnitude of 5-HT uptake between neuronal elements and astrocytes observed in the present study would then be due to differing levels of expression of the transporter between the two cell types.

There are conflicting views about the anatomical features of 5-HT release. Descarries et al. (1975) and Beaudet and Descarries (1978) analyzed sites that took up ^3H -5-HT following topical application by electron microscopy and found that less than 5% of serotonergic terminals in the rat cortex formed synapses with specialized junctions. In direct contrast, other reports (Molliver et al., 1982; Papadopoulos et al., 1987) suggest that 5-HT release occurs predominantly in sites that show junctional complexes and are synaptic in nature. If the latter view is correct, a significant fraction of the 5-HT released into the synaptic cleft would probably be taken back up by neuronal serotonergic terminals or by perisynaptic astrocytes. On the other hand, the existence of widely occurring transmitter/receptor mismatches

for several peptide, amino acid, and monoamine neurotransmitters including 5-HT in the rat brain (Herkenham, 1991) raises the possibility that 5-HT could also be released nonsynaptically. There would then be a greater likelihood of it diffusing some distance, a process referred to as volume transmission (Nicholson and Rice, 1991; see also Fuxe and Agnati, 1991) and thus would be taken up by astrocyte cell bodies and their extensive fine processes at some distance from the sites of release. In the absence of 5-HT uptake inhibitors selective for neurons or astrocytes, measurement of extracellular 5-HT levels by *in vivo* dialysis following stimulated release cannot yet provide an accurate assessment of neuronal versus astrocytic 5-HT uptake.

Since these cells were acutely isolated from rat brain and uptake studies were always performed within 4 hr of preparation with the cells kept in buffer without serum, it seems unlikely that we were seeing any expression of 5-HT transporters that were not normally expressed by these cells *in vivo*. This concern is a factor in studies on cultures, because such cells are prepared from 1–2-d-old rat pups and then kept in serum-containing medium in the absence of neurons for up to 3–4 weeks. Therefore, the 5-HT uptake seen in these cells could be a property of astrocytes at an early stage in development that could disappear later *in vivo*, or a property possibly induced by factors present in serum. The latter is a valid concern because we have shown that the level of expression of the 5-HT uptake system in cultured astrocytes is dependent on both the type and batch of fetal bovine serum used (Kimelberg et al., 1992), reminiscent of the findings of Barres et al. (1989) where Ca^{2+} channel expression by cultured astrocytes was similarly affected.

In summary, these results confirm the existence of a fluoxetine-sensitive 5-HT uptake system in astrocytes isolated acutely from 6- or 21-d-old rat cortex. The proportion of uptake that was fluoxetine sensitive was higher in the 21 d animals. We also found very high grain densities in half of the NF(+) structures studied, which were possibly serotonergic axonal processes with varicosities. These data suggest that a high-affinity uptake system could be functional in astrocytes *in vivo*, in addition to its known occurrence in axonal varicosities and terminals. Uptake by astrocytes *in vivo* is likely to be followed by metabolism by MAO, as described earlier for primary astrocyte cultures (Katz and Kimelberg, 1985; Kimelberg and Katz, 1986; Kimelberg, 1986, 1988; Fitzgerald et al., 1990). Psychiatric disorders potentially treatable with selective 5-HT uptake blockers such as fluoxetine (Prozac) are diverse and include major depression, obsessive compulsive neurosis, panic disorder, personality disorders with aggressive or impulsive features, eating disorders, substance use disorders, premenstrual tension, chronic pain, and dementia (Feighner and Boyer, 1991; Fuller, 1991; Boyer, 1992; Stahl, 1992). The effectiveness of selective 5-HT uptake blockers in such disorders could include the blockade of 5-HT uptake into astrocytes *in vivo*.

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