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Alterations in Glutamate Uptake in NT2-Derived Neurons and Astrocytes after Exposure to Gamma Radiation

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Abstract

Currently, the cellular and molecular mechanisms that underlie radiation-induced damage in the CNS are unclear. The present study began investigations of the underlying mechanism(s) for radiation-induced neurotoxicity by characterizing glutamate transport expression and function in neurons and astrocytes after exposure to γ rays. NTera2-derived neurons and astrocytes, isolated as pure cultures, were exposed to doses of 10 cGy, 50 cGy and 2 Gy γ rays, and transporter expression and function were assessed 3 h, 2 days and 7 days after exposure. In neurons, at 7 days after exposure, a significant increase was detected in EAAT3 after 50 cGy ($P < 0.05$) and a dose-dependent increase in GLT-1 expression was seen between doses of 10 and 50 cGy ($P < 0.05$). Functional assays of glutamate uptake revealed that neurons and astrocytes respond in a reciprocal manner after irradiation. Neurons responded to radiation exposure by increased glutamate uptake, an effect still evident at our last time (7 days) after exposure ($P < 0.05$). The astrocyte response to γ radiation was an initial decrease in uptake followed by recovery to baseline levels at 2 days after exposure ($P < 0.05$). The observations made in this study demonstrate that neurons and astrocytes, while part of the same multifunctional unit, have distinct functional and reciprocal responses. The response in neurons appears to indicate a protracted response with potential long-term effects after irradiation.

INTRODUCTION

Currently, the cellular and molecular mechanisms that underlie radiation-induced damage in the central nervous system (CNS) are unclear. For many years the CNS was considered to be relatively resistant to radiation given that its neuronal population is primarily post-mitotic (1). However, in recent years this view has changed dramatically as a result of studies using animal models of radiation-induced CNS damage as well as clinical data indicating that exposure of normal brain tissue to any form of ionizing radiation results in behavioral, cognitive and motor deficits that sometimes may not become evident for months or years. While high radiation doses can destroy tissue, lower doses can induce cognitive impairments without signs of obvious tissue damage (2). The manifestation of neurological deficits in the absence of overt neuropathology suggests that more subtle mechanisms of damage may be at work (3).

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Although the brain is composed of functionally different cell types, they form a syncytium in which each cell type depends on the others for normal brain function. Neurons and astrocytes, two principal cell types in the brain, coexist as an interdependent metabolic unit through the neurotransmitter glutamate. Excess glutamate that remains in the synaptic cleft after presynaptic release is cleared primarily by astrocytes by uptake. Glutamate, which is considered to be the major neurotransmitter in the mediation of excitatory neurotransmission in the CNS, is required for normal synaptic transmission. However, when glutamate release and uptake are unregulated, neurotoxic cascades, including excitotoxicity and oxidative glutamate toxicity, can occur. Consequently, extracellular glutamate must be cleared quickly, perhaps within 1 ms, to maintain glutamate below toxic levels (4). Glutamate transporters represent the only significant mechanism for the uptake of extracellular glutamate, and their importance for the long-term maintenance of low non-toxic glutamate concentrations is well documented (5).

Reuptake of glutamate is carried out by high-affinity, sodium-dependent glutamate transporters that belong to a family of integral membrane transport proteins. There are five mammalian isoforms of sodium-dependent glutamate transporters termed excitatory amino acid transporters or EAATs: EAAT1 (GLAST), EAAT2 (GLT-1), EAAT3 (EAAC1), EAAT4 and EAAT5 (5). In general, the predominant EAATs expressed by astrocytes are GLAST and GLT-1, whereas EAAT3 and EAAT4 are found on cortical neurons, and EAAT5 is found almost exclusively in retinal cells (4,6). The transporters also display regional expression patterns evident throughout the brain with abundant expression in cortex (EAAT 2 and 3), cerebellum (EAAT1, 3 and 4), hippocampus (EAAT1, 2 and 3), and striatum (EAAT2 and 3). Although neurons and astrocytes each preferentially express particular transporter isoforms, this does not exclude co-expression of other isoforms. For example, GLT-1 and EAAT3 have been shown to be expressed in neurons in the developing brain (7) as well as in the adult brain (8–10). Current reports in the literature suggest that both astrocytes and neurons express all isoforms. Although it is the transporters located on astrocytes that are believed to be responsible for the majority of glutamate uptake, recent studies have focused on the contribution of neuronal glutamate transporters, because these have been shown to contribute to the clearance of glutamate from the synapse (11). Given that glutamate transporters provide the main route by which glutamate is cleared, it can logically be predicted that an aberration in transporter expression and function may lead to toxic glutamate levels and thus promote neuronal degeneration (12).

Glutamate-triggered excitotoxicity is a major neurotoxic mechanism underlying many acute incidents such as brain ischemia and trauma, and it is believed to play a role in chronic neurodegenerative disorders (e.g., amyotrophic lateral sclerosis). It appears unlikely, given the highly integrated nature of the CNS and its dependence on cell-to-cell interactions, that radiation injury can be attributed to damage of only a single target or particular cell type (13). While the underlying mechanisms are presently unknown, several investigators have suggested that the observed deficits may not be due simply to precursor cell loss after exposure to ionizing radiation but may also be due in part to changes in the neurogenic microenvironment that alter intercellular cues (3,14). Understanding how low-dose exposure of neurons alters intercellular cues is key to uncovering the molecular mechanism(s) by which radiation deleteriously affects the brain (15).

Given that neurons and astrocytes are metabolically coupled by glutamate, we investigated the premise that ionizing radiation alters glutamate transport and metabolism, ultimately disrupting neuron-astrocyte coupling. In the present study, we began investigating the mechanism of radiation-induced neurotoxicity by characterizing glutamate transport expression and function after exposure to γ rays. Isolated pure cultures of neurons and astrocytes derived from NTera2/

D1 cells were used. Changes in transporter expression and function were assessed 3 h, 2 days and 7 days after exposure to 10 cGy, 50 cGy and 2 Gy γ rays.

MATERIALS AND METHODS

Cell Culture

Culture of NT2/D1 cells was carried out as described previously (16,17). NT2 cells were plated at a density of 2.5×10^6 cells per T-75 flask with 12 ml DMEM/F12 medium supplemented with 10% FBS, 5% penicillin- streptomycin, and 2 mM L-glutamine (complete medium). Cells were incubated at 37°C in 95% air, 5% CO₂ until they reached approximately 80% confluence (usually about 2 or 3 days) prior to initiating differentiation. *Differentiation*: During the differentiation period cells were cultured in 12 ml of complete medium containing 10 μ M retinoic acid (RA). Medium was replaced three times a week for a total of 4 weeks to induce the neural phenotype. *Replate 1*: After 4 weeks, cells were split 1:6 in complete medium and cultured for 48 h. *Replate 2 (neuron and astrocyte harvesting)*: Neurons (NT2/N) were separated from the underlying glial layer by mechanically dislodging neurons by gently striking the flask. Harvested neurons were then plated onto Matrigel-coated plates (BD Biosciences, San Jose, CA), while astrocytes (NT2/A) were replated 1:3 into new T-75 flasks. Neuron medium was replenished every 3–4 days with inhibition medium (DMEM/F12, 5% FBS, 1% penicillin- streptomycin, 10 μ M FRdU, 10 μ M Urd, 1 μ M araC) for a maximum of 2 weeks. Astrocytes were maintained in complete DMEM/F12 medium and replated every 7–10 days. Neurons and astrocytes were maintained in culture for 4 and 2 days, respectively, prior to use in the various experiments.

Human fetal astrocytes and all reagents for culture of these cells were purchased from ScienCell Research Laboratories (San Diego, CA). Fetal astrocytes were received as cryopreserved passage one cells. These cells were expected to divide approximately every 30 h and to undergo a maximum of three passages. Fetal astrocytes were plated at an initial seeding density of 7500 cells/cm² onto PDL-coated T-75 flasks. Cells were incubated at 37°C in 95% air, 5% CO₂. The medium was replaced every 2 days thereafter with 12.5 ml astrocyte medium (500 ml basal medium, 10 ml of fetal bovine serum, 5 ml of astrocyte growth supplement, and 5 ml penicillin/ streptomycin solution). Cells were subcultured when they reached 80% confluence and replated onto PDL-coated vessels and used at passage 3.

Irradiation Conditions

An upgraded Eldorado cobalt-60 irradiator unit (Atomic Energy of Canada, Commercial Products Division, Ottawa, Ontario) was used to supply the γ rays. The beam was directed vertically downward to project a 30 $\times$ 30-cm field at an isocenter 80-cm source-to-axis distance with a build-up layer of 5 mm. The dose was delivered in a single fraction at an approximate dose rate of 60 cGy/min. Dose calibration was performed using a Capintec PR-06G cylindrical ion chamber (SN = 5965) calibrated at an Accredited Dosimetry Calibration Laboratory that confirmed the delivered doses. Cell cultures were harvested for various assays at 3 h, 2 days or 7 days after irradiation (18).

Immunocytochemical Labeling of Cells

The protocol followed for immunocytochemical labeling has been described previously (19). Briefly, cells established on 25-mm round glass cover slips and fixed in 70% ethanol were primarily labeled with antisera/antibodies directed against the transporters: GLAST, GLT-1, EAAT3 and EAAT4 (anti-GLT-1, Abcam, Cambridge, MA; anti-GLAST, Abcam, Cambridge, MA; anti-EAAT3, Alpha Diagnostic International, San Antonio, TX; anti-EAAT4, Alpha Diagnostic International, San Antonio, TX). The primary antisera/antibody incubation was 16 h at 4°C followed by washing in PBS containing 0.05% Tween-20 (PBST). Antibodies were

secondarily labeled with Alexa-488 anti-rabbit or anti-mouse antibodies (Molecular Probes, Eugene, OR) for a minimum incubation period of 4 h at 25°C. After completion of the secondary antibody incubation, the nuclear stain propidium iodide (PI, 5 µg/ml) was added for 10 min. Excess secondary antibody and nuclear counterstain were removed by washing in PBST, and the cover slips were mounted to microscope slides using permafluor. The slides with cover slips were dried flat in the dark prior to photography and quantitative analysis on a laser scanning cytometer (LSC).

Quantification of Immunofluorescence by Laser Scanning Cytometry

The laser scanning cytometer (LSC) has an Olympus BX50 base con-figured with argon ion, helium-neon-ion and UV lasers for six-color analysis. Four sensors are digitized simultaneously at 625,000 Hz corresponding nominally to 0.5-µm spatial intervals along the scan. Fluorescence energy was collected by the objective, reflected by a partially silvered mirror, and steered through a scan lens to the scanning mirror. Four photomultiplier tubes, each capable of detecting a specific range of fluorescence wavelengths, support dichroic mirrors and optical interference filters. The fluorescence measurements and *x*, *y* coordinates are recorded digitally and stored.

Scanning parameters were adjusted by signal intensity to create a contour of the nuclear label. To quantify cells that were multicolored (secondary fluors) the gating parameters were set to contour on the nuclear label (*y* axis) and to sum (integrate) the fluorescence intensity of the proteins of interest (*x* axis) and/or additional colors set up separately in the display. Fluorescence intensity reflects a quantitative measure of the specific label in the irradiated and control cells. To confirm the gating parameters, cells in selected gates were inspected visually through a CCD camera attached to the microscope. Optimized protocol and display settings were stored as configuration files and used for all samples within a given experimental set. The average number of cells scanned per sample was $5,000 \pm 200$.

Image Capture and Processing

Images were taken on an Olympus BX-60-Based microscope (Horizon Optical, Temecula, CA). The microscope was equipped with a 100-W mercury lamp and epifluorescence filters with emission wavelengths of 470, 535 and 630 nm. The images were viewed through an OLY-750 analog color camera and captured with ImagePro Plus (Media Cybernetics, Silver Springs, MD).

Cell Viability

Cell viability and cell loss after irradiation were assessed using trypan blue dye exclusion. Astrocytes and neurons were plated in T-25 flasks at a density of 7.0×10^5 and 1.5×10^6 cells per flask, respectively. The medium was collected from cultures that underwent a medium change between the time of irradiation and the final times. Cell loss was assessed in the collected medium, and this value was added to the final calculation determining viability at each of the harvest times.

[³H]Glutamate Uptake

[³H]Glutamate uptake in cultures of isolated NT2/N and NT2/A cells was measured as described previously (20,21). Cells were plated at a density of 2.0×10^5 cells per well in six-well plates. Cells were washed twice in PBS and equilibrated in Krebs buffer (Sigma-Aldrich, St. Louis, MO) for 20 min at 37°C. Uptake was initiated by the addition of 1 ml L-[3,4-³H] glutamate (18.5 kBq/ml) (GE Healthcare, Piscataway, NJ). Up-take was terminated after 5, 20, 40, 60 and 90 min by adding 2 ml ice-cold PBS. Cell wells were then washed in ice-cold PBS and trypsinized with $1 \times$ trypsin. Trypsinized triplicate fractions were collected and intracellular

levels of [^3H]glutamate were determined using a model LS 6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA).

Western Blot Analysis

Protein samples were prepared by lysing the cells in ice-cold RIPA buffer (Sigma-Aldrich) containing protease inhibitors (Roche, Mannheim, Germany). The lysate was separated from insoluble material by centrifugation for 40 min at 14,000g. The soluble protein fractions were collected and quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Twenty micrograms of the corresponding proteins were separated on NuPAGE 10% Bis-Tris gels with 1.0-mm $\times$ 12 wells (Invitrogen, Carlsbad, CA) for 1 h at 100 mA. Transfer was carried out on PVDF 0.45- μm pore size membranes (Invitrogen) for 2.5 h at 190 mA. Membranes were blocked in 10 ml of blocking buffer (5% nonfat milk in wash buffer) for 1 h at room temperature. Hybridization of primary polyclonal antisera specific for the four glutamate transporters, GLT-1, GLAST, EAAT3 and EAAT4, was carried out overnight at 4°C. After over-night incubation, membranes were washed and secondary Cy5 antibodies (Chemicon, Temecula, CA) were added for an additional overnight incubation at 4°C. Visualization of fluorescence-labeled proteins was performed using a STORM model 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA) equipped with ImageQuant software. The scanned fluorescence signal was corrected for differences in the amount loaded using actin as a loading control.

Statistical Analysis

Statistical analysis was conducted after extensive consultation with and aid from the Loma Linda University Department of Epidemiology and Biostatistics. The data were compiled, compared and assessed using a variety of statistical tests. Two-way repeated-measures analysis of variance with one within-subjects factor (time) and one between-subjects factor (dose) and their interaction term was used to compare the effect of the different doses over time. Analysis of variance (ANOVA) with the Scheffe test for further multiple comparisons was used to compare the different doses with each other if the dose response was significant. A *P* value of < 0.05 was taken to indicate significant differences among groups. The analyses were performed using SPSS version 12 (SPSS, Inc., Chicago, IL).

RESULTS

Characterization of NT2-Derived Neurons and Astrocytes

The NTera2/D1 cell line is a well established human progenitor cell line that when induced with retinoic acid to differentiate will give rise to cells of two distinct lineages (16,17,22). We followed the procedure described by Sandhu *et al.* (17) to differentiate these stem cells into neurons and astrocytes. The procedure takes approximately 5 weeks; in the first 4 weeks, the cells were treated with all-trans-retinoic acid, followed by a replating without RA and then supplementation with mitotic inhibitors to suppress glial proliferation and enrich for neuronal cells. Samples of the resulting cultures were harvested and replated as individual cultures of distinct cell type, i.e., neurons or astrocytes. Microscopic inspection revealed that the separate cultures displayed the distinct morphologies characteristic of mature neurons and astrocytes (Fig. 1). Neuronal cultures displayed cells with extended processes that appeared to form networks when left in culture for more than 7 days. Astrocyte cultures contained cells displaying a stellate morphology similar to that of protoplasmic astrocytes rather than fibrous astrocytes. Immunocytochemical analysis was carried out to confirm that the differentiation process did in fact yield two types of cells with mature phenotypes.

Terminally differentiated cultures were immunolabeled for specific neuron and astrocyte markers. As shown in Fig. 2, the neuron-specific nuclear marker, NeuN, was used to label

neurons; GFAP was used to immunolabel astrocytes. In both cultures the nucleus was counterstained with pro-pidium iodide. Isolated cultures of each cell type were assessed for purity by verifying the crossreactivity of NeuN in astrocyte cultures and of GFAP in neuron cultures. Our observations indicated that the differentiation and harvesting method used produced neuron cultures that were at least 95% pure and astrocyte cultures with a purity close to 99%. Immunolabeling with NeuN and GFAP revealed that the differentiation process for the NT2 cells that we used yielded cells of mature neuron and astrocyte phenotypes, as had been reported previously (22). NT2-derived neurons were found to be post-mitotic, as are normal neurons in the CNS, and could not be subcultured once they reached this stage of terminal differentiation. NT2-derived astrocytes divided approximately every 48–55 h after differentiation, an observation that is in agreement with current literature reports that glia have the capacity to proliferate.

Protein Expression by Western Blot Analysis of Glutamate Transporters after Irradiation

Cultures of NT2/N and NT2/A cells were immunolabeled for each of the four transporters. The transporters were found to be localized in a general diffuse pattern in the plasma membrane (Fig. 3). NT2/N cells were found to express EAAT3 and EAAT4 as expected, but they were also positive for GLAST and GLT-1 (data not shown). NT2/A cells were found to express the transporters GLAST and GLT-1, which are reported to be expressed predominantly on astrocytes (5). Furthermore, the NT2/A cell cultures were also found to express low levels of EAAT3 and EAAT4 transporters traditionally designated to neurons. These findings corroborate recent reports that indicate that all isoforms of the transporters are expressed, although to varying degrees, by both neurons and astrocytes (7). Immunocytochemistry of transporter levels in neurons and astrocytes revealed variable transporter expression over time (Table 1). Transporter expression was measured at 3 h, 2 days and 7 days without irradiation and *after* irradiation in neurons and astrocytes.

Transporter protein expression was confirmed using Western blotting (Fig. 4). The raw pixel fluorescence values used in densitometric analysis are listed in Table 2. Protein expression of each of the four transporters was observed to vary over time in both cell types (Table 3). In astrocytes, the expression of GLAST, GLT-1 and EAAT4 was found to vary significantly at 3 h, 2 days and 7 days after irradiation. GLAST increased approximately 1.5-fold between 3 h and 2 days and about fivefold between day 2 and day 7. GLT-1 increased in a similar fashion but at about half the magnitude. Similar to GLT-1 and GLAST expression, EAAT3 showed increases over time but was expressed at levels 10 times lower than GLAST and GLT-1. In addition, expression of EAAT4, like that of EAAT3, was found to be 10 times lower than GLAST and GLT-1 in astrocytes with a twofold increase detected between 3 h and day after irradiation.

In neurons, transporter protein expression of each of the four transporters proteins was also found to change as function of time. GLAST showed a linear increase and appeared to double between times, which was perhaps a reflection of the maturity of the cultures. Transporters GLT-1, EAAT3 and EAAT4 exhibited a different pattern of protein expression over time: a plateau between 3 h and days and an increase between 2 days and 7 days.

Overall, dramatic changes in transporter expression were observed in astrocytes, but significant differences between doses of radiation were not detected. EAAT3 in astrocyte cultures increased 16-fold 7 days after 50 cGy irradiation. There was a similar effect of radiation at this time with Gy: a 12-fold increase in EAAT3. EAAT4 was found to increase 3 h after irradiation; however, continued elevation of EAAT4 was observed only with the highest dose of Gy 2 days after irradiation. In general, transporter expression in irradiated astrocyte cultures changed over time but did not change as a function of dose. Similar to astrocyte transporter expression, neuronal transporter expression changed as a function of time but did not change as a function

of dose, with the exception of differences in GLT- levels between 10 cGy and 50 cGy 7 days after irradiation.

Functional Effects of Radiation on Glutamate Transport

Immunocytochemical and immunoblotting analysis re-vealed normal fluxes in transporter levels as well as some changes in protein expression after irradiation. In general, glutamate transport is regulated by transporter expression as well as transporter activity. We therefore assessed the effects of radiation on transporter uptake activity by measuring the capacity of the transporters to take up labeled glutamate. [^3H]Glutamate was added to cell cultures 3 h, 2 days and 7 days after irradiation, and uptake of [^3H]glutamate was determined within a 90-min time course by liquid scintillation counts of intracellular levels of [^3H]glutamate.

Effect of Radiation on Glutamate Uptake in NT2/N Neurons

Maximal uptake of [^3H]glutamate was observed after approximately 60 min of initial incubation with [^3H]glutamate. Neurons were found to have a very low basal level of glutamate uptake, $73,000 \pm 13,000$ cpm/mg (Fig. 5). However, when neuronal cultures were exposed to 10 cGy, 50 cGy and 2 Gy γ rays, there was a 31-fold ($P < 0.05$, $n = 6$), 10-fold ($P < 0.05$, $n = 6$), and 14-fold ($P < 0.05$, $n = 6$) increase in uptake, respectively, that was detectable as early as 3 h after radiation exposure. At 2 days after irradiation, neuronal cultures that were exposed to 10 cGy had glutamate uptake levels comparable to those of control unirradiated cultures. However, neurons exposed to the two higher doses of 50 cGy and 2 Gy exhibited sustained increases in activity of 26-fold ($P < 0.05$, $n = 6$) and 24-fold ($P < 0.05$, $n = 6$), respectively. This dosedependent effect was still present at our last time, 7 days after irradiation with 50 cGy ($P < 0.05$, $n = 6$) and 2 Gy ($P < 0.05$, $n = 6$) γ rays (Fig. 5).

Effects of Radiation on Glutamate Uptake in NT2/A Astrocytes

In astrocytes, basal uptake activity of [^3H]glutamate was found to be moderate (Fig. 6). When astrocytes were exposed to 10 cGy γ rays, there was no detectable change in activity. However, at 50 cGy and 2 Gy, there was a significant decrease in uptake of fivefold ($P < 0.05$, $n = 6$) and sevenfold ($P < 0.05$, $n = 6$), respectively. By 2 days after irradiation with all three doses, uptake activity in astrocytes had returned to normal (baseline) levels and remained so 7 days after exposure.

To verify that the effects in uptake were not a phenomenon specific to the cell line, we carried out the same set of experiments using normal human fetal astrocytes (Fig. 7) and found effects similar to those measured in the NT2-derived astrocytes. Maximal uptake occurred approximately 40 min from the time glutamate was added to the cultures. Once again, we observed that at 3 h after irradiation, uptake was essentially the same as in unirradiated control cultures. However, with doses of 50 cGy and 2 Gy, there was a significant decrease in uptake ($P < 0.05$, $n = 3$). At 2 days after irradiation, glutamate uptake in astrocyte cultures exposed to doses of 50 cGy and 2 Gy returned to values similar to those in unirradiated fetal astrocyte cultures. Thus glutamate uptake in fetal astrocytes after irradiation confirmed that changes in glutamate uptake in NT2-derived astrocytes was a common astrocyte response to γ radiation. That is, the effect observed in NT2/A was not a phenomenon specific to this lineage of astrocytes.

DISCUSSION

Transporter Expression and the Effect of Radiation

Our investigation revealed that neurons and astrocytes both expressed all four glutamate transporters to varying degrees. It is known that astrocytes play the major role in glutamate

uptake (5). However, the fact that neurons express some GLAST and GLT-1 suggests that they may be capable of mediating glutamate uptake. This would suggest that neuronal GLAST and GLT-1, although not active under basal conditions, could serve as backup transporters in response to changes in the microenvironment caused by a stressor such as radiation. Expression of GLAST and GLT-1 in astrocytes and neurons varied at each of the times assayed in irradiated and unirradiated samples. The variable expression of GLAST and GLT-1 over time, which was found to be statistically significant, could be due to evolving *in vitro* conditions or factors. Expression of transporter protein has been reported to be differentially regulated, wherein each transporter subtype is responsive to multiple and at times opposing factors. For example, PKC activation is known to decrease GLT-1 and GLAST protein expression but to increase EAAT3 trafficking to the cell surface (23). Glutamate has opposite effects on GLT-1 and GLAST expression, increasing GLAST and decreasing GLT-1. In addition, cAMP can up-regulate GLAST but not GLT-1 (6). Thus the protein fluctuations we observed in each of the four transporters over time appear to reflect this very complex regulation of transporter expression. It is interesting to note that in both neurons and astrocytes the most dramatic change in protein expression was observed to be EAAT3, generally considered to be a minor player in glutamate uptake. However, the measured changes in transporter expression after irradiation do not appear to be driving the alteration in up-take in these cells, because we did not find a correlation between changes in transporter expression and changes in transporter activity. The increase in EAAT3 may be indicative of a subtle yet biologically significant event not necessarily tied to glutamate clearance. Recent reports (11) also implicate the presence and function of EAAT3 as involved in cellular redox homeostatic mechanisms, because EAAT3 may be the primary route of neuronal cysteine uptake. Furthermore, the existence of different pools of EAAT3 transporters has been suggested to explain phorbol-induced transport stimulation, which is rapid and independent of protein synthesis in C6 glioma cells (24). Our findings appear to be in agreement since we found the changes in transporter activity to be independent of transporter expression levels. Given the dual role and multilevel regulation of EAAT3, it is possible for EAAT3 to be involved in complementary functions independent of glutamate uptake that are initiated under non-basal conditions. Thus we believe it is an interesting candidate in delineating neural radiation-induced response mechanisms.

Neurons and astrocytes displayed various yet similar changes in transporter protein expression. Our data indicate that transporter expression fluctuated over time and that the differences in protein expression did not correlate to radiation dose at the doses used in this study. Certainly, it is possible to have changes in glutamate uptake without a direct change in protein expression. Therefore, based on our observations, we conclude that any alterations in glutamate uptake were due to changes in transporter activity and not to changes in transporter protein expression.

Differential Functional Response of Neurons and Astrocytes to Radiation

Glutamate uptake is regulated at several levels, primarily by expression of transporter protein and activity of expressed transporters. Our investigation of transporter expression indicates that radiation does not disrupt glutamate transport by altering the number or type of transporters expressed but rather does so by altering the *activity* of transporters. We expected that the function of the glutamate transporters would be affected by exposure to radiation, with higher doses causing more severe dysfunction, and/or dysfunction manifest earlier in time. Based on our results for transporter expression where we observed similar expression changes in neurons and astrocytes, we expected to see similar decreased response patterns in glutamate transporter functional assays. However, we saw quite opposite functional responses in the two cell types. Neurons appeared to respond to radiation exposure by increased up-take. This was an unexpected result because neuronal glutamate transporters generally are not believed to participate substantially in the uptake of glutamate.

The decrease in uptake observed in astrocytes was expected given that glutamate uptake is often suppressed after exposure to such stressors as hypoxia and generators of reactive oxygen species. It was encouraging to see that astrocyte uptake activity returned to normal relatively quickly, within 2 days after irradiation. However, what was perplexing was the unexpected increase in uptake observed in neurons. Furthermore, this change in activity persisted until the last measurement 7 days after exposure, which seems to indicate a protracted response in neurons and potential long-term effects after irradiation. It is uncertain whether these changes in activity are indicative of repair or injury processes. Whether such changes are tied to cellular redox homeostatic mechanisms is unclear. However, it does not seem likely that the relationship between radiation-induced oxidant levels and altered transporter activity are on a regulatory level directly because the two cell types displayed very different transporter responses to identical doses of radiation. However, this does not preclude a possible synergistic relationship between transporter activity and increased oxidative load.

We observed increases in EAAT3 in both neurons and astrocytes after irradiation, although not all increases were statistically significant. However, this general trend may be indicative of possible transporter involvement in other concomitant functional responses after irradiation. The current literature increasingly suggests a primary role for EAAT3 in glutathione synthesis, and therefore EAAT3 may be important in regulating cellular oxidant levels. Upon exposure to ionizing radiation, an increase in reactive oxygen species is expected due to radiolytic processes. It is possible that in the case of neurons the observed increase in transporter activity could occur in conjunction with intracellular oxidant changes induced by radiation. That is, the increase in neuronal transporter activity, in addition to clearing glutamate and thereby reducing excitotoxicity, could serve a dual function in combating radiation-induced increased oxidant load. Why then the reciprocal response in astrocytes with radiation? We believe that the reciprocal responses observed in neurons and astrocytes likely are not due to differences in their transporter protein expression profiles but rather are due to other factors subject to intracellular enzymatic differences. Astrocytes are generally believed to have a greater capacity to metabolize hydrogen peroxide given that they exhibit greater glutathione peroxidase and catalase activity than do neurons. This would make neurons more vulnerable to the effects of radiation than astrocytes. In fact, we observed this to be the case in our viability assays (Fig. 8). At each of the three times after irradiation, we found astrocytes to be relatively radioresistant compared to neurons. Neurons show increased vulnerability particularly to 50 cGy and 2 Gy γ rays at 2 and 7 days after irradiation. Thus it is logical to surmise that these two cell types, given their precise functional roles in the CNS and inherently distinct intracellular composition, would respond differently to a stressor such as radiation. Transporter expression and function are differentially regulated by various mechanisms. Among these mechanisms is phosphorylation. At present, the mechanism by which kinases influence uptake is unclear. It remains to be established whether changes in transporter activity are due to phosphorylation of the transporter itself or to changes in protein-protein interactions at the plasma membrane (11). With respect to transporter activity, PKC activation exerts opposing effects on different transporter isoforms, selectively increasing EAAT3 activity and contributing to reductions in GLT1/EAAT2 activity (25). Therefore, based on our findings and the dual effects mediated by PKC, we believe that signaling by PKC is a promising area of research to begin to understand the mechanism that underlies the reciprocal uptake response we observed in neurons and astrocytes after irradiation.

CONCLUSIONS

Much has been learned in recent years about the dynamic nature of the CNS. The bidirectional interaction between neurons and glia is complex. To understand this cellular interplay under physiological and pathological conditions, we must first have a better understanding of the distinct responses contributed individually by neurons and glia. The results of this study

indicate that neurons and astrocytes, while part of the same multifunctional unit, have distinct functional and reciprocal responses. A determination of whether the observed changes are indicative of positive or maladaptive responses cannot be made at this time. However, there is a certain degree of neuroplasticity that takes place in the brain, and these changes are often associated with positive processes like learning and memory and with synaptic reorganization after injury. There are several studies that demonstrate the effects of glutamate uptake inhibitors on excitatory postsynaptic currents and that suggest a role for glutamate uptake in synaptic plasticity (26,27). It is generally agreed that under physiological conditions astrocyte glutamate transporters are the dominant route of glutamate uptake. In our experiments we demonstrated that neuronal glutamate transporters are capable of significant glutamate uptake after exposure to a stressor such as radiation and therefore represent a probable alternative route of uptake. Therefore, it is reasonable to assume that the molecular and cellular effects that were observed after irradiation represent adaptive responses.

Our investigation revealed that neurons and astrocytes appear to respond in a reciprocal manner when exposed to ionizing radiation, and we can conclusively state that glutamate transporter activity in these two fundamental cell types are subject to the effects of γ radiation. We believe this finding warrants future study and that it may be key in deciphering the mechanism(s) of radiation-induced neurotoxicity. It is intriguing to think about the possible relationship between the observed changes in glutamate transport and radiation-induced ROS production because it is generally agreed that an inherent effect of photon-radiation exposure is increased production of ROS. Increased oxidant levels could directly influence astrocyte-neuronal coupling, because the generation of free radicals is believed to be an underlying mechanism in the neuropathology observed of many neurodegenerative disorders. In fact, in several neurodegenerative disorders (e.g., amyotrophic lateral sclerosis) oxidative stress and glutamate transport dysfunction occur in parallel. Given that much of the signaling taking place between neurons and astrocytes is tied to glutamate, future studies to examine radiation effects could focus on those elements of intercellular communication involving glutamate such as calcium signaling and calcium-dependent glutamate and ATP release. In addition, differential expression of purinergic receptors and ATP concentration in neurons and astrocytes provides an intriguing area of research to help delineate a more precise mechanism that would integrate observed changes in glutamate transport activity and global neuroglial dysfunction.

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FIG. 1. NT2/N and NT2/A cell morphology

Phase-contrast image of NT2-derived neurons and astrocytes. Panels a and b: NT2/N cells display classic neuron morphology with soma and extended processes. Panels c and d: NT2/A cells exhibit morphology characteristic of protoplasmic astrocytes. Original magnification 10 $\times$ in panels a and c and 20 $\times$ in panels b and d.

FIG. 2. Differentiated NT2 cells immunolabeled with markers of mature neuron and astrocyte phenotypes

Panel a: NT2-derived neurons labeled with the neuronal nuclear marker NeuN (green). Panel b: Astrocytes labeled with GFAP (green). In both images the nuclei are counterstained red with propidium iodide. Original magnification 40 $\times$.

FIG. 3. Immunolabeling of glutamate transporters in astrocytes

GLAST (panel a), GLT-1 (panel b), EAAT3 (panel c) and EAAT4 (panel d) were secondarily labeled green and the nuclei were counterstained red with PI. The images are representative of the diffuse immunolabeling pattern observed in these cultures. Original magnification 40 $\times$.

FIG. 4. Western blot of GLT1 glutamate transporter expression in astrocytes and neurons
The blot shows glutamate transporter EAAT-2 (GLT1) protein expression 2 days after exposure to γ rays at the doses indicated. RBL, rat brain lysate.

FIG. 5. Functional assessment of glutamate transporters in isolated neuron cultures after γ irradiation

Functional activity was assessed directly by measuring the uptake of $[^3\text{H}]\text{glutamate}$ in NT/N cultures 3 h, 2 days, and 7 days after irradiation. The time course for uptake ranged from 0 to 90 min after initial addition of $[^3\text{H}]\text{glutamate}$ to cultures. Data are the average values of six biological replicates $\pm$ SD.

FIG. 6. Functional assessment of glutamate transporters in isolated astrocyte cultures after γ irradiation

Functional activity was assessed directly by measuring the uptake of $[^3\text{H}]\text{glutamate}$ in NT/A cultures 3 h, 2 days and 7 days after irradiation. The time course for uptake ranged from 0 to 90 min after initial addition of $[^3\text{H}]\text{glutamate}$ to cultures. Data are the average values of six biological replicates $\pm$ SD.

FIG. 7. Functional assessment of glutamate transporters in normal fetal astrocytes after γ irradiation

Functional activity was assessed directly by measuring the uptake of $[^3\text{H}]\text{glutamate}$ in normal fetal astrocyte cultures 3 h and 2 days after irradiation. The time course for uptake ranged from 0 to 90 min after initial addition of $[^3\text{H}]\text{glutamate}$ to the cultures. Data are the average values of three biological replicates $\pm$ SD.

FIG. 8. Cellular viability in neurons and astrocytes after irradiation

Percentage cell loss was assessed 3 h, 2 days and 7 days after irradiation. Data are the average values of six biological replicates $\pm$ SD.

TABLE 1
Transporter Protein Expression Level as Quantified by Immunocytochemistry

	Astrocytes			Neurons		
	10 cGy	50 cGy	2 Gy	10 cGy	50 cGy	2 Gy
3 h						
GLAST	1.45 ± 0.34 ^a	1.39 ± 0.10	1.27 ± 0.03	1.54 ± 0.17	0.56 ± 1.13	0.98 ± 0.01
GLT1	1.27 ± 0.21	1.05 ± 0.54	1.08 ± 0.62	1.15 ± 0.33	1.14 ± 0.83	1.31 ± 0.23
EAAT3	1.03 ± 0.54	1.84 ± 0.05	1.22 ± 0.02	1.05 ± 0.54	1.23 ± 1.36	0.64 ± 1.06
EAAT4	0.59 ± 0.07	0.87 ± 0.27	0.98 ± 0.45	1.41 ± 1.02	0.70 ± 0.66	1.59 ± 0.05
2 days						
GLAST	1.23 ± 0.16	1.59 ± 0.05	1.31 ± 0.18	0.42 ± 0.15	0.81 ± 0.17	0.79 ± 0.19
GLT1	2.02 ± 0.19	1.07 ± 0.01	1.74 ± 0.27	1.00 ± 0.11	1.10 ± 0.34	0.57 ± 1.09
EAAT3	1.05 ± 0.45	0.89 ± 0.13	1.25 ± 0.13	1.10 ± 0.56	0.96 ± 1.08	0.83 ± 1.40
EAAT4	2.29 ± 0.64	1.32 ± 0.11	2.09 ± 0.09	0.85 ± 0.82	1.17 ± 0.28	1.15 ± 0.14
7 days						
GLAST	0.40 ± 0.03	0.63 ± 0.70	0.99 ± 0.29	1.01 ± 0.40	1.29 ± 10.65	1.48 ± 0.28
GLT1	1.08 ± 0.12	0.46 ± 0.14	1.20 ± 0.23	1.71 ± 0.07	1.32 ± 0.48	1.31 ± 0.09
EAAT3	0.79 ± 0.43	0.78 ± 0.06	1.52 ± 0.28	4.67 ± 1.36	8.61 ± 0.16 ^b	6.27 ± 0.34
EAAT4	1.42 ± 0.31	1.04 ± 0.03	0.95 ± 0.94	1.20 ± 0.85	0.87 ± 0.40	1.63 ± 0.06

Note. Transporter values represent relative levels changes in duplicate samples normalized unirradiated control values.

^aDuplicate values ± SD.

^bRepresents significant relative changes from unirradiated control values.

TABLE 2
Transporter Protein Expression Level as Quantified by Western Blotting

		Astrocytes				Unirradiated controls	Neurons		
		10 cGy	50 cGy	2 Gy	10 cGy		50 cGy	2 Gy	
3 h	GLAST	417746	472903	448081	616407	224738	168500	149589	106914
	GLT1	332868	374206	500786	539434	49573	50251	42048	50395
	EAAT3	117591	39082	35262	16863	35091	44004	48924	48357
	EAAT4	46318	44279	53675	84699	32668	39754	44522	16895
2 days	GLAST	1367933	1422512	936949	1212194	470154	539487	360933	391917
	GLT1	1590833	1426136	1286878	1548110	255306	298766	240859	101018
	EAAT3	29483	248964	502209	644449	22740	56984	17003	39986
	EAAT4	251411	407521	260179	325028	43172	58285	81448	125087
7 days	GLAST	5619724	5665803	5083730	5445996	638477	44745	679092	730074
	GLT1	2946644	3093864	2508564	2241228	1825999	1387607	1042016	1085261
	EAAT3	486656	417976	517640	506609	278108	16562	582779	262035
	EAAT4	261640	312849	310522	308862	553673	612828	643225	614680

Note. Transporter values represent raw pixel intensity values of triplicate samples.

TABLE 3
Western Blot Quantification of Transporter Protein Expression

	Astrocytes			Neurons		
	10 cGy	50 cGy	2 Gy	10 cGy	50 cGy	2 Gy
3 h						
GLAST	1.41 ± 1.21 ^a	1.16 ± 0.21	1.72 ± 0.95	0.77 ± 0.13	0.66 ± 0.34	0.59 ± 0.58
GLT1	1.62 ± 1.03	1.81 ± 0.71	1.55 ± 0.19	1.02 ± 0.28	0.79 ± 0.36	1.05 ± 0.66
EAAT3	1.00 ± 0.91	0.72 ± 0.50	0.52 ± 0.41	1.26 ± 0.14	1.31 ± 0.37	1.44 ± 0.38
EAAT4	1.81 ± 1.50	1.60 ± 0.80	2.88 ± 1.91	1.25 ± 0.54	1.18 ± 0.52	0.45 ± 0.30
2 days						
GLAST	1.05 ± 0.12	0.78 ± 0.30	0.91 ± 0.35	1.14 ± 0.26	0.79 ± 0.08	0.84 ± 0.07
GLT1	1.04 ± 0.29	0.95 ± 0.42	1.17 ± 0.38	1.05 ± 0.41	1.13 ± 0.39	0.68 ± 0.38
EAAT3	14.35 ± 23.89	28.63 ± 48.26	33.84 ± 54.04	3.81 ± 2.61	0.75 ± 0.95	1.09 ± 1.24
EAAT4	1.17 ± 0.82	1.11 ± 0.29	2.36 ± 1.87	1.30 ± 0.35	2.47 ± 1.20	4.61 ± 3.54
7 days						
GLAST	1.02 ± 0.06	0.92 ± 0.09	1.01 ± 0.22	1.17 ± 1.27	2.84 ± 2.87	1.51 ± 1.34
GLT1	1.22 ± 0.38	1.00 ± 0.33	0.84 ± 0.13	0.70 ± 0.34 ^b	7.87 ± 6.36 ^b	2.05 ± 2.22
EAAT3	1.02 ± 0.49	16.42 ± 27.31	12.51 ± 20.41	0.36 ± 0.56	18.46 ± 15.56	1.49 ± 0.74
EAAT4	1.16 ± 0.37	1.14 ± 0.29	1.19 ± 0.30	1.08 ± 0.08	1.25 ± 0.28	1.26 ± 0.49

Note. Transporter expression values represent relative levels of triplicate samples compared to unirradiated control levels.

^aRepresents triplicate values ± SD.

^bRepresents statistically significant relative changes.