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Title: Rapid Phosphorylation of Histone H2A.X Following Ionotropic Glutamate Receptor Activation

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Abstract

Excessive activation of ionotropic glutamate receptors increases oxidative stress, contributing to the neuronal death observed following neurological insults such as ischemia and seizures. Post-translational histone modifications may be key mediators in the detection and repair of damage resulting from oxidative stress, including DNA damage, and may thus affect neuronal survival in the aftermath of insults characterized by excessive glutamate release. In non-neuronal cells, phosphorylation of histone variant H2A.X (termed γ -H2AX) occurs rapidly following DNA double-strand breaks. We investigated γ -H2AX formation in rat cortical neurons (day *in vitro* 14) following activation of NMDA or AMPA/Kainate glutamate receptors using fluorescent immunohistochemical techniques. Moreover, we evaluated the co-localization of γ -H2AX “foci” with Mre11, a double-strand break repair protein, to provide further evidence for the activation of this DNA damage response pathway. Here we show that minimally cytotoxic stimulation of ionotropic glutamate receptors is sufficient to evoke γ -H2AX in neurons and that NMDA-induced γ -H2AX foci formation was attenuated by pretreatment with the antioxidant, Vitamin E, and the intracellular calcium chelator, BAPTA-AM. Moreover, a subset of γ -H2AX foci co-localized with Mre11, indicating that

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Abbreviations:

(AMPA)	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
(DSB)	double-strand break
(LDH)	lactate dehydrogenase
(LSD)	Least Significant Difference
(NMDA)	N-methyl-D-aspartic acid
(γ -H2AX)	phosphorylated histone H2A.X

at least a portion of γ -H2AX foci is damage-dependent. The extent of γ -H2AX induction following glutamate receptor activation corresponded to the increases we observed following conventional DNA damaging agents (i.e., non-lethal doses of gamma-radiation (1 Gy) and hydrogen peroxide (10 μ M)). These data suggest that insults not necessarily resulting in neuronal death induce the DNA damage-evoked chromatin modification, γ -H2AX, and implicate a role for histone alterations in determining neuronal vulnerability following neurological insults.

Keywords

histone modification; rat; cortical neurons; gamma-H2AX; glutamate receptors

In addition to DNA repair protein regulation, post-translational histone modifications can mediate the cellular response to DNA damage (Hassa & Hottiger, 2005). Although the importance of functional DNA-repair pathways is underscored by the abnormal neuronal development and vulnerability to injury observed in DNA repair-deficient mice (Vemuri et al., 2001; Culmsee et al., 2001; Meira et al., 2001; Laposa & Cleaver, 2001) and humans (Rolig & McKinnon, 2000; Brooks, 2002), DNA damage-related histone alterations remain relatively unexamined in neurons.

It is well documented in non-neuronal cells that histone variant H2A.X is phosphorylated on C-terminal serine residues (termed γ -H2AX) in response to DNA double-strand breaks (DSBs) (Fernandez-Capetillo et al., 2004). γ -H2AX occurs near the lesion site within minutes of DSB induction, forming nuclear “foci”. Immunohistochemical detection of γ -H2AX foci is a sensitive and quantitative intracellular index of DSBs; it has been suggested that each and all nuclear DSBs can be detected within a cell such that one γ -H2AX foci corresponds to a single DSB (Rogakou et al., 1998; Rogakou et al., 1999; Sedelnikova et al., 2002; Rothkamm & Lobrich, 2003; Fernandez-Capetillo et al., 2004). This marker has thus become the “gold standard” for examining DNA lesions at numbers subthreshold for detection by other methods such as pulse-field gel electrophoresis and the neutral COMET assay.

DNA damage can arise from glutamatergic activity during which increases in intracellular calcium and zinc levels lead to mitochondrial dysfunction and, in turn, the production of reactive oxygen species (Krieger & Duchon, 2002). Reactive oxygen species cause oxidative DNA damage, which contributes to the neuronal death observed following ischemia (Chan, 2001), prolonged seizures (Patel, 2004), and in neurodegenerative diseases (Alzheimer’s, Parkinson’s etc.) (Facchinetti et al., 1998; Barnham et al., 2004). The major form of oxidative DNA damage, DNA single-strand breaks, can be induced by glutamate receptor activation resulting in no or minimal cell death (Didier et al., 1996; Pieper et al., 2000). Closely-located single-strand breaks or the single-strand break repair process (Blaisdell et al., 2001) can, in turn, result in DSBs. It is thus possible that DNA damage-related chromatin modifications, such as γ -H2AX, occur in neurons following non-lethal insults known to evoke oxidative damage.

The purpose of this study was to investigate γ -H2AX formation in neurons following activation of ionotropic glutamate receptors, NMDA and AMPA/Kainate receptors, resulting in minimal cytotoxicity. Moreover, to examine the extent to which γ -H2AX formation is associated with DNA damage response pathways, we investigated the co-localization of γ -H2AX with the DNA repair-related protein, Mre11 (Paull et al., 2000; Mirzoeva & Petrini, 2001; Fernandez-Capetillo et al., 2004). To provide a baseline for comparisons between ionotropic glutamate receptor activation and conventional DNA-damaging agents, we also evaluated γ -H2AX induction following exposure to gamma-radiation, an exogenous source for evoking DSBs, and the prooxidant hydrogen peroxide.

Methods

Neuronal cultures

Cortical neuronal cultures were derived from rat embryonic cortices as described previously (Movsesyan et al., 2004). Briefly, cortices from 17-18 days old Sprague-Dawley rat embryos were cleaned from their meninges and blood vessels in Krebs-Ringers bicarbonate buffer containing 0.3% bovine serum albumin (Gibco, Gaithersburg, MD). Cortices were then minced and dissociated in the same buffer with 1800 U/ml trypsin (Sigma, St. Louis, MO) at 37°C for 20 minutes. Following the addition of 200 U/ml DNase I (Sigma) and 3600 U/ml soybean trypsin inhibitor (Sigma) to the suspension, cells were triturated through a 5 ml pipette. After the tissue settled for 5-10 minutes, the supernatant was collected and the remaining tissue pellet was re triturated as before. The combined supernatants were then centrifuged through a 4% bovine serum albumin layer. The cell pellet was resuspended in neuronal seeding medium, which consisted of Neurobasal Medium (Gibco), supplemented with 1.1% 100X antibiotic-antimycotic solution (Biofluids Inc., Rockville, MD), 25 μ M Naglutamate, 0.5 mM L-glutamine, and 2% B27 Supplement (Gibco). Cells were seeded at a density of 5×10^5 cells/ml onto 96-well tissue culture plates (Corning, Corning, NY) or on 12 mm glass coverslips (Fisher Scientific, Pittsburgh, PA) in 4-well plates (Nunc, Roskilde, Denmark) pre-coated with poly-D-lysine (70-150 kD, Sigma). On day 4 *in vitro*, feeding media (neuronal seeding medium without Na-glutamate and B27 supplement) in 1:2 proportion was added to cultures. This cell culture technique allowed to obtain rat cortical neuronal cultures as shown previously (91 ± 5 % of cells were neuron specific enolase-positive (Yakovlev et al., 2001)) and confirmed in this study using staining with neuronal nuclear antigen antibody (data not shown). All AMPA and NMDA experiments were performed on cultures at 14 days *in vitro* as functional AMPA/kainate and NMDA receptors are fully expressed at this time. Moreover, we also chose to irradiate cortical cultures at 14 days *in vitro* because we wanted to examine γ -H2AX in the absence of injury-induced cell death. *In vitro* data suggest that younger neurons may be susceptible to irradiation-induced cell death (Gobbel et al., 1998; Gobbel & Chan, 2001; Romero et al., 2003).

Hydrogen peroxide treatment

Hydrogen peroxide (10 μ M or 100 μ M, Fisher Scientific, Fairlawn, NJ) was added directly to the bathing media, and the cells were incubated for 10 min at 37°C before fixation.

Ionotropic glutamate receptor agonist treatment

Cells were exposed to α -amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid (AMPA; 25 μ M or 50 μ M, Research Biochemicals, Inc., Natick, ME) or N-methyl-D-aspartic acid (NMDA; 15 μ M or 50 μ M, Tocris Cookson, Baldwin, MO) and incubated for varying times before fixation (see Results). For all AMPA experiments, the NMDA-receptor antagonist, MK801 (20 μ M, Sigma), was added to the cultures prior to AMPA treatment to block secondary NMDA receptor-mediated events. Control cultures received either no drugs or were treated with receptor antagonists (NBQX or MK801) alone. To ensure that our observed results (i.e., γ -H2AX formation in response to AMPA or NMDA treatment) were receptor dependent, a group of sister cultures were pretreated with AMPA/kainate or NMDA receptor antagonists, NBQX (20 μ M, Sigma) or MK801 (20 μ M), respectively, 30 min prior to NMDA or AMPA application. For the pulse exposure to NMDA, MK801 (20 μ M) was added to the cultures 10 min following NMDA application. Cells were continuously exposed to the corresponding drugs at 37°C and 5% CO₂ for the durations indicated in the Results. BAPTA-AM (Sigma) and Vitamin E (Sigma) were prepared in DMSO and diluted in culture medium (final concentration of DMSO in cultures was < 0.01%). BAPTA-AM (50 μ M) or Vitamin E (50 μ M) was added 2 hr and again at 30 min (20 μ M and 50 μ M, respectively) prior to NMDA or

AMPA application. BAPTA-AM, Vitamin E, and vehicle (DMSO) alone were added to the respective control cultures.

Cell viability

Because neurons can die via necrosis or programmed cell death pathways following glutamate receptor activation (Nicotera et al., 1999), lactate dehydrogenase (LDH) release (Sinensky et al., 1995) and Trypan blue exclusion were used as measures of generic cell death. LDH release was assessed using the CytoTox-96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI) according to manufacturer's protocol. Relative absorbance was measured at 490 nm using a Multiskan Ascent microplate reader (Labsystems Inc., Helsinki, Finland). Control LDH levels were measured in cultures that had not received drug or irradiation treatment. Data were expressed as % cytotoxicity calculated as the amount of LDH released in response to treatment divided by maximum LDH release (full cell lysis with 0.1% Triton X). Trypan blue uptake was assessed by incubating cultures with 0.04% Trypan blue solution (Gibco) in culture medium for 10 min at room temperature. Images of the cultures were immediately captured using a Nikon CoolPix digital camera attached to an inverted light microscope and analyzed using Adobe Photoshop software (Adobe Systems, San Jose, CA). Consistent with reports from other studies (Lobner, 2000), LDH data were comparable with those from the Trypan Blue assay. Signs of apoptotic nuclear morphology were also monitored by DAPI staining (see below).

Irradiation

Cultures were gamma-irradiated with 1 Gy or 5 Gy using a J. L. Shepard Mark I ^{137}Cs irradiator (San Fernando, CA). Cells were incubated at 37°C and 5% CO_2 following irradiation for varying times as indicated in the Results.

Immunohistochemistry

Cells grown on poly-lysine covered coverslips in 4-well plates were placed on ice, and the medium was replaced with ice-cold phosphate buffered saline, pH 7.4, (PBS). The coverslips were briefly rinsed with ice-cold methanol, and the cells were fixed in methanol for 20 min at -20°C. After fixation, the coverslips were washed again for 20 min with ice-cold PBS. Cells were incubated in blocking solution (0.05% Triton-X, 5% normal goat serum (Sigma) in PBS) for 6 h at 4°C. Rabbit polyclonal anti-phospho-H2AX antibody (1:500; Upstate Biotechnology Inc., Lake Placid, NY) and mouse, monoclonal neuronal nuclear antigen antibody (1:500; Chemicon International, Temecula, CA) or mouse monoclonal anti-phospho-H2AX (1:300; Upstate Biotechnology Inc.) and rabbit polyclonal anti-Mre11 (1:300; Novus Biologicals, Littleton, CO) were added to the cultures, and the cells were incubated overnight at 4°C. Primary antibodies were omitted as a negative control for each treatment group. Coverslips were washed with blocking solution (3×10 min) on ice and then incubated for 1.5 h at room temperature with the corresponding secondary antibody. Secondary antibodies were Alexa Fluor 488-goat anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR), Alexa Fluor 568-goat anti-mouse IgG (1:500; Molecular Probes), and for neuronal nuclear antigen staining, goat anti-mouse Texas Red (1:250; Jackson Immunoresearch Inc., West Grove, PA) diluted in blocking solution. Cells were then washed with blocking solution (3×20 min) followed by PBS (3×10 min) and distilled water (3×10 min). Coverslips were mounted using Vectashield hard set mounting media with DAPI (Vector Laboratories, Burlingame, CA).

Image acquisition and γ -H2AX analysis

Images were visualized and captured using a Olympus IX-70 Fluoview Laser Confocal Scanning Microscope with a 100 $\times$ oil immersion lens. The laser intensity and gain were optimized and kept constant among all experimental groups. Images were captured sequentially

for each fluorescent label to minimize bleed-through between the channels. Foci were analyzed using Scion Image software (NIH). The optical density threshold for foci detection was determined by visual inspection and kept constant for all groups. A minimum of 50 cells were counted per group (images were taken from randomly chosen fields from 2 coverslips of sister cultures) for each experimental treatment. Nuclei were identified with DAPI staining, and foci were counted in neurons (neuronal nuclear antigen-positive cells) in which the entire nucleus was visible and free of apoptotic features.

Neurons having apoptotic morphology were excluded from the analyses. Bright nuclei (intense, complete coverage of the nucleus with γ -H2AX staining) were also excluded from the analysis as it has been suggested that this type of staining represents dying cells (Rogakou et al., 2000; Huang et al., 2004; Nowak et al., 2006). Although this pattern of γ -H2AX staining did not always coincide with the appearance of apoptotic nuclear morphology, we excluded all bright nuclei because apoptotic morphology can appear at later time points after maximal γ -H2AX induction. We did not observe significant numbers of bright nuclei (or apoptotic nuclear morphology) after irradiation, yet we observed a dose-dependent increase in this staining pattern following both hydrogen peroxide and 50 μ M NMDA treatment, consistent with the reported dose-dependent increases in neuronal death following treatment with these chemicals and our LDH and Trypan blue results following NMDA-treatment (data not shown). Both the average number of foci per nucleus and foci density (the sum of the pixels per nucleus occupied by foci) were calculated. Due to the confound of overlapping/touching foci, we obtained a more linear dose-response curve by analyzing foci density as compared to foci number following all treatments. Thus, data are expressed as average foci density (% mean untreated-control $\pm$ SEM). Foci distribution graphs (see Results for representative graphs) were generated by analyzing a minimum of 50 cells per group from a single experiment. Nuclei were classified as having 0-5, 6-10, 11-15, 16-20, 21-25, 26-30, or > 30 foci per nucleus, and data were expressed as the percent of the total number of cells counted for each time point.

Co-localization of Mre11 and γ -H2AX

Images were captured as detailed above in the analysis of γ -H2AX foci. Because of overlapping γ -H2AX foci in some cells and the diffuse staining pattern obtained with the Mre11 antibody, co-localization was semi-quantitative. Cells were scored positive for co-localization if more than 3 distinct γ -H2AX foci per cell colocalized with clear and distinct Mre11 foci.

Statistical analysis

The Kruskal-Wallis (KW) test was used to determine statistical significance for average foci density for each treatment. Non parametric analyses of foci density were performed since the data were not normally distributed and had unequal variances. Post-hoc analysis was done using the Least Significant Difference Mean Rank (LSD) test when analysis using the Kruskal-Wallis test yielded significance among groups. To determine statistical significance for LDH release, a one-way analysis of variance (ANOVA) was used followed by Tukey post hoc analysis for multiple comparisons. Significance was set at $p < 0.05$ for all tests.

Results

Gamma-radiation induces γ -H2AX in cortical neurons

Gamma-radiation causes DSBs both directly, by ionization of the DNA, and indirectly via the generation of free radicals (Gudkov & Komarova, 2003). To evaluate radiation-induced γ -H2AX in cortical neurons, we first investigated the time course of γ -H2AX formation following doses of gamma-radiation (i.e., 1 and 5 Gy). In untreated-control neurons, $> 90\%$ of the cells had fewer than 5 foci, with 68% having 0 foci (Fig 1A and 1B). This observation for controls is consistent with previous reports in both neurons (Kim et al., 2004; Nowak et al., 2006) and

non-neuronal cells (Sedelnikova et al., 2002; Sedelnikova et al., 2004). Following 1 Gy of radiation, we observed a rapid increase in γ -H2AX foci of varying size and intensity in a majority of neurons as early as 10 min post irradiation (Fig 1A-1C). This increase reached maximal levels 30 min after irradiation, during which > 90% of the neurons had foci levels greater than those of un-treated, control cultures (> 5 foci per nucleus). Foci density decreased by 4 hr after irradiation (Fig 1B and 1C), consistent with the rapid and transient γ -H2AX induction described in non-neuronal cells (Sedelnikova et al., 2002; Rothkamm & Lobrich, 2003). Post hoc analysis revealed that there was no significant difference in foci density between 4 and 24 hr following irradiation (Fig 1C). By 24 hr, 42% of cells had returned to having between 0 and 5 foci, yet a population of cells still had elevated-foci levels as compared to non-irradiated controls (> 5 foci per nucleus) (Fig 1B).

Although most populations of mature neurons are radiation resistant *in vitro* (Romero et al., 2003) and *in vivo* (Peissner et al., 1999; Tada et al., 2000; Nowak et al., 2006), there are reports of radiation-induced cell death *in vitro* in young, non-cycling neurons (Gobbel et al., 1998; Gobbel & Chan, 2001) and *in vivo* in neuronal progenitor cells and in the proliferative zones of the hippocampus (i.e., subgranular zone) and cerebellar granule cells (Herzog et al., 1998; Mizumatsu et al., 2003; Nowak et al., 2006). To confirm that cell death was not a significant contributor to either the radiation-induced γ -H2AX foci or to the disappearance of foci observed 4 hr after irradiation, we examined the nuclear morphology of foci-positive cells using DAPI staining. Consistent with the radiation resistance of mature neurons, foci-positive cells did not have nuclear features characteristic of programmed cell death pathways when nuclear morphology was visualized using DAPI staining. This absence of radiation-induced cell death was confirmed by the analysis of LDH release as a measure of cell viability. Although > 90% of the neurons had elevated numbers of γ -H2AX foci as compared to un-treated control neurons (≥ 5 foci) at 30 min following irradiation, there was no increase in LDH release in irradiated cultures as compared to non-irradiated controls up to 24 hr post irradiation (% cytotoxicity (% mean untreated control) = $105.8 \pm 4.2\%$).

It has previously been documented that the number of radiation-induced DSBs is proportional to the gamma-radiation dose in non-neuronal cells as measured by both pulse-field gel electrophoresis and γ -H2AX induction (Rothkamm & Lobrich, 2003). We next evaluated whether the observed radiation-induced γ -H2AX is dose-dependent by irradiating neuronal cultures with 5 Gy of gamma-radiation. We observed a dose-dependent increase in γ -H2AX formation 30 min following irradiation (Fig 1D; $p < 0.001$; KW; post hoc LSD, $p < 0.05$ among all groups).

To determine if the γ -H2AX foci that we observed following irradiation were correlated with recruitment of the DSB-related detection and repair pathways, we next analyzed the co-localization of γ -H2AX foci with the repair factor, Mre11 after 1 Gy of gamma-radiation. Clear co-localization was observed in a majority (> 60%) of the γ -H2AX positive cells, both 10 min and 4 hr post irradiation. Not all foci co-localized; a subpopulation of smaller, less intense γ -H2AX foci did not co-localize with Mre11 in some nuclei (Fig 2). Taken together, these results indicate gamma radiation induces γ -H2AX formation in mature cortical neurons in a dose-dependent manner consistent with the cellular response to radiation observed in non-neuronal cells.

γ -H2AX increases following treatment with hydrogen peroxide

Although a portion of radiation-induced DSBs are caused by reactive oxygen species, it is believed that the majority of DSBs are caused by direct ionization of the DNA. To verify if the pattern and extent of radiation-induced γ -H2AX is similar to that caused by reactive oxygen species, we analyzed γ -H2AX formation following the application of the pro-oxidant hydrogen peroxide, which generates hydroxyl radicals within cells.

There was a dose-dependent increase in γ -H2AX formation at 10 min following hydrogen peroxide application ($p < 0.001$; KW; post hoc LSD, $p < 0.05$ among all groups: Foci Density (% mean untreated-controls) = $100 \pm 128.8\%$, $1296 \pm 304.0\%$, and $3059 \pm 522.4\%$ for 0, 10, and 100 μ M hydrogen peroxide, respectively). These results indicate that in cortical neurons, increases in reactive oxygen species can result in the phosphorylation of H2A.X.

Ionotropic glutamate receptor activation induces γ -H2AX formation

To evaluate the effects of ionotropic glutamate receptor activation on γ -H2AX formation, we applied the ionotropic glutamate receptor agonists, NMDA or AMPA, to neurons to stimulate NMDA or AMPA/Kainate receptors, respectively. Because the evaluation of γ -H2AX formation can be complicated by excitotoxicity and the activation of cell death processes, we evaluated the dose-response of γ -H2AX induction to NMDA or AMPA concentrations causing minimal to moderate neuronal injury. We assessed NMDA -or AMPA-induced injury by measuring LDH release into the bathing medium and Trypan blue exclusion at various time points post-drug application.

There was a dose-dependent increase in LDH release as compared with controls 24 h after both AMPA and NMDA application (Fig 3A and 3B). Pretreatment with the NMDA antagonist, MK801, blocked the NMDA-evoked LDH release (Fig 3A'). The AMPA receptor antagonist, NBQX, only partially blocked AMPA-induced injury (Fig 3B'). Pretreating cells with NBQX and MK801 was required to completely prevent AMPA-induced LDH release (Fig 3B'), and, thus, cultures were pretreated with MK801 in all subsequent AMPA experiments to prevent the activation of NMDA receptors by secondary glutamate release.

NMDA

Following treatment with NMDA, we observed a rapid induction of γ -H2AX foci of varying sizes and intensities (Fig 4A). Foci-positive cells had normal nuclei free of apoptotic morphology (Fig 4B (top) and 4C) with the exception of a subset of brightly-stained nuclei, which were excluded from analysis (Fig 4B, bottom panel). We observed a maximal increase in γ -H2AX formation 10 min following both NMDA concentrations (Fig 5A and 5B). A maximal effect was achieved by treatment with 15 μ M NMDA. In both conditions, γ -H2AX returned to control levels by 4 h after drug application (Fig 5A-D). Although minimal cell death was observed using LDH assay at 24 h following application of 15 μ M NMDA (Cytotoxicity = $34.5 \pm 1.8\%$), we observed increases in γ -H2AX in a majority of neurons ($> 70\%$ had a more than 5 foci) following treatment with 15 μ M NMDA as compared to untreated-control neurons (fewer than 20% had > 5 foci) (Fig 5C). Pretreatment with MK801 prevented the observed NMDA-induced increases in γ -H2AX (foci density (% mean control $\pm$ SEM) = 134 ± 42.7 and 88 ± 36.7 for 10 min and 4 h post 50 μ M NMDA treatment, respectively).

Pulse exposure to NMDA (Wash-out)

It is possible that the observed decrease in NMDA-evoked γ -H2AX over time may be influenced by those cells that die following moderately injurious NMDA concentrations, as bright cells have been excluded from our analyses (see Methods). To examine this possibility, we assessed γ -H2AX levels following a pulse exposure to NMDA, which further limits NMDA-induced cell death (Fig 6). Because maximal γ -H2AX levels were observed 10 min post NMDA application, we blocked NMDA-induced glutamate receptor activation 10 min after NMDA treatment by adding MK801 to the cultures. This pulse exposure to NMDA treatment prolonged the robust induction of γ -H2AX up to 1 hr and resulted in maximal NMDA-induced γ -H2AX (Fig 5A' and 5B'). Similar to the observation following prolonged exposure to NMDA, γ -H2AX levels returned to those of the control by 4 hr post drug application.

AMPA

To assess the effect of non-NMDA receptor activation on γ -H2AX formation, we applied the AMPA/Kainate receptor agonist, AMPA, to the cultures. We observed an increase in γ -H2AX at 30 min after AMPA treatment as compared to untreated-controls (Fig 7A and 7B). There was a maximal induction of γ -H2AX following 50 μ M of AMPA, occurring by 1 h post drug application ($p < 0.001$; KW, post hoc LSD; $p < 0.05$). The number of cells having greater than control levels of foci (0-5 foci) following 25 μ M or 50 μ M AMPA treatment was greater than 40% and 80%, respectively (Fig 7C and 7D). This increase in foci-positive cells was greater than the increase in cell death observed 24 hr following AMPA treatment (% Cytotoxicity = $29.1 \pm 0.4\%$ and $31.4 \pm 0.5\%$ for 25 μ M and 50 μ M, respectively). Consistent with the minimal LDH release observed following AMPA treatment, neurons having AMPA-evoked γ -H2AX foci did not have apoptotic nuclear morphology when viewed using DAPI staining.

After treatment with 25 μ M AMPA, γ -H2AX foci density remained elevated as compared to untreated controls at 4 h post drug application (Fig 7A). The average foci density also remained elevated at 4 h following application of 50 μ M AMPA as compared to control cells, yet > 70 % of the neurons had returned to having 0 to 5 foci by this time point (Fig 7D). Pretreatment with the AMPA/Kainate receptor antagonist, NBQX, prevented the AMPA-induced increases in γ -H2AX (foci density (% mean control $\pm$ SEM) = 98.9 ± 11.6 and 89.3 ± 13.1 for 50 μ M AMPA at 30 min and 4 h after treatment, respectively).

Intracellular calcium levels affect γ -H2AX formation following ionotropic glutamate receptor activation

Increases in the concentration of intracellular calcium contribute to mitochondrial dysfunction and increases in reactive oxygen species (Krieger & Duchen, 2002). We investigated whether the NMDA- or AMPA-induced increase in γ -H2AX was dependent on intracellular calcium levels. Pre-loading the cells with the membrane permeable calcium chelator, BAPTA-AM, attenuated the NMDA-mediated increases in γ -H2AX (Fig 8A). Although it appeared that BAPTA-AM also attenuated the AMPA-evoked γ -H2AX formation, the decrease did not reach threshold for significance (Fig 8B).

Vitamin E attenuates γ -H2AX formation following ionotropic glutamate receptor activation

If the observed increase in γ -H2AX formation was due to an increase in oxidative stress, attenuating the oxidative stress should inhibit γ -H2AX formation. To test this, we pretreated cells with the antioxidant, Vitamin E. Vitamin E attenuated NMDA-induced γ -H2AX formation (Fig 8A). The same trend was observed for AMPA-evoked γ -H2AX following pretreatment with Vitamin E, yet post hoc analysis revealed that Vitamin E did not significantly reduce- γ -H2AX formation following treatment with AMPA (Fig 8B).

Co-localization of Mre11 and γ -H2AX following ionotropic glutamate receptor activation

To determine whether the observed increases in γ -H2AX following AMPA or NMDA treatment corresponded to the activation of DSB detection and repair pathways, we next assessed co-localization of Mre11 with γ -H2AX foci. Both the large, intense γ -H2AX foci and the smaller, less intense foci co-localized with Mre11 at 10 min following NMDA application (Fig 9A) and 1 h after application of AMPA (Fig 9B). We observed colocalized foci in >50% of the γ -H2AX positive cells following NMDA or AMPA treatment. There was a population of small foci that did not appear to co-localize with γ -H2AX foci. Although the majority of large, discrete γ -H2AX foci co-localized with Mre11 foci, the reverse was not the case. We observed Mre11 foci that did not co-localize with γ -H2AX foci in the nucleus and, to a lesser extent, in the cytoplasm of a subset of both treated and un-treated control neurons.

Discussion

Our results demonstrate a rapid and transient induction of γ -H2AX, a DNA damage-related histone modification, following ionotropic glutamate receptor activation and in response to a direct DNA damaging agent (gamma-radiation) and hydrogen peroxide, which causes indirect DNA damage by reactive oxygen species generation (Deng et al., 1999; Liu & Martin, 2001; Martin & Liu, 2002). To our knowledge, our results provide the first evidence for γ -H2AX formation in neurons following *physiologically* relevant insults (i.e., glutamate receptor activation) not necessarily resulting in cell death.

We investigated γ -H2AX following insults causing minimal injury (i.e. mildly injurious concentrations of AMPA, pulse exposure to NMDA, and 1 Gy of radiation) to avoid the confounding influence of cell death processes on γ -H2AX formation. γ -H2AX can coincide with early stages of endonucleolytic DNA cleavage (DNA fragmentation) (Rogakou et al., 2000; Huang et al., 2004; Nowak et al., 2006), and the progression of programmed cell death pathways can result in a disappearance of γ -H2AX (Huang et al., 2004). Notably, more than 90% of irradiated neurons had ≥ 5 foci per nucleus, exceeding the fraction of cells that die following a dose of 1 Gy as evidenced by our LDH data and by previous studies of radiation-induced neuronal death in cortical cultures (Gobbel et al., 1998). We observed a similar phenomenon following mildly-injurious NMDA or AMPA treatments in which the number of cells positive for γ -H2AX exceeded the number of dying cells up to 24 hr after treatment. This indicates that cell death can not exclusively account for the radiation- or glutamate receptor-evoked γ -H2AX.

The γ -H2AX and Mre11 co-localization observed following treatment with radiation, NMDA, or AMPA, indicates that at least a portion of γ -H2AX foci is DNA-damage *dependent* and suggests that DSB repair pathways are mobilized following γ -H2AX formation. It has been proposed that the disappearance of γ -H2AX foci indicates successful DSB repair (Rothkamm & Lobrich, 2003; Riballo et al., 2004). Thus, it would be attractive to speculate that both the co-localization of γ -H2AX and Mre11 and the transient γ -H2AX induction following injury indicates successful DSB repair. There is evidence, however, that the kinetics of foci disappearance and DSB repair are disparate (Forand et al., 2004), precluding us from drawing conclusions regarding the successful completion of repair.

The failure of some γ -H2AX to co-localize with Mre11 is consistent with the rapid nature of the DSB detection/repair processes resulting in transient co-localization immediately following injury (Mirzoeva & Petrini, 2001). Moreover, because primary cortical cultures are comprised of heterogeneous neuronal populations, it is possible that co-localization occurs at different time points among each subpopulation. It should be noted that the detection of γ -H2AX and Mre11 co-localization is reportedly difficult due to some technical limitations in resolving Mre11 foci without removal of the nucleoplasmic pool of Mre11 by in situ cell fractionation procedures (Mirzoeva & Petrini, 2001).

Although γ -H2AX is considered to be a sensitive and quantitative measure of DSBs in non-neuronal cells, in post-mitotic neurons, which are characterized by high rates of gene transcription, γ -H2AX may have a novel function following injury. We can not exclude the possibility that the failure of some γ -H2AX foci to colocalize with Mre11 may indicate that a population of γ -H2AX foci are DSB-*independent*. Recently, a population of small γ -H2AX foci not co-localizing with DNA repair factors was reported in non-neuronal cell types, and it has been suggested that this foci population is DSB-independent (McManus & Hendzel, 2005). The number of γ -H2AX foci we observed following glutamate receptor activation was relatively low (comparable to that observed following 1 Gy of gamma-radiation), not reaching the minimal threshold for detection of other DSB induction and repair assays (i.e., COMET,

PFGE, end-joining assay). Therefore, we cannot independently verify whether the functional significance of injury-evoked γ -H2AX in neurons is comparable to that in non-neuronal cell types. Because a DSB-independent role for γ -H2AX induction has only been described in the mouse XY body (Fernandez-Capetillo et al., 2003), it is difficult to speculate what novel functions γ -H2AX may serve in the brain. It is reasonable to suggest that this histone modulation may alter chromatin structure and, in turn, gene regulation in response to neuronal injury.

Low levels of γ -H2AX foci in the untreated-control neurons indicate that this histone modification is not common in neurons under normal physiological conditions *in vitro*. Background γ -H2AX foci have been reported in other cell types (Balajee & Geard, 2004; Sedelnikova et al., 2004), and may be caused by the cell culture process itself. Alternatively, persistent γ -H2AX foci may represent chronic chromatin modifications or DSBs in numbers not reaching the level necessary to trigger their detection/repair. It is attractive to speculate that because neurons do not divide, they may have an inherently-higher tolerance for DNA damage than proliferating cells given that the cellular response to such damage is linked to defined checkpoints in the cell cycle (Kruman, 2004). This may explain the radiation resistance observed in mature neuronal populations (Peissner et al., 1999; Tada et al., 2000; Romero et al., 2003).

The significance of persistent γ -H2AX is unknown. Increases in persistent γ -H2AX foci have been reported during aging of both non-neuronal cell types *in vitro* and in the brain *in vivo* (Sedelnikova et al., 2004). Accumulated damage could arise from deficient repair as there is evidence suggesting that DSB repair-efficiency decreases in the brain during normal aging (Ren & de Ortiz, 2002) and in age-related pathologies (Davydov et al., 2003). Because it has been suggested that neurons attempt to re-enter the cell cycle before dying (Park et al., 2000; Kruman, 2004) or during pathological conditions such as Alzheimer's Disease (Raina et al., 2004), accumulated DNA damage or persistent injury-evoked chromatin alterations may be detected upon re-activation of the cell cycle machinery, triggering neurodegeneration (Nospikel & Hanawalt, 2003). Moreover, persistent damage or chromatin alterations could change the transcription of genes necessary for neuronal function. It is therefore possible that persistent γ -H2AX may affect long-term neuronal functioning and/or survival.

Although we observed a dose-dependent increase in γ -H2AX following most of the treatment regimens (AMPA, IR, and hydrogen peroxide), maximal γ -H2AX induction was reached after prolonged exposure to 15 μ M NMDA. Pharmacological wash-out of NMDA with MK801 (pulse application), however, resulted in a dose-dependent increase in γ -H2AX similar to that observed following AMPA treatment, suggesting that the more severe extent of damage resulting from prolonged exposure to 50 μ M NMDA masked the detection of the γ -H2AX response. Our exclusion of brightly-stained nuclei from analyses (as they are indicative of cell death (Rogakou et al., 2000; Huang et al., 2004; Nowak et al., 2006), may have omitted the most severely damaged cells. Additionally, overlapping foci observed in cells having many foci made it difficult to precisely quantify γ -H2AX, resulting in a threshold for foci detection that may be surpassed with increasingly-injurious drug concentrations.

Increases in intracellular calcium and subsequent reactive oxygen species production are known to contribute to the neurodegeneration following excitotoxic insults (Krieger & Duchen, 2002). This prompted us to investigate whether blocking calcium and/or reactive oxygen species increases would prevent the γ -H2AX formation observed following ionotropic glutamate receptor activation. The increases in γ -H2AX following NMDA treatment were attenuated by pretreatment with the antioxidant, Vitamin E, and by the intracellular calcium chelator, BAPTA-AM, supporting the role of calcium and/or reactive oxygen species production as triggers for γ -H2AX. A similar pattern of attenuation was observed for AMPA-

evoked γ -H2AX following pretreatment with Vitamin E or BAPTA-AM, yet the attenuation of γ -H2AX did not reach the threshold for statistical significance using non-parametric analysis.

It should be noted that none of the compounds *completely* protected neurons from the effects of glutamate receptor agonists on γ -H2AX formation. It is possible that both Vitamin E and BAPTA-AM did not fully penetrate the cell membrane because of their lipophilic nature, resulting in a partial reduction of intracellular reactive oxygen species and calcium, respectively. Also, additional mediators of oxidative stress, such as zinc influx through calcium permeable NMDA or AMPA receptors (Sensi & Jeng, 2004), or oxidative-damage-independent mechanisms may contribute to γ -H2AX induction. This may be particularly true following AMPA treatment. Activation of calcium permeable AMPA/kainate receptors can lead to reactive oxygen species production (Carriedo et al., 1998; Carriedo et al., 2000). These receptors desensitize rapidly, however, and the extent of AMPA-induced reactive oxygen species production in the absence of receptor desensitization inhibitors is unclear. Although AMPA-evoked γ -H2AX was receptor dependent, it is possible that γ -H2AX may result from secondary glutamate release acting on metabotropic glutamate receptors or non-receptor mediated mechanisms. This may explain the delay in maximal γ -H2AX induction following AMPA treatment as compared to NMDA treatment.

Our results provide the first clear evidence that stimulation of ionotropic glutamate receptors in mature neurons results in rapid phosphorylation of H2A.X. It is attractive to speculate that because γ -H2AX is a sensitive and specific marker for DSBs in non-neuronal cells, our data suggest that minimally injurious glutamate receptor activation can induce DSBs in neurons. Furthermore, given that deficiencies in this cellular response to chromatin damage cause genomic instability and can suppress necessary repair mechanisms (Bassing & Alt, 2004; van Attikum & Gasser, 2005), our data suggest that γ -H2AX may contribute to neuronal vulnerability following neurological conditions characterized by excessive glutamatergic activity and/or increases in oxidative stress.

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Figure 1.

Increased γ -H2AX formation in cortical neurons following ionizing radiation. **A-C**, Time course for γ -H2AX formation following 1 Gy of radiation. **A**, Representative confocal images of γ -H2AX immunostaining in untreated-control neurons (left panel) and 10 min (middle) and 4 hr (right) after 1 Gy of radiation. White dots of varying sizes are γ -H2AX "foci". Cells were identified with neuronal nuclear antigen staining (not shown) and nuclei were visualized with DAPI and outlined with dashed lines on γ -H2AX images. Nuclei having an apoptotic morphology were excluded from analysis. Scale bar, 10 μ m. **B**, Distributions of the percent of neurons having 0-5, 6-10, 11-15, 16-20, 21-25, 26-30, and > 30 foci (expressed as a percentage of the total number of DAPI stained cells counted for each group). **C**, γ -H2AX increased transiently following 1 Gy of radiation. Average foci density was calculated as a percent of the mean value for untreated-control neurons (N = 100-110 neurons per group). **D**, Increases in γ -H2AX foci density following irradiation were dosedependent (% mean untreated controls $\pm$ SEM; N = 80, 60, and 55 for 0, 1, and 5 Gy, respectively). Neurons were irradiated and incubated for 30 min before measuring γ -H2AX foci density. * $p < 0.05$ as compared to untreated controls; ■ $p < 0.05$ as compared among time points or treatments; Kruskal-Wallis followed by post hoc LSD rank.

Figure 2. γ -H2AX and Mre11 co-localization 1 hr following 1 Gy of ionizing radiation. Confocal images of the representative co-localization of γ -H2AX foci (red; left panel) and Mre11 foci (green; middle panel) following irradiation. Merging the images revealed that the majority of discrete, radiation-induced γ -H2AX foci co-localized with Mre11 foci (white arrows; yellow foci; right panel). Scale bar, 10 μ m

Figure 3.

LDH release measurements of neuronal death following ionotropic glutamate receptor activation. NMDA (A) and AMPA (B) caused a dose-dependent increase in LDH release 24 hr after treatment. LDH results are expressed as % cytotoxicity (AMPA- or NMDA-evoked LDH release divided by LDH release following total cell lysis with 0.1% Triton-X). A', Pretreatment with 20 μM MK801 blocked the NMDA-induced LDH release following treatment with 50 μM NMDA. B', Pretreatment with 20 μM NBQX attenuated the AMPA-induced LDH release following treatment with 250 μM of AMPA, yet it did not completely block the AMPA-induced injury. Co-application of NBQX (20 μM) and MK801 (20 μM) prior to AMPA exposure completely blocked AMPA-induced injury. (N = 6 for all groups, *p < 0.05)

and $p^{**} < 0.01$ as compared to controls; ■ $p < 0.05$ and ■ ■ $p < 0.01$ as compared among treatments; ANOVA followed by post hoc Tukey).

Figure 4.

γ -H2AX foci patterns following NMDA receptor activation. *A*, Representative patterns shown are of neurons treated with 50 μ M NMDA. Right-most picture showing bright γ -H2AX immunostaining covering the entire nuclear region represents neurons that were excluded from the analyses (see Methods). 25 μ M and 50 μ M AMPA and 15 μ M NMDA treatment produced similar foci patterns. *B and C*, DAPI (left column) and γ -H2AX (right column) staining revealed that foci-positive cells had normal nuclear morphology. *B*, Foci-positive cells did not have apoptotic nuclear morphology (top) with the exception of a population of nuclei that were completely filled with bright γ -H2AX foci (bottom, white arrow). Although not all bright nuclei appeared apoptotic, all cells with this staining pattern were excluded from the analysis. *C*, By 4 hr following 15 μ M NMDA treatment, the number of γ -H2AX foci returned to that of untreated control neurons in a majority of neurons. Both foci-positive cells (white arrow) and those neurons having control foci levels (< 5 foci) had normal nuclei free of apoptotic features. Scale bar, 10 μ m

Figure 5.

Effects of NMDA on γ -H2AX formation in cortical neurons. *A* and *B*, Average density of γ -H2AX foci following treatment with 15 μ M (*A*) or 50 μ M (*B*) NMDA (% mean untreated controls $\pm$ SEM; $N = 51$ -68 for 15 μ M; $N = 50$ -58 for 50 μ M). *A'* and *B'*, the average foci density per nucleus following a pulse application of 15 μ M (*A'*) or 50 μ M (*B'*) of NMDA ($N = 50$ for all groups). MK801 (20 μ M) was added 10 min after NMDA application. *C* and *D*, Distributions of the percent of neurons having 0-5, 6-10, 11-15, 16-20, 21-25, 26-30, and > 30 foci following application of 15 μ M (*C*) or 50 μ M (*D*) NMDA (expressed as a percentage of the total number of DAPI stained cells counted for each group). * $p < 0.05$ as compared to untreated controls; ■ $p < 0.05$ as compared among treatments; Kruskal-Wallis followed by post hoc LSD rank

Figure 6.

LDH release measurements of neuronal death following continuous or a pulse exposure to NMDA. LDH release was measured 24 hr after applying NMDA and is expressed as % cytotoxicity (NMDA-evoked LDH release divided by total LDH release following cell lysis with 0.1% Triton-X). For the pulse exposure to NMDA (patterned bars), MK801 (20 μM) was added to the bathing media 10 min after NMDA application. (N = 6 for all groups, * p < 0.05 and ** p < 0.01 as compared to controls; ■ p < 0.05 and ■■ p < 0.01 as compared among treatments; ANOVA followed by post hoc Tukey)

Figure 7.

AMPA-induced γ -H2AX formation in cortical neurons. *A and B*, Average density of γ -H2AX following treatment with 25 μ M (*A*) or 50 μ M (*B*) AMPA (% mean untreated controls $\pm$ SEM; $N = 50$ -52 for 25 μ M; $N = 59$ -67 for 50 μ M). *C and D*, Distributions of the percent of neurons having 0-5, 6-10, 11-15, 16-20, 21-25, 26-30, and > 30 foci following application of 25 μ M (*C*) or 50 μ M (*D*) AMPA (expressed as a percentage of the total number of DAPI stained cells counted for each group). Cultures were pretreated with MK801 (20 μ M) 30 min prior to AMPA exposure to block the effects of secondary glutamate release on NMDA receptors. * $p < 0.05$ as compared to untreated controls; ■ $p < 0.05$ as compared among treatments; Kruskal-Wallis followed by post hoc LSD rank

Figure 8.

Pretreatment with BAPTA-AM or vitamin E attenuates NMDA-evoked γ -H2AX foci. Average γ -H2AX foci density per nucleus following NMDA (A) or AMPA (B) treatment (% mean vehicle (DMSO)-treated controls $\pm$ SEM; N = 71 for controls; N = 60-72 for AMPA-treated groups; N = 85-105 for NMDA-treated groups). DMSO, Vit E (vitamin E), or BAPTA-AM were added prior to adding glutamate receptor agonists (see Methods), and γ -H2AX was measured at 10 min or 1 hr after NMDA or AMPA treatment, respectively. For all AMPA experiments, cultures were pretreated with MK801 (20 μ M) to block the effects of secondary glutamate release on NMDA receptors. * p < 0.05 as compared to untreated controls; ■ p < 0.05 as compared among treatments; Kruskal-Wallis followed by post hoc LSD rank

Figure 9.

Co-localization of γ -H2AX and Mre11 foci following ionotropic glutamate receptor activation. *A and B*, Immunoreactivity for γ -H2AX (red, left panels) and Mre11 (green, middle panels) 10 min following 15 μ M NMDA treatment (*A*) or 1 hr post 25 μ M AMPA application (*B*). Merged images (right panels) show colocalization of γ -H2AX and Mre11 foci (yellow foci). Mre11 staining could be observed throughout the nucleoplasm with the exception of the nucleolus and, to a lesser extent, was observed in the cytoplasm of some cells. The majority of large, discrete γ -H2AX foci co-localized with Mre11. There was a population of smaller, less intense γ -H2AX foci that did not co-localize with Mre11 at the time points investigated. There was a population of nuclear Mre11 foci that did not co-localize with γ -H2AX. Scale bar, 10 μ m.