

Innate Gender-based Proclivity in Response to Cytotoxicity and Programmed Cell Death Pathway*

Received for publication, May 17, 2004, and in revised form, June 30, 2004
Published, JBC Papers in Press, July 1, 2004, DOI 10.1074/jbc.M405461200

Lina Du‡, Hülya Bayir‡, Yichen Lai‡, Xiaopeng Zhang‡, Patrick M. Kochanek‡§¶, Simon C. Watkins||, Steven H. Graham**, and Robert S. B. Clark‡§¶¶

From the Departments of ‡Critical Care Medicine, §Pediatrics, ||Cell Biology and Physiology, and **Neurology, Safar Center for Resuscitation Research, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15260 and ¶Children's Hospital of Pittsburgh, Pittsburgh, Pennsylvania 15213

Many central nervous system (CNS) diseases display sexual dimorphism. Exposure to circulating sex steroids is felt to be a chief contributor to this phenomenon; however, CNS diseases of childhood and the elderly also demonstrate gender predominance and/or a sexually dimorphic response to therapies. Here we show that XY and XX neurons cultured separately are differentially susceptible to various cytotoxic agents and treatments. XY neurons were more sensitive to nitrosative stress and excitotoxicity versus XX neurons. In contrast, XX neurons were more sensitive to etoposide- and staurosporine-induced apoptosis versus XY neurons. The responses to specific therapies were also sexually dimorphic. Moreover, gender proclivity in programmed cell death pathway was observed. After cytotoxic challenge, programmed cell death proceeded predominately via an apoptosis-inducing factor-dependent pathway in XY neurons versus a cytochrome *c*-dependent pathway in XX neurons. This gender-dependent susceptibility is related to the incapacity of XY neurons to maintain intracellular levels of reduced glutathione. *In vivo* studies further demonstrated an incapacity for male, but not female, 17-day-old rats to maintain reduced glutathione levels within cerebral cortex acutely after an 8-min asphyxial cardiac arrest. This gender difference in sensitivity to cytotoxic agents may be generalized to non-neuronal cells, as splenocytes from male and female 16–18-day-old rats show similar gender-dependent responses to nitrosative stress and staurosporine-induced apoptosis. These data support gender stratification in the evaluation of mechanisms and treatment of CNS disease, particularly those where glutathione may play a role in detoxification, such as Parkinson's disease, traumatic brain injury, and conditions producing cerebral ischemia, and may apply to non-CNS diseases as well.

Sexual dimorphism exists in the normal developing mammalian brain, particularly in sex steroid-concentrating regions such as the hypothalamus (1). However, there is also evidence of innate gender differences at the cellular level, dependent

upon the brain region, neuronal subtype, and developmental stage. For instance, phenotypic differences in XY and XX brain cells appear to be independent of gonadal phenotype (2), and cultured dopaminergic neurons from female rat fetuses are morphologically distinct and have increased capacity for dopamine uptake versus neurons from males (3). Cell death in certain brain regions can be different in both magnitude and duration between male and female rats, supporting the notion that divergent pathways of cell death occur between genders (4). Innate sexual dimorphism independent of circulating sex steroids would have significant clinical implications in central nervous system (CNS)¹ diseases with regard to both mechanisms and treatments.

Many CNS diseases also display sexual dimorphism, specifically a predilection for one gender (5, 6) or a gender-dependent response to treatment (7, 8). Exposure to circulating sex steroids is felt to be a chief contributor to this phenomenon; however, sexual dimorphism is also exhibited in many CNS diseases afflicting children and the elderly. Studies in children show that girls have more favorable neurologic outcome after traumatic brain injury (9) and respond more favorably to treatment for medulloblastoma (10) compared with boys but that girls demonstrate worse outcome after stroke (11). Elderly men have a higher incidence of Parkinson's disease and do not respond as well to surgical intervention compared with elderly females (6, 7). Elderly men with Alzheimer's disease have an earlier onset and increased mortality compared with elderly females (12, 13). In a study examining patients after cardiac arrest where the mean age was 67 years, male gender, but not age, was associated with poor outcome (14).

To determine whether innate gender differences independent of circulating sex steroids could contribute to gender-related differences in CNS disease prevalence and outcome, we developed a model system where male and female embryologic day (ED) 17 rats were segregated, and XY and XX neurons were cultured separately. The response to various cytotoxic agents and treatments and proclivity in programmed cell death pathway was then evaluated in this model system.

EXPERIMENTAL PROCEDURES

Reagents and Materials—Neurobasal medium, RPMI 1640, and B27 supplement were from Invitrogen. Peroxynitrite (ONOO⁻) was from

* This work was supported by NINDS, National Institutes of Health Grants RO1 NS38620 and P50 NS30318, NICHD, National Institutes of Health Grant T32 HD40686, and by the Children's Hospital of Pittsburgh. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Safar Center for Resuscitation Research, 3434 Fifth Ave., Pittsburgh, PA 15260. Tel.: 412-383-1900; Fax: 412-624-0943; E-mail: clarkrs@ccm.upmc.edu.

¹ The abbreviations used are: CNS, central nervous system; ED, embryologic day; INH2BP, 5-iodo-6-amino-1,2-benzopyrone; z-VAD, *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone; RT, reverse transcription; AIF, apoptosis-inducing AIF factor; ELISA, immunosorbent assay; SH, sulfhydryl; MTT, 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate; AMPA, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid; NAC, *N*-acetylcysteine; DIV, days *in vitro*; PND, post-natal day; PI, propidium iodide.

Cayman Chemical, Ann Arbor, MI. The poly-ADP-ribose polymerase inhibitor 5-iodo-6-amino-1,2-benzopyrone (INH2BP) was a kind gift from Dr. Csaba Szabó, Inotek Pharmaceuticals, Inc., Beverly, MA. The pancaspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (z-VAD) was from Enzyme Systems Products, Livermore, CA. The reverse transcription (RT)-PCR primers for rat *SRY3* and actin were synthesized and purchased from Invitrogen. The antibody against apoptosis-inducing factor (AIF) was from Santa Cruz Biotechnology, Santa Cruz, CA. The anti-caspase-3 antibody that recognizes both the intact zymogen and the cleaved p17 fragment was from Cell Signaling, Beverly, MA. The anti-cytochrome *c* oxidase and anti-histone III antibodies were from BD Pharmingen. The cytochrome *c* enzyme-linked immunoabsorbant assay (ELISA) was from R&D Systems, Minneapolis, MN. ThioGlo-1 reagent for measurement of reduced glutathione (GSH) and protein sulfhydryls (SHs) were from Convalent Associates, Inc., Woburn, MA. The 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT) assay, hydrogen peroxide (H₂O₂), L-glutamate, *N*-methyl-D-aspartate (NMDA), kainate, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), staurosporine, etoposide, 17 β -estradiol, MK801, catalase, superoxide dismutase, *N*-acetylcysteine (NAC), and remaining reagents were from Sigma, unless otherwise noted.

Gender-segregated Neuronal Cultures, Cytotoxicity, and Pharmacological Studies—Male and female Sprague-Dawley rat fetuses (ED 16–17) were separated by visual inspection, and neurons were harvested and cultured separately using standard methods (15, 16). Male and female fetuses can be distinguished by visualization of the dorsal penile vessels and sex cords (Fig. 1a) (3). Dissociated cell suspensions from cerebral cortices were placed in 96-well plates (5×10^4 cells/well) or in plastic dishes coated with poly-D-lysine (1.3×10^7 cells/well). Cortical neuron-enriched cultures were achieved by using neurobasal medium with serum- and estrogen-free B27 supplement (17). Neuron survival was optimized by replacing glutamine with GlutaMax. Cells were incubated at 37 °C in a humidified chamber containing 5% CO₂. On the second and sixth days *in vitro* (DIV) the culture media was replaced with fresh media. At 10 DIV, cultures consisted primarily of neurons with minimal glial cells detectable (>95% microtubule associated protein-2-immunoreactive cells, <5% glial fibrillary acidic protein immunoreactive cells analyzed in 8–10 fields/well, collected from 6 independent experiments). The majority of experiments were performed on the 10th DIV, with the exception of the excitotoxicity studies, which were performed on days 8–14. Primary hippocampal neuron cultures were prepared in similar fashion, with the exceptions that dissociated cell suspensions were prepared from hippocampi and glia were removed by treating cultures on DIV 2 with 5 μ M cytosine arabinoside for 72 h.

Nitrosative/Oxidative Stress—Neuron-enriched cultures were exposed to varying concentrations of ONOO⁻ or H₂O₂ in phosphate-buffered saline for 10 min, whereupon fresh media was replaced. For excitotoxicity, neuron-enriched cultures were exposed to varying concentrations of L-glutamate with 5 μ M glycine for 30 min, NMDA for 20 h or Kainate or AMPA for 30 min in culture media. Glycine was not added in the NMDA, AMPA, or kainate experiments. For apoptosis, neuron-enriched cultures were exposed to varying concentrations of staurosporine or etoposide in culture media for 4 h.

For pharmacological studies cultures were pretreated with either 17 β -estradiol (10–60 nM for 24 h on DIV 7, 10, and 14), the poly-ADP-ribose polymerase inhibitor INH2BP (100 μ M), the NMDA antagonist MK801 (1–5 μ M), the antioxidant catalase (5000 units), or the pancaspase inhibitor z-VAD (100 μ M) dissolved in 20% dimethyl sulfoxide (Me₂SO) or the antioxidants superoxide dismutase (300 units) or NAC (5 mM) dissolved in distilled H₂O. High concentration stock solutions were diluted in culture media. Although poly-ADP-ribose polymerase inhibitors are notoriously nonspecific, INH2BP at the concentration used here significantly inhibited poly-ADP-ribosylation in neurons (15) and was considerably more potent and selective than 3-aminobenzamide (18).

Gender-segregated, Isolated Splenocytes—Spleens were isolated from male and female post-natal day (PND) 16–18 Sprague-Dawley rats. Splenocytes were obtained by squeezing the organ in culture medium (RPMI 1640 containing 10% fetal bovine serum, 1% glutamine, 100 units of penicillin, and 100 mg/ml streptomycin). After centrifugation at $500 \times g$ for 10 min at 4 °C, erythrocytes were removed using red blood cell lysing buffer (Sigma). Splenocytes were again centrifuged, and the pellet was washed twice with culture medium. Isolated gender-segregated splenocytes were resuspended in culture medium and cultured in 5% CO₂ at 37 °C for 24–48 h at a concentration of 1×10^6 cells/ml in 96-well plates. For cytotoxicity assays varying concentrations of ONOO⁻ or staurosporine were added to culture media, and the MTT assay was performed at 24 and 48 h.

RT-PCR for *SRY3*—RT-PCR was performed using genomic DNA harvested from neurons in culture or brain tissue from male and female rats using standard methods (19). Total RNA was extracted using TRIzol reagent (Invitrogen), and the concentration was determined using a spectrophotometer. One μ g of total RNA from each sample was reverse transcribed with 200 units of Superscript II reverse transcriptase (Invitrogen) for 50 min at 42 °C primed with 0.5 μ g of random hexamer. RT products were then digested with RNase H at 37 °C for 30 min. cDNA samples (1 μ l) were subjected to PCR amplification using primers specific for rat *SRY* gene (*forward*, 5'-GCTCAACAGAATCCAGCAT-3'; *reverse*, 5'-TTTGTGAGGCAACTTCACG-3') or primers for rat actin with a Robocycler Gradient 96 PCR system (Stratagene, La Jolla, CA) using a Plantinum Taq PCRx kit (Invitrogen). Reagents were assembled in a final volume of 50 μ l as follows: 1 μ l of first-strand cDNA, 1 μ M each primer, 1 \times PCR amplification buffer, 2.5 mM MgSO₄, 1 \times PCR digoxigenin-labeling mix (Roche Applied Science), and RNase free water to volume. Samples were initially denatured at 94 °C for 3 min, and 2 units of Taq DNA polymerase were then added. Thermocycling parameters were 1 min at 94 °C, 1 min of annealing at 55 °C and 1 min of extension at 72 °C repeated for 30 cycles with a final extension step at 72 °C for 10 min. PCR products were then separated on a 1.2% agarose gel and transferred onto nylon membranes overnight in 20 \times SSC (1 \times SSC = 0.15 M NaCl and 0.015 M sodium citrate). After air drying and UV cross-linking, the membrane was blocked at room temperature for 1 h in 3% blocking reagent in 1 \times maleic acid buffer (Roche Applied Science) and incubated for 30 min at room temperature with alkaline phosphate-conjugated anti-digoxigenin at a concentration of 1:10,000 in 1% blocking reagent. A CDP-Star chemiluminescence reagent (PerkinElmer Life Sciences) was applied before exposing the membrane to x-ray film.

Assessment of Cell Viability—Cell viability was assessed using the MTT assay, lactate dehydrogenase release, and/or flow cytometry. Duplicate 100- μ l supernatant samples from 96-well plates were used for the MTT assay (15, 16). Values are expressed as the percentage of control (untreated) neurons and represent 5–8 separate experiments. In some paradigms, lactate dehydrogenase release was measured in duplicate 20- μ l supernatant samples.

Flow cytometry labeling of propidium iodide (PI) uptake and annexin V binding was also used to evaluate cell viability after ONOO⁻, glutamate/glycine, and staurosporine cytotoxicity (15). Neurons were harvested using trypsin-EDTA, washed once in ice-cold phosphate-buffered saline, and resuspended in 1 ml of annexin V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). 1×10^5 cells were stained with 5 μ l of annexin V-fluorescein isothiocyanate and 5 μ g/ml PI in binding buffer at 4 °C. After 20 min, 400 μ l of binding buffer was added to each tube, and samples were analyzed using a FACSCalibur flow cytometer. Unstained cells were used to determine background fluorescence, and thresholds for normal (unlabeled) cells were determined. The percentage of cells in each quadrant was calculated. For statistical comparison, the percentage of normal cells defined as PI and annexin V for each cytotoxicity paradigm were analyzed.

Subcellular Fractionation and Western Blot Analysis—Cellular proteins were separated into mitochondrial, nuclear, and cytosolic fractions (15, 16). Neurons at 0, 2, and 24 h after cytotoxic treatment were lysed in buffer. Samples were centrifuged at $600 \times g$ for 15 min at 4 °C. The supernatants were centrifuged at $17,200 \times g$ for 20 min at 4 °C. These supernatants were used for assessment of cytosolic proteins. The pellets containing mitochondria were lysed in buffer and sonicated. The initial pellet was re-suspended in lysis buffer then centrifuged at $16,000 \times g$ for 25 min at 4 °C with the supernatant used for assessment of nuclear proteins. Protein concentration was determined using bicinchoninic acid (Pierce).

Protein samples (50 μ g) were then boiled in loading buffer for 5 min followed by chilling on ice, then electrophoretically separated on SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Bio-Rad). After blocking overnight in 5% nonfat milk in phosphate-buffered saline, the membranes were incubated in 1:100 dilutions of antibodies against AIF or caspase-3 at room temperature for 1 h, washed, then incubated in appropriate secondary antibody. Protein bands were visualized using a chemiluminescence detection system (PerkinElmer Life Sciences) and exposed to x-ray film. The relative optical density of detected peptides was semi-quantified using an Eastman Kodak Co. imaging system. To determine the purity of individual cellular compartments, immunoblotting for cytochrome *c* oxidase and histone III was performed *pre hoc*.

Cytochrome *c* Enzyme-linked ELISA—Cellular proteins were separated into mitochondrial and cytosolic fractions (15, 16). Cytochrome *c*

concentrations were determined by ELISA within 96-well plates using triplicate samples as per manufacturer's instruction.

DNA Analysis—Chromosomal DNA samples were prepared in agarose plugs using a CHEF Mammalian Genomic DNA Plug Kit (Bio-Rad). Samples were homogenized in cell suspension buffer, mixed with preheated (50 °C) 2% low melting point agarose, and transferred into agarose plug molds. After solidification at room temperature the plugs were incubated with 1 mg/ml proteinase K overnight at 50 °C without agitation. Deproteinized DNA-containing agarose plugs were washed in wash buffer, added to wells, and sealed with low melting point agarose. Pulsed field gel electrophoresis was performed as described (16) using a CHE DR II pulsed field gel electrophoresis system (Bio-Rad). Fragments were separated on a 1.2% agarose gel at 14 °C for 20 h. Field strength was set at 6 V/cm, and initial switching time was set at 60 s for 15 h followed by final switching time 90 s for 9 h. The gel was stained with ethidium bromide and visualized under UV light. DNA laddering was performed using standard methods.

Fluorescence Assay of GSH and Protein-SH—The concentrations of GSH and protein-SH were determined using ThioGlo-1, a maleimide reagent that produces a highly fluorescent product upon its reaction with sulfhydryl groups (20), modified for use in culture and brain tissue. A standard curve was established using 0.04–4 μM GSH in 50 mM Na₂PO₄ buffer, pH 7.4, containing 10 μM Thio-Glo-1. GSH content was estimated by an immediate fluorescence response observed upon the addition of ThioGlo-1 to each sample. Levels of total protein-SH were determined as an additional fluorescence response after adding of 4 mM SDS to the same sample. A Cytofluor 2350 fluorescence plate reader (Millipore Corp., Marlborough, MA) was used to detect fluorescence at excitation and emission wavelengths of 388 and 500 nm, respectively.

Asphyxial Cardiopulmonary Arrest in Post-natal Day 17 Rats—Cortical brain tissue samples were obtained from PND 17 rats after 8-min of asphyxial cardiac arrest. This model produces hypoxic-ischemic injury at a time when circulating levels of reproductive hormones are similar between genders (21). At 6 or 24 h after asphyxia or in sham controls, rats were perfused with ice-cold saline, and cerebral cortices were dissected and frozen. Gender was verified at necropsy. The concentrations of GSH and protein-SH were determined as above. For analysis, 6- and 24-h samples were combined for each gender.

Data Analysis—Data are presented as the mean ± S.D. Comparisons between genders, treatment groups, and time points were made using two-factor analysis of variance with Tukey's *post hoc* tests unless otherwise specified. If data failed tests of normality and/or equal variance, data were ranked before analysis. For cell viability studies, 3–9 wells/condition and a minimum of 3 independent experiments were performed. For Western blot, ELISA and DNA gels 3–6 wells/condition and a minimum of 2 independent experiments were performed. DNA gels were qualitatively analyzed. A *p* < 0.05 was considered significant.

RESULTS

Gender Proclivity in Response to Cytotoxicity—There were no obvious gross morphologic differences between XY and XX neurons. Accurate gender separation was verified using RT-PCR for the Y-chromosome gene *SRY3* (Fig. 1*b*). The sensitivity of XY and XX neurons to multiple cytotoxic insults is shown in Fig. 1*c*. The conversion of MTT to formazan as the indicator of cell viability was used because the assay could be performed in a high-throughput fashion. Cytotoxicity models of nitrosative/oxidative stress using exogenous ONOO⁻ or H₂O₂, excitotoxicity (supraphysiologic glutamate and glycine), cell-signal mediated apoptosis (staurosporine), and apoptosis mediated via topoisomerase II inhibition (etoposide) were tested. Remarkably, for most cytotoxicity paradigms, XY and XX neurons were differentially susceptible. XY neurons were more sensitive to nitrosative stress and excitotoxicity, whereas XX neurons were more sensitive to etoposide and staurosporine. Responses to H₂O₂ were similar between genders. Because ONOO⁻ and other oxidants affect mitochondrial function thereby influencing the results of the MTT assay, other measures of cell viability were performed. Gender-dependent responses to nitrosative stress, excitotoxicity, and staurosporine were confirmed by flow-cytometric analysis using PI and Annexin V binding as markers (Fig. 1*d* and *e*), as well via measurement of lactate dehydrogenase release (data not shown).

Hippocampal neurons at ED 17 are also non-sex steroid

producing (1). Similar gender-dependent sensitivity to ONOO⁻, glutamate/glycine, and staurosporine was seen in hippocampal neurons compared with cortical neurons (Fig. 1*g*). However, both XY and XX hippocampal neurons were more sensitive to similar concentrations of glutamate/glycine and staurosporine than their cortical neuron counterparts.

To determine whether results in neurons could be generalized to non-neuronal cells, the sensitivity of splenocytes to nitrosative stress and staurosporine-induced apoptosis was also examined. Remarkably, similar gender-dependent sensitivity to ONOO⁻ and staurosporine was seen in splenocytes as was seen in neurons (Fig. 1*h*; representing *n* = 10 wells/condition and 2 independent experiments). However, splenocytes of both genders appeared more sensitive to equivalent concentrations of ONOO⁻ and staurosporine versus cortical neurons. In addition, gender differences were not detected in splenocytes after staurosporine treatment until 48 h after exposure (versus 2 h in neurons).

Gender Proclivity in Programmed Cell Death Pathway—Pathways of programmed cell death were examined by measuring mitochondrial release of cytochrome *c* and AIF. We and others have previously reported that nitrosative/oxidative stress induces programmed cell death in mixed-gender neurons via an AIF-mediated, poly-ADP-ribose polymerase-dependent and caspase-independent pathway (15, 22). After ONOO⁻, nuclear translocation of AIF was more prominent in XY neurons in contrast to cytochrome *c* release, which was more prominent in XX neurons (Fig. 2, *a–c*). Similar to our previous studies, a concomitant reduction in mitochondrial AIF was not detected using this dose of ONOO⁻ (15). With higher concentrations of ONOO⁻, a reduction in mitochondrial AIF is seen (16). The predilection for XY neurons to undergo an AIF-mediated programmed cell death pathway was confirmed by the detection of 50-kbp DNA fragments. Large scale DNA fragments are the biochemical hallmark of AIF-mediated cell death (23) and were increased in XY neurons to a greater degree than in XX neurons after ONOO⁻ exposure (Fig. 2*d*). These data show that under conditions of nitrosative stress, AIF-mediated cell death predominates over cytochrome *c* mediated cell death in XY versus XX neurons and vice versa.

Mitochondrial release of cytochrome *c*, caspase-3 proteolysis, and oligonucleosomal DNA fragmentation are phenotypic hallmarks of caspase-mediated programmed cell death culminating in apoptosis (24). After staurosporine exposure, XX neurons demonstrated more cytochrome *c* release, caspase-3 proteolysis, and oligonucleosomal DNA fragmentation versus XY neurons (Fig. 2, *e–g*). The magnitude of differences between XY and XX neurons were significant but less striking with staurosporine compared with ONOO⁻ treatment. These data show that XX neurons are more sensitive to staurosporine-induced apoptosis versus XY neurons.

Gender-dependent Responses to Treatments—A variety of antioxidants were tested in neurons after ONOO⁻ exposure. XY neurons were protected from nitrosative stress by pretreatment with catalase and NAC but not superoxide dismutase, whereas XX neurons were protected only by pretreatment with catalase (Fig. 3*a*). After nitrosative stress, both XY and XX neurons are protected and respond in similar magnitude to the poly-ADP-ribose polymerase inhibitor INH2BP; however, XY neurons benefited more from the NMDA antagonist MK801 than XX neurons (Fig. 3, *b* and *c*). These differences are unlikely to be related to estrogen, as exogenous 17-β-estradiol did not protect neurons from ONOO⁻-toxicity, and in fact exacerbated cell death in XX neurons (Fig. 3*d*).

XY neurons were more sensitive than XX neurons to glutamate/glycine whether tested on DIV 8, 10, or 14, suggesting

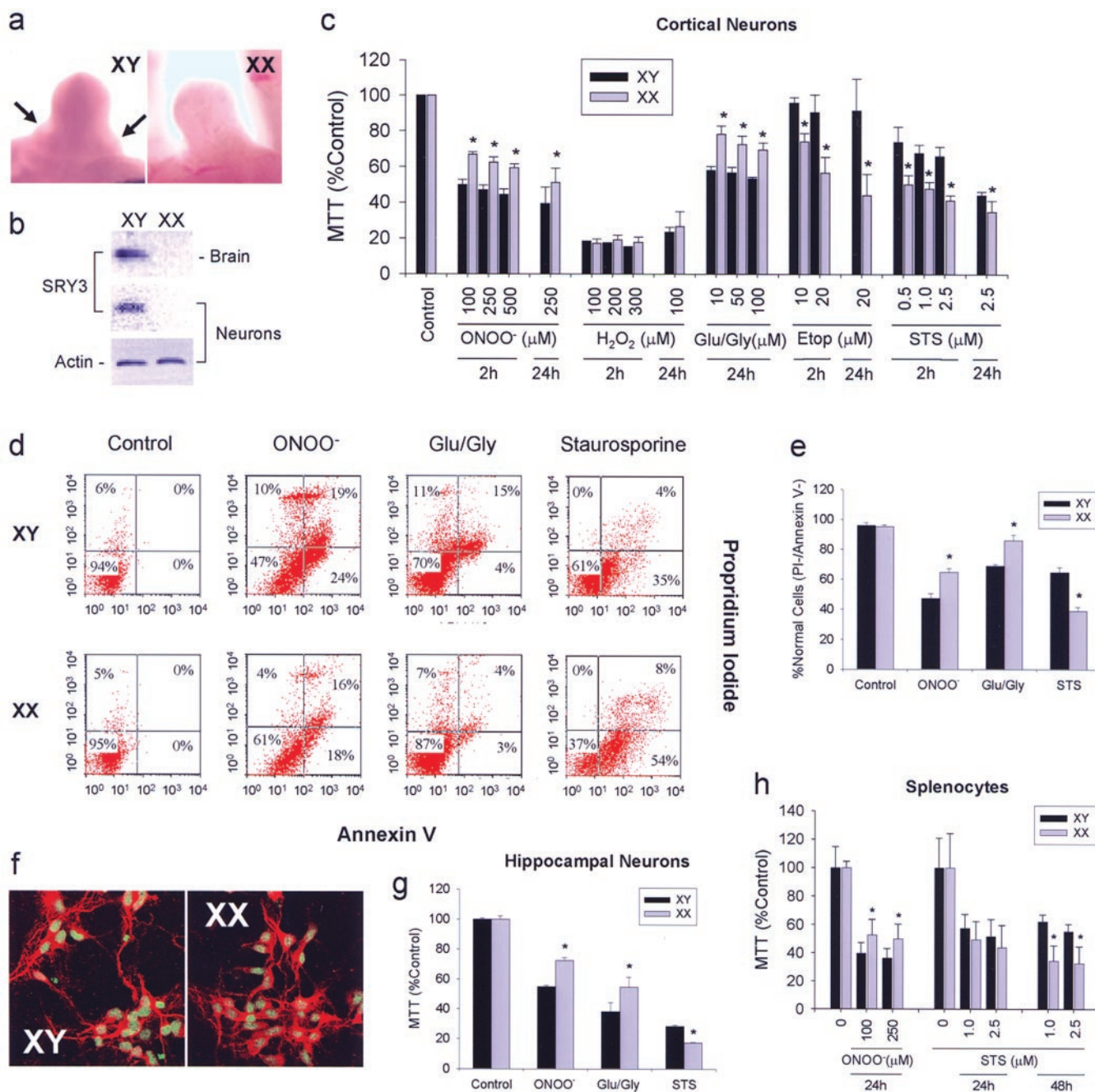


FIG. 1. Gender segregation and proclivity in response to cytotoxicity. *a*, prominence of sex cords (arrows) and penile vessels were used to separate ED 17 males from females. *b*, accuracy of gender segregation in primary cortical neurons verified using RT-PCR for the Y-chromosome gene *SRY3*. *c*, differential sensitivity of XY and XX cortical neurons to multiple cytotoxic insults. Cell viability was determined using the MTT assay with results presented as % of control. XY cortical neurons were more sensitive to ONOO⁻ and glutamate (+5 μM glycine) excitotoxicity versus XX cortical neurons. XX cortical neurons were more sensitive to staurosporine (STS) and etoposide (Etop) versus XY cortical neurons. Similar sensitivity to H₂O₂ was seen in both XY and XX cortical neurons. *d*, flow cytometry at 0 and 24 h after ONOO⁻, Glu/Gly, or staurosporine exposure (x axis: PI, y axis: annexin V binding). *e*, quantification of the percentage of normal cortical neurons, defined as PI-/annexin V-, for each condition. *f*, primary hippocampal XY and XX neurons at base-line labeled with an antibody against microtubule associated protein-2 (red) and the nuclear dye Sytox Green. *g*, XY hippocampal neurons were more sensitive to 250 μM ONOO⁻ and 10 μM glu/5 μM glycine versus XX hippocampal neurons. In contrast, XX hippocampal neurons were more sensitive to 2.5 μM staurosporine versus XY hippocampal neurons. *h*, differential sensitivity of XY and XX splenocytes to cytotoxic insults. Similar to neurons, XY splenocytes were more sensitive to ONOO⁻ versus XX splenocytes, and XX splenocytes were more sensitive to staurosporine versus XY splenocytes. *, *p* < 0.05 versus XY cells; *n* = 3–10 wells/condition; independent experiments were performed 2–4 times for each.

that gender-dependent differences are not simply due to different *in vitro* rates of glutamate receptor maturation between genders (Fig. 3e). However, because gender dependent differences were most prominent on DIV 14, subsequent excitotoxicity experiments were performed at this time point. The response to multiple ionotropic glutamate receptor agonists was also tested. XY neurons were consistently more sensitive than

XX neurons over multiple concentrations of NMDA, kainate, and AMPA (Fig. 3f). XY neurons treated with the NMDA antagonist MK-801 were protected from excitotoxicity in a dose-dependent manner (Fig. 3g). In XX neurons cell viability was reduced after treatment with 1 μM MK801 but increased after treatment with 5 μM MK801 versus vehicle after glutamate/glycine. The increased sensitivity of XX versus XY neurons to

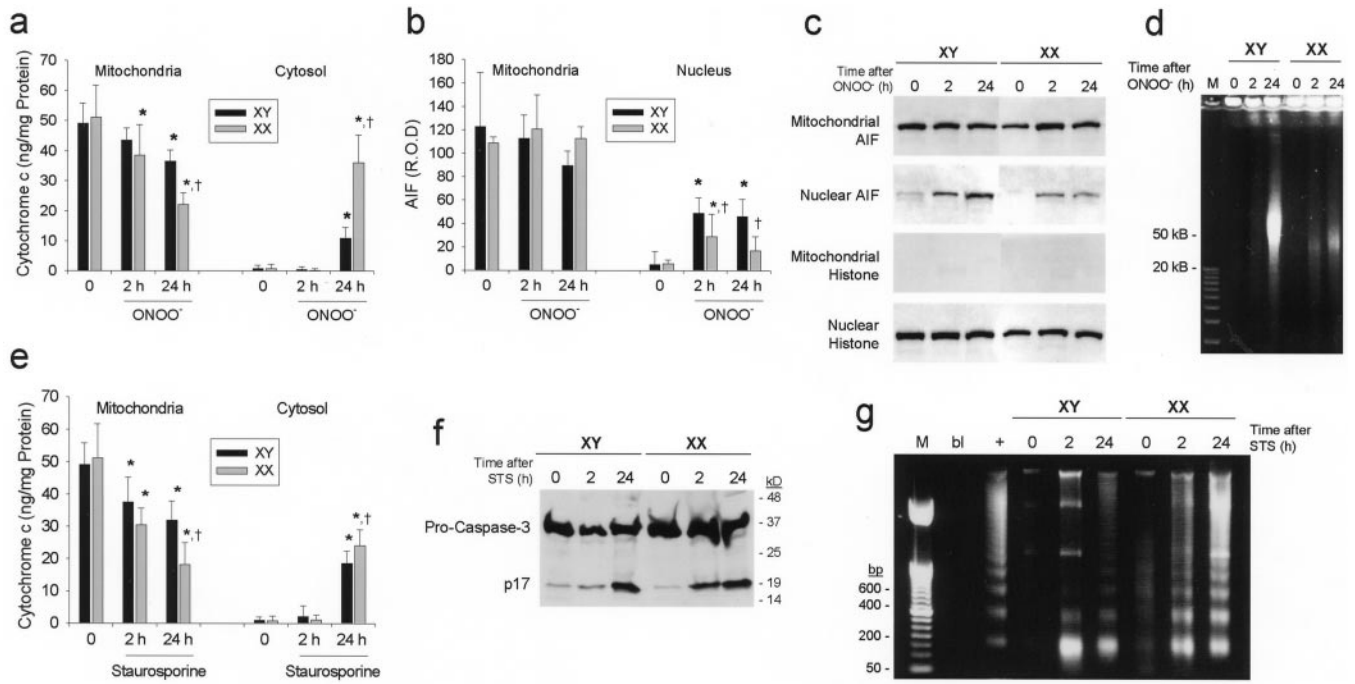


FIG. 2. Gender proclivity in programmed cell death pathway in primary cortical neurons. *a*, mitochondrial and cytosolic cytochrome *c* levels measured by ELISA after 250 μM ONOO⁻. *b* and *c*, Western blot for AIF in mitochondrial and nuclear cell fractions. An anti-histone antibody was used to verify nuclear separation. Relative optical densities (*R.O.D.*) were used for quantification. *d*, large scale DNA fragmentation detected using pulsed field gel electrophoresis. *e*, mitochondrial and cytosolic cytochrome *c* levels after 2.5 μM staurosporine (*STS*). *f*, caspase-3 proteolysis. *g*, oligonucleosomal DNA fragmentation (*M*, marker; *bl*, blank; +, positive control). *, *p* < 0.05 versus gender control; †, *p* < 0.05 XY neurons; *n* = 3–9 wells/group.

MK801 *in vitro* is consistent with work by others showing increased neuronal necrosis in female versus male rats injected with NMDA antagonists *in vivo* (25). Treatment with the poly-ADP-ribose polymerase inhibitor INH2BP and the caspase inhibitor z-VAD had no effect in either gender. Similar to XX neurons under conditions of nitrosative stress, 17- β -estradiol exacerbated cell death after excitotoxicity.

In contrast to nitrosative stress and excitotoxic insults, XX neurons were more sensitive to staurosporine-induced injury. XX neurons showed a more robust response to treatment with the caspase inhibitor z-VAD versus XY neurons (Fig. 3*h*). Similar to experiments shown in Fig. 1*c*, the magnitude of differences between XY and XX neurons were less striking at 24 h after staurosporine compared with the other cytotoxicity paradigms tested.

GSH Depletion in XY Neurons and Male Rat Brain; a Potential Mechanism?—Because XY but not XX neurons responded to treatment with NAC, presumably via restoration of intracellular GSH levels, we examined GSH levels after exposure to ONOO⁻. Intracellular GSH levels were reduced in XY but not XX neurons 24 h after ONOO⁻ and were partially restored in XY neurons with NAC treatment (Fig. 4*a*). These alterations could not be explained by cellular release of GSH in XY neurons (data not shown). Intracellular levels of protein-SH were not different between genders (Fig. 4*b*), suggesting that the reduction in GSH was not simply a reflection of total cellular sulfhydryls or a greater degree of oxidative/nitrosative stress in the XY neurons. Base-line concentrations for GSH are at the upper limit of other published data (~20–65 nmol/mg of protein) (26, 27). A similar gender-dependent difference in the capacity to maintain intracellular levels of GSH was also seen *in vivo* utilizing a model of asphyxial cardiac arrest in sexually immature rats (21). Brain levels of GSH within cerebral cortex 6–24 h after asphyxial cardiac arrest in PND 17 rats were lower in male versus female rats (Fig. 4*c*). Similar to studies *in vitro*,

protein-SH concentrations were not different between genders (Fig. 4*d*).

DISCUSSION

The novel findings of this study are as follows. 1) XY and XX neurons are differentially susceptible to a variety of cytotoxic agents, with XY neurons more sensitive to nitrosative stress and excitotoxicity compared with XX neurons and XX neurons more sensitive to apoptosis-inducing agents compared with XY neurons; 2) in XY neurons an AIF-mediated pathway of programmed cell death predominates, whereas in XX neurons a cytochrome *c*-mediated pathway of programmed cell death predominates given similar cellular insults; 3) responses to therapies targeting nitrosative stress, excitotoxicity, and caspase-mediated apoptosis are also gender-dependent; 4) in terms of nitrosative stress and ischemia, one mechanism contributing to this gender proclivity is the incapacity of XY neurons to maintain intracellular levels of GSH; and 5) splenocytes from male and female PND 16–18 rats show similar gender-dependent responses to nitrosative stress and staurosporine-induced apoptosis, suggesting that these results may be generalized to non-neuronal cells *in vitro*. These findings have important clinical ramifications related to CNS and potentially non-CNS diseases, where nitrosative stress, excitotoxicity, and apoptosis contribute to pathology and where both genders are affected.

Nitrosative stress is felt to play important roles in many CNS diseases including neurodegenerative diseases, cerebral ischemia, and traumatic brain injury (28). Consistent with the *in vitro* data in the present study, men are more susceptible than women to both Parkinson’s and Alzheimer’s diseases (6, 7, 12, 13) and ischemia after cardiac arrest (14). In addition, boys have worse outcome after traumatic brain injury than girls (9), and men demonstrate a greater degree of lipid peroxidation than women after traumatic brain injury (29). Our data do not discount the importance of circulating sex steroids and their

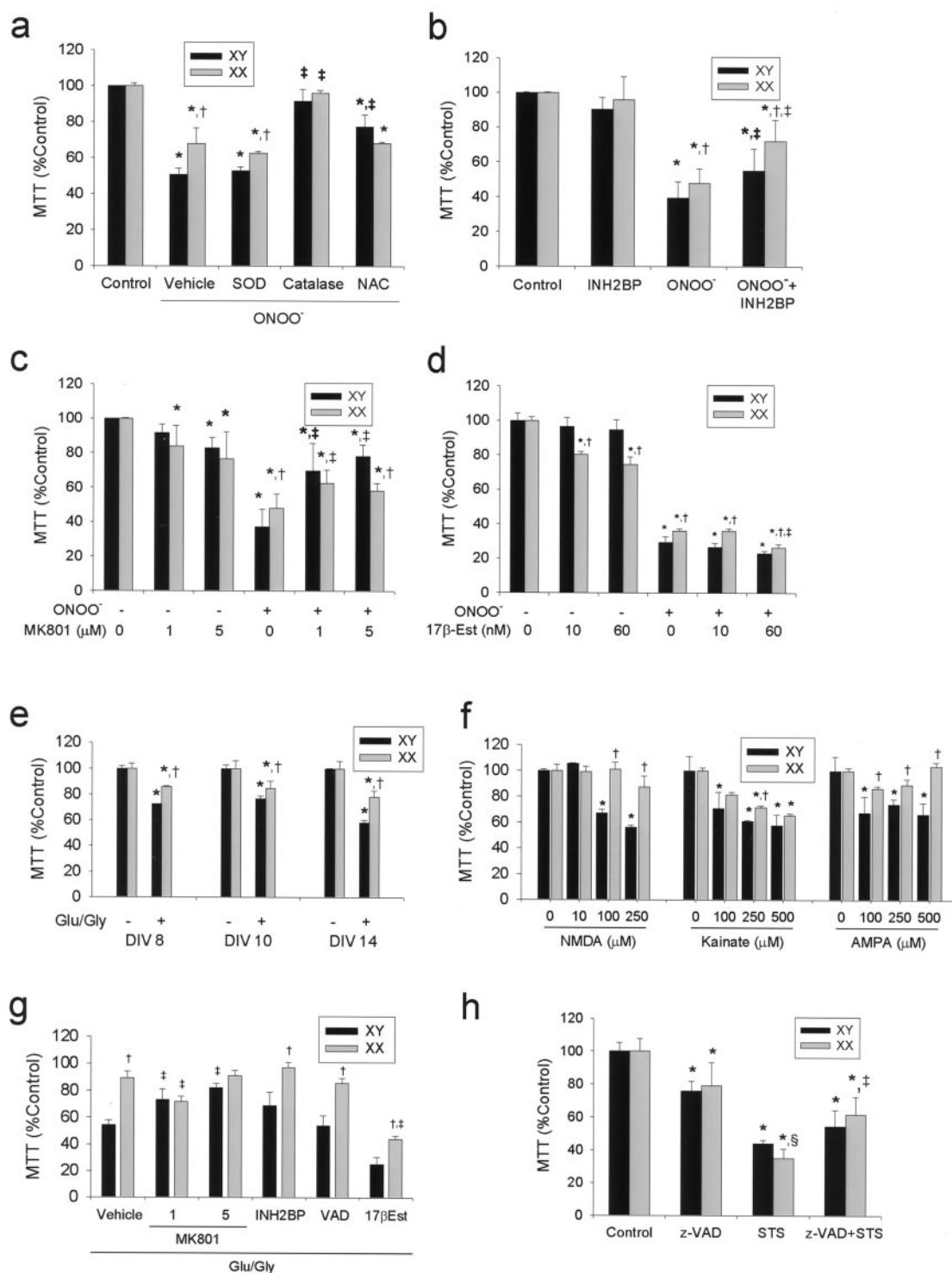
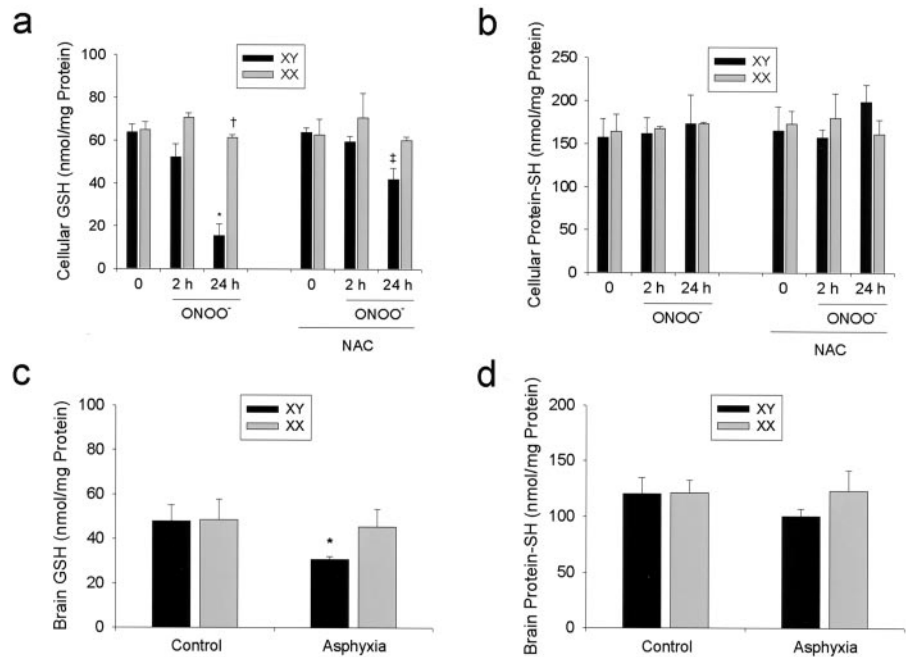


FIG. 3. Gender-dependent responses to treatments in primary cortical neurons. Response of XY and XX neurons to superoxide dismutase (*SOD*) (300 units), catalase (5000 units), and NAC (5 mM) (*a*), 100 μ M INH2BP (*b*), 1 or 5 μ M MK801 (*c*), or 17 β -estradiol (*d*) 24 h after 250 μ M ONOO⁻, assessed using MTT. *e*, sensitivity to 10 μ M L-glutamate + 5 μ M glycine, tested on DIV 8, 10, or 14. *f*, sensitivity to increasing concentrations of the glutamate receptor agonists NMDA, kainate, and AMPA. *g*, sensitivity to 10 μ M L-glutamate + 5 μ M glycine (tested on DIV 14) and response to treatment with 1 μ M MK801, 100 μ M INH2BP, 100 μ M z-VAD, or 50 nM 17 β -estradiol assessed using MTT at 24 h. *h*, sensitivity of XY and XX neurons to 2.5 μ M staurosporine (*STS*) and response to treatment with 100 μ M z-VAD. *, $p < 0.05$ versus gender control; †, $p < 0.05$ versus XY neurons; ‡, $p < 0.05$ versus vehicle; §, $p < 0.05$ versus XY neurons, Mann-Whitney rank sum test.

influence on neurological outcome in these disease states (8). Furthermore, our data do not address the issue of sex steroid imprinting and decades of exposure to sex hormones. As such, these data may be less applicable to diseases of the elderly versus childhood. However, our data do identify an important role for innate gender differences, independent of circulating

sex steroids. Although exogenous 17 β -estradiol is generally protective in models of oxidative stress and excitotoxicity (30), our data demonstrated a lack of benefit under conditions of nitrosative stress and indeed high dose 17 β -estradiol increased ONOO⁻ toxicity in XX neurons (Fig. 3D). We are unaware of previous studies testing the therapeutic effect of 17 β -estradiol

FIG. 4. GSH depletion in XY cortical neurons and male rat brain. *a* and *b*, intracellular GSH and protein-SH concentrations after 250 μM ONOO⁻ in neurons treated with NAC or vehicle ($n = 4$ wells/group performed independently 3 times). *c* and *d*, brain GSH and protein-SH concentration obtained from cerebral cortices of PND 17 rats after 8-min asphyxial cardiac arrest or sham control ($n = 3-6$ rats/group). *, $p < 0.05$ versus gender control; †, $p < 0.05$ versus XY neurons; ‡, $p < 0.05$ versus vehicle.



under similar conditions; however, estrogens are capable of stimulating NO production by NO synthases (31), potentially exacerbating ONOO⁻-mediated toxicity.

Excitotoxicity is another major contributor to the pathological consequences of many CNS diseases including cerebral ischemia, traumatic brain injury, and seizures, and outcomes from these diseases are also gender-dependent including those observed in children and the elderly (9, 11). The present data suggest that males would be more susceptible than females to diseases where excitotoxicity contributes to the overall pathology and that males may benefit more from treatments targeting excitotoxicity than females. These data may help explain the disappointing results observed in clinical trials testing NMDA antagonists after acute brain injury (32). Similar to nitrosative stress, we found that treatment with 50 nM 17 β -estradiol was not protective under conditions of excitotoxicity and indeed exacerbated toxicity in XX neurons (Fig. 3g). This may be related to our use of higher doses (33) or long durations (34) of 17 β -estradiol compared with other studies showing neuroprotection in cortical neurons, possibly related to the generation of toxic estrogen metabolites such as 2-methoxyestradiol (35).

The present data, showing that XX neurons and XX splenocytes are more sensitive than XY neurons and XY splenocytes to apoptosis-inducing agents, are also consistent with clinical studies where girls responded better than boys to treatment for leukemia (36) and medulloblastoma (10). Our data are also congruent with clinical stroke studies, where girls demonstrate worse outcome than boys (11), but premenopausal women are at lower risk for stroke than age-matched men (8). The latter suggests that high levels of female reproductive hormones predominate over innate gender differences but that innate gender differences may be unmasked when sex hormone levels are similar between sexes. Compared with the other cytotoxicity paradigms tested in the present study, gender differences in both sensitivity to apoptosis-inducing agents and response to treatment were less striking, suggesting a more prominent role for sex steroids *versus* innate gender differences related to apoptosis. Indeed, estrogen induces expression of apoptosis-suppressing genes and is protective in experimental stroke (37).

Gender proclivity for different cell death pathways was also

identified. Nitrosative stress is a key component of glutamate neurotoxicity (38), and both trigger predominately caspase-independent cell death either via AIF-mediated apoptosis or necrosis (15, 22). In contrast, etoposide and staurosporine produce cell death by triggering caspase-dependent apoptosis (24). XY neurons showed proclivity for AIF-mediated cell death, whereas XX neurons showed proclivity for cytochrome *c* release and caspase-dependent apoptosis and a robust response to treatment with a caspase inhibitor. Previous studies examining mechanisms of cell death in neuronal cultures have utilized primarily mixed gender neurons. Our data identify potentially important mechanistic differences between XY and XX neurons in culture.

What are the potential mechanisms that could explain this gender proclivity? GSH is a prominent intracellular defense against oxidative/nitrosative stress, and the capacity to maintain GSH levels under stressful conditions is gender-dependent. For example, mitochondria from female rats contain higher glutathione peroxidase activity than those from males (39), and GSH and glutathione *S*-transferase content are higher in human lymphocytes obtained from women compared with men (40). Interestingly, intramitochondrial AIF functions as an antioxidant (41) and has structural homology to glutathione reductase (42). The present *in vitro* and *in vivo* data show that GSH levels diminish in XY neurons after ONOO⁻ and in male brain after ischemia at a time when circulating levels of sex steroids are similar but are maintained in both XX neurons and female brain under similar conditions, identifying one biologically plausible contributory mechanism for this gender proclivity. Another possibility is that the reason males are more sensitive to oxidative/nitrosative stress than females is multi-factorial and that reduced GSH levels in XY neurons and male brain simply represent a global reduction in antioxidant reserves, although protein-SH levels were maintained. Further investigation defining the role of glutathione in gender proclivity is warranted.

In conclusion, primary neurons from the developing male rat brain are more sensitive to nitrosative stress and excitotoxicity than their female counterparts, whereas primary neurons from the developing female rat brain are more sensitive to cytotoxic agents that produce caspase-dependent apoptosis than their male counterparts. In cerebral cortex and hippocampus, this

likely represents innate differences between XX and XY neurons rather than differences in available sex steroids, and the ability to maintain GSH appears to be a contributing factor toward this gender proclivity *in vitro* and *in vivo*. These data have implications in many CNS and perhaps non-CNS diseases and their related therapies.

Acknowledgments—We are grateful to Lan Shao, Henry Alexander, and Christina Marco for expert technical assistance and Dr. Joseph Carcillo for valuable suggestions.

REFERENCES

- MacLusky, N. J., and Naftolin, F. (1981) *Science* **211**, 1294–1302
- Carruth, L. L., Reisert, I., and Arnold, A. P. (2002) *Nat. Neurosci.* **5**, 933–934
- Reisert, I., Engele, J., and Pilgrim, C. (1989) *Cell Tissue Res.* **255**, 411–417
- Nunez, J. L., Lauschke, D. M., and Juraska, J. M. (2001) *J. Comp. Neurol.* **436**, 32–41
- Sacco, R. L. (2001) *Neurology* **5**, Suppl. 2, S31–S34
- Van Den Eeden, S. K., Tanner, C. M., Bernstein, A. L., Fross, R. D., Leimpeter, A., Bloch, D. A., and Nelson, L. M. (2003) *Am. J. Epidemiol.* **157**, 1015–1022
- Hariz, G. M., Lindberg, M., Hariz, M. I., and Bergenheim, A. T. (2003) *Acta Neurol. Scand.* **108**, 28–37
- Hurn, P. D., and Macrae, I. M. (2000) *J. Cereb. Blood Flow Metab.* **20**, 631–652
- Donders, J., and Hoffman, N. M. (2002) *Neuropsychology* **16**, 491–499
- Weil, M. D., Lamborn, K., Edwards, M. S., and Wara, W. M. (1998) *J. Am. Med. Assoc.* **279**, 1474–1476
- Hurvitz, E. A., Beale, L., Ried, S., and Nelson, V. S. (1999) *Pediatr. Rehabil.* **3**, 43–51
- Ueki, A., Shinjo, H., Shimode, H., Nakajima, T., and Morita, Y. (2001) *Int. J. Geriatr. Psychiatry* **16**, 810–815
- Gambassi, G., Lapane, K. L., Landi, F., Sgadari, A., Mor, V., and Bernabie, R. (1999) *Neurology* **53**, 508–516
- Vukmir, R. B. (2003) *J. Women's Health* **12**, 667–673
- Du, L., Zhang, X., Han, Y. Y., Burke, N. A., Kochanek, P. M., Watkins, S. C., Graham, S. H., Carcillo, J. A., Szabo, C., and Clark, R. S. B. (2003) *J. Biol. Chem.* **278**, 18426–18433
- Zhang, X., Chen, J., Graham, S. H., Du, L., Kochanek, P. M., Draviam, R., Guo, F., Nathaniel, P. D., Szabo, C., Watkins, S. C., and Clark, R. S. (2002) *J. Neurochem.* **82**, 181–191
- Brewer, G. J. (1995) *J. Neurosci. Res.* **42**, 674–683
- Szabo, C., Virag, L., Cuzzocrea, S., Scott, G. S., Hake, P., O'Connor, M. P., Zingarelli, B., Salzman, A., and Kun, E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3867–3872
- Zhang, X., Graham, S. H., Kochanek, P. M., Marion, D. W., Nathaniel, P. D., Watkins, S. C., and Clark, R. S. (2003) *FASEB J.* **17**, 1367–1369
- Bayir, H., Kagan, V. E., Tyurina, Y. Y., Tyurin, V., Ruppel, R. A., Adelson, P. D., Graham, S. H., Janesko, K., Clark, R. S., and Kochanek, P. M. (2002) *Pediatr. Res.* **51**, 571–578
- Fink, E. L., Alexander, H., Marco, C. D., Dixon, C. E., Kochanek, P. M., Jenkins, L. W., Lai, Y., Donovan, H. A., and Clark, R. S. (2004) *Pediatr. Crit. Care Med.* **5**, 139–144
- Yu, S. W., Wang, H., Poitras, M. F., Coombs, C., Bowers, W. J., Federoff, H. J., Poirier, G. G., Dawson, T. M., and Dawson, V. L. (2002) *Science* **297**, 259–263
- Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebbersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) *Nature* **397**, 441–446
- Budd, S. L., Tennesi, L., Lishnak, T., and Lipton, S. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 6161–6166
- Fix, A. S., Wozniak, D. F., Truex, L. L., McEwen, M., Miller, J. P., and Olney, J. W. (1995) *Brain Res.* **696**, 194–204
- Wang, X. F., and Cynader, M. S. (2000) *J. Neurochem.* **74**, 1434–1442
- Canals, S., Casarejos, M. J., Rodriguez-Martin, E., de Bernardo, S., and Mena, M. A. (2001) *J. Neurochem.* **76**, 56–68
- Szabo, C. (1996) *Brain Res. Bull.* **41**, 131–141
- Bayir, H., Marion, D. W., Puccio, A. M., Wisniewski, S. R., Janesko, K. L., Clark, R. S. B., and Kochanek, P. M. (2004) *J. Neurotrauma* **21**, 1–9
- Behl, C. (2002) *Nat. Rev. Neurosci.* **3**, 433–442
- Weiner, C. P., Lizasoain, I., Baylis, S. A., Knowles, R. G., Charles, I. G., and Moncada, S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5212–5216
- Ikonomidou, C., and Turski, L. (2002) *Lancet Neurol.* **1**, 383–386
- Singer, C. A., Figueroa-Masot, X. A., Batchelor, R. H., and Dorsa, D. M. (1999) *J. Neurosci.* **19**, 2455–2463
- Harms, C., Lautenschlager, M., Bergk, A., Katchanov, J., Freyer, D., Kapinya, K., Herwig, U., Megow, D., Dirnagl, U., Weber, J. R., and Hortnagl, H. (2001) *J. Neurosci.* **21**, 2600–2609
- Picazo, O., Azcoitia, I., and Garcia-Segura, L. M. (2003) *Brain Res.* **990**, 20–27
- Ishii, E., Eguchi, H., Matsuzaki, A., Koga, H., Yanai, F., Kuroda, H., Kawakami, K., Ayukawa, H., Akiyoshi, K., Kamizono, J., Tamai, Y., Kinukawa, N., and Okamura, J. (2001) *Med. Pediatr. Oncol.* **37**, 10–19
- Alkayed, N. J., Goto, S., Sugo, N., Joh, H. D., Klaus, J., Crain, B. J., Bernard, O., Traystman, R. J., and Hurn, P. D. (2001) *J. Neurosci.* **21**, 7543–7550
- Zhang, J., Pieper, A., and Snyder, S. H. (1995) *J. Neurochem.* **65**, 1411–1414
- Borras, C., Sastre, J., Garcia-Sala, D., Lloret, A., Pallardo, F. V., and Vina, J. (2003) *Free Radic. Biol. Med.* **34**, 546–552
- van Lieshout, E. M., and Peters, W. H. (1998) *Carcinogenesis* **19**, 1873–1875
- Klein, J. A., Longo-Guess, C. M., Rossmann, M. P., Seburn, K. L., Hurd, R. E., Frankel, W. N., Bronson, R. T., and Ackerman, S. L. (2002) *Nature* **419**, 367–374
- Mate, M. J., Ortiz-Lombardia, M., Boitel, B., Haouz, A., Tello, D., Susin, S. A., Penninger, J., Kroemer, G., and Alzari, P. M. (2002) *Nat. Struct. Biol.* **9**, 442–446

Innate Gender-based Proclivity in Response to Cytotoxicity and Programmed Cell Death Pathway

Lina Du, Hülya Bayir, Yichen Lai, Xiaopeng Zhang, Patrick M. Kochanek, Simon C. Watkins, Steven H. Graham and Robert S. B. Clark

J. Biol. Chem. 2004, 279:38563-38570.

doi: 10.1074/jbc.M405461200 originally published online July 1, 2004

Access the most updated version of this article at doi: [10.1074/jbc.M405461200](https://doi.org/10.1074/jbc.M405461200)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 41 references, 10 of which can be accessed free at <http://www.jbc.org/content/279/37/38563.full.html#ref-list-1>