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Glutamate-mediated excitotoxicity in neonatal hippocampal neurons is mediated by mGluR-induced release of Ca⁺⁺ from intracellular stores and is prevented by estradiol

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Abstract

Hypoxic/ischemic (HI) brain injury in newborn full-term and premature infants is a common and pervasive source of life time disabilities in cognitive and locomotor function. In the adult, HI induces glutamate release and excitotoxic cell death dependent on NMDA receptor activation. In animal models of the premature human infant, glutamate is also released following HI, but neurons are largely insensitive to NMDA or AMPA/kainic acid (KA) receptor-mediated damage. Using primary cultured hippocampal neurons we have determined that glutamate increases intracellular calcium much more than kainic acid. Moreover, glutamate induces cell death by activating Type I metabotropic glutamate receptors (mGluRs). Pretreatment of neurons with the gonadal steroid estradiol reduces the level of the Type I metabotropic glutamate receptors and completely prevents cell death, suggesting a novel therapeutic approach to excitotoxic brain damage in the neonate.

Keywords

excitatory amino acids; imaging; metabotropic receptors; neuroprotection; perinatal brain injury; steroids

Introduction

Hypoxic/ischemic (HI) brain injury occurs in 2–4 full-term neonates per 1000 births and approximately 50% of premature babies in the United States (<http://www.marchofdimess.com/peristats>; Thornberg *et al.*, 1995). Severe HI injury is associated with a 50% mortality rate, with half of the deaths occurring within the first month of life, and a complication rate of 80% in survivors (Thornberg *et al.*, 1995; Toh, 2000). Infants who survive severe HI injury frequently exhibit long-term behavioural abnormalities such as attention deficit hyper-activity disorder, cerebral palsy, mental retardation, and epilepsy

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(Robertson & Finer, 1993; Lou, 1996; Simon, 1999) as well as long-term cognitive impairments, including deficits in attention span, short-term recall, and problem solving (Amiel-Tison C, 1986; Biagas, 1999; Simon, 1999; Fotopoulos *et al.*, 2001). Despite these facts, relatively little is known regarding the mechanisms mediating ischemic brain injury in newborn and premature infants.

In adult brain, HI injury induces excessive release of the excitatory amino acid, glutamate, activating AMPA and NMDA receptors, thereby massively elevating intracellular Ca^{++} and initiating a cellular cascade culminating in cell death (Berridge, 1984; Choi, 1988a; 1988b; Siesjo, 1989; Choi & Rothman, 1990; Choi, 1990; Mattson, 1992; Pivovarov *et al.*, 2004). Exogenous administration of glutamatergic agonists that activate AMPA/kainate or NMDA receptors to living animals or to cultured primary neurons has proven a useful model system for studying the mechanisms of excitotoxic brain injury of mature neurons (McGeer & McGeer, 1981; Cook & Crutcher, 1986; Azcoitia *et al.*, 1998). The same treatment of neonatal rats produces small amounts of regionally selective and sex specific damage (Ben-Ari *et al.*, 1984; Wolf & Keilhoff, 1984; Hilton *et al.*, 2003). We previously explored kainic acid (KA)-induced excitotoxicity in newborn rat pups and found only modest increases in intracellular calcium concentrations, which were slightly attenuated by pretreatment with the neuroprotective gonadal steroid, estradiol (Hilton *et al.*, 2004; 2005). Thus the primary trigger of excitotoxic cell death in the very young brain remains largely unknown.

We have now explored the effects of the endogenous ligand, glutamate, on cell death and Ca^{++} dynamics in immature hippocampal neurons and have found that, in contrast to the adult, glutamate caused excitotoxic cell death by triggering release of Ca^{++} from internal stores via activation of Type I metabotropic glutamate receptors. Importantly, pretreatment with estradiol decreased glutamate-induced intracellular calcium responses, reduced metabotropic receptor protein levels and completely prevented glutamate-induced cell death. Combined, these findings represent a novel mechanism of excitotoxic cell death in immature hippocampal neurons and one of the most potent neuroprotective effect of estradiol reported to-date.

Materials and methods

Materials

Glutamate receptor antagonists—NMDA receptor antagonist, D(-)-2-amino-5-phosphonopentanoic acid (AP5); AMPA receptor antagonist, 2,3-dihydro-6-nitro-7-sulphamoyl-benzo(f)-quinoxaline (NBQX); selective GluR2-lacking AMPA receptor channel blocker, 1-naphthylacetylspermine trihydrochloride (NAS); nonselective metabotropic glutamate receptor (mGluR) antagonist at micromolar concentrations, but selective mGluR Type II antagonist at nanomolar concentrations, (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495) (Kingston *et al.*, 1998); mGluR Type I antagonist, (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA); mGluR Type III antagonist, (RS)-alpha-methylserine-O-phosphate (MSOP).

Calcium compounds—L-type voltage-sensitive calcium channel blocker, diltiazem; cell impermeant Ca^{++} chelator, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA); cell permeant Ca^{++} chelator, 1,2-bis (2-amino-5-fluorophenoxy) ethane-N,N,N',N'-tetra acetic acid tetrakis (acetoxymethyl) ester (BAPTA-AM); Ca^{++} ATPase inhibitor, thapsigargin.

Metabotropic glutamate receptor agonists—mGluR Type I, (S)-3,5-dihydroxyphenylglycine (DHPG); mGluR Type II, (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV); mGluR Type III, L-(+)-2-amino-4-phosphonobutyric acid (L-AP4). DHPG, DCH-IV, L-AP4, AP5, AIDA and MSOP were

obtained from Tocris Bioscience (Ellisville, MO); diltiazem, EGTA, Thapsigargin and NBQX were obtained from Sigma–Aldrich (St. Louis, MO); and BAPTA-AM was obtained from Molecular Probes (Eugene, OR). NAS was a gift from Akira Isokawa (Daicel Chemical Industries, Ltd, USA).

Primary cultures of rat hippocampal neurons

All animal procedures were approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee, and followed National Institute of Health guidelines. Timed pregnant Sprague–Dawley female rats purchased from Charles River Laboratories (Wilmington, MA, USA) were killed with carbon dioxide on E18 and the hippocampi dissected from all fetuses. Fetal hippocampi were dissociated using trypsin digestion and triturated to give a single cell suspension. The suspension was incubated in a water bath for 15 min at 37 °C to allow the cells to settle, and the supernatant was removed. Five millilitres of Hanks balanced salt solution (HBSS+) was added and the cells again allowed to settle for 5 min. This process was repeated two more times. Cells were dissociated by pipetting and DNAase treatment. Cell count and viability were determined using a haemocytometer and Trypan Blue staining before cells were plated at a density of 300 000 cells per coverslip on 25 mm poly L-lysine coated coverslips. These were then placed in 60-mm dishes containing 4 mL plating medium (86 mL MEM, 10 mL horse serum, 3 mL filter sterilized 20% glucose, 1 mL pyruvic acid, 100 mM). Cells were placed in a 5% CO₂ incubator for 4 h at 37 °C. Coverslips were transferred from the plating media to dishes containing 3 mL Neurobasal media (1 mL B-27 supplement, 1 mL antibiotic/antimycotic 100× liquid, 125 µL L-glutamine to a volume of 50 mL with Neurobasal). In order to mimic the physiologic hormonal environment that exists *in vivo*, 17β-estradiol dissolved in DMSO or vehicle (DMSO 0.01%) for a final concentration of 1 nM was added to hippocampal cultures on days *in vitro* (DIV) 0, 2 and 4. Previous data from our laboratory has demonstrated that this concentration approximates physiological levels in developing brain for up to 24-h posttreatment (Nunez & McCarthy, 2003). Vehicle treated controls received the same volume of DMSO. Hippocampal cell cultures were again treated with estradiol or vehicle on DIV2 and DIV6. On DIV3 and 7, 1 mL of medium was removed from each culture dish and replaced with 1 mL of fresh Neurobasal medium.

Tunel staining/cresyl violet

Estradiol or vehicle was added to the medium of cultured hippocampal neurons generated from E18 embryos as described above. Glutamate (10 µM), LY341495 (10 µM), a Type I metabotropic glutamate receptor antagonist, glutamate plus LY341495, or saline vehicle was administered on the morning of DIV4, followed by another treatment four hours later. The regimen was repeated the following day and cells fixed by immersion in 4% paraformaldehyde for 25 min at room temperature the morning of DIV6. Cells were permeabilized by immersion in 0.2% Triton X-100 solution in PBS, and stained using the standard protocol provided in the DeadEnd Colorimetric TUNEL system (Promega, Madison, WI) and TUNEL positive cells visualized using diaminobenzidine (DAB). Cells were lightly counter-stained with the Nissl stain cresyl violet, dehydrated and mounted on slides. TUNEL positive cells and neurons were counted in five fields per quadrant of each coverslip using the Neurolucida program (Microbrightfield version 2.01; Microbrightfield Co., Colchester, VT, USA). Twenty fields were averaged in order to obtain mean cell number per coverslip ($n = 4–9$ coverslips). In order to ensure that cell distribution was uniform across all coverslips and treatments, the number of cresyl violet stained cells were counted and the number of TUNEL positive cells normalized to overall cell number.

Calcium imaging of cultured hippocampal neuron

Cells used for calcium imaging were generated from E18 embryos and imaging was performed on DIV5. On the day of imaging, hippocampal neurons were incubated with Fura 2AM in DMSO and processed as described previously (Hilton *et al.*, 2006). Baseline measurements of resting calcium concentrations for individual cells were obtained during which images were captured every 30 s. In initial experiments, glutamate was perfused for 90 s with image capture occurring every 5 s. Physiologic saline solution (PSS) was then superfused over cells in order to ensure calcium transients returned to baseline with image capture every 30 s. In order to determine the source of increased intracellular calcium, coverslips were treated with ionotropic or metabotropic glutamate receptor antagonists. In these experiments, 10 μ M glutamate was perfused over the cells initially, followed by PSS. After a return to baseline, the antagonist was perfused for 3 min, followed immediately by concurrent application with the antagonist and glutamate. In experiments with BAPTA, thapsigargin, and EGTA, the solutions were infused for 40 s prior to the introduction of glutamate, followed by concurrent application with the blocking solution and glutamate for 3 min. Experiments with EGTA were carried out in calcium free buffer. In experiments with the metabotropic receptor agonists, solutions were infused for 40 s. All antagonists were administered at a dose determined to saturate all receptors (Bleakman & Lodge, 1998; Dobrev *et al.*, 1999; Semyanov & Kullmann, 2000; Chen *et al.*, 2004; Wu *et al.*, 2004; Baskys *et al.*, 2005). Parameters evaluated were similar to those previously explored (Hilton *et al.*, 2005; Nunez *et al.*, 2005; Hilton *et al.*, 2006) and include: (i) percentage of cells responding; (ii) maximal per cent increase in intracellular calcium concentration above baseline; (iii) time for intracellular calcium to rise to greater than 10% above baseline; (iv) time for the calcium transient to reach its maximal response, and (v) the time for the calcium transient to return to 50% of its maximal response. A total of 50–217 neurons from three to ten coverslips spanning three to eight separate cultures from different litters were analysed per treatment group.

A Zeiss Axiovert 100 inverted microscope with illumination by a Til Photonics Polychrome II Monochromator was used (Applied Scientific Instrumentation, Eugene, OR, USA), with image acquisition and analysis as previously described (Hilton *et al.*, 2006). Calcium calibration was performed using a calcium calibration buffer kit (Molecular Probes) and analysis was performed on the raw free calcium concentration data after baseline was subtracted.

Western blot analysis of cultured hippocampal neurons

Hippocampal cultures were treated with estradiol on DIV0, 2 and 4 with tissue collection on DIV5 for analysis of estradiol effects early in development (E2 DIV0–5) or were treated with estradiol on DIV5, 7 and 9 with tissue collection on DIV10 for analysis of estradiol effects at a slightly older time period (E2 DIV5–10) in development. Coverslips containing DIV5 and DIV10 hippocampal cells were placed on ice and 75 μ L of ice-cold lysis buffer containing 50 mM Tris-HCl, 1% Na-deoxycholate, 0.25% NP-40, 150 mM NaCl, 1 mM EDTA and protease inhibitors (1 μ g/mL of aprotinin, leupeptin, and pepstatin; 1 mM phenylmethylsulphonyl fluoride) added to each dish. Cells were collected from two separate preparations, with 12 samples each from the estradiol and control groups. For each sample, 20 μ g protein was electrophoresed on a SDS-polyacrylamide gel (8–16% Tris glycine; Invitrogen, Novex, Carlsbad, CA) before transfer to a Sequi-Blot PVDF membrane (Bio-Rad laboratories, Hercules, CA). Membranes were incubated with rabbit polyclonal antibody generated against rat mGluR1, mGluR2/3 and mGluR5 protein, at dilutions of 1 : 1000 (Upstate Biotechnology Lake Placid, NY) for 3 h at room temperature, followed by incubation in mouse anti-rabbit HRP-linked secondary antibody (1 : 2500, Cell Signalling Technology, Beverly, MA) for 30 min. Immunoreactive bands were detected using an enhanced chemiluminescence kit (ECL kit, New England Biolabs, Beverly, MA) and membranes exposed to film (Hyperfilm-ECL,

Amersham Pharmacia Biotech, Arlington Heights, IL). The following day, membranes were incubated in a primary antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA) at 1 : 5 000 000 for 2 h at room temperature, rinsed with Tris-buffered saline with Triton, incubated for 30 min in anti-mouse at 1 : 3000, and visualized as above. GAPDH is a housekeeping protein used to control for variation in loading of protein. The mGluR5 protein was detected as a single band at molecular mass 130 kDa, mGluR1 was detected at 140 kDa, mGluR2/3 was detected at 96 kDa and GAPDH was detected at 30 kDa. The integrative grayscale pixel area-density was captured with a CCD camera and analyses were performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available at <http://rsb.info.nih.gov/nih-image/>). Data are expressed as the ratio of mGluR to GAPDH signal.

Statistical analysis

One-way analyses of variance ($_{ANOVA}$) were performed on baseline calcium levels, glutamate-induced peak intracellular calcium concentration, rise time, peak time, and recovery time followed by *posthoc* Tukey's or Newman-Keuls comparisons, with a level of $P < 0.05$ required to obtain statistical significance. Numbers of TUNEL positive cells were normalized to number of neurons counted and a nonparametric Kruskal-Wallis performed. Data are presented as the ratio of TUNEL positive cells to cresyl violet stained cells per coverslip. A one-way $_{ANOVA}$ was performed on the mGluR, GAPDH ratios from the Western blots with a level of $P < 0.05$ required to obtain statistical significance.

Results

The effect of glutamate on cell death and intracellular calcium in immature hippocampal neurons

Primary cultures of hippocampal neurons derived from embryos at E18 and treated with glutamate (10 μM) on DIV4 and DIV5 exhibited a two-fold increase compared to vehicle treatment in the number of TUNEL positive cells on DIV6 (Kruskal-Wallis, $P < 0.05$). There was a baseline level of cell death of ~18%, consistent with other studies of hippocampal neurons at this age (Fig. 1A).

Using neurons derived in the same manner, calcium imaging was performed on DIV5 using the fluorometric calcium dye FURA-2AM ($n = 207\text{--}217$ cells). Bath applied glutamate (10 μM) increased the intracellular calcium concentration from a resting level of 42 nM to a peak amplitude of ~300 nM. The same dose of KA (10 μM) also increased intracellular calcium but only to a concentration of ~115 nM (Fig. 1B). The latency to the peak level of intracellular calcium in response to glutamate (time to peak) was markedly and significantly faster than KA (51.52 ± 2.88 vs. 80.7 ± 8.53 s; $F_{1,58} = 139.67$; $P < 0.0001$, $n = 30$ neurons/treatment; Fig. 1C and D). More importantly, the peak response following glutamate was ~40 s prior to the offset of glutamate, followed by a precipitous drop. In contrast, calcium levels rose gradually throughout the duration of KA application, reaching peak levels ~10 s before offset and declining only when KA was removed. The different temporal responses and shapes of the calcium transients suggests the release and depletion of intracellular stores induced by glutamate vs. continuous influx of extracellular calcium induced by KA.

The source of the glutamate-induced increase in intracellular calcium in immature hippocampal neurons

There are numerous routes by which glutamate administration can result in increased intracellular calcium, including influx through NMDA and calcium permeable AMPA receptors or indirectly via depolarization induced opening of voltage-sensitive calcium channels (VSCCs). We employed various antagonists and blockers alone, and in combination,

to determine the source of the glutamate-induced increase in free intracellular calcium in hippocampal neuronal cultures. Antagonists of ionotropic glutamate receptors and blockers of VSCCs only modestly reduced glutamate-induced calcium responses ($F_{6,432} = 24.37$; $P < 0.0001$; $n = 50$ – 75 neurons/treatment group; Fig. 2A). Antagonizing the NMDA receptor with AP5 ($100 \mu\text{M}$) decreased glutamate-induced calcium by 20% ($P < 0.05$), while the AMPA receptor antagonist, NBQX ($100 \mu\text{M}$), and the L-type VSCC blocker, diltiazem ($100 \mu\text{M}$), had no significant effect. Combinations of ionotropic glutamate receptor antagonists and VSCC blockers, including AP5/NBQX, AP5/diltiazem, and NBQX/diltiazem still only reduced the calcium transient modestly (data not shown), with a maximal reduction of 38% induced by the combination of all antagonists/blockers together ($P < 0.005$). The selective blocker of polyamine-sensitive AMPA receptors, NAS ($100 \mu\text{M}$), also slightly but significantly reduced calcium responses to glutamate ($F_{3,214} = 17.63$; $P < 0.001$), as did combinations of NAS with other receptor antagonists (data not shown). We conclude that calcium influx via the L-type VSCC, NMDA receptor and GluR2-lacking AMPA receptor each represent a distinct yet minor component (each ranging from 10 to 20%) of the route via which glutamate leads to increased intracellular calcium in immature hippocampal neurons. Moreover, even when combined, these pathways account for at most one-third of the Ca^{++} influx induced by glutamate.

We next asked what the remaining source of the calcium signal might be by applying a Ca^{++} -free extracellular solution containing the calcium chelator, EGTA ($500 \mu\text{M}$); the cell permeant calcium chelator, BAPTA-AM ($1 \mu\text{M}$); or the cell permeant calcium store re-uptake inhibitor, thapsigargin ($5 \mu\text{M}$). An overall effect of treatment was observed ($n = 50$ – 75 neurons per treatment group; $F_{3,212} = 42.37$; $P < 0.001$; Fig. 2B). *Posthoc* analyses indicated superfusion with BAPTA-AM or thapsigargin significantly reduced the glutamate-induced increase in intracellular calcium concentration by 92–95% ($P < 0.001$), whereas EGTA was without effect ($P > 0.6$), suggesting the bulk of the glutamate-induced Ca^{++} transient was derived from intracellular Ca^{++} stores.

Effect of metabotropic glutamate receptor activation on intracellular calcium and cell death

Glutamate can cause Ca^{++} release from intracellular stores via activation of metabotropic GluRs. We therefore tested the effect of selective and nonselective mGluR antagonists on glutamate-induced Ca^{++} signals. The nonselective metabotropic glutamate receptor antagonist, LY341495, was administered in doses ranging from 10 nM to $10 \mu\text{M}$. At high concentrations, LY341495 blocks all mGluR subtypes (Kingston *et al.*, 1998) and here reduced the magnitude of glutamate-induced calcium transients by 90% at $10 \mu\text{M}$ and 25% at $1 \mu\text{M}$ ($F_{5,374} = 18.237$, $P < 0.0001$; $n = 60$ – 70 neurons per treatment group; Fig. 3A). At lower concentrations (10 nM), the mGluR antagonist, LY341495, is selective for Type II mGluRs and at either the 10 nM or 100 nM doses it had no significant effect on the magnitude of the glutamate-induced increase in intracellular calcium. The mGluR Type I specific antagonist, AIDA ($100 \mu\text{M}$), reduced glutamate-induced calcium increases by 40% ($P < 0.05$), while the mGluR Type III specific antagonist, MSOP ($100 \mu\text{M}$), reduced glutamate-induced calcium increases by approximately 60% ($P < 0.001$).

Given that mGluR antagonists were effective at attenuating glutamate-induced increases in intracellular Ca^{++} , we assessed mGluR agonists for their ability to increase intracellular Ca^{++} in immature hippocampal neurons. A large increase in intracellular free Ca^{++} was induced by the Type I mGluR agonist DHPG ($F_{3,267} = 122.42$; $P < 0.001$; $n = 60$ – 70 per group; Fig. 3B), but the Type II and Type III mGluR agonists DCG-IV and L-AP4 ($10 \mu\text{M}$ each) had no effect on intracellular free Ca^{++} concentration. The DHPG-induced increase in intracellular Ca^{++} was of similar magnitude to that induced by glutamate, indicating that activation of Type I receptors by glutamate induces mobilization of Ca^{++} from internal stores in immature hippocampal neurons. There was no significant difference in the time to the peak of the calcium

transient induced by DHPG compared to glutamate. Taken together, these results suggest that glutamate acts primarily at Type I mGluRs to induce mobilization of calcium from intracellular stores in immature neurons.

Given that high doses of the metabotropic glutamate receptor antagonist, LY341495, completely blocked the glutamate-induced increase in intracellular Ca^{++} , we next asked whether it would also block glutamate-induced cell death. Glutamate treatment induced a 74% increase in the number of TUNEL positive cells compared to treatment with vehicle ($K-W = 11.41$, $P < 0.01$). Pretreatment of cells with LY341495 5 min prior to glutamate, however, significantly decreased the number of TUNEL positive cells ($P < 0.05$). The administration of LY341495 alone did not affect the number of TUNEL positive cells (Fig. 3C). We conclude that glutamate-induced cell death in immature hippocampal neurons is mediated primarily by mGluRs and subsequent metabotropic Ca^{++} release from intracellular stores.

The effect of estradiol on the glutamate-induced calcium transient and cell death via the Type I metabotropic glutamate receptor

Hippocampal neurons were treated with estradiol (1 nM) or vehicle for four DIV prior to calcium imaging at a concentration that approximates physiologic levels in developing brain (Nunez & McCarthy, 2003). There was no effect of hormone treatment on resting intracellular calcium (data not shown). Pretreatment with estradiol significantly attenuated the magnitude of the maximal calcium response to glutamate (10 μM) compared to cells treated with vehicle ($F_{1,423} = 10.534$; $P < 0.001$; Fig. 4A and B). There was no effect of pretreatment with estradiol on time to peak, recovery time, or the number of responding cells (data not shown). We next pretreated cells with estradiol in the same dose and time course as above and assessed glutamate-induced cell death. Estradiol pretreatment completely prevented the increase in TUNEL positive cells seen in response to glutamate treatment ($P < 0.005$; Fig. 5A and B). Moreover, estradiol alone resulted in 57% fewer TUNEL positive cells compared to vehicle ($P < 0.005$), consistent with prior data demonstrating that estradiol reduces apoptotic cell death (Liu *et al.*, 2001; Zhang *et al.*, 2003) and, early in development, may exert an effect on cell number even in the absence of an insult (Hilton *et al.*, 2003).

Lastly, the same dose and time course of estradiol pretreatment of cultured hippocampal neurons prepared in the same manner as above (DIV0–5), revealed a hormonally induced significant decrease in protein levels of the Type I metabotropic receptors, mGluR1 ($F_{2,11} = 24.054$, $P < 0.0001$) and mGluR5 ($F_{2,11} = 41.182$, $P < 0.0001$) but not the Type II receptors, mGluR2/3, compared to nontreatment neurons. We next asked whether this hormonally induced decrease in metabotropic glutamate receptor protein is unique to immature neurons or could be induced in slightly older neurons. In order to examine this possibility, we administered estradiol later in neuronal development, at DIV5, 7 and 9. We found that estradiol, when administered later in development, still induced a decrease in Type I metabotropic receptor protein, as well as a decrease in Type II receptor protein, compared to nontreated neurons, suggesting that the effect of estradiol on Type I receptors endures for at least the first 10 days postnatal and begins to affect Type II receptors as development progresses ($F_{2,11} = 7.274$, $P = 0.013$; Fig. 6). The Type I metabotropic receptors (mGluR1 and mGluR5) are found throughout the hippocampus on the day of birth with a considerably higher expression of mGluR5 early in development compared to mGluR1 (Lopez-Bendito *et al.*, 2002). A decrease in mGluR1 and mGluR5 protein would be predicted to lead to decreased Type I metabotropic glutamate receptor activity and could account for the reduction of glutamate-induced Ca^{++} release from internal stores. The reduced expression of Type I metabotropic receptors could thus in part account for the neuroprotective action of estradiol during early development.

Discussion

The immature human brain is susceptible to injury from hypoxic/ischemic insults and seizures. Damage following these events affects the hippocampus and the cerebral cortex – two brain regions crucial for normal cognition and behaviour. A well-established model of neonatal brain injury mimicking the cellular events and pathologic deficits observed during HI involves excessive activation of the glutamatergic system and, thereby, elevation of the intracellular Ca^{++} concentration (Vannucci & Vannucci, 1997). Attenuating or buffering the elevated intracellular Ca^{++} that occurs following neonatal HI and other types of early insult is a potential therapeutic approach for achieving neuroprotection. We report here that neonatal cultured hippocampal neurons are vulnerable to excitotoxic cell death induced by glutamate and that pretreatment with estradiol completely blocks this cell death.

Antecedent to establishing the neuroprotective mechanism of estradiol is determining the source of the increased free intracellular Ca^{++} induced by glutamate in these immature neurons. Contrary to what is observed in mature neurons (Choi, 1988a; Monaghan *et al.*, 1989), the cumulative contribution of the NMDA receptors, calcium-permeable AMPA receptors, and VSCCs to the increased free intracellular Ca^{++} induced by glutamate was only ~20–40% of the total. Instead, the vast majority of the increase was through mobilization of Ca^{++} from internal stores. Incubation with either the cell permeant Ca^{++} chelator, BAPTA-AM, or depletion of Ca^{++} stores with the re-uptake inhibitor, thapsigargin, resulted in a greater than 90% reduction in the glutamate-induced increase in intracellular Ca^{++} , while incubation with calcium-free saline containing EGTA, reduced the glutamate-induced Ca^{++} response by less than 10%.

The importance of metabotropic glutamate receptors in adult brain injury remains uncertain. Agonists of Type II and III mGluRs are neuroprotective in models of brain injury (Zwienenberg *et al.*, 2001; Brazel *et al.*, 2005). Conversely, Type I mGluR antagonists are neuroprotective via numerous mechanisms, including prevention of the elevated IP_3 release and intracellular calcium release from intracellular stores that accompanies traumatic brain injury, resulting in inhibition of apoptosis following hypoxia–ischemia (Bao *et al.*, 2001; Lyeth *et al.*, 2001; Brazel *et al.*, 2005). However, there are also reports in adults of mGluR Type I agonists attenuating susceptibility to NMDA-induced excitotoxicity and HI/injury via enhancement of phospholipase C activation, suppression of NMDA receptor currents and enhancement of GABA_A receptor currents and GABA release (Cozzi *et al.*, 2002; Baskys *et al.*, 2005). The role of metabotropic receptor activation or antagonism in the neonate is not as studied, although metabotropic glutamate receptors are present in hippocampus and cortex early in development (Romano *et al.*, 1996; Defagot *et al.*, 2002; Lopez-Bendito *et al.*, 2002; Di Giorgi Gerevini *et al.*, 2004). Type II receptor antagonism results in neuroprotection against homocysteic acid-induced excitotoxicity and HI in some neonatal models of brain injury (Cai *et al.*, 1999; Brazel *et al.*, 2005; Folbergrova *et al.*, 2005). To date, antagonism of the Type I metabotropic glutamate receptor in the neonate has not been explored.

We found that nonspecific antagonism of metabotropic receptors with a high concentration of LY341495 resulted in a 95% reduction in glutamate-induced intracellular Ca^{++} and prevented excitotoxic cell death. Treatment with specific Type I and Type III metabotropic receptor antagonists also reduced glutamate-induced Ca^{++} increase from 40 to 60%. However, only the Group I agonist, DHPG, was able to increase intracellular Ca^{++} , with the increase in magnitude equivalent to that observed following glutamate alone. We conclude that glutamate-induced cell death in the neonatal brain is due to activation of mGluR and that Type I receptors are likely a major source of the increase in intracellular calcium.

Embryonic and neonatal brains are exposed to high levels of estradiol originating from maternal circulation, placenta, fetal gonads (Weisz & Ward, 1980; Rhoda *et al.*, 1984) and potentially synthesized *de novo* by the hippocampus (Prange-Kiel *et al.*, 2003; Amateau *et al.*, 2004). Estradiol is neuroprotective in both *in vitro* and *in vivo* models of ischemic and excitotoxic brain injury in the adult and aged rat, with levels of protection usually on the order of a prevention of 20–60% of the damage (Weaver *et al.*, 1997; Culmsee *et al.*, 1999; Dubal *et al.*, 1999; Alkayed *et al.*, 2000; Sampei *et al.*, 2000; Harms *et al.*, 2001). Mechanistic aspects of estradiol-mediated neuroprotection have been well defined in the adult striatum, cortex and hippocampus where estradiol can induce genomic/transcriptional effects such as increased levels of Bcl-2 and Apaf-1 (Dubal *et al.*, 1999), or activate signal transduction pathways that are neuroprotective, such as MAP kinase (Wade & Dorsa, 2003). Estradiol can also act directly on NMDA receptors to minimize excitatory amino acid binding (Weaver *et al.*, 1997) and decrease the release of amino acids (Ritz *et al.*, 2002), or on L-type VSCCs to regulate calcium influx (Mermelstein *et al.*, 1996; Kurata *et al.*, 2001). To date, there are no studies supporting a role for estradiol in direct modulation of metabotropic glutamate receptor protein levels although steroids can activate PKA and PKC through a G-coupled pathway (Kelly & Wagner, 1999). In CA1/CA3 cultures from postnatal rat pups estradiol increases CREB phosphorylation through the Type-I metabotropic glutamate receptor, mGluR1, but not mGluR5, as well as through Type-II and Type-III mGluRs, but the effect on protein levels was not explored (Boulware *et al.*, 2005). Our data suggest that estradiol decreases both mGluR1 and mGluR5 protein in immature hippocampal neurons. The effect of estradiol endures for at least 10 days postnatal given that estradiol administered on DIV5–10 continues to suppress both mGluR1 and mGluR5 protein, exhibiting an even greater effect on mGluR5 compared to DIV0–5 treatment. In addition, estradiol treatment on DIV5–10 decreases mGluR2/3 protein, an effect not observed with earlier estradiol exposure. These results suggest that the ability of estradiol to modulate metabotropic glutamate receptor protein persists throughout the early period of hippocampal development and may increase in breadth and magnitude as the hippocampus matures.

Neuroprotective effects of estradiol in models of paediatric brain damage have been relatively unexplored. We have reported that estradiol reduces KA-induced damage in neonatal females (Hilton *et al.*, 2004), but enhances GABA-induced damage (Nunez & McCarthy, 2003). The current results indicate a complete protective effect of estradiol against glutamate-induced cell death in immature hippocampal neurons and reveals a potential novel mechanism that contributes at least in part to this protection, the down-regulation of the Type I metabotropic glutamate receptors and a reduction in the glutamate-induced increase in intracellular Ca^{++} . Most reported neuroprotective effects of estradiol involve up regulation of molecules implicated in neurotropic activity, neurogenesis, and prevention of apoptosis (Favit *et al.*, 1991; Dubal *et al.*, 1999; Miller *et al.*, 2005) or scavenging of free radicals (Culmsee *et al.*, 1999). Mechanisms involving estradiol-mediated neuroprotection via down-regulation of key proteins or mRNA are relatively few (Cardona-Gomez *et al.*, 2004).

Conclusion

In summary, we report that glutamate induces cell death in neonatal hippocampal neurons via the release of calcium from internal stores following activation of metabotropic glutamate receptors, predominantly Type I. We further report a complete protective effect of pretreatment with estradiol against the excitotoxic effects of glutamate, and evidence that this is via a down regulation of Type I receptor protein. These results are the first demonstration of a possible mechanistic basis for the neuroprotective effects of estradiol against glutamate-mediated excitotoxicity in the neonatal brain.

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Abbreviations

AIDA	(<i>RS</i>)-1-amonoindan-1,5-dicarboxylic acid
AP5	D(-)-2-amino-5-phosphonopentanoic acid
BAPTA-AM	1,2-bis(2-amino-5-fluorophenoxy) ethane- <i>N,N,N',N'</i> -tetra acetic acid tetrakis (acetoxymethyl) ester
DHPG	(<i>S</i>)-3,5-dihydroxyphenylglycine
DIV	days <i>in vitro</i>
EGTA	ethylene glycol-bis(2-aminoethylether)- <i>N,N,N',N'</i> -tetra acetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GluR	glutamate receptor
HI	hypoxic/ischemic
KA	kainic acid
L-AP4	L-(+)-2-amino-4-phosphonobutyric acid
LY341495	(2 <i>S</i>)-2-amino-2-[(1 <i>S</i> ,2 <i>S</i>)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid
mGluR	metabotropic glutamate receptors
MSOP	(<i>RS</i>)-alpha-methylserine- <i>O</i> -phosphate
NAS	1-naphtylacetylspermine trihydrochloride
NBQX	2,3-dihydro-6-nitro-7-sulphamoyl-benzo(f)-quinoxaline
VSCC	voltage-sensitive calcium channels

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Fig. 1.

Glutamate induces cell death and increases intracellular calcium in immature hippocampal neurons. (A) Glutamate treatment ($10 \mu\text{M}$) of cultured hippocampal neurons on DIV4 and 5 significantly increases the ratio of TUNEL positive to cresyl violet stained cells on DIV6 when compared to vehicle treatment (Kruskal Wallis, $P < 0.05$; $n = 4-9$ coverslips). (B) Mean ($\pm$ SEM) peak intracellular Ca^{++} concentration following glutamate or kainic acid application. Baseline intracellular calcium levels are indicated by the horizontal dotted line. (C) Representative traces of the calcium transients induced by glutamate and kainic acid. Note the different temporal profiles and shapes of the calcium transients induced by glutamate vs. KA, suggesting release from and depletion of internal stores by glutamate and extracellular influx by KA (D) There is a significant difference in rise time to peak calcium and decay time following glutamate administration compared to KA (ANOVA; $*P < 0.0001$).

Fig. 2.

Glutamate increases intracellular calcium via release from internal stores. (A) Antagonism of the AMPA receptor with NBQX and blocking of L-type VSCCs with diltiazem (Dil) had no significant effect on the magnitude of glutamate-induced Ca^{++} transients. The GluR2 lacking-AMPA receptor channel blocker, NAS, had a small but significant effect, as did the NMDA receptor antagonist, AP5 (~20% reduction). The combination of AP5, NBQX and diltiazem reduced glutamate-induced intracellular Ca^{++} by only 38% ($100 \mu\text{M}$ each; $n = 50\text{--}75$ neurons per treatment group; one-way ANOVA, $*P < 0.05$, $**P < 0.005$ compared to glutamate at 100%). (B) Pretreatment with Ca^{++} -free extracellular saline containing the calcium chelator, EGTA ($500 \mu\text{M}$) had no effect on the magnitude of glutamate-induced increase in free intracellular

Ca⁺⁺. In contrast, the glutamate-induced calcium transient was almost completely blocked by pretreatment with the intracellular calcium pump re-uptake inhibitor, thapsigargin (5 μ M), or preincubation in calcium free medium containing the cell permeant calcium chelator, BAPTA-AM (1 μ M; $n = 50$ – 75 neurons per treatment group; ANOVA; $*P < 0.0001$ compared to glutamate at 100%).

Fig. 3. Metabotropic glutamate receptor antagonists block glutamate induced Ca^{++} and cell death. (A) Antagonists to the metabotropic glutamate receptors reduced the increase in intracellular Ca^{++} subsequent to glutamate administration, but the greatest inhibition was by the general metabotropic glutamate receptor antagonist LY341495 (10 μM). At this high concentration, LY341495 is a nonspecific metabotropic glutamate receptor antagonist and was almost 90% effective at blocking glutamate-induced increases in free intracellular Ca^{++} (*** $P < 0.0001$ compared to glutamate at 100%). LY341495 at a 1 μM concentration reduced intracellular calcium by approximately 25% (* $P < 0.05$ compared to glutamate at 100%). At low concentrations, LY341495 is a specific antagonist of the Type II mGluRs, and did not block

glutamate-induced intracellular calcium. Type I and Type III metabotropic glutamate receptor inhibitors, AIDA and MSOP (100 μM), also significantly blocked glutamate induced increases in intracellular Ca^{++} ($n = 60\text{--}70$ neurons per treatment groups; ANOVA , $*P < 0.05$, $**P < 0.001$, respectively, and compared to glutamate at 100%), as did combinations of antagonists (data not shown). (B) Activation of the Type I metabotropic glutamate receptor with the agonist DHPG (10 μM) significantly increased free intracellular Ca^{++} ($n = 60\text{--}70$ neurons per group; ANOVA , $*P < 0.001$), to a level equivalent to that induced by glutamate. Neither the Type II nor Type III metabotropic glutamate receptor agonists DCG-IV or L-AP4 (10 μM) had any appreciable effect on intracellular Ca^{++} . Data are presented as the per cent of glutamate-induced intracellular Ca^{++} response. Glutamate alone is set at 100% for comparison purposes. (C) Glutamate significantly increases the number of TUNEL immunoreactive cells in hippocampal cultures compared to treatment with vehicle (ANOVA ; $*P < 0.05$). Treatment with the nonspecific metabotropic antagonist LY341495 (10 μM) has no significant effect on TUNEL positive cell number and pretreatment with LY341495 prior to glutamate completely prevents the increase in TUNEL positive cells seen following glutamate alone ($n = 4\text{--}9$ coverslips). Data are presented as the per cent of TUNEL positive cells from total cells.

Fig. 4. Pretreatment with estradiol decreases intracellular calcium concentration. (A) Mean ($\pm$ SEM) peak amplitude of the calcium transient induced by glutamate ($10 \mu\text{M}$) application to cultured hippocampal neurons imaged on DIV5. Pretreatment with estradiol on DIV0, 2 and 4 significantly attenuates the peak amplitude of the glutamate-induced calcium response ($*P < 0.001$). (B) Representative pseudocolored image of fluorescence emitted in vehicle (1) and estradiol (2) treated hippocampal neurons following glutamate perfusion.

Fig. 5. Pretreatment with estradiol decreases cell death. (A) Representative micrograph of TUNEL positive cells. Arrows point to selected TUNEL positive cells in vehicle treated (a), estradiol treated (b), glutamate treated (c) and estradiol plus glutamate treated cultures (d). Images are at 40 $\times$ (scale bar, 30 μ m). (B) Glutamate significantly increases the number of TUNEL positive cells compared to vehicle (* $P < 0.05$; $n = 4-9$ coverslips) and pretreatment with estradiol on DIV0, 2, and 4 prior to glutamate exposure completely prevents this increase. There are also significantly fewer TUNEL positive cells in cultures pretreated with estradiol compared to vehicle and estradiol plus glutamate (# $P < 0.005$; $n = 4-9$ coverslips), suggesting estradiol is neuroprotective of cultured hippocampal neurons even in the absence of an excitotoxic insult.

Fig. 6.

Pretreatment with estradiol decreases Type 1 metabotropic receptor protein. Estradiol pretreatment early in development (DIV0–5) significantly decreased protein levels of mGluR5 and mGluR1, compared to nontreated cells, but had no effect on mGluR2/3 protein levels. Estradiol administration on DIV5–10 decreased mGluR1 and mGluR5 protein as well as mGluR2/3 compared to nontreated cells ($n = 4$; $*P < 0.05$ compared to no estradiol). In addition, estradiol treatment on DIV5–10 decreased mGluR5 compared to treatment on DIV0–5 ($**P < 0.01$ compared to DIV0–5). Each sample represents cells collected from two coverslips, across two separate culture preparations. Representative photomicrographs of Western Blot

detection of metabotropic receptor protein are noted above each histogram, with each lane representing one sample.