High and low concentration of 17α-estradiol protect cerebellar granule neurons in different time windows

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Abbreviations: CGN, cerebellar granule neurons; ER, estrogen receptor; GCL, glutamate cysteine ligase; GSH, glutathione; NMDA, *N*-methyl-D-aspartate; NOS, nitric oxide synthase; ROS, reactive oxygen species

Abstract

17α-estradiol is a hormonally inactive isomer of 17β-estradiol, but with similar potency as neuroprotector. However, we have previously reported that pretreatment with high concentration (10 μM) of both estrogens abolishes their neuroprotection in rat cerebellar granule neurons. Here, we have examined neuroprotective properties of 17α-estradiol against glutamate-induced excitotoxicity in chicken cerebellar granule neurons using low (1 nM) and high concentration.17α-Estradiol, 1 nM, was neuroprotective when glutamate was administered after a pretreatment period of 24 h, but not when coadministered with glutamate. In contrast, 10 μM was protective when coadministered with glutamate, but was not efficient when glutamate was administered after a pretreatment period. The difference in protection was linked to a stronger calcium response during glutamate exposure in the non-protective treatments. In addition, the pretreatment period with 10 μM was accompanied by increased protein level of the N-methyl-ᴅ-aspartate receptor subunit NR2B and reduced glutathione level. Thus, 17α-estradiol has a concentration and time dependent protective effect against glutamate-induced cell death.

Keywords: Calcium; estradiol; glutamate-induced toxicity; neuroprotection; NR2B

Introduction

It has long been accepted that estrogen has neuroprotective properties [1, 2] which may be mediated through estrogen receptors (ERs) [3, 4], or through non-ER mechanisms [5]. Non-ER mechanisms have led to the development of non-feminizing estrogens that exert neuroprotection without activation of ERs [5, 6]. 17α-Estradiol is a stereoisomer of 17β-estradiol with >200 fold less estrogen activity, but with similar potency as a protector [7]. However, we have previously reported that pretreatment with both 17β-estradiol and 17α-estradiol at high concentration (10 μM) abolishes their neuroprotection from glutamate-induced cell death in rat cerebellar granule neurons (CGNs) due to down-regulation of glutathione (GSH) [8].

In stroke and neurodegenerative diseases, enhanced extracellular glutamate results in neurodegeneration and calcium dependent cell death in neurons [9] through *N*-methyl-D-aspartate (NMDA) receptors [10]. Further, production of nitric oxide (NO) is linked to NMDA receptor-mediated calcium influx through a structural link between the neuronal nitric oxide synthase (nNOS) and NMDA receptors [11]. NMDA receptors can have a synaptic or extrasynaptic localization. However, NMDA receptor stimulation is dramatically different between synaptic and extrasynaptic NMDA receptor as the extrasynaptic NMDA receptor is identified to mediate the excitotoxicity through excess levels of glutamate while synaptic NMDA receptors stimulation have anti-apoptotic activity [12]. NR2B subunit is found preferentially in extrasynaptic NMDA receptors and NR2A in the synaptic NMDA receptors [13]. Moreover, one of the mechanisms that 17β-estradiol protects neurons from glutamate-induced cell death is documented to be mediated through NMDA receptors [14, 15]. In addition, in micromolar concentrations, estrogen molecules work as reactive oxygen species (ROS) scavengers in glutamate-mediated neuronal toxicity due to its phenolic hydroxyl group [16]. The aim of the present study was therefore to investigate how 17α-estradiol works as a

neuroprotector against glutamate-induced toxicity in cultures of chicken CGNs using both high (10 μM) and low (1 nM) concentrations. We reveal that pretreatment with 1 nM of 17α-estradiol protects against glutamate-induced cell death, while pretreatment with 10 μM fails to protect. However, acute treatment with 17α-estradiol results in protection only at 10 μM, giving the molecule a concentration and time dependent protective effect against glutamate-induced cell death.

Material and methods

Materials

Basal Eagle's medium (BME) and L-glutamine were purchased from Lonza BioWhittaker (Verviers, Belgium). Penicillin-streptomycin (PenStrep) was acquired from Gibco, Life technologies (Paisley, UK). Chicken serum was obtained from Gibco and Biowest (Nuaillé, France). Dimethyl sulfoxide (DMSO), 17α-estradiol, dihydrorhodamine 123 (DHR), monochlorobimane (mBCl), cytosine β-D-arabinofuranoside (Ara-C), and anti-β-actin antibody were all obtained from Sigma-Aldrich (St. Lois, US). Anti-NMDAR2B antibody (ab65783) was purchased from Abcam (Cambridge, UK), donkey anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, US) and secondary antibody goat anti-mouse (HRP) was obtained from Bio-Rad (Hercules, California, US). Pierce® BCA Protein Assay Kit was from Thermo Scientific (Rockford, US) while Luminata Crescendo/Forte was acquired from Merck Millipore (Temecula, US). Fura-2, AM was purchased from Invitrogen™ Molecular Probes (Eugene, US).

Cell cultures and treatments

Eggs (*Gallus gallus*, 50-60 g) were obtained from Nortura Samvirkekylling (Våler, Norway) and incubated at 37.5° C in 45 % humidity in an OvaEasy 380 Advance EXII Incubator (Brinsea, Weston-super-Mare, UK). On embryonic day 17 eggs were anesthetized in crushed ice for 7 min before cultures of granule neurons were prepared from cerebella and isolated as previously described [17]. Cells were seeded out in BME supplemented with chicken serum (7.5%), KCl (25 mM), L-glutamine (2 mM), insulin (100 nM) and PenStrep (1%) with a density of 1.9 x 10^6 cells/mL in dishes or 96 wells plates. After 24 h medium was replaced with serum-free BME as previously described [18] supplemented with Ara-C (10 µM) to prevent growth of non-neuronal cells. For exposure treatments, DMSO (0.1%), 17α-estradiol (1 nM or 10 µM), or DHR (0.1 µM) was used. After 4 days *in vitro* (DIV4) cultures were treated as in (i) or (ii). (i) Acute treatment, A: cultures were pre-incubated in physiological buffer described in [8] containing exposure treatments for 15 min. Then cultures were washed before exposed to 100 µM glutamate together with 10 µM glycine as coagonist in the absence or presence of exposure treatments in physiological buffer for 15 min. Cells serving as controls were untreated or treated with physiological buffer added 1.2 mM $MgCl_2$. The glutamate exposure was removed and cultures were incubated in serum-free BME for 24 h. (ii) Pretreatment, P: on DIV3 cultures were given exposure treatments. On DIV4, cultures were exposed to physiological buffer supplemented with glutamate as in (i). Control cells were treated as described in (i) for 15 min. Cell death was measured by trypan blue exclusion on DIV5 as described in [19].

ROS measurements

CGNs were seeded out in black 96 wells plates and treated with DMSO or 17α-estradiol, or left untreated, on DIV3. On DIV4, cultures were loaded with DHR (0.1 µM) in the medium for 30 min (37° C). Then the cells were washed and glutamate exposed using experimental

buffer from [20] containing 2 mM $CaCl_2$ with DMSO or 17α-estradiol as described in (i) or with experimental buffer only (ii), *Cell culture and treatments*. Control cells were treated with experimental buffer. Five minutes before the end of glutamate exposure, oxidation of DHR was recorded at excitation and emission wavelengths of 485 nm (15 nm bandwidths) and 535 nm (20 nm bandwidths) using a CLARIOstar® plate reader (BMG Labtech, Ortenberg, Germany). The buffer with treatments was removed and cells were washed before replaced with new buffer and incubated further at 37° C. Oxidation of DHR was recorded at 1 h before Hoechst 33342 (0.2 µg/ml) was added and incubated for 5 min before the buffer was removed and replaced. Hoechst was measured at excitation and emission wavelengths of 350 nm (22 nm bandwidths) and 461 nm (36 nm bandwidths), respectively. DHR fluorescence arbitrary units were corrected with Hoechst fluorescence arbitrary units after subtraction of blank values.

Glutathione measurements

CGNs were seeded out in black 96 wells plates and treated with DMSO or 17α-estradiol, or left untreated, on DIV3. For cells treated as (i) and (ii) in *Cell culture and treatments* on DIV4, experimental buffer was used for glutamate exposure. Control cells were treated with experimental buffer only. Medium was removed 24 h after glutamate treatment and cells were incubated with serum-free BME containing 40 µM mBCl for 30 min (37° C). Then medium was replaced with experimental buffer and fluorescence was recorded using CLARIOstar® plate reader at excitation and emission wavelengths of 380 nm (15 nm bandwidths) and 478 nm (21 nm bandwidths), respectively. Next, Hoechst 33342 was added as described in *ROS measurements*. mBCl fluorescence arbitrary units were corrected with Hoechst arbitrary units after subtraction of blank values.

Calcium measurements

CGNs were seeded out in CELLBIND® 96-Well Microplates, Corning® (Corning Incorporated, Kennebunk, ME, US) and treated with DMSO or 17α-estradiol, or left untreated, on DIV3. On DIV4, cells were loaded in medium with the membrane permeable Ca^{2+} -binding fluorescent probe Fura-2/AM (5.2 µM) for 40 min (37° C). Then cells were washed and added experimental buffer supplemented with 1 mM of $MgSO_4$ for de-esterification and incubated for 15 min. The buffer was then changed to experimental buffer in the presence of DMSO or 17α-estradiol as described in (i) or with experimental buffer only (ii), *Cell culture and treatments*. A CLARIOstar® plate reader equipped with two injectors was used for calcium measurements. One injector was loaded with experimental buffer and the other with experimental buffer containing glutamate and glycine diluted to 100 µM and 10 µM by injection in each well, respectively. Injection volume ranged between 11-25 µl between experiments and injection speed was set to 100 [µl/s]. The plate was read in well mode at excitation wavelengths 335 nm and 380 nm (12 nm bandwidths) and emission at 510 nm (30 nm bandwidths) using orbital averaging. Interval time was 1.4 s per sampling point. Total measurement in each well was at least 12 s and glutamate was injected after 2 s. First reading after injection was 2.6 s due to movement of the plate. Some wells were injected only with experimental buffer. Average of background autofluorescence in wells not loaded with Fura-2/AM were subtracted from excitations at 335 nm and 380 nm before 335/380 ratio in each well was used to calculate changes in intracellular calcium.

Western blot analysis

CGNs were unexposed or exposed to DMSO or 17α-estradiol on DIV3. After 24 h, the cultures were washed twice in ice cold PBS and harvested in 2% SDS with protease inhibitors

PMSF (300 µM), leupeptin (5 µg/ml), pepstatin A (1 µg/ml), and phosphatase inhibitor Na_3VO_4, (100 µM). Pierce® BCA Protein Assay Kit was used to determine total protein concentration before proteins were analyzed with a standard western blot protocol described previously [19] using primary antibodies against NMDAR2B (1:1000) or β-actin (1:5000), and then with HRP conjugated secondary antibody (1:10 000). Immunoreactive protein was detected using a chemiluminescence gel documentation system (Syngene, Cambridge, UK) with Luminata HRP detection substrates.

Statistical methods

Statistical analyses were performed in SigmaStat 3.5 software (Systat Software) and results are presented as mean ± SEM. Multiple comparisons were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test. Kruskal-Wallis ANOVA on ranks followed by Dunn's post hoc test or Tukey test was applied on non-parametric data. Student's *t*-test was used for comparison between two groups. A p value of <0.05 was considered significant.

Results

We first investigated protection by 17α-estradiol against glutamate-induced cell death in chicken CGNs after 24 h (Figure 1). 17α-estradiol was either given 24 h prior to glutamate insult (pretreatment, P) or treated 15 minutes before and 15 minutes together with glutamate (acute, A). Glutamate exposure for 15 minutes resulted in cell death. Cells acutely treated with 10 µM concentration prior to glutamate exposure protected the cells from the glutamate-induced cell death. However, pretreatment with 10 µM 24 h before glutamate insult did not protect the cells. Since estrogens most probably exert their protection through antioxidant activity at 10 µM [21, 22] we wanted to expose the cells to a concentration where it is unlikely to act as an antioxidant, but rather through ligand binding activity. Hence, cells

treated with 1 nM 17α-estradiol responded differently to glutamate exposure. Pretreatment with 1 nM concentration protected the cells from the glutamate-induced cell death. However, when 1 nM was given acutely with glutamate it did not protect the cells.

Since ROS is involved in the glutamate-induced cell death, neurons were pretreated or acutely treated with DHR (Figure 2A). Exposure with DHR resulted in a small reduction in glutamate-induced cell death, suggesting that ROS is involved to some extent. We further investigated peroxynitrite production at the time of glutamate exposure and after 1 h in cells pretreated or acutely treated with 17α-estradiol (Figure 2B). Glutamate caused a significant 2-fold increase in ROS production immediately during glutamate exposure and after 1 h. Surprisingly, only cells pretreated with 10 μM of 17α-estradiol had significantly increased levels of ROS production 1 h after glutamate exposure. All the other treatments decreased the glutamate-induced ROS production.

Since reduction in GSH levels is involved in glutamate-induced cell death [8], we investigated if this reduction also is seen in chicken CGNs. Glutamate exposure reduced the GSH levels after 24 h (Figure 3). Similar reduction was seen in cells pretreated with both concentrations of 17α-estradiol and given glutamate. In addition, a reduction was seen in cells that were only pretreated with 10 μM 17α-estradiol for 24 h. Pretreatment with 1 nM did not affect the GSH levels. Surprisingly, with acute treatment of both 1 nM and 10 μM of 17α-estradiol together with glutamate there was no reduction in the GSH level.

Glutamate-induced neurotoxicity is calcium dependent and changes in calcium levels during glutamate exposure were examined (Figure 4A-F). Injection of glutamate gave a rapid increase in intracellular calcium which lasted for at least 20 s (Figure 4A), while injection of buffer did not induce a response (data not shown). Pretreatment with 10 μM 17α-estradiol for 24 h also had a significant increase in calcium levels with glutamate exposure (Figure 4B and D), immediately after injection and after 10 s. However, pretreatment with 1 nM 17α-estradiol

did not give a significant increase. Interestingly, when 17α-estradiol was present in the buffer (acute treatment), only 1 nM caused a significant glutamate-induced rise in calcium (Figure 4C and D). Thus, significant increase in calcium levels with treatments of 17α-estradiol is in consistency with reduced protection against glutamate-induced cell death.

To further find the mechanism behind the calcium response in neurons pretreated with 17α-estradiol, we examined the expression of the NMDA receptor unit NR2B in the neurons before the cells were exposed to glutamate (Figure 4E-F). Cells pretreated with 10 µM 17α-estradiol for 24 h had a significant 7-fold increase in NR2B protein level on DIV4. Thus, NR2B levels may explain the difference in glutamate-induced calcium levels between pretreatment with 1 nM and 10 µM of 17α-estradiol.

Discussion

Non-feminizing estradiol analogs are reported to be equally or more potent than 17β-estradiol against glutamate toxicity [5]. In the present work we demonstrate that 10 uM 17α-estradiol, but not 1 nM, is neuroprotective when administered together with glutamate, most likely because of the lower calcium level. On the other hand, pretreatment with 1 nM 17α-estradiol protects, whereas 10 uM 17α-estradiol fails to provide neuroprotection because of upregulation of NR2B, no decrease in calcium level, and a reduction in GSH levels. Our previous findings in rat and mice CGNs showed that a pretreatment period with 10 µM of 17α-estradiol abolished the acute neuroprotection against glutamate-induced toxicity in CGNs [8], consistent with our present study in chicken CGNs. Also, these results were linked to reduced GSH levels. The present results suggest that 17α-estradiol acts through different mechanisms with low and high concentration. Since estrogens work as ROS scavengers we confirmed this by investigating the levels of peroxynitrite. NO is enhanced due to NMDA receptor activation of nNOS during glutamate exposure [11] and NO reacts fast with

superoxide to form peroxynitrite. All treatments except pretreatment with 10 µM 17α-estradiol reduced the ROS levels after glutamate exposure. This suggests that other mechanisms are involved in the glutamate-induced death since the non-protective acute treatment with 1 nM also reduced the ROS levels. The reduced ROS levels by acute treatment were also accompanied by no reduction in GSH levels. Further, glutamate exposed cells pretreated with 1 nM and 10 µM had similar reduction in GSH levels as glutamate alone. This fails to explain the protective effect of pretreatment with 1 nM. However, pretreatment with 10 µM for 24 h reduced the GSH levels. Pretreatment with 10 µM did not affect the synthesis of the glutamate cysteine ligase catalytic subunit GCLC of the rate limiting enzyme GCL in GSH synthesis (data not shown), suggesting that reduced activity of the enzyme and/or depletion of GSH are responsible for the reduced GSH levels [23]. A sufficient level of the key antioxidant GSH is needed for neuroprotective actions of 17β-estradiol and 17α-estradiol in nM and µM doses [24]. Thus, the neurons are likely to be more vulnerable for an excitotoxic stimulus if GSH levels are decreased at the time point for administration because of reduced capacity to scavenge excess ROS production, and this probably caused the glutamate-induced increase in ROS levels after 1 h with pretreatment 10 µM 17α-estradiol.

To further investigate the non-protective and protective effect of 17α-estradiol against glutamate-induced death we examined glutamate-induced calcium response. Interestingly, only the non-protective treatments gave a significant response in the glutamate-induced calcium levels. Glutamate induces strong calcium signaling through NMDA receptors. Minor changes in intracellular calcium induced by estrogen are linked to biological response [25], and mirrors neuroprotection in the present work.

Elevated levels of glutamate in the extracellular space and increases in the number of NMDA receptors located extrasynaptically are two pathological states which characterize several neurological diseases [26]. The subunit NR2B is primarily found in extrasynaptic sites [13].

We therefore examined the expression of NR2B subunit in the CGNs after a pretreatment period with 17α-estradiol. Pretreatment with 10 μM 17α-estradiol enhanced this expression. Thus, the non-protective effect by pretreatment with 10 μM could be related to increased NR2B levels. Further, delayed neuronal disintegration which occurs hours after glutamate exposure, depends on presence of extracellular calcium [27], and increased levels of NR2B could amplify this process.

Research suggests that ERs are involved in the neuroprotection of estrogen against glutamate-induced neurotoxicity through attenuation of glutamate-induced calcium overload by modulating NMDA receptors [15]. However, 17α-estradiol has weak estrogen activity and may therefore influence calcium influx during glutamate excitotoxicity through other mechanisms, but its weak estrogenic activity at higher concentrations should be taken into consideration. An ER-independent neuroprotection of 17α-estradiol against glutamate-induced toxicity in cortical neurons via protein phosphatase preservation and through extracellular signal-regulated kinase 1/2 has also been reported [28]. Thus, further studies will help to understand the mechanisms behind the concentration and time dependent neuroprotection by 17α-estradiol in cerebellar neurons.

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Figure legends

Figure 1

Pretreatment with 1 nM 17α-estradiol protects cerebellar granule neurons against excitotoxicity while 10 µM protects during acute treatment. Cells were either treated with 17α-estradiol or DMSO for 24 h (black bars), or treated with 17α-estradiol or DMSO for 15 min (white bars) and then in the presence of glutamate for 15 min. Control cells were treated similarly, except in the absence of glutamate (black and white bars). Cell death was counted after 24 h. Data are mean ± SE from 3-5 independent experiments based on duplicates or triplicates and presented as percent of respective DMSO control. *$p<0.05$ compared to corresponding DMSO control, black or white bars (ANOVA on ranks, Dunn's *post hoc* test). #$p<0.05$ compared to glutamate bar; black bar (one-way ANOVA, Dunnett's multiple comparisons test) and white bar (ANOVA on ranks, Dunn's *post hoc* test).

Figure 2

Pretreatment with 1 nM and acute treatment with 1 nM or 10 µM of 17α-estradiol reduce glutamate-induced peroxynitrite production. (A) Cells were either treated with DHR (0.1 µM) or DMSO for 24 h before exposure to glutamate (black bars), or treated with DHR (0.1 µM) or DMSO for 15 min and then in the presence with glutamate (white bars). Cell death was counted after 24 h. Data are mean ± SE from 2 independent experiments performed in triplicates and presented as percent of DMSO control. *$p<0.05$ compared to DMSO control (one-way ANOVA, Dunnett's multiple comparisons test). #$p<0.05$ showing difference between respective glutamate control and DHR, black and white bars (Student's t-test). (B) Neurons were either untreated or treated with 17α-estradiol or DMSO for 24 h before loading with DHR for 30 min or loaded with DHR for 30 min prior to pre-incubation with 17α-estradiol or buffer for 15 min before and during glutamate exposure. Cells were exposed to

glutamate for 15 min. Oxidation of DHR was measured at time point 10 min during glutamate exposure (first bars) and after 1 h (second bars). The fluorescent intensity of DHR was corrected with the fluorescent intensity of Hoechst 3342. Bars represent mean ± SE from 4-5 independent experiments based on 3-8 replicates in each experiment. *$p<0.05$ compared to corresponding DMSO control; start (ANOVA on ranks, Dunn's *post hoc* test). #$p<0.05$ compared to corresponding DMSO control, 1 h (one-way ANOV, Dunnett's multiple comparisons test).

Figure 3

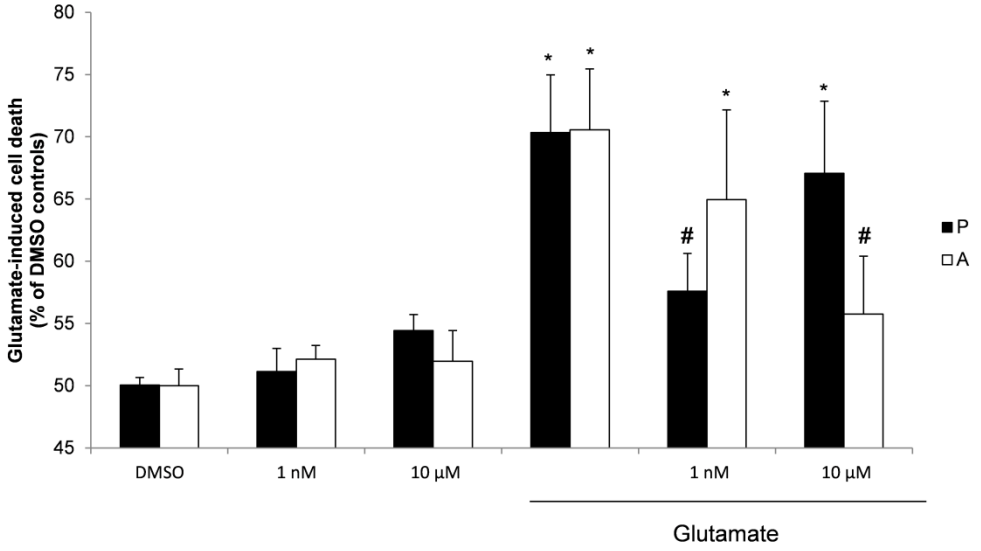
Glutamate treatment and pretreatment with 10 µM 17α-estradiol reduce intracellular glutathione levels. Neurons were untreated (white bars) or treated with 17α-estradiol 24 h (black bars) before they were treated with buffer, DMSO, glutamate or with glutamate and 17α-estradiol (black and white bars). Total GSH levels were measured 24 h after glutamate exposure. GSH levels are corrected against fluorescent intensity with Hoechst 3342 and are shown as percent of buffer control. Bars represent mean ± SE from 3-4 independent experiments based on 6-16 replicates. *$p<0.05$ compared to buffer control when normalized to buffer control (ANOVA on ranks, Dunn's *post hoc* test).

Figure 4

17α-Estradiol changes intracellular glutamate-induced calcium response and NR2B expression. (A) Calcium response in neurons stimulated with glutamate. Arrow indicates injection of glutamate. One representative experiment is shown. (B-C) Calcium response in glutamate treated neurons pretreated with 17α-estradiol (B) and acutely treated with 17α-estradiol (C). Representative graphs based on the mean from 5 independent experiments are shown. (D) Calcium response in glutamate stimulated neurons pretreated or acutely treated with 17α-estradiol after glutamate injection (first bar) and after 10 s (second bar). Buffer

control was not given glutamate. Bars represent mean ± SE from 6 independent experiments. *p<0.05 significantly different from buffer control when normalized to glutamate, start (ANOVA on ranks, Tukey test). #p<0.05 significantly different from buffer control when normalized to glutamate, 10 s (ANOVA on ranks, Tukey test). (E) Cells were untreated or treated with 17α-estradiol or DMSO for 24 h before harvested for western. One representative blot with NR2B and β-actin is shown. (F) Protein levels of NR2B was quantified and shown relative to DMSO treated cells. Bars represent mean ± SE from 4 independent experiments. *p<0.05 compared to DMSO control (ANOVA on ranks, Dunn's *post hoc* test).

Figure 1


Glutamate-induced cell death
(% of DMSO controls)
80
75
70
65
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50
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DMSO
1 nM
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Figure 2

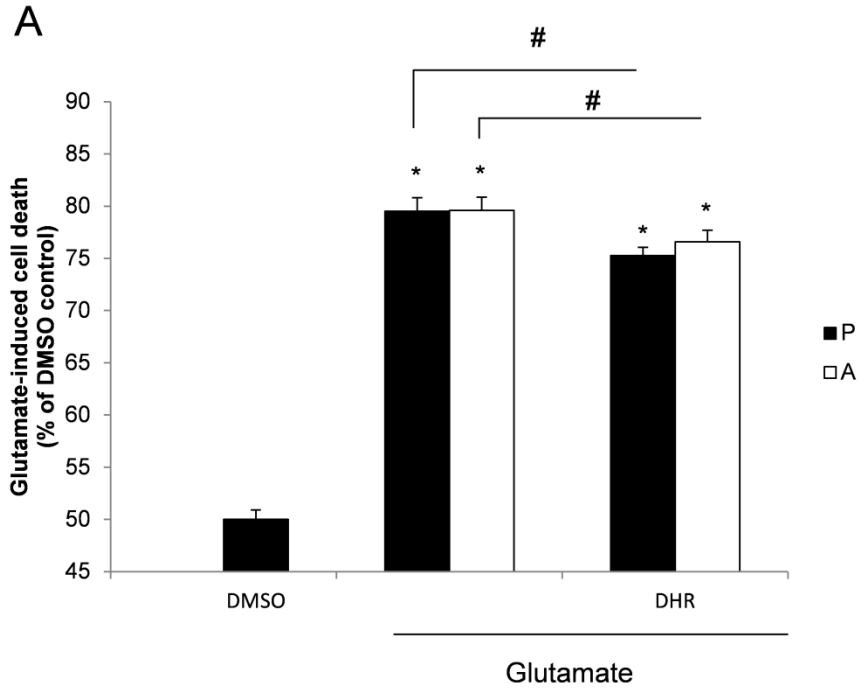


Figure 2

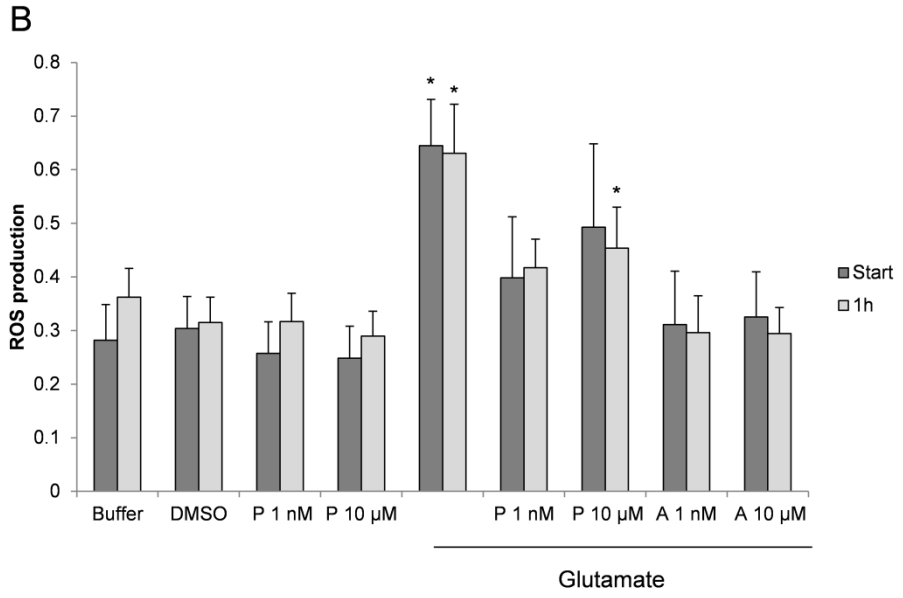


Figure 3

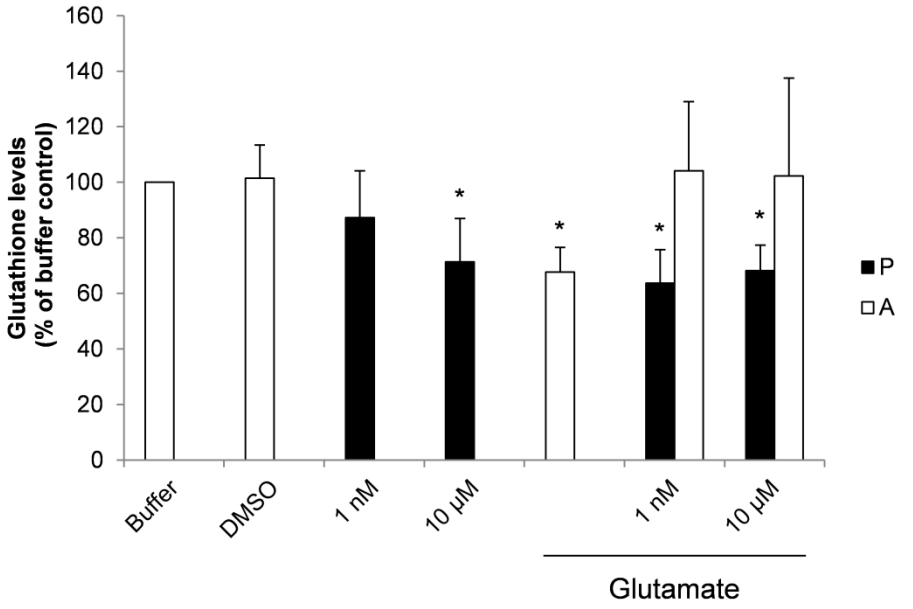

Glutathione levels
(% of buffer control)
160
140
120
100
80
60
40
20
0
Buffer
DMSO
1 nM
10 µM
1 nM
10 µM
Glutamate
P
A
*
*
*
*

Figure 4

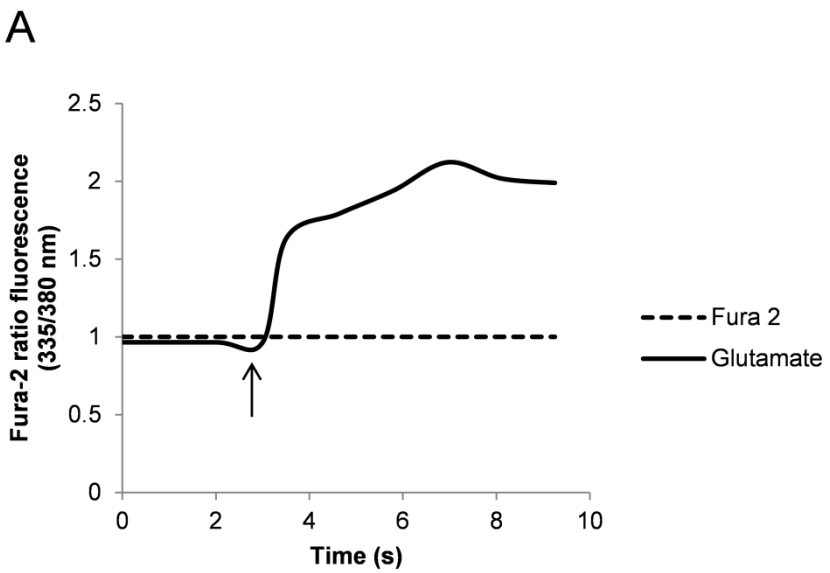


Figure 4

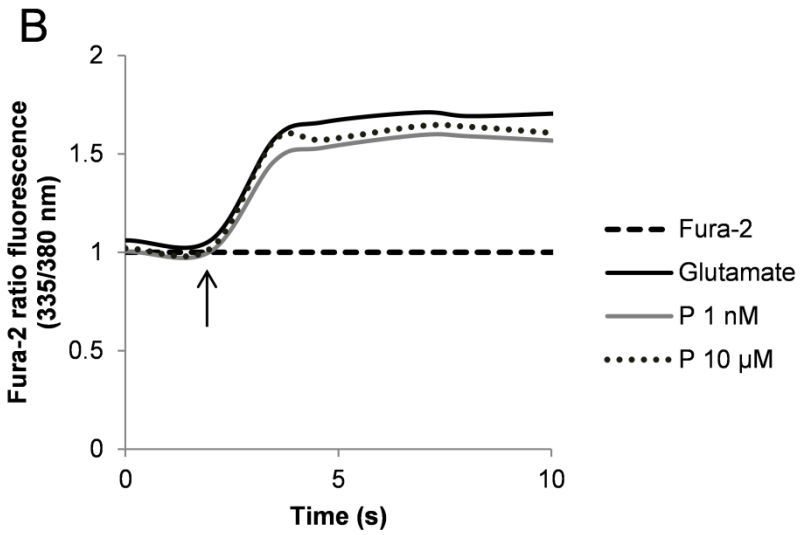


Figure 4

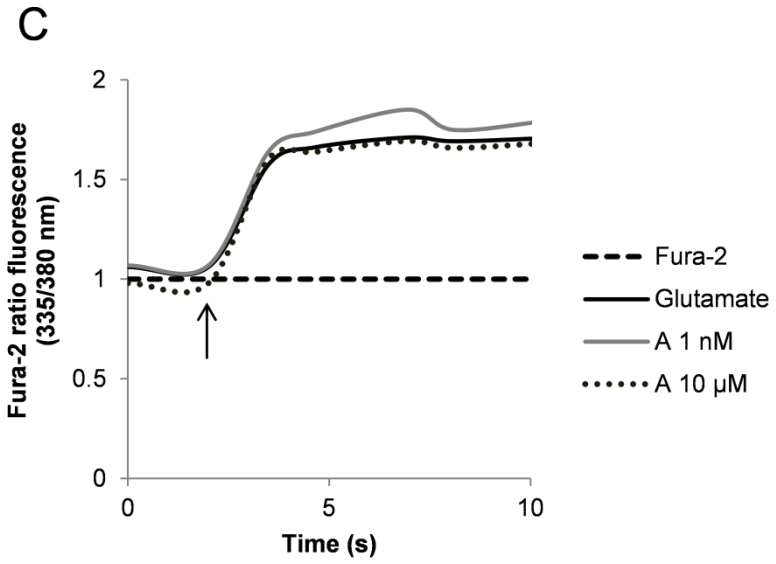


Figure 4

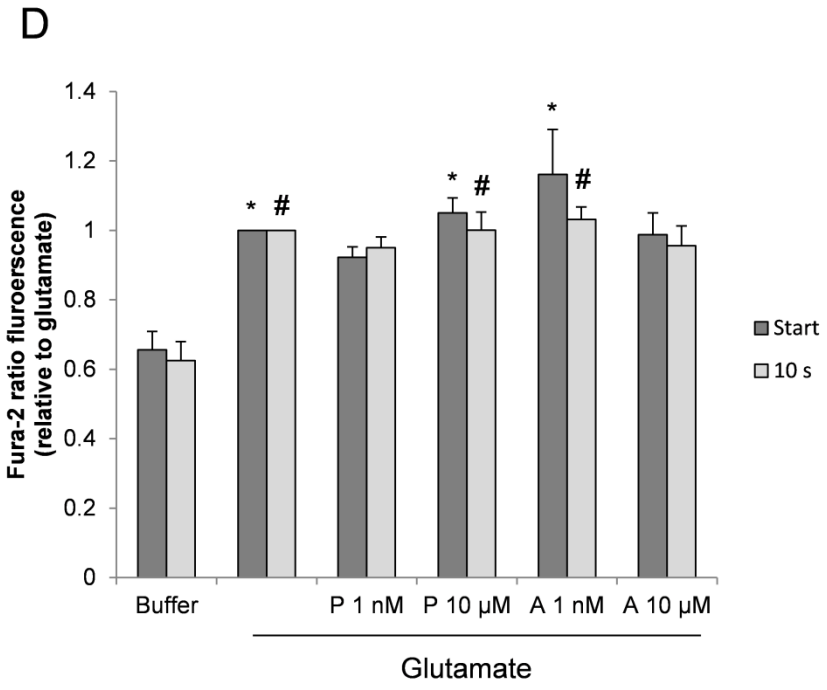
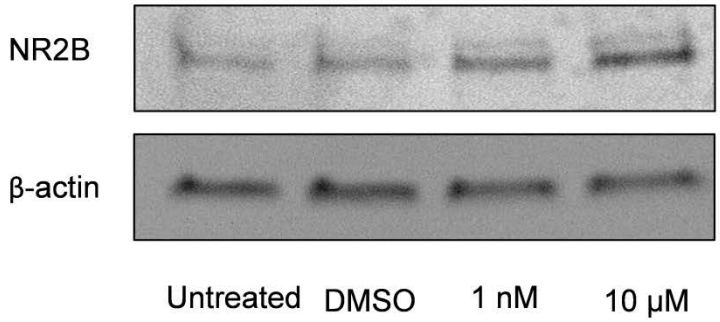
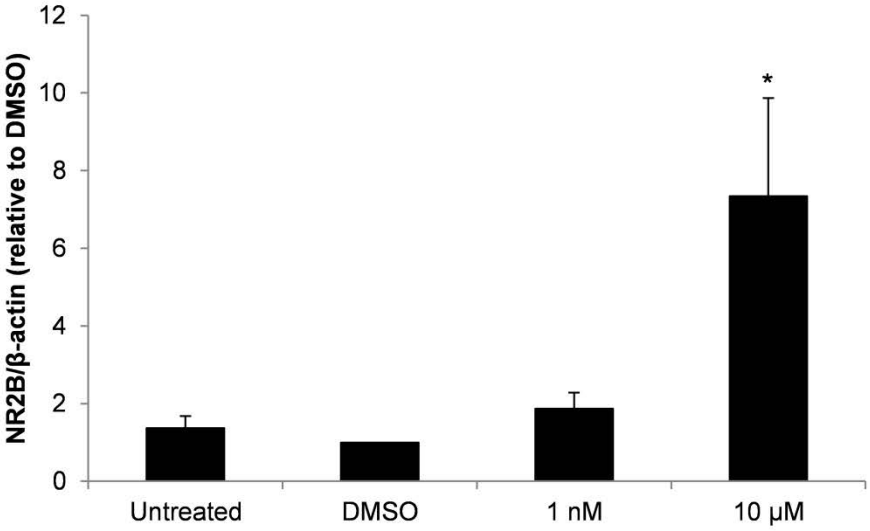


Figure 4

E


NR2B
β-actin
Untreated
DMSO
1 nM
10 μM

F


NR2B/β-actin (relative to DMSO)
12
10
8
6
4
2
0
*
Untreated
DMSO
1 nM
10 μM