

# Oestrogen effects on kainate-induced toxicity in primary cultures of rat cortical neurons

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**Abstract.** Oestrogens protect neurons against excitatory amino acid-induced toxicity; however data on their interaction with particular subtype of glutamate receptors are sparse. Therefore in the present study we investigated oestrogen effects on kainate neurotoxicity in primary cortical neurons. The data showed that both oestradiol-17 $\beta$  and oestrone (100 nM and 200 nM) reduced kainate toxicity by ca. 40%. Since tamoxifen only partly inhibited the above effects, we suggest that both genomic and non-genomic mechanisms are involved in the anti-kainate action of oestrogens.

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**Key words:** oestradiol-17 $\beta$ , oestrone, tamoxifen, kainate neurotoxicity, primary culture, cortical neurons

It is generally accepted that oestrogens affect neuronal outgrowth, differentiation and survival by either classic actions - when they activate transcription *via* nuclear receptors, or non-genomic actions - when they rapidly activate signal transduction pathways. A large number of effects develop *via* the genomic pathway on which oestrogen binds to the intracellular receptor -  $\alpha$  or  $\beta$ . The oestrogen receptor (ER) is a ligand-activated transcription factor that binds to specific DNA sequences - oestrogen response elements (EREs). However, oestrogens may also affect the expression of genes whose promoters do not contain EREs, but respond to factors by acting through other response elements such as AP-1 and SRE sites (Waters et al. 1997). A novel pathway of action of gonadal steroids on the central nervous system was found by showing rapid and reversible changes in the membrane excitability after steroid application. There is evidence that a rapid action may involve membrane or intracellular receptors that are coupled to ion channels. In this regard, the interaction between oestrogens and membrane excitatory amino acid (EAA) receptors seems particularly interesting. Recently it has been reported that there exists a distinct oestrogen-binding site, which appears to be coupled to AMPA/kainate receptors by a cAMP-dependent phosphorylation process (Gu et al. 1999).

Local administration of oestradiol potentiates excitatory responses to glutamate and quisqualate in Purkinje cells (Smith 1991), and to kainate in hippocampal neurons (Gu and Moss 1996). Accordingly, oestradiol lowers the seizure threshold in both women (Backstrom et al. 1990) and experimental animals (Wilson 1992). On the other hand, an increasing body of evidence indicates that oestrogens may reduce the EAA-induced neuronal injury (Goodman et al. 1996, Singer et al. 1999).

The majority of studies on protective effects of oestrogen against EAA toxicity concerns NMDA and glutamate, whereas little is known about functional interactions between oestrogens and kainate receptors. Therefore, we addressed that problem of oestrogen effects on kainate-induced toxicity in rat cortical neurons in primary cultures. In addition to oestradiol-17 $\beta$ , oestrone was tested, as it occurs in abundance in human brain (Lanthier and Patwardhan 1986). In order to verify involvement of intracellular oestrogen receptors, the non-steroid antioestrogen tamoxifen was used.

Primary cortical neurons were prepared, as described previously (Koch and Choi 1987, Dawson et al. 1991), from rat embryos at 16-17 days gestation. Briefly, corti-

ces were removed under a dissecting microscope, washed, minced, and finally dissociated using a nylon mesh (120  $\mu$ M and 30  $\mu$ M in size). Cells were then placed on poly-L-lysine-coated plates at a density of  $1 \times 10^6$  cells per 2 cm<sup>2</sup> well. They were cultured for 11 days prior to experimentation in Eagle's Minimum Essential Medium (Lab. Sera and Vaccines, Lublin, Poland) containing a heat-inactivated 10% foetal calf serum (Hungarpol, Hungary) and an oestrogen-free insulin-transferrin-selenium supplement (Gibco). In order to discourage non-neuronal cell proliferation, cytosine  $\beta$ -D-arabino-furanoside (Sigma; 1  $\mu$ M) was applied. Thus the level of astrocytes identified by their content of the intermediate filament protein GFAP (glial fibrillary acidic protein) did not exceed 10%. The cultures were kept at 37° C in a humidified atmosphere containing 10% CO<sub>2</sub>.

After 11 days of culturing, the cells were pre-treated with oestradiol-17 $\beta$  (Sigma; 50 nM, 100 nM, 200 nM), oestrone (Sigma; 50 nM, 100 nM, 200 nM), and tamoxifen (TOCRIS; 100 nM), a non-steroidal oestrogen receptor antagonist. Twenty four hours later the cells were exposed for another 24 h to kainate (TOCRIS; 150  $\mu$ M) in a medium containing 21 mM glucose instead of serum. Both oestrogens and tamoxifen were dissolved in water solution of 20% 2-hydroxypropyl- $\beta$ -cyclodextrin (Research Biochemicals International) and added in volume of 10  $\mu$ l per 1 ml of culture medium.

Kainate neurotoxicity was detected by an efflux of lactate dehydrogenase (LDH) into culture media 24 h after treatment with kainate. We used a Sigma colorimetric method (Sigma Procedure No. 500), according to which the amount of colored hydrazone, formed in a reaction of pyruvic acid with 2,4-dinitrophenylhydrazine, is inversely proportional to the LDH activity in the sample and can be quantified by measuring the wave-length absorbance at 400-550 nm.

We normalized data to the amount of LDH released from vehicle-treated cells receiving kainic acid (100%) and expressed it as a percentage of LDH from 8-10 separate platings  $\pm$  SEM. Statistical comparisons were carried out using the Student's one-tailed *t*-test.

We observed that a 24 h exposure of primary cortical neurons to kainate resulted in a dose-dependent increase in cell damage, as detected with a cell lysis and lactate dehydrogenase (LDH) efflux into the culture media (Fig. 1). This finding is in accordance with the results of Koch and Choi (1988) who demonstrated that 5-min. treatment with kainate, 200  $\mu$ M, destroyed most of the

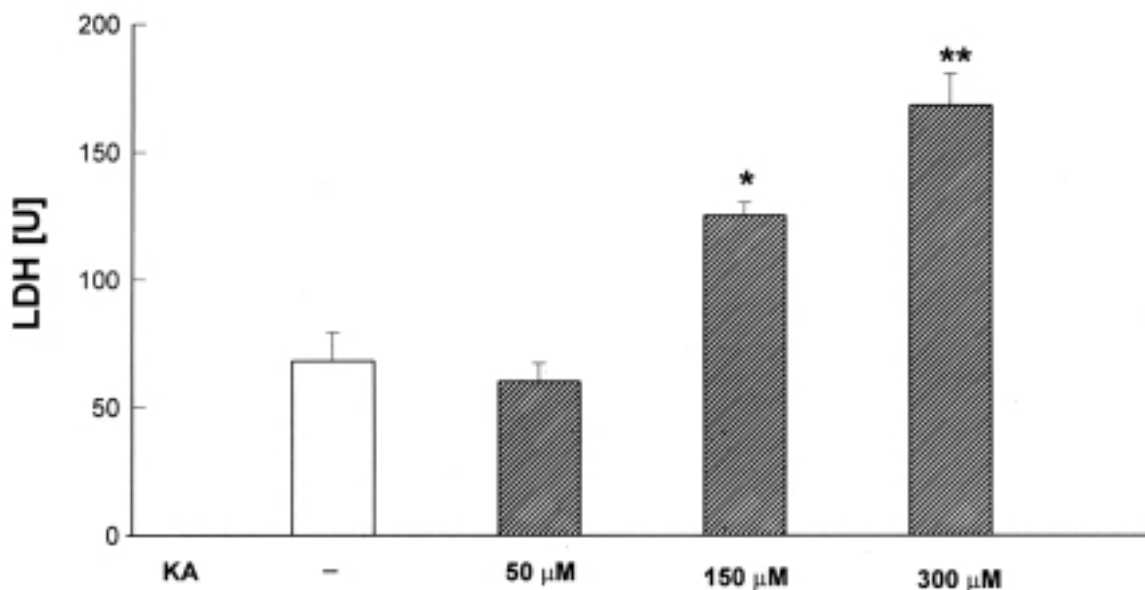


Fig. 1. The effect of kainate (KA) on LDH efflux from rat cortical neurons in primary cultures. The results are shown as mean values from 8-10 separate platings ( $1 \times 10^6$  cells/ $2 \text{ cm}^2/\text{ml}$ )  $\pm$  SEM. Asterisks indicate statistically significant effects (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

NADPH-phosphate diaphorase-containing neurons. Those authors showed that prolongation of the kainate exposure time up to 24 h increased the neuronal loss up to 95%.

In our study both oestradiol-17 $\beta$  (100 and 200 nM) and oestrone (200 nM) protected cells from kainate neurotoxicity, since they inhibited LDH release by about 40% (Fig. 2). The only available report on oestrogen interaction with kainate, (Regan and Guo 1997), showed that continuous 24-h exposure of mouse cortical cells to kainate resulted in the death of about 80% of neurons. When the cells were exposed to kainate together with oestrogens, the neurotoxic effect was partly blocked. It is noteworthy that these authors observed protective effects of oestrogens at concentrations more than 60 times higher than those used in our study. The higher efficiency of oestradiol-17 $\beta$  in our experiment may possibly be due to the 24-h pretreatment which allows sufficient time to activate a hormone-mediated protective mechanism.

Protective effects of oestrogens against glutamate were previously observed by Goodman et al. (1996). Those authors indicated that 2-h pretreatment with 100 nM – 10 mM oestradiol-17 $\beta$  or oestrone resulted in significant protection against glutamate toxicity, as well as against glucose deprivation,  $\text{FeSO}_4$  and amyloid beta-peptide toxicity. Some of those effects were also

observed by Singer et al. (1996, 1999) who showed that 24-h pretreatment with 15 and 50 nM oestradiol-17 $\beta$  significantly reduced LDH efflux from primary cortical neurons exposed for 5 min. to glutamate. Moreover, they demonstrated that tamoxifen blocked the protective effects of oestradiol-17 $\beta$ , having suggested that activation of the classic oestrogen receptor was necessary for oestrogen neuroprotection against glutamate toxicity.

We observed that tamoxifen partly inhibited oestrogen effects (the level of LDH released into the culture media was 18% to 34% higher in comparison to the effects of oestrogens alone (Fig. 2)), which supports the hypothesis about involvement of both genomic and non-genomic mechanisms in the protective effects of oestrogens. It should be mentioned that the lower concentration of tamoxifen (50 nM) which was used in the preliminary study did not affect oestrogen neuroprotective effects. The 100 nM concentration of tamoxifen chosen for the present study was the lowest effective dose which by itself had no effect on LDH level in control cultures.

The potentially beneficial genomic effects of oestrogens on neurons include increased expression of neurotrophins and their high-affinity receptors, induction of calcium binding proteins, and stabilization of microtubules (Miranda et al. 1994). It is also suggested

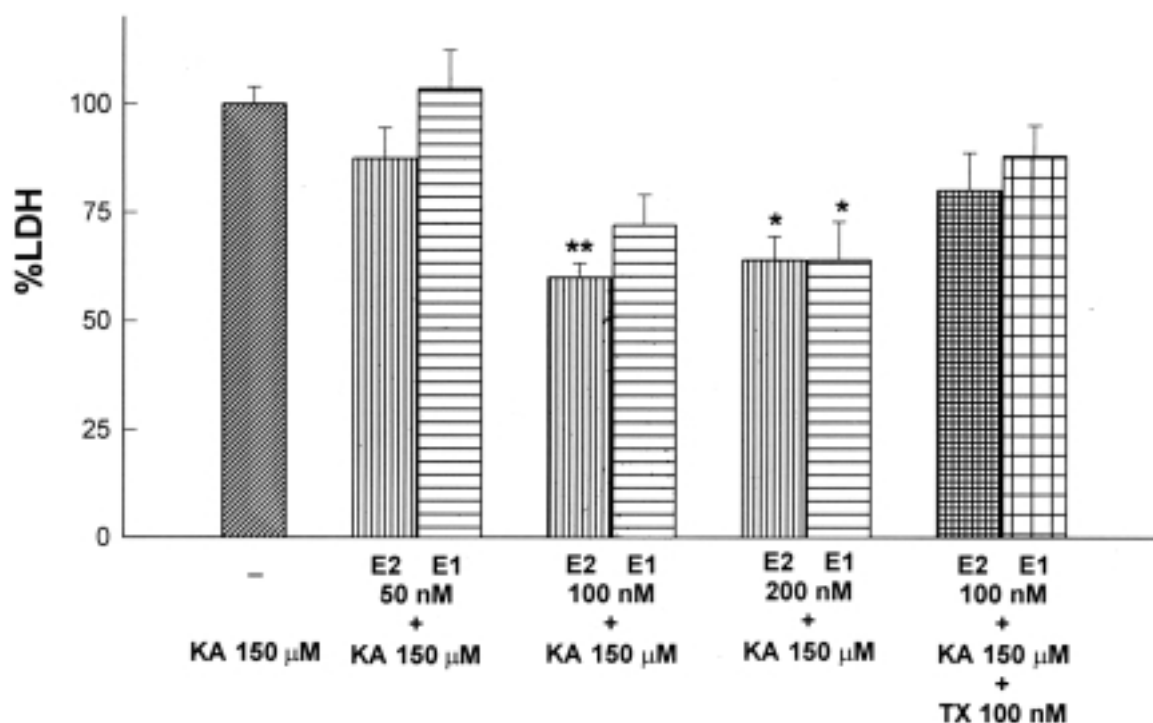


Fig. 2. Effects of oestradiol-17 $\beta$  (E2), oestrone (E1), and tamoxifen (TX) on the kainate (KA)-induced toxicity in rat cortical neurons in primary cultures. The results are shown as a percentage of LDH from 8-10 separate platings ( $1 \times 10^6$  cells/2 cm<sup>2</sup>/ml)  $\pm$  SEM. Asterisks indicate statistically significant effects (\* $P$ <0.05; \*\* $P$ <0.01).

that oestrogens significantly enhance neuronal viability by increasing expression of antiapoptotic proteins Bcl-2 and Bcl-xl (Singer et al. 1998, Pike 1999). Oestrogens may also protect neurons through some non-genomic mechanisms. Such oestrogen effects have been demonstrated for a variety of cytotoxic insults. Most of them, especially regarding amyloid beta-protein, hydrogen peroxide, and glutamate, have been suggested to be due to free radical scavenging (Green et al. 1998). Weaver et al. (1997) observed that the oestrogen neuroprotection against NMDA toxicity was not blocked by tamoxifen. Additionally, it was demonstrated that oestrogens blocked the vascular and neuronal L-type voltage-gated calcium channels and could thus attenuate the cellular injury associated with calcium influx *via* those channels (Mermelstein et al. 1996).

In conclusion, our study shows that oestradiol-17 $\beta$  and oestrone attenuate kainate neurotoxicity in primary cultures of rat cortical neurons. We suggest that the neuroprotective effects of oestrogens against kainate neurotoxicity are partly mediated by intracellular hormone receptors.

Supported by the State Scientific Research Committee (KBN), grant no. 4 P05A 035 14.

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*Received 30 November 1999, accepted 10 March 2000*