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Zearalenone alters the excitability of rat neuronal networks after acute *in vitro* exposure

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ABSTRACT

Zearalenone (ZEA) is a mycotoxin produced by *Fusarium* species, detectable in various cereals and processed food products worldwide. ZEA displays a significant estrogenic activity, thus its main health risk is the interference with sexual maturation and reproduction processes. However, in addition to being key hormonal regulators of reproductive function, estrogenic compounds have a widespread role in brain, as neurotrophic and neuroprotective factors, and they may influence the activity of several brain areas not directly linked to reproduction, as well. Therefore, in the present study, acute effects of ZEA were studied on certain neuronal functions in rats.

Experiments were performed on rat brain slices or live rats. Slices were incubated in ZEA-containing (10–100 μM) solution for 30 min. Electrically evoked and spontaneous field potentials were studied in the neocortex and in the hippocampus. At higher concentrations, ZEA incubation of the slices altered excitability and the pattern of epileptiform activity in neocortex and inhibited the development of LTP in hippocampus.

For the verification of these *in vitro* results, *in vivo* electrophysiological and immunohistochemical investigations were also performed. ZEA was administered systemically (5 mg/kg, i.p.) to male rats and somatosensory evoked potentials and neuronal activation studied by c-fos expression were analyzed. No neuronal activation could be demonstrated in the hippocampus within 2 h of the injection. In the somatosensory cortex, ZEA did not change *in vivo* evoked potential parameters, but the activation of a small neuronal population could be demonstrated with the c-fos technique in this brain area. This result could be associated with the ZEA-induced alteration of epileptiform activity observed *in vitro*.

Altogether, the toxin altered the excitability and plasticity of neuronal networks after direct treatment in slices, but the effects were less prominent on the given brain areas after systemic treatment *in vivo*. A probable explanation for the partial lack of *in vivo* effects may be that after a single injection, ZEA did not cross the blood-brain barrier at sufficient rate to allow the build-up of comparable concentrations in the investigated brain areas. However, in case of compromised blood-brain barrier functions or long-term repeated exposure, alterations in cortical and hippocampal functions cannot be ruled out.

1. Introduction

Zearalenone (ZEA) is a mycotoxin produced by *Fusarium* species, it can be detected in cereals, feed and processed food products worldwide. The health risks presented by ZEA are mainly linked with its estrogenic activity, it may interfere with sexual maturation and reproductive

Abbreviations: α-ZAL, α-zearalanol; ACSF, artificial cerebrospinal fluid; AUC, area under the curve; BBB, blood-brain barrier; CA1, Cornu Ammonis 1 region of the hippocampus; CNS, central nervous system; DMSO, dimethyl sulfoxide; E2, 17β-estradiol; EFP, evoked field potential; EPSP, excitatory postsynaptic potential; ER, estrogen receptor; GPER1, G-protein-coupled estrogen receptor 1; I-O curve, input-output curve; i.p., intraperitoneal; LEC, lateral entorhinal cortex; LTP, long-term potentiation; MFR, magnesium-free ACSF; POP-spike, population spike; S1, primary somatosensory cortex; S2, secondary somatosensory cortex; s.c., subcutaneous; T, voltage threshold of stimulation; TBS, theta burst stimulation; ZEA, zearalenone.

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functions (Zinedine et al., 2007). At high dietary concentrations, it may cause decreased fertility, resorption or deformities of fetuses and abortion in farm animals, the pig being the most sensitive species (Steinberg, 2013). Consumption of ZEA-contaminated cereals has been linked with premature sexual development and early telarche in young girls (Szuets et al., 1997).

Estrogens are steroid hormones that include estrone, estriol, and 17β-estradiol (E2), the last of which is the most potent endogenous estrogenic compound (Heldring et al., 2007). Estrogens act *via* three types of estrogen receptors (ERs) that are known as ERα, ERβ and the structurally unrelated G-protein-coupled ER1 (GPER1) (Almey et al., 2015). According to the traditional view, ERα and ERβ are located in the cytoplasm and functioning as nuclear receptors (Parikh et al., 1987). However, recent studies indicated that all three types of receptors may be localized in the cell membrane, allowing rapid non-genomic effects such as altering membrane permeability and activating second messenger cascades (Boonyaratanakornkit and Edwards, 2007; Almey et al., 2015).

ERs are expressed in central nervous system (CNS) structures associated with reproductive functions such as the hypothalamus, but also in various other brain areas such as the hippocampus, amygdala, cerebellum and different cortical regions in both sexes (Almey et al., 2015). Estrogens are important trophic factors for neurons, enhancing synaptogenesis during ontogenesis, but their neuroprotective effects have been demonstrated also in adulthood. The role of ERs has been demonstrated in cognitive functions and memory processes (Heldring et al., 2007; Lu et al., 2019). The effects of E2 and other estrogenic compounds on neuronal excitability and plasticity has been studied most extensively in the CA1 region of the hippocampus (Sheppard et al., 2019). It was demonstrated that E2 increased glutamate-mediated neuronal excitability in hippocampal rat brain slices (Teyler et al., 1980; Foy et al., 1999; Oberlander and Woolley, 2016) and in cultured hippocampal rat neurons *in vitro* (Zadran et al., 2009). E2 treatment of rats subsequently increased Ca^{2+}-currents in CA1 hippocampal slices (Joels and Karst, 1995). Based on various *in vivo* epilepsy models, estrogens are considered to be proconvulsant agents (Scharfman and MacLusky, 2006; Younus and Reddy, 2016).

It is known that ZEA acts as an agonist on both α and β type estrogen receptors, although with a lower affinity than E2 (Takemura et al., 2007). For interaction of ZEA and GPER1, only indirect data are available to date (Yip et al., 2017). ZEA effects on the CNS are not well known, but due to its estrogenic properties, it may affect neuronal network functions. ZEA treatment of immature female rats caused precocious puberty and increased expression of the neuropeptide kisspeptin in the hypothalamus (Kriszt et al., 2015). Very few studies focused on ZEA-mediated functional nervous system alterations not linked to reproduction. It has been shown that α-zearalanol (α-ZAL), a metabolite of ZEA ameliorates memory impairment in ovariectomized mice (Dong et al., 2013). α-ZAL has been tested also *in vitro*, on rat hippocampal neuronal cultures, and its neuroprotective effect against amyloid-β induced neurotoxicity has been demonstrated (Dong et al., 2007). However, α-ZAL displays a higher affinity to ERs than the parent compound ZEA (Takemura et al., 2007).

The aim of the present study was to test the acute effects of ZEA on CNS neuronal networks with direct *in vitro* and with *in vivo* administration. We focused on brain areas which are not traditionally linked to reproductive functions, *i.e.* the somatosensory cortex and the CA1 region of the hippocampus. Brain slices of untreated rats were incubated in ZEA-containing solution, and cortical and hippocampal field potentials were studied. On the other hand, after systemic administration of a single dose of ZEA *via* i.p. injection, c-fos immunohistochemistry was applied to identify the brain areas in which ZEA caused neuronal activation. To test the acute effect on neuronal excitability and information processing *in vivo*, somatosensory evoked potentials were recorded with the stimulation of the tibial nerve in urethane-anesthetized rats, before and after ZEA i.p. injection.

2. Materials and methods

2.1. Animals

Male Wistar rats (Toxi-coop Ltd., Budapest, Hungary) weighing 150−280 g or 300−470 g were used for the *in vitro* and *in vivo* experiments, respectively. Experiments were carried out in accordance with the Hungarian Act of Animal Care and Experimentation (1998, XXVIII) and with the directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Experimental protocols were approved by the Animal Care and Use Committee of Eötvös Loránd University and Budapest Animal Health Care Authority. All possible efforts were made to minimize the number of animals used and to minimize animal suffering. Rats were kept under constant 12 h light/dark cycle and controlled temperature (22 ± 2 °C). Standard pellet food and tap water were available *ad libitum*.

2.2. Chemicals

Zearalenone (ZEA) was purchased from Bio-Techne R&D Systems Ltd. (Budapest, Hungary). All other compounds were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary) if not stated otherwise. ZEA was dissolved in dimethyl sulfoxide (DMSO) and artificial cerebrospinal fluid (ACSF) and stored as 500 μM stock solution at −20 °C for *in vitro* experiments or DMSO and sterile physiological saline and stored as 4.156 mM stock solution at −20 °C for *in vivo* experiments.

2.3. In vitro electrophysiology

Rats (n = 40) were anesthetized with chloral-hydrate (350 mg/kg i.p.), then decapitated with guillotine. The brain was quickly removed and 400 μm thick horizontal slices were cut with a vibratome (EMS-4000, Electron Microscopy Sciences, Hatfield, USA). The slices were allowed to regenerate for at least an hour at room temperature in ACSF saturated with carbogene (5 % CO_2 and 95 % O_2). The composition of the ACSF was (in mM) 126 NaCl, 26 $NaHCO_3$, 1.8 KCl, 1.25 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 10 glucose (pH 7.2–7.4). Then, individual slices were kept in a small, special incubating chamber in 2 mL standard ACSF or ZEA-containing ACSF (10 μM, 50 μM, 100 μM) for 30 min. To test the effect of the vehicle, a DMSO control group was also included; here, DMSO concentration was equivalent to that of the 100 μM ZEA group (0.83 V/V%). Applied toxin concentrations were set up based on other, *in vitro* cell culture studies (Abid-Essefi et al., 2004; Venkataramana et al., 2014). After pretreatment, the slices were placed into an Oslo-type recording chamber (FST Ltd., Vancouver, Canada), heated to 33 ± 1 °C and perfused with carbogenated ACSF (2.5 mL/min). Slices were selected for hippocampal/cortical recording based on the rat brain stereotaxic atlas (Paxinos and Watson, 1998).

In the hippocampus, electrically evoked field potentials were recorded in the CA1 region: a bipolar stimulation electrode was placed at the Schaffer collaterals and square voltage pulses of 100 μs width delivered (BioStim, Supertech Ltd., Pécs, Hungary). Two extracellular glass microelectrodes (5−15 MΩ) filled with 1 M NaCl were positioned into the *stratum radiatum* and the *stratum pyramidale* to register excitatory postsynaptic potentials (EPSPs) and population spikes (POP-spikes), respectively (Fig. 1D). In the cortex, the two recording electrodes were placed into layer 2/3 of the lateral entorhinal (LEC) and secondary somatosensory (S2) cortices and the stimulation electrode was positioned in layer 6 of the S2, below the recording electrode. Evoked potentials were recorded from the latter area and spontaneous activity elicited with magnesium free ACSF (MFR) was recorded from both cortical regions (Fig. 1A). Signals were processed by an Axoclamp 2B (Axon Instruments, Union City, CA, United States), band-pass filtered (0.16 Hz-1 kHz) and amplified 1000x (BioAmp, Supertech Ltd., Pécs, Hungary) and digitized by an NI-6023E A/D card (National Instruments, Austin,

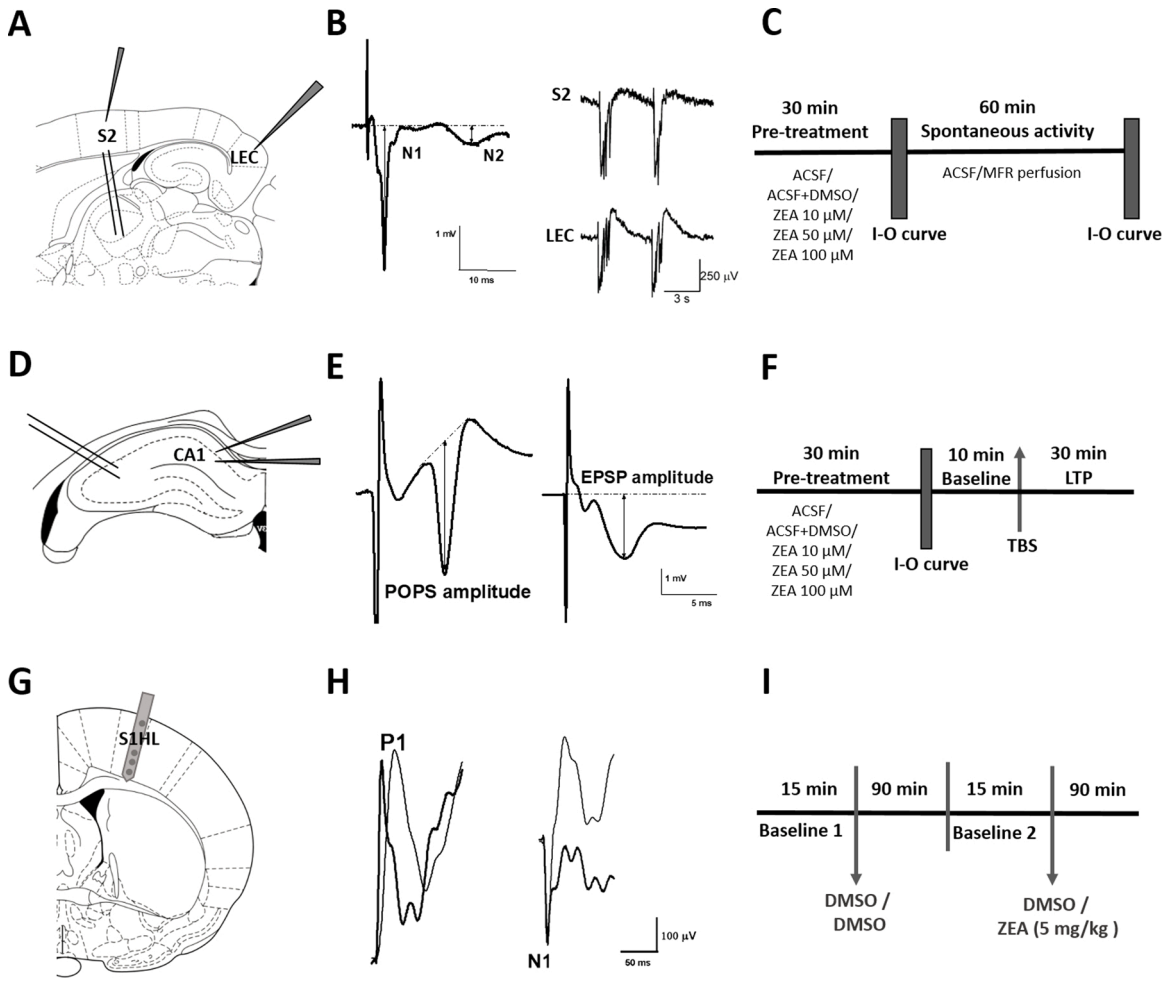

Fig. 1. Protocol for *in vitro* and *in vivo* electrophysiological experiments.
(A) Positioning of the electrodes in the neocortex slice: stimulation in layer 6 of the secondary somatosensory cortex (S2) and recording in layer 2/3, an additional recording electrode in layer 2/3 of the lateral entorhinal cortex (LEC). (B) Representative evoked EPSP, with the analyzed parameters (left trace) and representative epileptiform bursts recorded during MFR perfusion (right traces). (C) Timeline of experiments for neocortex slices. (D) Positioning of the electrodes in the hippocampus slice: stimulation at the Schaffer-collaterals and recording in *stratum radiatum* and *stratum pyramidale* of CA1. (E) Representative evoked POPS (left trace) and EPSP (right trace) with the analyzed parameters. (F) Timeline of experiments for hippocampus slices. (G) Positioning of the 16-channel vertical electrode array in the hindlimb area of the primary somatosensory cortex of anesthetized rats. (H) Representative EFPs recorded in two different rats, in layer 2/3 (left panel) and in layer 5 (right panel), with analyzed peaks. (I) Timeline of *in vivo* experiments.

Texas) for off-line analysis. Processed signals were recorded and analyzed with the SPEL Advanced Intrasys computer program (Experimetria Ltd., Budapest, Hungary) and custom-written Matlab-based software (by Sándor Borbély) respectively.

Basic network activity was tested by determining the voltage threshold of the evoked field potentials (T). Then an input-output (I-O) curve was recorded by gradually increasing stimulation intensity until 4 T in cortex and 3 T in hippocampus. In hippocampus, this was followed by evoking long-term synaptic potentiation (LTP), with a stimulation intensity necessary to evoke 70 % of the maximal POP-spike. A 10-min baseline was recorded, then theta burst stimulation (TBS) was applied, followed by an additional 30-min recording (Fig. 1F). In the cortex, following I-O curve recording in S2, a one-hour long field potential measurement was recorded without stimulation in both cortical areas either with ACSF or MFR perfusion to detect spontaneous seizure-like activity. Finally, another I-O curve was recorded (Fig. 1C).

In the hippocampus, the amplitude of EPSPs and POP-spikes were analyzed (Fig. 1E). For cortical evoked potentials, the amplitudes of an early (N1) and a late (N2) negative peak were measured (Fig. 1B). Spontaneous seizure-like activity was characterized by latency time, number and burst length of events. The latter parameters were quantified during the last 5 min of the MFR perfusion. From the number of events, average frequency was calculated (n/300 s). Active time percentage was calculated as (average burst length in sec * number of bursts)/300 s.

One-way ANOVA (with Levene's test for homogeneity of variances and Tukey post hoc test, $p < 0.05$) or repeated measures ANOVA (with Sidak's test for multiple comparisons, $p < 0.05$) were used for statistical analysis to estimate significant differences between control and treated groups. Data are presented as means ± S.E.M.

2.4. *In vivo electrophysiology*

Rats (n = 12) were anesthetized with urethane (1.2 g/kg, i.p.) and fixed in a stereotaxic frame (David Kopf) with the top of the skull set horizontal to comply with the rat brain atlas (Paxinos and Watson, 1998). A 4 × 3 mm craniotomy centered 1.5 mm posterior to Bregma and 1.5 mm lateral to the midline was made in the right hemisphere. Rectal temperature was maintained at 37 °C.

Hindlimb area of the primary somatosensory cortex (S1) was localized as described previously (Borbély et al., 2016). A 16-channel vertical electrode array (Neuronelektród Ltd., Budapest, Hungary) was lowered

to a depth of 1–1.2 mm below the dura to record evoked field potentials (EFPs) from different cortical layers (interelectrode distance: 150 μm; contact diameter: 40 μm Pt-Ir) (Fig. 1G). A 1.1 mm stainless steel reference electrode (Fine Science Tools, USA) was placed above the cerebellum. Two stimulating needle electrodes were inserted near the tibial nerve to deliver square voltage pulses (1 ms duration, 0.1 Hz) (Master8, A.M.P.I., Jerusalem, Israel). Stimulation intensity corresponding to the double of threshold voltage (9.68 ± 1.29 V) was used for recording.

Recordings lasted for 210 min. Rats were split into two experimental groups in a randomized fashion. Double control group (n = 6) received vehicle (DMSO)-containing saline i.p. two times, at 15 min and 120 min. In treated group (n = 6), DMSO injection was administered at 15 min, then ZEA (5 mg/kg, dissolved in DMSO and saline) was injected at 120 min. Pre-injection periods (between 0−15 min and 105−120 min, respectively) served as baseline. Duration of the recordings after DMSO or ZEA injections (90 min) was selected to ensure compatibility with the parallelly running c-fos activation experiments (Fig. 1I).

The applied dose of ZEA (5 mg/kg) was chosen based on previous similar *in vivo* rat studies (Turcotte et al., 2005; Shin et al., 2009).

EFP signals amplified (Supertech Ltd., Pécs, Hungary), conditioned (filter: 0.1 Hz – 1000 Hz, gain: 5000x) then digitalized at 2048 Hz with 16-bit resolution (Labview; National Instruments, Austin, TX, USA).

Among the EFPs recorded by various contacts, the wave with shortest latency and largest amplitude was selected for EFP analysis. Single EFPs were averaged in 15 min long blocks. Slope of the responses was calculated based on the first 3.5 ms long period of the ascending component of P1 waves for layer 2/3 EFPs and descending component of N1 waves for layer 5 responses (Fig. 1H). Areas under the curves (AUC; integral) were also calculated.

Amplitude, latency, slope and AUC differences between baseline and post-injection values were compared statistically by one-way ANOVA followed by Student-Newman-Keuls post-hoc test ($p < 0.05$). Data are presented as means ± S.E.M.

At the end of the experiment, position of the recording array was marked using current injections to evoke small electrolytic lesions. Then, rats were perfused transcardially and coronal brain sections (50−60 μm) were cut, then stained in gallocyanine solution. Bright-field light-microscopy was used to locate recording sites. Histology verified that recording arrays were placed in the hindlimb area of the S1 (data not shown).

2.5. Detection of neuronal activation with c-fos immunohistochemistry

ZEA was injected to 6 rats i.p. at a dose of 5 mg/kg, while control rats (n = 6) received physiological saline injections containing the vehicle, DMSO. The animals were sacrificed and transcardially perfused 2 h later. Brains were removed, postfixed for a day in 0.1 M phosphate buffer (PB) containing 4 % paraformaldehyde, and then transferred to PB containing 20 % sucrose for an additional day for cryoprotection.

Then, 50 μm thick coronal sections of brain tissue were prepared with a cryostat (Leica CM1520). The sections were stored at 4 °C. Every fifth 50-μm-thick free-floating brain section of ZEA injected and control injected animal was processed for c-Fos immunohistochemistry as described previously (Cservenák et al., 2017). Sections were pre-treated in PB containing 0.3 % hydrogen peroxide for 15 min for quenching of endogenous peroxidase activity. Then, sections were incubated in PB containing 0.5 % Triton X-100 and 3 % BSA for 1 h. Sections were then incubated in anti-c-Fos antiserum (1:2000, Santa Cruz, sc-52) at room temperature for two nights. Then, sections were incubated in biotin-conjugated anti-rabbit secondary antibody (1:800; Jackson Immuno Research, 711-065-152) for 1 h and then in the avidin–biotin–peroxidase complex (ABC; 1:500; Vector Laboratories) for 1 h. The labelling was visualized by nickel-DAB peroxidase technique for 20 min. Sections were covered with DPX Mountant (depex, Sigma 06522) after drying.

Brain areas were identified using the Paxinos & Watson stereotaxic atlas of the rat brain (Paxinos and Watson, 1998). Photomicrographs were taken with a light microscope equipped with a digital camera (Nikon Eclipse Ni, 25.4 2 M P Slider Camera, Spot RT3 software). The numbers of Fos labelled neuronal cell bodies were counted in the hippocampus and the somatosensory cortex using ImageJ software, version 1.50i (ImageJ, RRID SCR_003070, Wayne Rasband, National Institute of Health, Bethesda, MD). The density of labelled neurons was expressed as cell number/mm^2.

The density of c-Fos-positive neurons in response to ZEA was compared to control injections using two-sample t-tests in each brain region. All statistics were performed with Prism 5 for Windows (GraphPad Software, La Jolla, CA). Data are presented as means ± S.E.M.

3. Results

3.1. In vitro electrophysiology after incubation of brain slices with ZEA

Pretreatment with DMSO-containing ACSF did not alter any parameters in any experiment compared to the ACSF control group. Voltage thresholds necessary to evoke field EPSPs or POP-spikes in somatosensory cortex and hippocampus are summarized in Table 1.

3.1.1. Basic network excitability in neocortex

In the somatosensory cortex, there was no difference in voltage threshold values in the different pretreatment groups (Table 1). Concerning the amplitude of the early component of the cortical field EPSPs recorded after preincubation, ZEA pretreatment at lower and medium concentrations significantly modified the shape of the I-O curve, compared to DMSO controls (p-values are 0.0014 and 0.0005, respectively). ZEA pretreatment at 100 μM modified the I-O curve shape in a similar way, but here, the difference was not statistically significant ($p = 0.2090$). In case of treated slices, the curve saturates at lower stimulation intensities compared to the DMSO and ACSF controls, indicating an inhibitory effect. The vehicle, DMSO itself did not influence the shape of the I-O curve significantly ($p = 0.3826$) (Fig. 2A). In case of the late component of the EPSPs, there was no significant difference between the ZEA-treated and the DMSO control groups (Fig. 2B). P-values for late component amplitudes compared to the DMSO control group are 0.9889, 0.3111, 0.8879 and 0.9999 for the ACSF control group and the 10, 50 and 100 μM ZEA preincubation groups, respectively.

I-O curves were recorded again after 1 h perfusion with standard ACSF or MFR, here, the amplitudes of the evoked components recorded

Table 1
The average voltage stimulation thresholds for evoking neocortical and hippocampal evoked potentials are summarized. Data are presented as mean ± S.E.M. In neocortex, n = 12, 12, 11, 11 and 11 for the control, DMSO, 10 μM, 50 μM and 100 μM treated groups respectively. In hippocampus, n = 10, 8, 6, 7 and 6 for the control, DMSO, 10 μM, 50 μM and 100 μM treated groups respectively. There was no significant difference among voltage threshold values compared to the DMSO control group.

	Neocortex	Hippocampus	
	EPSP threshold [V]	EPSP threshold [V]	POP-spike threshold [V]
Control	2.64 ± 0.12	2.67 ± 0.19	3.00 ± 0.20
	($p = 0.1556$)	($p = 0.6316$)	($p = 0.7427$)
DMSO	2.33 ± 0.07	2.38 ± 0.12	2.75 ± 0.16
10 μM	2.45 ± 0.15	2.42 ± 0.24	2.58 ± 0.30
ZEA	($p = 0.8607$)	($p = 0.9997$)	($p = 0.9468$)
50 μM	2.45 ± 0.11	2.17 ± 0.17	2.25 ± 0.11
ZEA	($p = 0.8607$)	($p = 0.8692$)	($p = 0.2897$)
100 μM	2.45 ± 0.08	2.25 ± 0.28	2.17 ± 0.25
ZEA	($p = 0.8607$)	($p = 0.9756$)	($p = 0.1771$)

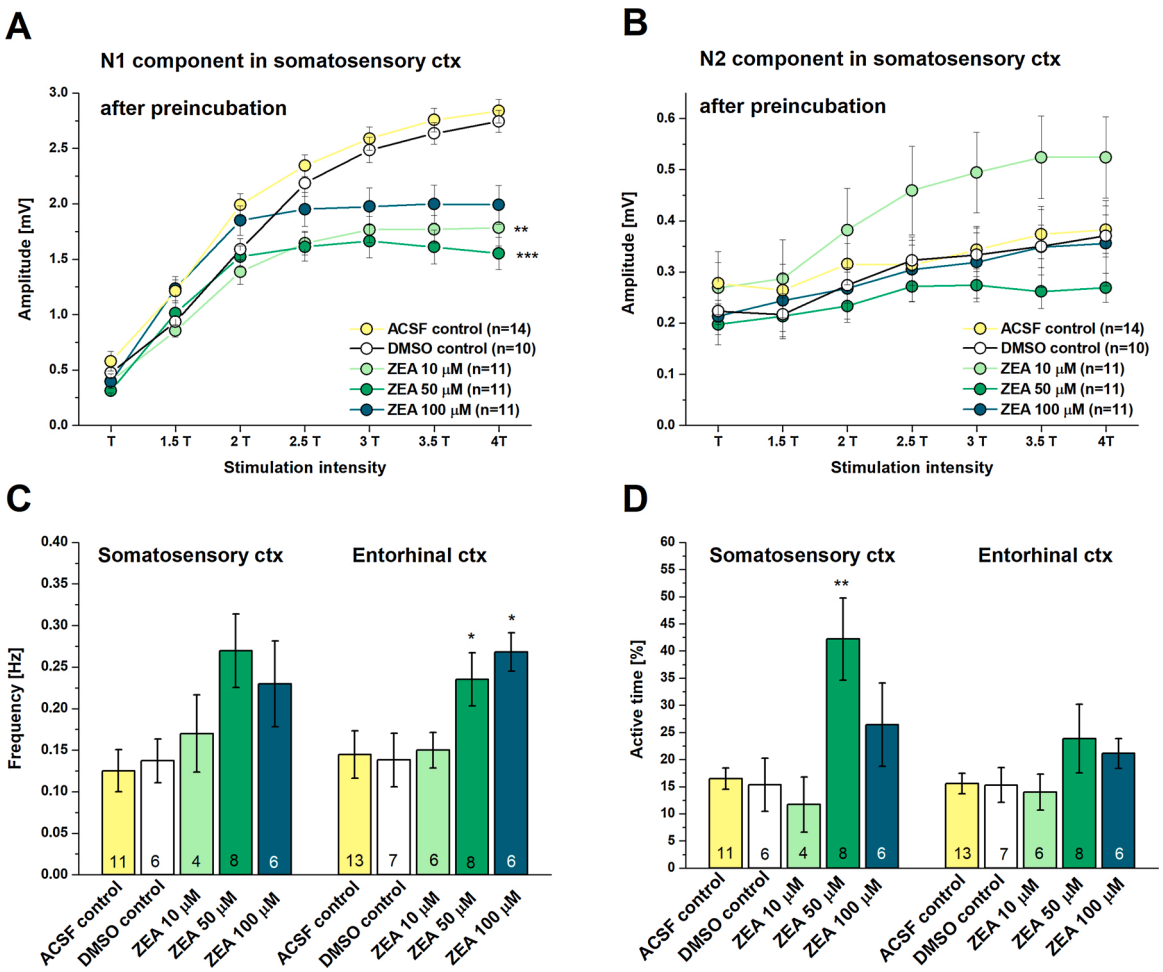

Fig. 2. ZEA incubation of neocortical slices altered network excitability and epileptiform activity in a dose-dependent manner. (A) I-O curves showing the amplitude of the early component of the field EPSP right after 30 min ACSF, DMSO or ZEA preincubation. (B) I-O curves showing the amplitude of the EPSP late component right after ACSF, DMSO or ZEA preincubation. (C) Frequency of the occurrence of epileptiform bursts during the last 5 min of the MFR perfusion in the two analyzed brain areas. (D) Time ratio spent with epileptiform bursts during the last 5 min of the MFR perfusion. Numbers inside the columns indicate sample numbers. (Statistical analysis: repeated measures or one-way ANOVA; *, ** and *** mean significant differences compared to the DMSO control group, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.).

with 2 T stimulation are presented in Table 2. In control slices, ACSF perfusion caused no change in the amplitude of the EPSP early component, while DMSO and lower concentrations of ZEA non-significantly enhanced it, there was a rather high variability among the data. After 1 h perfusion with MFR, the amplitude of the EPSP early component was not changed significantly compared to the baseline measurement.

As for the late component, in control slices, 1 h perfusion with ACSF caused an approximately 3-fold increase in the amplitude of the late component. In ZEA treated slices, this increase was dose-dependently smaller, but there is no significant difference among the groups due to the high variability of data. After MFR perfusion, the amplitude of the EPSP late component increased approximately 5-fold in control slices, which can be explained by the fact that this component is mediated primarily by NMDA receptors. Preincubation with ZEA attenuated this enhancement of the late component, but the differences between the treatment groups were not statistically significant (Table 2).

Table 2
The average amplitudes of neocortical evoked potentials at medium stimulation intensity (2 T, double threshold) are summarized. Data are presented as mean ± S.E.M. Sample numbers were n = 14, 10, 11, 11 and 11 for the control, DMSO, 10 μM, 50 μM and 100 μM treated groups respectively. There were no significant differences among the amplitudes of the 2 T field EPSP before and after ACSF or MFR perfusion, compared to the DMSO control group.

	After pretreatment		After 60 min ACSF perfusion		After 60 min MFR perfusion	
	Early component [mV]	Late component [mV]	Early component [mV]	Late component [mV]	Early component [mV]	Late component [mV]
Control	1.94 ± 0.09 (p = 0.0819)	0.23 ± 0.03 (p = 0.8965)	2.97 ± 0.35 (p = 0.9999)	0.60 ± 0.20 (p = 0.9394)	1.87 ± 0.39 (p = 0.9960)	1.32 ± 0.21 (p = 0.3572)
DMSO	1.59 ± 0.09	0.27 ± 0.03	2.98 ± 0.56	0.76 ± 0.25	2.00 ± 0.47	0.93 ± 0.28
10 μM ZEA	1.39 ±0.11 (p = 0.5293)	0.38 ± 0.08 (p = 0.4011)	3.08 ± 0.64 (p = 0.9997)	0.65 ± 0.22 (p = 0.9888)	1.18 ± 0.19 (p = 0.2884)	0.45 ± 0.08 (p = 0.2099)
50 μM ZEA	1.52 ± 0.09 (p = 0.9821)	0.23 ± 0.03 (p = 0.9483)	2.51 ± 0.43 (p = 0.9087)	0.45 ± 0.20 (p = 0.6945)	1.13 ± 0.28 (p = 0.4142)	0.66 ± 0.12 (p = 0.6739)
100 μM ZEA	1.85± 0.13 (p = 0.3067)	0.27 ± 0.06 (p = 0.9999)	2.25 ± 0.51 (p = 0.6995)	0.21 ± 0.112 (p = 0.2533)	2.37 ± 0.35 (p = 0.8573)	0.82 ± 0.17 (p = 0.9792)

3.1.2. Epileptiform activity in neocortex

Spontaneous activity of the slices during 1 h ACSF perfusion was negligible, a few small-amplitude events were observed in both S2 and LEC, but these events were usually not synchronous between the two cortical areas. ZEA pretreatment of slices did not change the number or the pattern of these events, suggesting that ZEA does not act as a convulsant in these conditions (data not shown).

On the other hand, perfusion with MFR elicited spontaneous rhythmic epileptiform bursts usually initiating in LEC and propagating to S2 (Fig. 1B right panel). Burst parameters were analyzed during the last 5 min of the MFR perfusion when the activity pattern was totally stable. Here, ZEA preincubation increased the frequency of bursts and this effect was dose-dependent; at higher concentrations (50 and 100 μM ZEA), changes were significant (Fig. 2C). The increase in burst frequency often went parallel with the decrease in burst length, so the time ratio spent with bursting did not change considerably. However, in the 50 μM ZEA group in case of the somatosensory cortex, burst frequency increased while burst length remained constants, so the active time was significantly higher than in control slices (Fig. 2D). See numerical data of all parameters with statistical values in Supplementary Table 1.

3.1.3. Basic excitability in hippocampus

In the CA1 region of the hippocampus, the threshold for evoking field EPSP-s and POP-spikes was similar in all treatment groups (Table 1). However, the amplitude of field EPSPs was significantly lower in slices treated with 10 and 100 μM ZEA, compared to the DMSO control group (p-values are 0.0191 and 0.0027, respectively). The ACSF control group and the slices treated with 50 μM ZEA did not differ significantly from the DMSO control group (p-values are 0.1027 and 0.1955, respectively). As for the POPS amplitude, ZEA treatment did not affect this parameter, and the shape of the I-O curve was similar in all treatment groups. P-values for POPS amplitudes compared to the DMSO control group are 0.9712, 0.9999, 0.9781 and 0.9735 for the ACSF control group and the 10, 50 and 100 μM ZEA preincubation groups, respectively (Fig. 3A and B).

3.1.4. Long-term potentiation in hippocampus

In ACSF and DMSO control slices, TBS elicited a significant increase in POPS amplitude ($p = 0.0048$ and 0.0919) and EPSP amplitude ($p = 0.0495$ and 0.0496), compared to the baseline. Increase in POPS amplitude was inhibited by ZEA treatment in a dose-dependent manner, the difference compared to DMSO controls was statistically significant at 50 and 100 μM ZEA concentrations (p-values are 0.0058 and 0.0165, respectively). ACSF control and slices treated with 10 μM ZEA did not differ significantly from DMSO controls (p-values are 0.6450 and 0.3806, respectively). ZEA preincubation also prevented the increase in EPSP amplitude, but due to relatively high variance of data, there were no significant differences among the treatment groups here. P-values for the development of EPSP amplitude compared to the DMSO control group are 0.8279, 0.5909, 0.4951 and 0.2142 for the ACSF control group and the 10, 50 and 100 μM ZEA preincubation groups, respectively. (Fig. 3C and D).

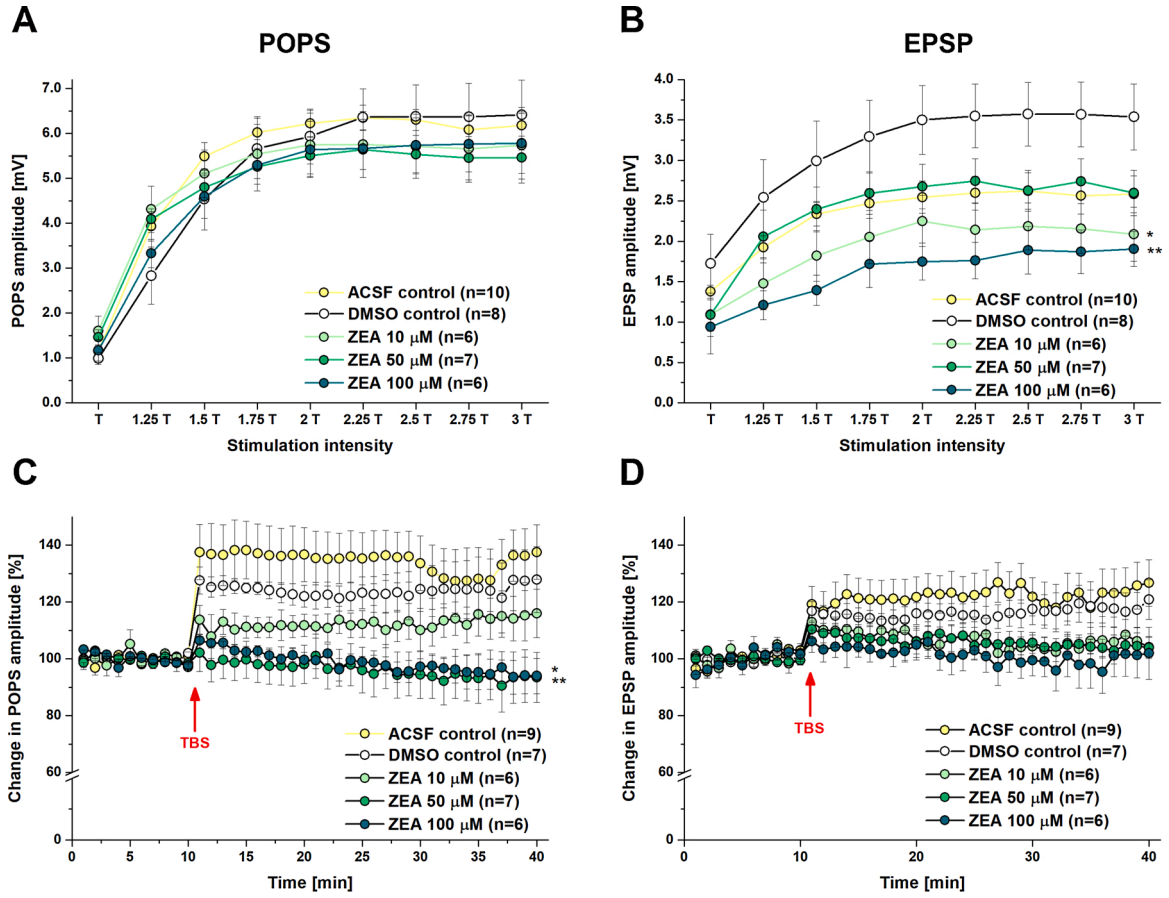

Fig. 3. ZEA incubation of hippocampal slices altered synaptic plasticity studied by LTP without significantly affecting basic network excitability. (A) I-O curves showing the POPS amplitude right after 30 min ACSF, DMSO or ZEA preincubation. (B) I-O curves showing the EPSP slope right after 30 min ACSF, DMSO or ZEA preincubation. (C) Change in EPSP amplitude expressed as % of the 10-min baseline (recovery timepoint was taken from the last 1 min as an average of the last 6 data points). Arrow indicates theta burst stimulation (TBS). LTP induction was followed for 30 min after TBS. (D) Change in POPS amplitude expressed as % of the 10-min baseline. (Statistical analysis: repeated measures ANOVA; * and ** mean significant differences compared to the DMSO control group, $p < 0.05$ and $p < 0.01$, respectively.).

3.2. In vivo somatosensory EFP recording after ZEA injections

Tibial nerve stimulation evoked characteristic EFPs in anesthetized rats both after control (DMSO) and ZEA injections. Short latency (<50 ms) responses in layer 2/3 of the somatosensory cortex showed a marked positive peak (P1) with a highly variable duration (Fig. 1H, left panel). In contrast, layer 5 EFPs were highly uniform in shape; they consisted of a high-amplitude negative wave (N1) followed by a sequence of 2–7 smaller waves falling into the gamma frequency range (Fig. 1H, right panel).

Analyzed EFP parameters were not significantly changed by either DMSO or ZEA injections compared to the corresponding (pre-injection) baseline values. Neither EFP latency ($p = 0.8152$ for layer 2/3; $p = 0.9811$ for layer 5), slope ($p = 0.6241$ for layer 2/3; $p > 0.9999$ for layer 5) nor AUC ($p = 0.9997$ for layer 2/3; $p = 0.4081$ for layer 5) did show significant alterations (see Supplementary Table 2. for numerical data). Small, non-significant deviations in layer 2/3 ($p = 0.9768$) and layer 5 ($p = 0.9974$) EFP amplitudes could be observed in both control rats (Fig. 4A and C) and ZEA-injected rats (Fig. 4B and 4D). These changes can probably be explained by slight fluctuations of anesthetic depth during the experiment.

3.3. Detection of neuronal activation with c-fos immunohistochemistry after ZEA injections

Neuronal activation pattern was studied 2 h after DMSO (vehicle control) or ZEA injection. In the somatosensory cortex, the labelling was rather sparse (Fig. 5A) and a significant increase ($p = 0.0306$) in the density of c-fos positive cells following ZEA injection occurred (Fig. 5B). In the hippocampus, the number of c-Fos labelled cells was very low in both control and treated rats (Fig. 5C). In the ZEA-injected group, a non-significant reduction ($p = 0.0941$) in the density of immunopositive cells was observed, compared to the control group (Fig. 5D).

4. Discussion

ZEA is a mycotoxin mainly produced by various *Fusarium* species, representing a risk for human and animal health. It is also known as a mycoestrogen, because in animals, its main molecular targets are estrogen receptors; this leads to its interference with reproductive functions. However, estrogens play an important role in the central nervous system, influencing the survival and the functioning of neurons in several brain regions not directly linked to reproduction. The aim of the present study was to investigate the effects of ZEA on rat neocortex and hippocampus after short-term, high-dose exposure *in vitro* and *in vivo*.

Applied *in vitro* concentrations (10–100 μM) used for the incubation of brain slices were chosen based on previous *in vitro* studies. In rat

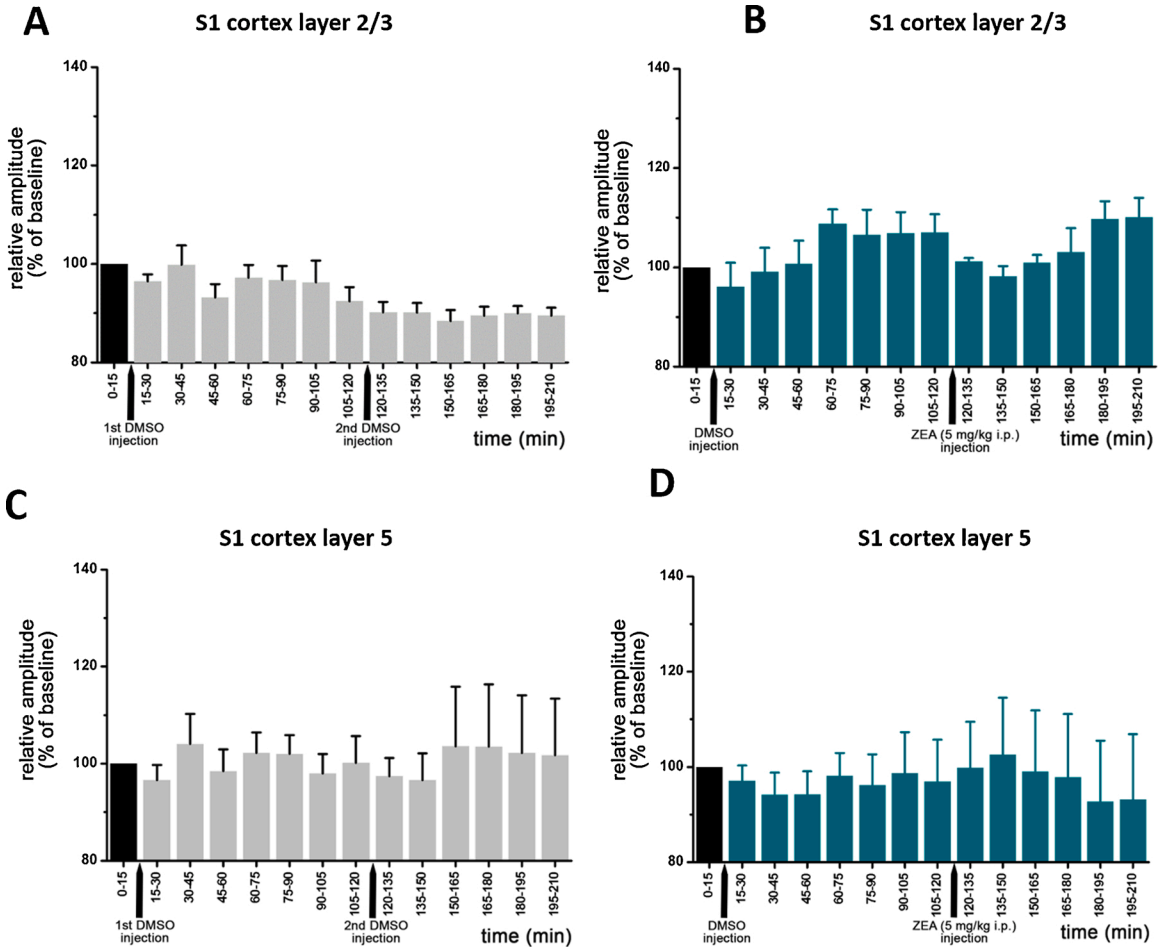

Fig. 4. ZEA injection did not alter relative amplitude changes of somatosensory cortex EFPs recorded in anesthetized rats. (A) EFP amplitude changes in cortical layer 2/3 of the double control group who received DMSO injections twice, at 15 min and 120 min of the experiment. (B) EFP amplitude changes in cortical layer 2/3 of the treated group who received DMSO at 15 min and ZEA at 120 min. (C) EFP amplitude changes in cortical layer 5 of the double control group. (D) EFP amplitude changes in cortical layer 5 of the ZEA-treated group. Baseline (pre-injection period) data were taken as 100 % and post-injection data averaged in 15-min long epochs are expressed as percent of the baseline. No significant difference was indicated by one-way ANOVA.

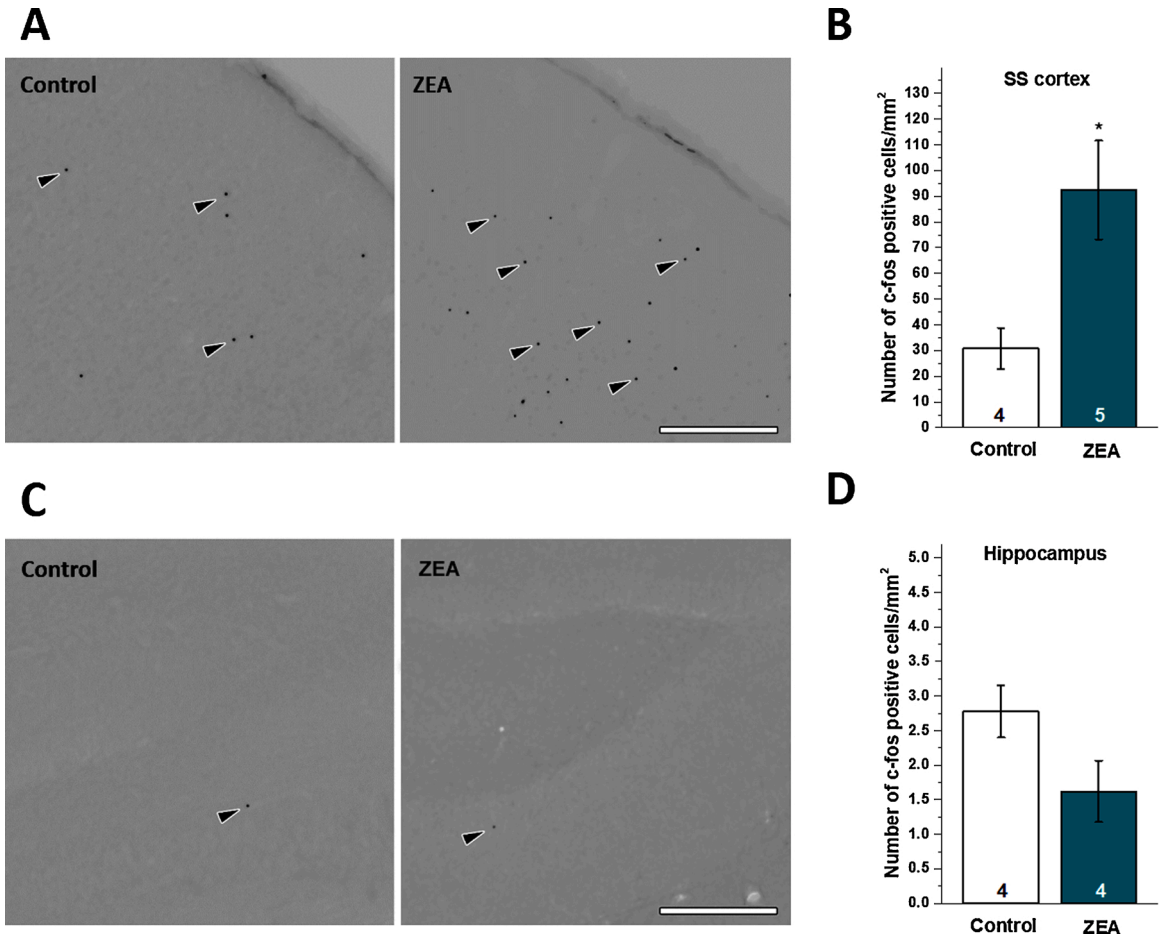

Fig. 5. ZEA injection induced activation of neurons in somatosensory cortex, but not in hippocampus, as determined with c-Fos immunohistochemistry. (A) Representative photomicrographs showing the somatosensory cortex of control (DMSO-injected, left panel) and treated (ZEA-injected, right panel) rats. Activated cells are visible as black dots, some of them marked with arrowheads. Scale bar: 200 μm. (B) The density of activated cells is expressed as cell/mm^2 in the somatosensory cortex. Numbers inside the columns indicate sample numbers. (C) Representative photomicrographs showing the hippocampus of control (left panel) and treated (right panel) rats. Scale bar: 200 μm. (D) The density of activated cells expressed as cell/mm^2 in the hippocampus. (Statistical analysis: two-sample t-test, * means significant difference compared to the control group, $p < 0.05$.).

primary cerebellar culture, already 0.1 nM ZEA treatment for 6−16 h modified the expression of thyroid hormone receptors and estrogen receptors (Kiss et al., 2018; Jócsák et al., 2019). On human neuroblastoma cells, cytotoxic effects were observed at 24 h ZEA incubation above 25 μM (Venkataramana et al., 2014). However, these studies were carried out on cell cultures, where the cell monolayer is exposed to a higher degree to chemicals present in the medium than neurons inside a brain slice. For our brain slices, also the treatment time was considerably shorter (30 min) than the several hours usually applied for cell cultures. Nevertheless, the concentrations applied on the brain slices are still considerably higher than those expected after *in vivo* exposure, according to pharmacokinetic data, these would be in the nanomolar range (Shin et al., 2009).

Based on *in vitro* receptor binding assays, ZEA is a full agonist on ERα and a partial agonist on ERβ (Kuiper et al., 1998; Takemura et al., 2007). However, at larger *i.e.* micromolar concentrations, ZEA may act as antagonist on both receptor subtypes (Mueller et al., 2004). The binding of ZEA to GPER1 has not been demonstrated so far, but a 24 h-long ZEA exposure modifies the expression of this receptor type as well, suggesting an effect on GPER1 (Yip et al., 2017).

ERα is sparsely expressed in neocortex, while ERβ expression is rather strong in the somatosensory cortex with prominent expression in the pyramidal cells and large interneurons located in layer IV and V (Shughrue and Merchenthaler, 2001). Subcortical components of the sensory pathways lack both ERα and ERβ expression in rats of both sexes (Simerly et al., 1990; Shughrue and Merchenthaler, 2001). In hippocampus CA1 region, both ERα and ERβ are sparsely expressed (Simerly et al., 1990). Membrane-associated ERα was mostly localized to *stratum radiatum* CA1 interneurons, while ERβ to dendritic spines of CA1 pyramidal cells (Almey et al., 2015). GPER1 is widely distributed in the brain, with high expression in the hippocampus (Brailoiu et al., 2007).

Experimental data about the rapid neuronal effects of ZEA are lacking, so excitability alterations caused by E2 will be discussed to interpret the results of the present study.

After short *in vitro* treatment of rat brain slices with ZEA, in the neocortical area, we saw only slight alterations in network excitability, indicated by changes in I-O curve shape. The most conclusive result was a higher seizure susceptibility: 50 and 100 μM ZEA caused an increase in epileptiform burst frequency in magnesium-free convulsant solution. This is consistent with the idea that estrogens have an overall proconvulsant effect (Scharfman and MacLusky, 2006), but no other *in vitro* study has investigated the convulsant effect of ZEA or E2 in the neocortex. In contrast, the short-term direct effects of female sexual steroids on synaptic plasticity have been tested in rat neocortical slices: micromolar concentrations of E2 increased LTP efficiency without any change in baseline excitability (Sachs et al., 2007). This implicates an enhancement in NMDA-receptor function which would also lead to enhanced epileptiform activity in MFR.

In hippocampus slices, a significant decrease of the EPSP amplitude was observed in slices treated with 10 and 100 μM ZEA, without any

change in the EPSP stimulation threshold, indicating a decreased excitatory input to CA1 pyramidal cells. Concerning synaptic plasticity, we saw a marked dose-dependent inhibition of LTP development by ZEA in these slices. This finding is in contrast with the results of similar *in vitro* studies investigating E2 effects in the CA1 area. It has been reported that perfusion with 0.1 nM E2 enhanced the amplitude of POPS in CA1 area of hippocampal slices originating from male rats (Teyler et al., 1980). The effect seems to be mediated by both AMPA and NMDA receptors, and in addition to the increase in baseline excitability, E2 perfusion also leads to enhanced LTP (Foy et al., 1999). According to a more recent study in female mouse hippocampus *in vitro*, this enhancement of neuronal excitability caused by estrogen is mediated by all three estrogen receptors types (ERα, ERβ and GPER1), but the contribution of GPERs is the most significant (Kumar et al., 2015). This fact may explain the discrepancy between our results with ZEA and the previous results with E2, as there is no data about the binding of ZEA to GPERs. Also, in most studies, the E2 concentrations applied were much lower that the ZEA concentrations applied by us. ERs are present both in excitatory and inhibitory neurons in hippocampus, thus E2 may influence both transmission types and the ratio of these may depend on the exact concentration (Almey et al., 2015). Thus, the outcome in neuronal excitability may vary according to agonist concentration. This effect is probably also true for ZEA, the exact effects may depend on brain area, effective concentration and time parameters as well. The fact that in the neocortex, the observed effects of ZEA did not show a linear dose-dependence, supports this idea. *E.g.* in case of the late component of the field EPSP, the only apparent change occurred at 10 μM ZEA treatment, while higher concentrations had no effect on this parameter. Also, in case of the epileptiform activity, the most prominent change in time spent with activity was observed in somatosensory cortex after 50 μM ZEA, while 100 μM had no significant effect here. Multiple possible explanations exist for these peculiar concentration-dependent results. As ERs are present both on excitatory and inhibitory neurons (Almey et al., 2015), it is possible that lower concentrations of the agonist are required to activate those on excitatory neurons then those on inhibitory neurons. It has been demonstrated that ZEA may act as agonist at lower concentrations, but antagonist at higher concentrations (Mueller et al., 2004). The presence of different types and ratios of ERs in different brain areas and on different cell types may account for the observation that different concentrations influence significantly different measured parameters (Almey et al., 2015; Lecomte et al., 2017; Shughrue et al., 1997). Finally, the possibility cannot be ruled out that 50 and 100 μM ZEA treatments affect the viability of the slices and this manifests itself *e.g.* during the somewhat lengthy LTP development protocol in the hippocampus; these concentrations proved to induce oxidative stress and to be cytotoxic in a cell culture study (Venkataramana et al., 2014).

After acute *in vivo* treatment of male rats (a single ZEA injection i.p. at dose of 5 mg/kg) somatosensory EFPs and neuronal activation pattern with c-Fos staining were studied. In the electrophysiological test, no changes were seen in ZEA-treated rats in any of the analyzed parameters of the EFPs compared to controls. In the c-Fos experiment, significant activation of a small neuronal subpopulation was detected after ZEA injection in the somatosensory cortex, while no effect was observed in the hippocampus. The cell activation seen in the somatosensory cortex could be associated with the epileptogenic effect observed in this brain area after *in vitro* ZEA treatment of brain slices. In the c-Fos study, the density of cells activated was rather sparse, with the majority of neurons unaffected. Therefore, it is plausible that the effect was not detectable with field potential recording, which would require the alteration of activity of a larger neuronal population located around the recording array. It is also possible that the sparsely labelled c-fos positive neuronal population represents a particular subtype of neurons which does not influence EFP parameters significantly. However, the activation of a particular neuronal population may be sufficient to significantly alter network activity in the presence of a convulsant, which could be in the background of the epileptogenic effect observed in somatosensory cortex slices preincubated with ZEA and treated with MFR.

In a study by Turcotte et al. (2005), a similar ZEA dose was used to examine potential estrogenic effects of ZEA in female rats *in vivo*. ZEA injections (2 mg/rat s.c.) were made for 3 days and ZEA administration evoked increased progestin receptor expression comparable to that seen after the injection of E2 benzoate. These data suggest that ZEA acts as an E2 receptor agonist in the dose used in the present study. Although the effect of ZEA and estrogenic compounds is stronger on female reproductive functions, estrogenic compounds have significant effects on the brain functions of both sexes, and in general, no sex-related expression pattern differences were found for ERs in the brain areas examined in our study (Hutson et al., 2019). Thus, the partial lack of ZEA effect cannot be attributed to the sex of the experimental animals used *in vivo*. The fact that male rats were used for *in vitro* tests also, and here, changes could be observed after ZEA treatment, supports this idea.

The discrepancy between *in vitro* and *in vivo* results may be explained by insufficient concentrations in the examined brain areas after the injection. Access of ZEA through the BBB was not verified directly in this study. Using a physiologically-based toxicokinetic model developed with *in vivo* data (Shin et al., 2009), ZEA tissue-to-blood partition coefficient was found to be low for the brain tissue, showing limited bioavailability of the toxin for the CNS (Mukherjee et al., 2014). A recent *in silico* study using prediction tools (Agahi et al., 2020) also supports low BBB access of ZEA, however, certain metabolites have a much higher probability of access to the brain. In our study, i.p. administration was applied which decreases the importance of metabolic processes compared to oral administration. Despite the low brain transfer, several studies reported changes in neuroendocrine functions after systemic ZEA injections and these changes presume the interaction of ZEA and intracellular ERs in neurons (*e.g.* Kriszt et al., 2015). However, even if ZEA can access through the BBB, its kinetics is also unknown. It can be hypothesized that brain penetration of ZEA is slow and during the recording period after ZEA application (90 min), buildup of effective ZEA concentration was not possible in the somatosensory cortex to affect a sufficiently large neuronal population necessary to modify evoked field potentials.

To conclude, our *in vivo* electrophysiological and immunohistochemistry results support the previous information about the low BBB permeability of ZEA after acute treatment. It seems that under these conditions, information processing in cortical and hippocampal areas are not affected. However, our *in vitro* findings indicate that in case of high-concentration direct, acute exposure of brain slices, ZEA can alter the excitability and synaptic plasticity phenomena in these brain areas. Interestingly, some of our findings are in contrast with estrogen effects on neuronal excitability reported so far, mostly concerning the hippocampus area. The explanation could be the lack of binding of ZEA to GPERs in neurons, which are probably responsible for an important fraction of E2 effects. More detailed dose-response relationship studies concerning neuronal effects of ZEA are also lacking, and presumably, even the direction of neuronal excitability alterations may depend on the exact concentration. It is also possible that ZEA has other targets in neurons which could mediate acute effects. Future studies are warranted to address these questions.

Conflict of Interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Veronika Bódi: Investigation, Visualization, Writing - original draft, Writing - review & editing. **Vivien Csikós:** Investigation, Visualization. **Tímea Májer:** Investigation, Visualization. **Attila Tóth:** Investigation, Visualization, Writing - original draft, Writing - review & editing. **Árpád Dobolyi:** Conceptualization, Supervision, Methodology. **Ildikó Világi:** Conceptualization, Supervision, Methodology, Funding acquisition,

Project administration. **Petra Varró:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2021.08.001.

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