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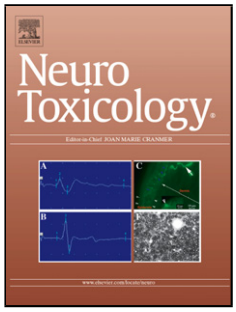


Short-term neuronal effects of Fumonisin B1 on neuronal activity in rodents

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# Short-term neuronal effects of Fumonisin B1 on neuronal activity in rodents

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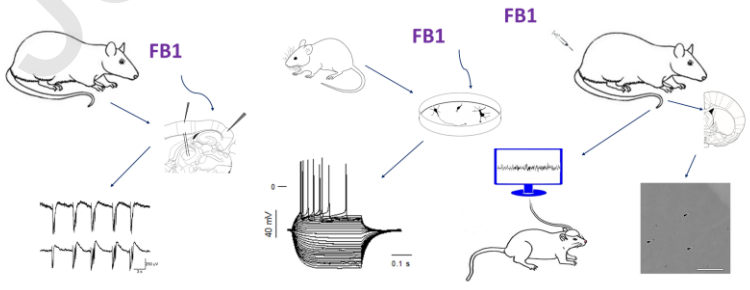
## Graphical abstract



Highlights:

- Acute effects of the mycotoxin FB1 on neuronal activity were investigated
- FB1 was applied directly on cultures and slices or administered *in vivo*
- Electrophysiological and immunohistochemical measurements were carried out
- FB1 increased the excitability of neurons and neuronal networks treated directly
- FB1 had no effects on neocortical or hippocampal activity *in vivo*

**Abstract**

Fumonisin B1 (FB1) is a mycotoxin produced by microscopic fungi (mostly *Fusarium* species), which may infect our major crops. The toxin inhibits the development of these plants and may also have harmful effects on animals and humans consuming the infected crops.

FB1 inhibits sphingolipid biosynthesis which leads to altered membrane characteristics and consequently, altered cellular functions. There are some indications that the toxin has inhibitory effects on neuronal activity in case of repeated consumption, presumably due to sphingolipid depletion. However, according to new literature data, FB1 may have acute excitatory neural effects, too, via different mechanisms of action. Therefore, in the present study, we addressed the neuronal network effects of FB1 following acute treatment, using different electrophysiological techniques *in vitro* and *in vivo*.

Acute treatments with FB1 (10-100 µM) were carried out on brain slices, tissue cultures and live animals. After direct treatment of samples, electrically evoked or spontaneous field potentials were examined in the hippocampus and the neocortex of rat brain slices and in hippocampal cell cultures. In the hippocampus, a short-term increase in the excitability of neuronal networks and individual cells was observed in response to FB1 treatment. In some cases, the initially enhanced excitation was reversed presumably due to overactivation of neuronal networks. Normal spontaneous activity was found to be stimulated in hippocampal cell cultures. Seizure susceptibility was not affected in the neocortex of brain slices.

For the verification of the results caused by direct treatment, effects of systemic administration of FB1 (7.5 mg/kg, i.p.) were also examined. Evoked field potentials recorded *in vivo* from the somatosensory cortex and cell activation measured by the c-fos technique in

hippocampus and somatosensory cortex were analyzed. However, the hippocampal and cortical stimulatory effect detected *in vitro* could not be demonstrated by these *in vivo* assays. Altogether, the toxin enhanced the basic excitability of neurons and neuronal networks after direct treatment but there were no effects on the given brain areas after systemic treatment *in vivo*. Based on the observed *in vitro* FB1 effects and the lack of data on the penetration of FB1 across the blood-brain barrier, we assume that *in vivo* consequences of FB1 administration can be more prominent in case of perturbed blood-brain barrier functions.

Keywords: neurotoxicity, electrophysiology, c-Fos, mycotoxin, field potential, brain slice, tissue culture

**Abbreviations**

| | |
|---|---|
| ACSF | Artificial cerebrospinal fluid |
| BBB | Blood-brain barrier |
| EFP | Extracellular field potential |
| EPSP | Excitatory postsynaptic potential |
| FB1 | Fumonisin B1 |
| LEC | Lateral entorhinal cortex |
| MEA | Multielectrode Array |
| MFR | Magnesium-free Ringer |
| POP-spike | Population spike |
| S1 | Primary somatosensory cortex |
| S1HL | Hindlimb area of the primary somatosensory cortex |
| S2 | Secondary somatosensory cortex |
| TDI | Tolerable daily intake |

## 1. Introduction

Agricultural crops are exposed to infections of microscopic fungi throughout the world, which can interfere with growing and reproduction of the plant. Moreover, these fungi can release different toxic secondary metabolites, so called mycotoxins into the plant tissues. The toxins are usually chemically and heat stable compounds, so they can be present in processed food and feed, and cause a risk for human and animal health. To avoid these negative effects, authorities define legal limits for toxin contamination of grain-based nutrition (Stockmann-Juvala and Savolainen, 2008).

Maize is one of the most commonly consumed grains in developing countries, thanks to its easy cultivation, high yield and aptitude to adaptation. Several studies suggest that corn may be highly infected with different microscopic fungi, such as *Fusarium* species, found worldwide (Ariño et al., 2007; Bryła et al., 2013; Scott, 2012). These fungi produce several types of mycotoxins in various composition; among them the most commonly found in food samples are fumonisins, deoxynivalenol and zearalenone (Wentzel et al., 2017). Fumonisin B1 (FB1) is the most abundant among the different types of the fumonisin group and it has been detected in corn in all parts of the world since its identification in the late 1980-ies (Stockmann-Juvala and Savolainen, 2008). The tolerable daily intake (TDI) for FB1 is 2 µg/kg bw/day, whereas the maximum tolerable limit for FB1 in maize is 1 ppm. In countries where food consumption is based on maize, human exposure may highly exceed TDI (Wild and Gong, 2010).

FB1 is hepatotoxic, nephrotoxic and neurotoxic in different animal species (Stockmann-Juvala and Savolainen, 2008). Although the International Agency for Research on Cancer has classified FB1 as a potential human carcinogen (group 2B), it does not seem to be mutagenic, but possibly a cancer promoter (Bryła et al., 2013; Wentzel et al., 2017). Epidemiological data have linked FB1 consumption to a higher incidence of human esophageal cancer in South Africa and other regions, but the connection is debated (Stockmann-Juvala and Savolainen, 2008). The main mechanism of FB1 toxicity should be based on its ability to inhibit ceramide synthase, leading to the accumulation of sphinganine and sphingosine which can be pro-apoptotic and cytotoxic (Escrivá et al., 2015). Moreover, due to the enzyme inhibition, depletion of complex sphingolipids may occur, and the plasma membrane structure of the cells may be altered.

We have sporadic information about how FB1 toxicosis affects the nervous system. In equine species, leukoencephalomalacia (necrotic degeneration of brain white matter) may develop due to consumption of FB1-contaminated feed (Ross et al., 1992). FB1-contamination has been shown to increase the risk of neural tube defects in several species; however, the effect

seems to be mediated indirectly via the inhibition of folate uptake (Ross et al., 1992). In turn, some data suggest the direct neuronal effect of FB1; repeated treatment of rats causes functional changes in nervous system e. g. alters the excitability and plasticity of neuronal networks and epileptic activity in the cortex *in vitro* (Banczerowski-Pelyhe, 2008; Banczerowski-Pelyhe et al., 2002b; Banczerowski et al., 2008), or decreases nerve conduction velocity and alters spinal reflexes *in vivo* (Banczerowski-Pelyhe et al., 2002a). The latter data are in concordance with the finding that FB1 interferes with axonal growth and myelinization in cell cultures (Harel and Futerman, 1993; Monnet-Tschudi et al., 1999).

Besides the relatively long-term effects, which may be explained by altered sphingolipid metabolism, it was described more recently that FB1 may have short-term effects on neuronal tissue. Acute treatment with FB1 modifies the features of glia and neuroblastoma cells in brain cell cultures. It was detected that FB1 interferes with the mitochondrial complex I causing depolarization of the mitochondrial membrane and elevation of intracellular calcium level (Domijan and Abramov, 2011). As a consequence, the toxin may strengthen the stimulating effects of glutamate causing an even higher rise in intracellular calcium concentration (Domijan et al., 2012). This phenomenon cannot be explained with the impairment of sphingolipid metabolism because the time-scale of these effects is in the minute-range, which is not enough for the manifestation of the consequences of ceramide synthase inhibition (Stockmann-Juvala and Savolainen, 2008).

To date there are no experimental data about the direct, short-term electrophysiological effects of FB1 on neuronal networks. Hence the present study was designed to examine the acute effects of FB1 on neural network activity evaluated in *vitro* and *in vivo*. Based on the above findings, our aim was to examine whether the stimulatory effects of FB1 could be detected on the level of complex neuronal networks. FB1 was applied *in vitro* on rat brain slices or mouse neuronal cultures and network and cellular functions were characterized with field potential and patch clamp recordings, respectively. Finally, FB1 was directly administered to rats and the effects on cortical somatosensory evoked potentials and c-fos expression assessed *in vivo*.

## 2. Materials and methods

### *2.1. Animal maintenance and treatment*

Young adult, male Wistar rats (150-280 g for *in vitro* or 300-465 g for *in vivo* experiments, Toxi-coop Ltd., Budapest, Hungary) and CD1 mice (embryonic day 17-18) were used for the experiments. 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 and mice 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*

Fumonisin B1 purchased from Bio-Techne R&D Systems Ltd. (Budapest, Hungary) was dissolved in artificial cerebrospinal fluid (ACSF) and stored as 500 µM stock solution at -20 ºC for *in vitro* patch clamp and slice experiments. This was then diluted in ACSF to reach 100, 50, 40 or 10 µM final concentrations. For Multielectrode Array (MEA) experiments, 1 mM FB1 (dissolved in 10% DMSO) stock solution was diluted into the culture medium. Final concentrations of 100 µM, 50 µM or 20 µM FB1 were applied, control cultures received only the DMSO solvent (1%, 0.5% or 0.2%, respectively). For *in vivo* experiments, FB1 was dissolved in sterile physiological saline and stored as 4,156 mM stock solution at -20 ºC. All other compounds were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary).

### *2.3. Electrophysiological recordings on hippocampal cultures*

Primary cultures of embryonic hippocampal cells were prepared from CD1 mice on embryonic day 17-18, according to Czöndör et al., 2019.

For MEA recordings dissociated hippocampal cells were seeded onto 4-well or single-well *MEA (*Qwane Biosciences or Multichannel Systems) multi-electrode chambers at $4x10^4$ or $4-5x10^5$ cells/well densities, respectively. Prior to seeding the cells, surface of the multi-electrode arrays was coated by poly-L-lysine (2 µg/cm$^2$) and laminin (4.2 µg/cm$^2$). MEA recordings were carried out 10-11 days following planting.

For patch clamp recordings $1.3x10^5$ hippocampal cells were seeded onto poly-L-lysine and laminin coated glass coverslips in 24-well plates. Electrophysiological recordings were performed 14-17 days after plating when the neurons typically exhibited robust spontaneous bursting activity, mature cellular properties and synaptic connections.

#### *2.3.1. Patch clamp studies*

The experiments were performed in whole-cell patch clamp conditions at room temperature (21-23 °C) using a MultiClamp 700B amplifier (Molecular Devices). The composition of the

extracellular solution (artificial cerebrospinal fluid, ACSF) was (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5, D-glucose 10; pH 7.45. 10 µM CNQX, 40 µM AP-5 and 20 µM bicuculline was included in the bath to assure synaptic isolation of the neurons. The patch electrodes were pulled from borosilicate glass, had 6-8 MΩ resistance and filled with the following solution (in mM): K-gluconate 100, KCl 10, KOH 10, $MgCl_2$ 2, NaCl 2, HEPES 10, EGTA 0.2, D-glucose 5; the pH was set to 7.3. Current step protocols included constant level current steps of 350 ms duration, starting at -150 pA and incremented by 4 pA until robust firing responses were reached. The current threshold of spiking was used to determine rheobase. The input-output curve of the neuron was obtained and spikes counted for each current level up to +200 pA (*Fig. 1A,B*). Physiological parameters extracted from the subthreshold voltage traces included the membrane resistance, voltage sag, membrane time constant, and afterdepolarization. The voltage responses of the same neurons were recorded before and after the application of FB1 (40 µM). Cells were held in FB1 solution for 25-50 minutes.

For statistical analysis paired t-test was used, statistical significance was accepted at the $p < 0.05$ level.

### *2.3.2. MEA recordings*

Spontaneous extracellular field potentials were recorded at 37 °C (Temperature Controller TC01102, Multichannel Systems) using a USB-1060 INV Microelectrode Array System (Multichannel Systems) with a MEA Amplifier with Blanking Circuit (Multichannel Systems) amplifier at a sampling rate of 20 kHz/channel. Signals were high-pass filtered at 300 Hz and low-pass filtered at 3000 Hz. Spontaneous firing was recorded for 10 minutes (control) and repeated right after FB1 or solvent treatment for another 10 minutes (*Fig. 2A*). Recorded spikes were detected by a threshold crossing method and were sorted by the k means algorithm. The spike timestamps were extracted using the Plexon Offline Sorter (Plexon Inc.). Analysis of the spike timestamps, including characterizing spontaneous firing and burst activity was performed by the NeuroExpress software, developed by A. Szűcs.

Statistical analysis was performed with repeated-measures two-way ANOVA with Tukey's post hoc test for normal data to estimate the significance of differences between control and FB1 treated groups. Statistical significance was accepted at the $p < 0.05$ level.

## *2.4. In vitro electrophysiological recordings on brain slices*

Rats (n=71) were decapitated under deep chloral-hydrate anesthesia, the brains were isolated and cut with a vibratome (Electron Microscopy Sciences, Hatfield, USA). 400 µm thick horizontal slices were incubated for an hour at room temperature in ACSF bubbled with carbogen. The composition of ACSF was (in mM): 126 NaCl; 26 $NaHCO_3$; 1.8 KCl; 1.25 $KH_2PO_4$; 1.3 $MgSO_4$; 2.4 $CaCl_2$; 12 glucose, pH 7.2-7.4. After this incubation period, individual slices (n=118) were incubated in a small, special incubating chamber in 2 ml standard ACSF or FB1-containing ACSF (10 µM, 50 µM, 100 µM) for another 30 minutes. Applied concentrations were set up based on other, *in vitro* cell culture studies (Domijan et al., 2012; Osuchowski et al., 2005a; Osuchowski and Sharma, 2005; Stockmann-Juvala et al., 2004).

After that the slices were placed into an Oslo-type recording chamber (FST Ltd., Vancouver, Canada) in which the temperature was held in 33±1 $^oC$ and through which ACSF or magnesium free ACSF (MFR) was perfused, which were also bubbled with carbogen.

In the hippocampus, with a bipolar tungsten stimulation electrode placed at the Schaffer collaterals field potential responses were electrically evoked (square voltage pulses of 100 µs width, BioStim, Supertech Ltd., Pécs, Hungary) in the CA1 region. Two extracellular glass microelectrodes filled with 1 M NaCl (5-15 MΩ) were positioned into the *stratum radiatum* and the *stratum pyramidale* to record excitatory postsynaptic potentials (EPSPs) and population spikes (POP-spikes), respectively (*Fig. 3A*). In the neocortex, two recording electrodes were placed into the layer 2/3 of the lateral entorhinal (LEC) and secondary somatosensory (S2) cortical areas. The stimulation electrode was positioned below the recording electrode in the S2 at the border of layer 6 and the white matter (*Fig. 3C*). Evoked field potentials were recorded from the latter area and spontaneous activity elicited via MFR was recorded from both cortical regions. Signals were band-pass filtered (0.16 Hz-1 kHz) and amplified 1000x (BioAmp, Supertech Ltd., Pécs, Hungary). Evoked and spontaneous potentials were recorded and analyzed with SPEL Advanced Intrasys computer program (Experimetria Ltd., Budapest, Hungary) and custom-written Matlab-based software (by Sándor Borbély), respectively.

Basic synaptic functions were tested by determining the voltage threshold of evoked field potentials (T). In the hippocampus this was followed by testing long-term synaptic potentiation (LTP). Stimulus intensity was set up to evoke 70% of the maximal amplitude of POP-spikes. After recording a 10-min baseline, repetitive stimuli (theta burst stimulation, TBS) were applied with 10 bursts containing 4 pulses at 100 Hz repeated at 200 ms intervals (i.e. at 5 Hz) for inducing LTP. Then, recording was continued for 30 min (*Fig. 3B*). In the

neocortex, following test stimulation with 2T intensity in the S2 area, a one-hour-long activity recording was carried out in both cortical areas without any stimulation. During this, slices were perfused with ACSF or MFR for eliciting seizure-like events. Active time was calculated from the frequency and the length of bursts. Then, a second test stimulation with 2 T was carried out (*Fig. 3D*).

In the hippocampus, the amplitude of POP-spike and the slope (derived quantity from amplitude and rising time) of EPSP, in the neocortex the amplitude of the early and late components of EPSP and the active time during spontaneous epileptiform activity were analyzed (*Fig. 4A,D* and *Fig. 5A,D*).

One-way ANOVA (with Levene's test for homogeneity of variances and Tukey's post-hoc test, $p<0.05$) was performed for statistical analysis to estimate the significance of differences between control and treated groups.

### *2.5. In vivo electrophysiological recordings*

Rats (n=12) were anesthetized with urethane (1.2 g/kg, i.p.), fixed in a stereotaxic frame (David Kopf) and maintained at 37 °C.

Hindlimb area of the primary somatosensory cortex (S1) was localized as described previously (Borbély et al., 2016; Toth et al., 2008) (*Fig. 3E*). Evoked extracellular field potentials (EFPs) were recorded by a 16-channel vertical electrode array (Neuronelektród Ltd., Budapest, Hungary) and two stimulating needle electrodes were inserted near the tibial nerve. Stimulus strength twice the threshold (8.35 V ± 0.37 V) was used with 1 ms duration and 0.1 Hz rate. A total of 1260 EFPs were recorded. EFP signals were conditioned (filter: 0.1 Hz–1000 Hz, gain: 5000x, Supertech Ltd., Pecs, Hungary) then digitalized at 2048 Hz with 16-bit resolution.

Recordings lasted for 210 minutes. Rats were split into two separate experimental groups in a randomized fashion. The first group (double control, n=6) received saline injections two times, at 15 minutes and 120 minutes to rule out possible drug-independent time effects as recordings lasted relatively longer. In the second group (n=6), saline injection was administered first at 15 minutes then FB1 was injected at 120 minutes. Pre-injection periods (between 0-15 minutes and 105-120 minutes, respectively) served as baseline.

FB1 and respective saline solutions were injected i.p. at body temperature in a volume of 2 ml/kg. FB1 was applied in a dose of 7.5 mg/kg, based on previous similar *in vivo* rat studies (Bondy et al., 1995; Shephard et al., 1992) (*Fig. 3F*).

Parameters of the EFPs were calculated from layer 2/3 and layer 5 responses, respectively (*Fig. 6A,C)*. Single EFPs were averaged in 15 min long blocks containing 90 responses each. Amplitude and latency values of the characteristic waves were determined in these blocks and expressed as mean and standard error of the means (S.E.M). Slope of the responses was calculated based on the amplitude of the first 3.5 ms long period of the ascending component of the P1 waves in case of the layer 2/3 EFPs and descending component of the N1 waves in case of the layer 5 responses (*Fig. 6A,C*). The areas under the curves (integral) were also calculated.

Amplitude, latency, slope and under curve area differences between baseline (pre-injection) and post-injection values in the consecutive 15-minutes long blocks were compared statistically by two-way ANOVA followed by Student-Newman-Keuls post-hoc test. Data from matching time points after saline vs. FB1 injections for layer 2/3 and layer 5, respectively, were also compared by the same test. Statistical significance was accepted at the $p < 0.05$ level.

At the end of the experiment, position of the recording array was marked using direct current injections and confirmed by histological analysis. Bright-field light-microscopy was used to locate recording sites, which were marked on the appropriate plates of the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson, 1998). Histology verified that recording arrays were placed in the hindlimb area of the S1 (data not shown).

### 2.6. *Immunohistochemistry*

FB1 was injected intraperitoneally to rats (n=6) in a dose of 7.5 mg/kg, while control animals (n=6) received physiological saline solution. The animals were sacrificed with urethane and perfused transcardially with 4% paraformaldehyde 2 hours later. 4% paraformaldehyde was prepared in 0.1 M phosphate buffer (PB; pH=7,4). Brains were removed, postfixed in 4% paraformaldehyde for a day, and then transferred to PB containing 20% sucrose for an additional day for cryoprotection. 50 µm thick serial coronal sections were cut with cryostat (Leica CM1520). Sections were collected in PB in 5 parallels used separately for individual staining. The sections were stored at 4 °C until immunohistochemical procedures started.

Every fifth 50-µm-thick free-floating brain section from FB1 injected and control injected animal was processed for c-Fos immunohistochemistry as described previously (Cservenák et al, 2017). Briefly, sections were pre-treated in PB containing 0.3% hydrogen peroxide for 15 min for quenching of endogenous peroxidase activity. Then the sections were incubated in PB containing 0.5% Triton X-100 and 3% bovine serum albumin for 1 hour. Sections were then

incubated in anti-c-Fos antiserum (1:2000, Santa Cruz, sc-52) at room temperature for two nights. Following the primary antibody, the sections were incubated in biotin-conjugated anti-rabbit secondary antibody (1:800; Jackson Immuno Research, 711-065-152) for 1 hour and then in avidin–biotin–peroxidase complex (ABC; 1:500; Vector Laboratories) for 1 hour. The labelling was visualized by nickel-2'-diaminobenzidine peroxidase technique for 20 min. Sections were coverslipped with Depex mounting medium (Sigma, catalogue number: 06522). Brain areas were identified using the Paxinos & Watson stereotaxic atlas of the rat brain. The brain areas containing Fos-immunoreactive cells were detected and captured with a light microscope equipped with a digital camera (Nikon Eclipse Ni, 25.4 2 MP Slider Camera, Spot RT3 software). The numbers of Fos labelled neuronal cell bodies were counted in the hippocampus and the somatosensory cortex after FB1 injection. Data from 6 animals per group ("FB1 injected" and "saline injected" groups) were analysed, and the density of labelled neurons was expressed as the cell number/mm$^2$. The total number of Fos-immunoreactive neurons in activated regions was counted using ImageJ software, version 1.50i (ImageJ, RRID SCR_003070, Wayne Rasband, National Institute of Health, Bethesda, MD) in photomicrographs (*Fig. 7A,C*). Statistical analysis was performed using 2-way ANOVA (using brain region and treatment) followed by Bonferroni post-hoc tests when the effect of FB1 injection was examined on the number of labelled neurons. All statistics were performed with Prism 5 for Windows (GraphPad Software, La Jolla, CA).

## 3. Results

### *3.1. In vitro electrophysiology on hippocampal cultures*

#### *3.1.1. Patch clamp recordings*

The voltage responses of the neurons were recorded before and after the application of FB1 (40 µM) to identify potential changes in the passive or active membrane properties of the neurons. While subthreshold voltage traces indicated no change in membrane resistance (*Fig. 1C,E*) or the voltage sag, we observed a slight increase of the firing at suprathreshold current levels (*Fig. 1D*). Also, the rheobase of the neurons was decreased slightly (*Fig. 1F*). Action potential amplitude was also slightly reduced after FB1 application which indicates the increase of the intrinsic excitability of the neurons.

#### *3.1.2. MEA recordings*

Changes in the spontaneous network activity of hippocampal cultures were analysed by comparing burst parameters before and after drug or solvent administration. The ratio of spikes during bursts did not change upon DMSO or FB1 treatments (data not shown). In case of solvent treatments, even 1% DMSO did not evoke significant alterations within the measured burst parameters (data not shown). While lower concentrations (20 and 50 µM) of FB1 did not influence network burst activity, 100 µM FB1 evoked a significant increase in burst frequency and burst length, as well (*Fig. 2B* and *2C*, respectively).

### *3.2. In vitro electrophysiology on brain slices*

#### *3.2.1. Hippocampus*

The average voltage threshold (T) of stimulation and the slope of EPSP or amplitude of POP-spike during stimulation test is summarized in *Table 1*. There was no significant difference among voltage threshold values. Treatment with the highest concentration of FB1 caused a significant increase in both the amplitude of POP-spikes and the slope of EPSPs, compared to the control slices *(Fig. 4B,E*).

| EPSP | Threshold [V] | Slope evoked with 2T intensity [mV] | POP-spike | Threshold [V] | Amplitude evoked with 2T intensity [mV] |
|---|---|---|---|---|---|
| control | 2.97±0.23 | 2.38±0.20 | control | 3.43±0.22 | 3.37±0.50 |
| 10 µM | 2.5±0.19 | 3.13±0.20 | 10 µM | 2.5±0.19 | 4.67±0.29 |
| 50 µM | 3.27±0.31 | 3.41±0.45 | 50 µM | 3.32±0.29 | 4.48±0.44 |
| 100 µM | 2.73±0.08 | 3.70±0.32 * | 100 µM | 3.00±0.14 | 6.21±0.45 * |

*Table 1. The average voltage stimulation threshold of hippocampal evoked potentials and the average slope of EPSPs and amplitude of POP spikes evoked with 2T stimulation intensity in hippocampal slices are summarized. Data are presented as mean ± S.E.M. N=15, 8, 11 and 11 of the control, 10 µM, 50 µM and 100 µM treated groups respectively. There was no significant difference among voltage threshold values. *: 100 µM FB1-treatment caused significant increases in EPSP slope and POP spike amplitude, compared to the control group.*

LTP induction via TBS was effective, in the control group EPSP slope and POP-spike amplitude increased by 23.93±3.64% and 44.90±0.29%, respectively. The increase of the responses was statistically significant, compared to baseline. LTP induction was similarly effective in the groups treated with 10 and 50 µM FB1. However, in case of the group treated with 100 µM FB1, there was no significant increase of EPSP slope and POP-spike compared to baseline, probably due to already increased excitability. After LTP, the amplitude of evoked responses was higher in all treated groups than in the control group (*Fig. 4B,E*).

However, when comparing the increases as percent of the baseline, there were no significant differences among the toxin treatment groups *(Fig. 4C,F).*

*3.2.2. Lateral entorhinal and secondary somatosensory cortices*

The average voltage threshold values and the average amplitudes of EPSPs evoked in the S2 are shown in *Table* 2. There was no significant difference among voltage threshold values. In neocortex, we analyzed the early and late components of the EPSPs. The first stimulation test with 2T intensity was recorded right after the FB1 pre-treatment. The amplitude of early component was significantly increased by the pre-treatment with FB1 (10 and 100 µM) compared to the control slices while it did not seem to have any impact on the amplitude of late component (*Fig. 5B,C*).

After the stimulation test, a one-hour-long spontaneous activity recording took place during which the slices were perfused either with ACSF or MFR, then stimulation test was carried out again. There were no significant differences in the amplitude of the early component during the second test stimulation compared to the first test regardless to the solution of perfusion. The late component was not altered either in the groups perfused with ACSF. In the groups perfused with MFR, as an effect of the activation of NMDA receptors, the amplitude of the late component drastically increased in the control group and in the treatment groups (*Table 2*).

| | Threshold [V] | 1st test stimulation | | 2nd test stimulation | | | |
|---|---|---|---|---|---|---|---|
| | | ACSF | | ACSF | | MFR | |
| | | Early comp. [mV] | Late comp. [mV] | Early comp. [mV] | Late comp. [mV] | Early comp. [mV] | Late comp. [mV] |
| control | 2.77±0.20 | 1.15±0.10 | 0.25±0.04 | 1.68±0.27 | 0.60±0.17 | 1.64±0.33 | 0.85±0.24 |
| 10 µM | 1.71±0.10 | 2.02±0.13 * | 0.37±0.08 | 3.28±0.3 | 0.67±0.08 | 2.51±0.26 | 1.41±0.17 |
| 50 µM | 2.20±0.14 | 1.72±0.17 | 0.28±0.06 | 2.42±0.44 | 0.64±0.17 | 1.39±0.23 | 0.78±0.16 |
| 100 µM | 2.60±0.22 | 2.57±0.17 * | 0.29±0.07 | 2.79±0.13 | 0.42±0.09 | 2.59±0.46 | 1.01±0.30 |

*Table 2. The average voltage threshold of cortical evoked potentials and the average amplitude of EPSPs evoked with 2T intensity in cortical slices are summarized. Data are presented as mean ± S.E.M. N=10, 8, 9 and 5 of the ACSF perfusion and 11, 8, 11 and 6 of the MFR perfusion of the control, 10 µM, 50 µM and 100 µM treated groups respectively. There was no significant difference among voltage threshold values. *: 10 and 100 µM FB1-treatment caused significant increases in EPSP amplitude, compared to the control group.*

During the one-hour ACSF-perfusion, there was only negligible spontaneous activity in the control and all the treated groups as well, thus we can confirm that the toxin itself does not

have convulsive effect. During MFR-perfusion, 1-2-second-long bursts appeared with a frequency of 0.15-0.25 Hz but no differences caused by the pre-treatment could be observed between groups. Time ratio spent in bursting activity during the last five minutes of MFR-perfusion was in the S2 16.48±1.90%, 19.24±4.42%, 16.29±2.54% and 16.65±2.73% and in the LEC 15.57±1.85%, 21.25±1.85%, 16.20±2.86% and 14.95±2.86% for control and 10, 50, 100 µM FB1-treated groups, respectively (*Fig. 5E*).

### *3.3. In vivo electrophysiology*

Tibial nerve stimuli elicited clear EFPs after control (saline) as well as FB1 injections. Short latency responses (up to 50 ms) in layer 2/3 EFPs showed a characteristic positive peak (P1) which was highly variable in duration (*Fig. 6A*). Layer 5 EFPs were highly uniform in shape. These consisted of a high-amplitude negative wave (N1) which was followed by a sequence of negative-positive waves falling into the gamma frequency range (2-7 waves; frequency: 40-45 Hz) in some cases (*Fig. 6C*).

No significant changes were found in any of the EFP parameters analyzed (amplitude, latency, slope, under curve area) after saline or FB1 injections (7.5 mg/kg i.p.) compared to corresponding baseline (pre-injection) values in any experimental rat groups (n=6 in both groups). Statistical analysis comparing matching time points after saline vs. FB1 injections in layer 2/3 and layer 5, respectively, also showed lack of effect after FB1 exposure. Small, non-significant deviations of the analyzed EFPs parameters can be attributed to the fluctuations of anesthetic depth over time (*Fig. 6B,D*).

### *3.4. c-Fos activation*

C-Fos activation pattern was determined in the hippocampus and the somatosensory cortex 2 hours after FB1 or saline injections (7.5 mg/kg i.p.). Distribution of labelled neurons was very sparse in hippocampus *(Fig. 7A)* and somewhat denser in somatosensory cortex *(Fig. 7C)*. In the analyzed brain areas, *in vivo* FB1 treatment did not change significantly the number of Fos-positive neurons, compared to the corresponding brain areas in control rats (*Fig. 7B,D*).

## 4. Discussion

Fumonisin B1 is a mycotoxin produced by *Fusarium* species that represents a risk for human and animal health. We know that it can interfere with *de novo* sphingolipid biosynthesis in case of chronic consumption and possibly it has an impact on the liver and the kidney. In this

study we have investigated whether the toxin had a specific effect on the nervous system caused by acute exposure (a single, high-dose effect).

Applied doses and concentrations in the current study were chosen according to previous literature. For *in vivo* exposure FB1 was applied in a dose of 7.5 mg/kg, based on previous similar *in vivo* rat studies (Shephard et al., 1992; Bondy et al., 1995). For *in vitro* exposure we determined the applied concentrations according to previous experiments carried out on cell cultures where a wide range of concentrations from 0.1 µM to 200 µM was used. As our experiments were performed on cultures and brain slices, we had to consider the fact that in slices, neurons are organized in several layers and target cells are not located on the surface. In the absence of data about the speed and mechanism of diffusion of FB1 in brain tissue we supposed that higher concentrations may be needed for brain slices compared to cell cultures. Also the exposure time was considerably shorter in our case than for typical cytotoxicity tests. In the mentioned studies the lowest efficient concentration applied for the shortest time was 50 µM, so we used this concentration to test the effects and a lower concentration (10 or 20 µM) to test ineffectiveness; according to some authors FB1 applied in 100 µM concentration is cytotoxic, thus we have defined 100 µM as the highest concentration for our experiments (Stockmann-Juvala et al., 2004; Osuchowski and Sharma, 2005; Domijan et al., 2012). In our study, applied doses and concentrations are all higher than physiologically relevant levels, considering the TDI of 2 µg/kg/day.

To summarize our experimental results, *in vitro* exposure of brain slices or neuronal cultures to various concentrations of FB1 exerted a mainly stimulatory effect on the functioning of networks and individual cells. The amplitude of evoked field potentials increased in hippocampus and neocortex slices and spontaneous bursting activity was enhanced in cultures. Cultured hippocampal neurons exhibited increased firing in response to current injections. However, a single, relatively high-dose i.p. injection of FB1 to rats did not cause any significant short-term change in the neuronal activation pattern in hippocampus or neocortex, nor did it alter cortical evoked field potentials recorded in anesthetized animals.

There are scarce experimental data so far about the direct, short-term electrophysiological effects of FB1 on neuronal networks. It has been demonstrated that a low-dose i.p. FB1 injection to mice lowers the threshold dose of epileptic activity evoked with the convulsive agent pentylenetetrazole (Poersch et al., 2015). This observation is in accordance with our results on enhanced excitability, although the models and the exposure modes are different. Some experiments aiming to elucidate its subchronic effects were carried out previously at our department, with similar methods as in the present study. In adult rats fed with toxin-

containing feed, the amplitude of evoked potentials in the auditory and somatosensory cortex decreased, moreover, seizure-like events elicited with the convulsant 4-aminopyridine were inhibited (Banczerowski-Pelyhe et al., 2002b). However, after prenatal exposure, FB1 increased evoked field potentials, seizure-like event length and the degree of LTP (Banczerowski-Pelyhe, 2008; Banczerowski et al., 2008). This contradiction may suggest that repeated FB1-intake can enhance the basic excitability of neuronal networks during a sensitive stage of the nervous system development but after consumption in adulthood, the effects are distinct as dependent on quite different mechanisms. However, our present experimental protocols cannot be directly compared to those of the above-mentioned studies as in the previous experiments, animals were exposed to the toxin for several days either prenatally or as an adult. This type of treatment permits the development of effects linked to ceramide synthase inhibition, in contrast with the acute treatment applied now which is not prolonged enough for this. Considering this, we can conclude that in our experiments, different background mechanisms may lead to alterations in neuronal functions.

According to the literature, acute effects of FB1 on the brain were investigated only on cell cultures using calcium imaging (Domijan et al., 2012). The results showing that the toxin can enhance the excitatory effect of glutamate on cells are in concordance with our observations about increased excitability in cultured cells and brain slices as well. Nevertheless, some inhibitory effects of FB1 could also be observed in our study, notably the tendency for impaired hippocampal LTP in the 100 µM FB1 treatment group, and the smaller increase in cortical field potentials in response to MFR perfusion. MFR activates NMDA type glutamate receptors which increases the amplitude of the late component of the field EPSPs (Gean, 1990). This effect was smaller in the treatment groups of 50 and 100 µM FB1. It is possible that in a system already overactivated by the removal of magnesium, the toxin may cause an even greater dysfunction. In another study carried out on neuronal cell cultures, acute FB1 treatment induced the depolarization of the mitochondrial membrane. As a consequence, mitochondrial calcium uptake is inhibited which renders cells more vulnerable to physiological and pathological calcium stimuli (Domijan and Abramov, 2011). Treating cell cultures with MFR and FB1 simultaneously causes an extremely elongated increase in the intracellular calcium level of cells as MFR also increases the intracellular calcium levels via activating NMDA receptors (Domijan et al., 2012). Based on these findings we suggest that the observed decrease of evoked potentials in the slices treated with high-concentration of FB1 is caused by the overactivation of neurons that drives them into refractory stage. The possibility of cell death caused by this overactivation cannot be excluded.

For the verification of *in vitro* results, treatment of live rats was also applied in the present study. The effects of systemic administration of FB1 (7.5 mg/kg, i.p.) on neuronal cell activation measured by the expression of immediate early gene c-fos and evoked field potentials in urethane-anesthetized rats were tested. In these models, no significant alterations were found within 2 hours of FB1 treatment. One possible reason for this is likely the insufficient transfer of FB1 across the blood-brain barrier (BBB). However, some literature data about brain effects of FB1 in rodents after *in vivo* exposure are available. In addition to the previously mentioned study on the increased seizure susceptibility of mice treated with FB1 (Poersch et al., 2015), alterations of brain neuromodulator levels have also been reported. After dietary exposure to FB1, the levels of 5-hydroxyindoleacetic acid (5-HIAA) and the ratio of 5-HIAA/serotonin in the whole brain of rats increased, suggesting a possible dysfunction induced by fumonisin in either 5-HT metabolism or 5-HIAA elimination (Porter et al., 1990). A subsequent study by the same investigators reported decreased norepinephrine to dopamine ratio (Porter et al., 1993). These changes in the transmitter or transmitter metabolite content of the brain tissue were considered as implicit evidences for the brain access of the FB1. Also, the previously mentioned electrophysiological experiments done in our department demonstrated subtle alterations in brain excitability and nerve conduction velocity (Banczerowski-Pelyhe et al., 2002b, 2002a). However, presence of FB1 in the neural tissue and/or in the liquor cerebrospinalis was not measured in any of the above-mentioned studies.

In developing rats with incomplete BBB, FB1 treatment evoked an increase in sphinganine concentration and sphinganine/sphingosine ratio in the brain, suggesting successful BBB transfer (Kwon et al., 1997). However, in adult mice, FB1 transfer across the BBB measured via the inhibition of sphingolipid biosynthesis was found only when BBB integrity was compromised by lipopolysaccharide treatment (Osuchowski et al., 2005b). No study exists to date to prove the presence of FB1 in liquor cerebrospinalis after systemic FB1 administration in rats (Shier, 2000).

Another reason for the discrepancy between *in vitro* and *in vivo* results may be the metabolism of FB1 *in vivo*. However, FB1 metabolism in rats is not prominent, in addition to the production of hydrolyzed FB1 by gut microbiota, only one type of metabolite has been recently identified. N-acyl-FB1 was found at very low levels after five days FB1 i.p. administration (Harrer et al 2015) and there is no data about the presence of this molecule after single acute FB1 administration nor about its brain effects.

To summarize, our *in vivo* EFP and immunohistochemistry data support the hypothesis that in normal circumstances, BBB is only minimally permeable for FB1, at least, in acute conditions. This may be one possible cause of the lack of FB1 effects on c-fos expression and somatosensory EFPs recorded *in vivo*. However, *in vitro* findings show that FB1 did effectively alter field potential parameters and increase neuronal excitability when BBB was not present and FB1 could directly diffuse near the neurons. Here, the applied FB1 concentrations are presumably higher than the brain concentrations that may develop after systemic administration in an animal with intact BBB. Future studies should address the question regarding the central nervous system penetration of FB1 by using longer toxin exposure times. Even if brain penetration of the toxin is negligible, it may be effective in the peripheral nervous system and this hypothesis should also be tested by future experiments.

## Author contributions

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## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Conflict of interest

None.

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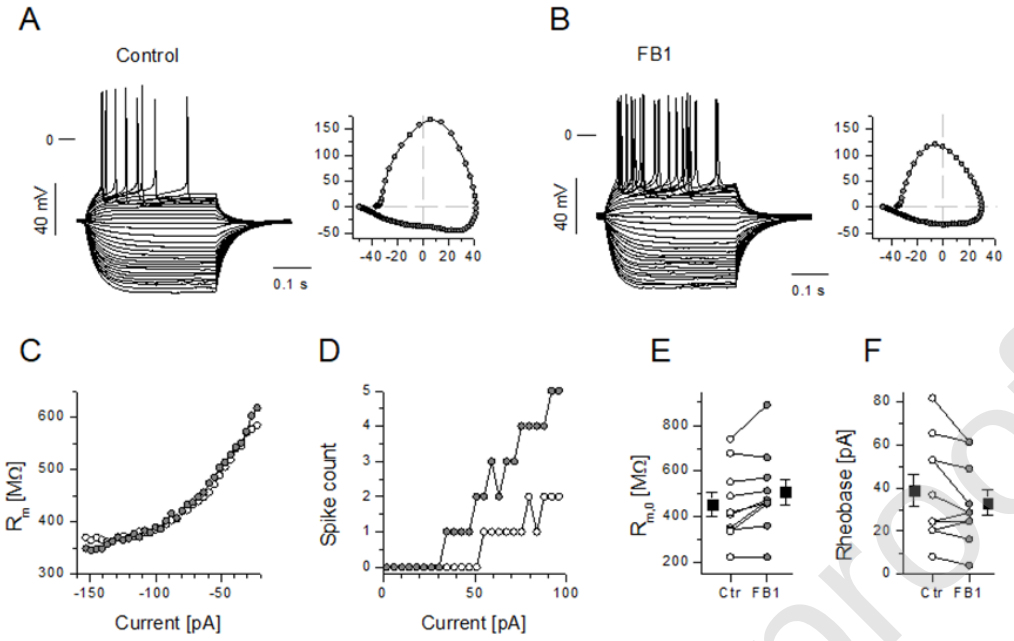


*Figure 1.* Acute FB1 application slightly increases the excitability of individual cultured hippocampal neurons. Voltage responses of a hippocampal neuron were measured before (A) and after (B) the application of FB1 (40 µM) using gradually increasing depolarizing current steps. In both examples the last trace shows the responses at +96 pA current level. The inserts are the phase portraits of the first action potential above rheobase. (C) Membrane resistance plotted as a function of the input current. Here, FB1 application caused no noticeable change. (D) Input-output functions in control and after FB1 application. The firing of this neuron becomes more intense after the mycotoxin application. Input membrane resistance (E) and rheobase (F) are shown for all the experiments (n=10). Averages are expressed as lines. Slight reduction of the rheobase after FB1 indicates the increase of the intrinsic excitability of the neurons.

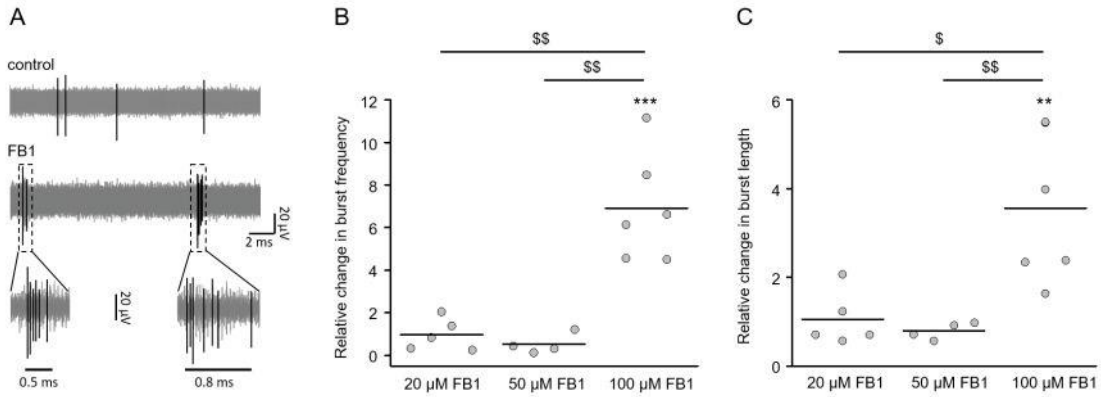


*Figure 2.* Acute FB1 application significantly increased the excitability of cultured hippocampal neuronal networks. Spontaneous firing of hippocampal neurons was measured before and after the application of FB1. (A) Representative pattern of spontaneous firing before (control, upper recording) and after (FB1, lower recording) the administration of 100 µM FB1. Burst activity is indicated by the horizontal lines below the enlarged recordings segments, with the elapsed times indicated below. (B) Relative burst frequency and (C) burst length values after FB1 treatments. 100 µM FB1 significantly increased burst activity in cultures. Averages of individual data points are expressed as horizontal lines. (Statistical analysis: repeated-measures two-way ANOVA with Tukey's post hoc test; * and $ mean significant difference compared to the control or FB1-treated values, respectively; $p<0.05$ $, $p<0.01$ ** or $$, $p<0.001$ ***.)

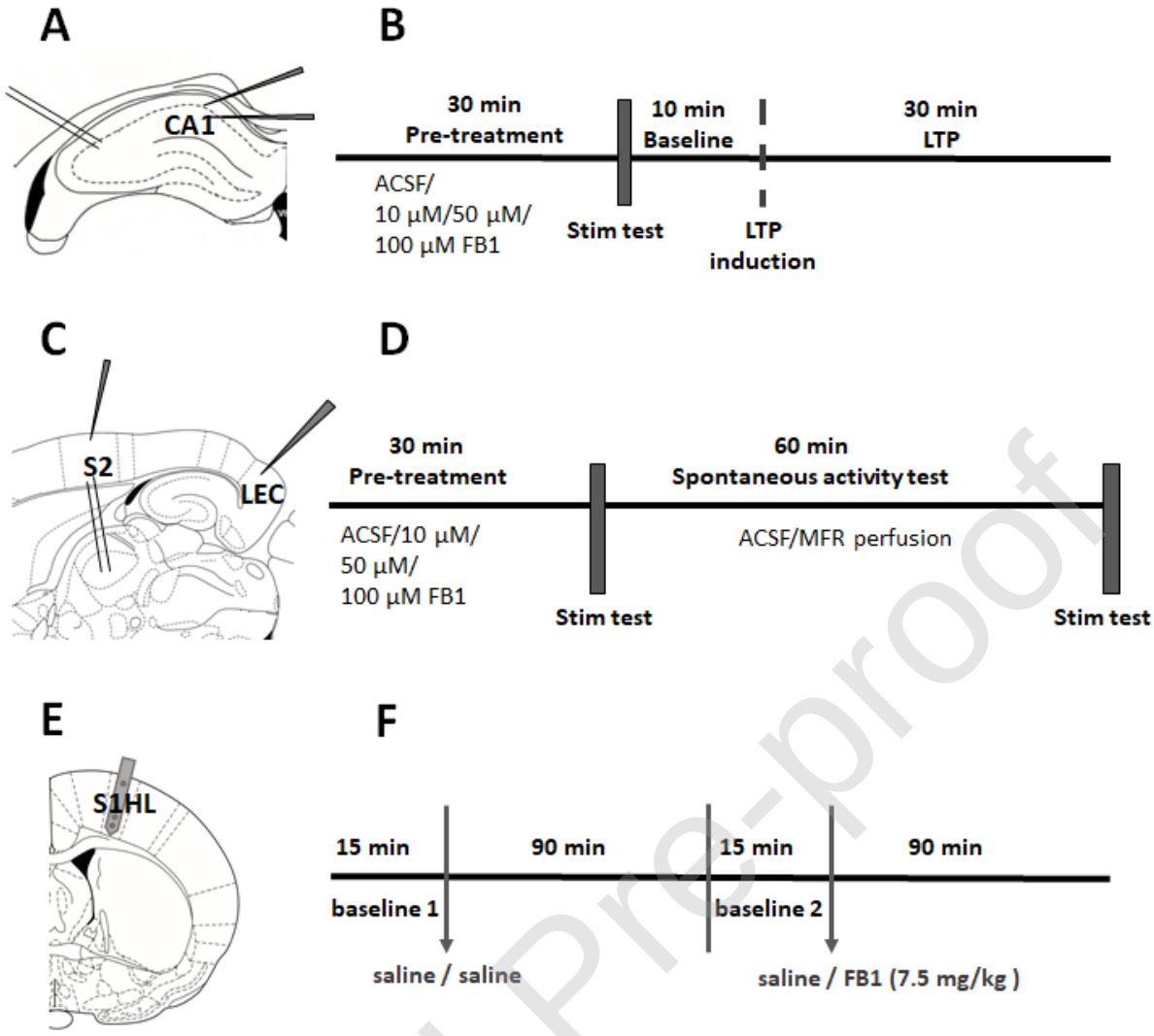


*Figure 3.* Experimental protocol for slice experiments and *in vivo* electrophysiology. (A) Positioning of the electrodes in the hippocampus: stimulation at the Schaffer-collaterals and recording in the *stratum radiatum* and the *stratum pyramidale*. (B) Timeline of experiments for hippocampus slices. (C) Positioning of the electrodes in the neocortex: stimulation in the secondary somatosensory cortex, at the border of the gray and white matter and recording in the 2/3 layer, an additional recording electrode in the 2/3 layer of the lateral entorhinal cortex. (D) Timeline of experiments for neocortex slices. (E) Positioning of the 16-channel vertical electrode array in the hindlimb area of the primary somatosensory cortex. (F) Timeline of *in vivo* experiments.

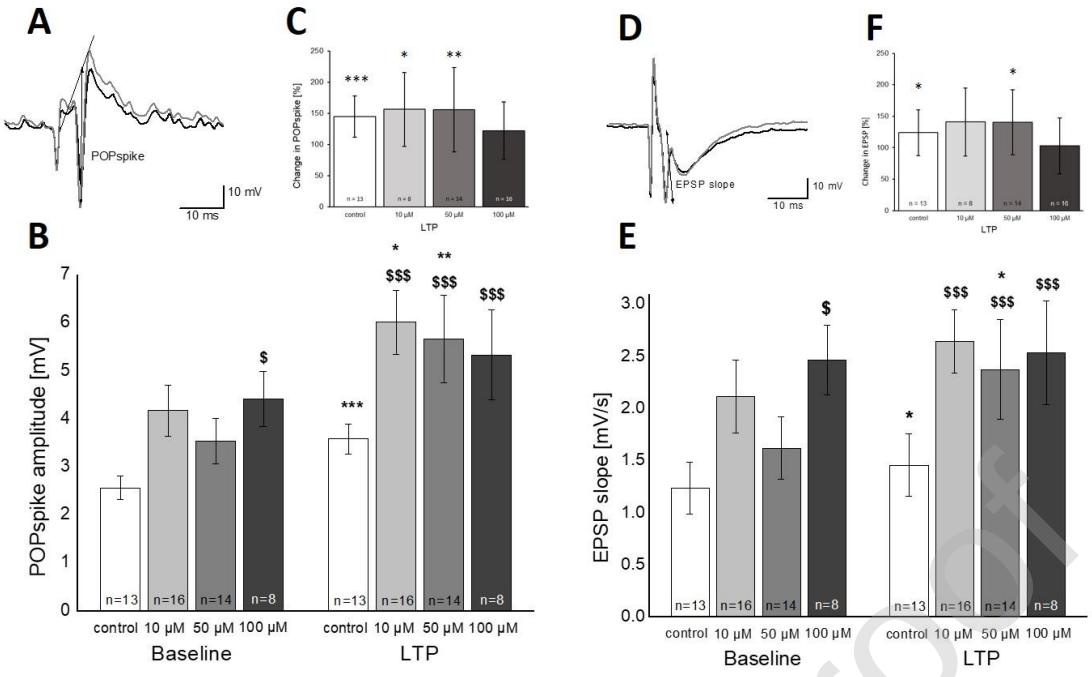


*Figure 4.* Excitatory effect of FB1 treatment on evoked potentials in hippocampal slices. (A) Representative evoked POP-spike from *stratum pyramidale* before (black line) and after (gray line) the LTP induction. POP-spike amplitude was analyzed. (B) The average amplitude of the POP-spikes recorded before LTP induction (baseline) and 30 minutes after LTP induction. (C) The average change in POP-spike amplitude 30 minutes after LTP induction compared to the baseline. (D) Representative evoked EPSP from *stratum radiatum* before (black line) and after (gray line) the LTP induction. Initial slope of the EPSPs was analyzed. (E) The average slope of the EPSPs recorded before LTP induction (baseline) and 30 minutes after LTP induction. (F) The average change in EPSP slope 30 minutes after LTP induction compared to the baseline. Data are expressed as mean and S.E.M. (Statistical analysis: One-way ANOVA for the differences between groups; $ means significant difference compared to the control group, $p<0.05$; paired t-test for the change of POP-spike amplitude or EPSP slope compared to baseline, * means significant difference compared to the baseline, $p<0.05$.)

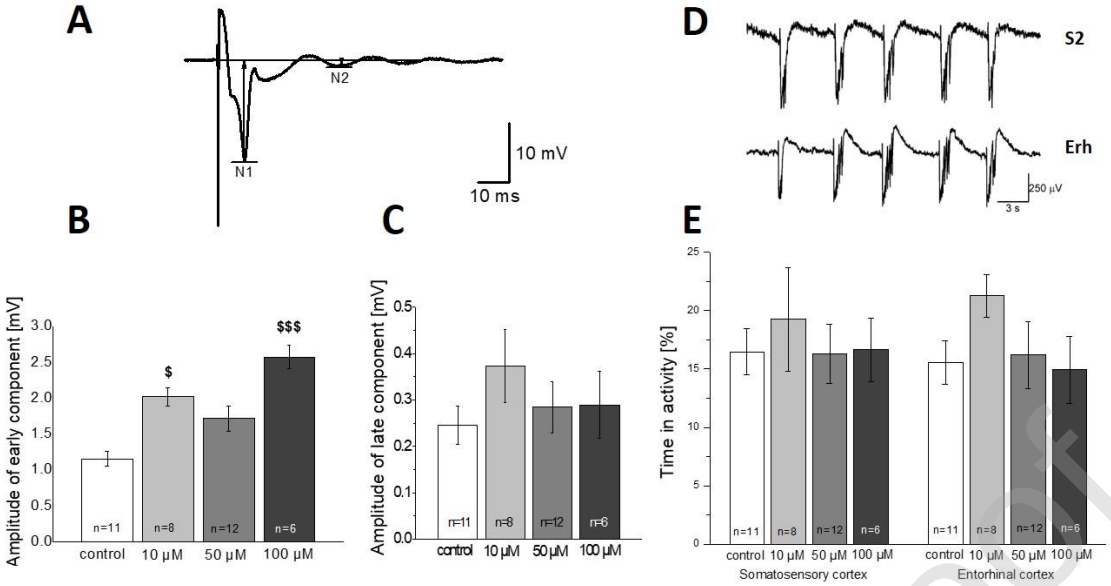


*Figure 5.* Effects of FB1 on evoked potentials and epileptiform activity in neocortical slices. (A) Amplitude of early (N1) and late (N2) component of evoked EPSPs in the secondary somatosensory cortex (S2) were analyzed. (B) Amplitude of N1 evoked in the S2 after ACSF or FB1 pre-treatment. (C) Amplitude of N2 evoked in the S2 after ACSF or FB1 pre-treatment. (D) Representative spontaneous epileptiform activity recorded in MFR in the somatosensory and entorhinal cortices. (E) Ratio of time in spontaneous activity during the last 5 minutes of the MFR-perfusion. Data are expressed as mean and S.E.M. (Statistical analysis: One-way ANOVA; $ means significant difference compared to the control group; $p<0.05$.)

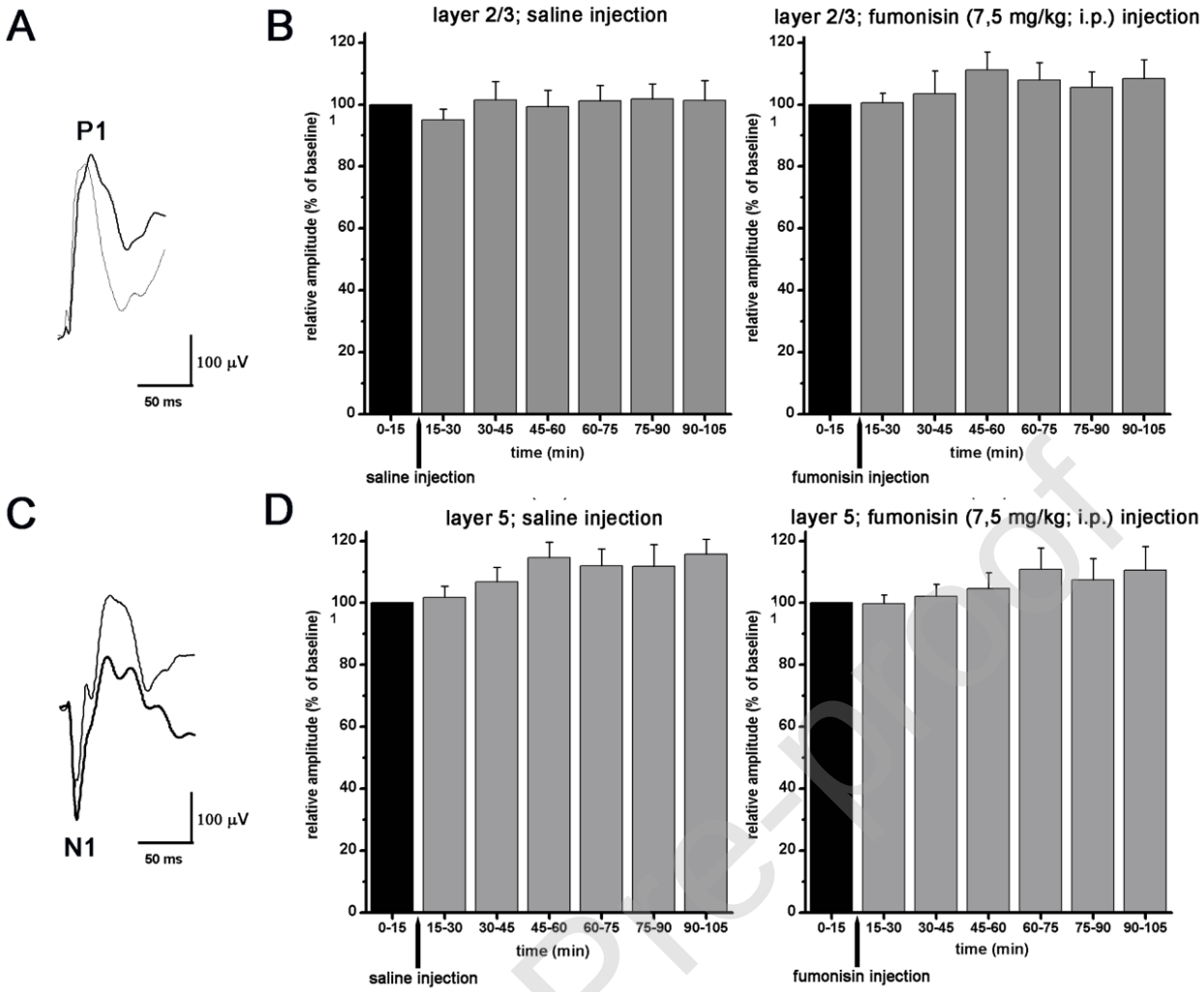


*Figure 6.* FB1 i.p. injections (7.5 mg/kg) did not change significantly the amplitude of evoked potentials measured *in vivo* in the somatosensory cortex of anesthetized rats. (A) Representative evoked EFP waves recorded in layer 2/3 of the HL area of the S1 after electrical stimulation of the contralateral tibial nerve. (B) Amplitude changes in layer 2/3, after saline injection at 15$^{th}$ min and FB1 injection at 120$^{th}$ min of the experiment. Baseline (pre-injection period) data were taken as 100% and post-injection data in 15-minutes long epochs are expressed as percent of the baseline. (C) Representative evoked EFP waves recorded in layer 5. (D) Amplitude changes in layer 5. Data are expressed as mean and S.E.M.

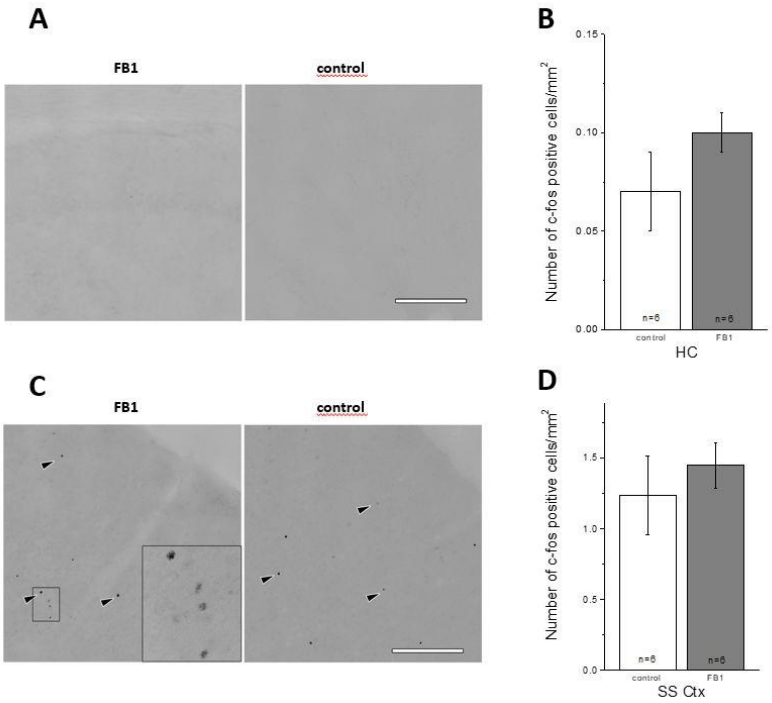


*Figure 7.* FB1 i.p. injection (7.5 mg/kg) administered to rats did not evoke specific activation of neurons examined with c-Fos immunohistochemistry. (A) Representative images of hippocampus sections from treated (left panel) and control (right panel) animals (scale bar represents 200 µm). No activated cells are present in the area. (B) The number of activated cells/mm$^2$ in the hippocampus. (C) Representative images of somatosensory cortex sections from treated (left panel) and control (right panel) animals. Activated cells appear as black dots (scale bar: 200 µm). Some of the labeled neurons are indicated by black arrowheads. The magnification of the squared area in the left panel is shown in an inlet to demonstrate the fine morphology of c-Fos labeling within the nuclei. (D) The number of activated cells/mm$^2$ in the somatosensory cortex. Data are expressed as mean and S.E.M.