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Toxic effects of midazolam on differentiating neurons in vitro as a consequence of suppressed neuronal Ca^{2+}-oscillations

Barbara Sinner[a,*], Oliver Friedrich[b], York Zausig[a], Thomas Bein[a], Bernhard M. Graf[a]

[a] Department of Anesthesiology, University of Regensburg, Regensburg, Germany
[b] Institute of Medical Biotechnology, Friedrich-Alexander University Erlangen-Nuremberg, Germany

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ABSTRACT

Background: In immature neurons anesthetics induce apoptosis and influence neuronal differentiation. Neuronal Ca^{2+}-oscillations regulate differentiation and synaptogenesis. We examined the effects of the long-term blockade of hippocampal Ca^{2+}-oscillations with midazolam on neuronal synapsin expression.
Material and methods: Hippocampal neurons were incubated at day 15 in culture with the specific $GABA_A$ receptor agonist muscimol (50 μM) or with midazolam (100 and 300 nM), respectively, for 24 h. TUNEL and activated-Caspase-3 staining were used to detect apoptotic neurons. Ca^{2+}-oscillations were detected using the Ca^{2+}-sensitive dye FURA-2 and dual wavelength excitation fluorescence microscopy. Synapsin was identified with confocal anti-synapsin immunofluorescence microscopy.
Results: Muscimol, when applied for 24 h, decreased the amplitude and frequency Ca^{2+}-oscillations significantly. Midazolam concentration-dependently suppressed the amplitude and frequency of the Ca^{2+}-oscillations. This was associated by a downregulation of the synapsin expression 24 h after washout.
Conclusion: Neuronal Ca^{2+}-oscillations mediate neuronal differentiation and are involved in synaptogenesis. By acting via the $GABA_A$ receptor, midazolam exerts its toxic effect through the suppression of neuronal Ca^{2+}-oscillations, a reduction in synapsin expression and consecutively reduced synaptic integrity.

1. Introduction

Benzodiazepines are widely used for sedation in neonatal and pediatric intensive care (Anand and Hall, 2006). In extensive animal trials various anesthetics have been found to induce neuronal apoptosis (Jevtovic-Todorovic et al., 2003; Nikizad et al., 2007). The results for midazolam are rather conflicting: e.g., in a model of neonatal rats or in neuronal cell cultures from rats, midazolam did not induce apoptosis (Vutskits et al., 2005). In contrast, in a neonatal mouse model midazolam induced neuronal apoptosis (Young et al., 2005).

In the immature brain neuronal development is regulated by Ca^{2+}-oscillations. Ca^{2+}-oscillations are present during a time period of high plasticity (Adelsberger et al., 2005; Allene and Cossart, 2010). They trigger the regulation of gene expression, neuronal differentiation and synaptogenesis and are therefore fundamental components of neuronal network development and plasticity (Adelsberger et al., 2005; Allene and Cossart, 2010). Local Ca^{2+} influx via ligand or voltage-gated Ca^{2+} channels results in an increase in the intracellular Ca^{2+} concentration which triggers Ca^{2+} release from the endoplasmic reticulum (ER) via inositol triphosphate (IP_3)- and ryanodine receptors. This local ER Ca^{2+} efflux triggers Ca^{2+} release via neighbouring IP3 and RY receptors (Ca^{2+}-induced Ca^{2+}-release). Increased intracellular Ca^{2+}-concentration is rapidly restored back to baseline levels by reuptake into the ER. Periodical increase and decrease of the intracellular Ca^{2+}-concentration causes Ca^{2+}-oscillations. In the immature brain, neuronal Ca^{2+}-oscillations are present during the period of extensive neuronal differentiation. Glutamate is the most important neurotransmitter in the developing brain and is has been shown that neuronal Ca oscillations are mediated by glutamate (Allene and Cossart, 2010; Garaschuk et al., 1998; Sinner et al., 2005). GABA (gammabutyric acid) is the most important inhibitory transmitter in the adult brain. During neuronal differentiation the $GABA_A$ receptor undergoes a distinctive change: in the initial period, the activation of the $GABA_A$ receptor results in a depolarization. In rodents, during the period of excessive synaptogenesis, the activation of the $GABA_A$ receptor changes to hyperpolarization (Adelsberger et al., 2005; Khazipov et al., 2008). Both neurotransmitters affect Ca^{2+}-oscillations and might thereby, influence neuronal development and differentiation (Adelsberger et al., 2005; Garaschuk et al., 1998, 2000; Khazipov et al., 2008; Mantelas

* Corresponding author at: Department of Anesthesiology, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany.
Tel.: +49 941 944 7801; fax: +49 941 944 7802.
E-mail address: barbara.sinner@klinik.uni-regensburg.de (B. Sinner).

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et al., 2003; Sinner et al., 2005, 2006). Neuronal Ca^{2+} oscillations activate various signaling pathways which are directly or indirectly involved in neuronal development and differentiation, e.g., Ca^{2+} calmodulin kinases, extracellular-signal-regulated kinases (ERK1/2) or cAMP element binding protein (CREB) (de Koninck and Schulman, 1998; Zhou et al., 2009).

Here, we examined the role of the $GABA_A$ receptor and the impact of long-term application of midazolam on neuronal Ca^{2+}-oscillations and the consequences for synapsin expression. We tested the hypothesis that long-term activation of the $GABA_A$ receptor influence Ca^{2+}-oscillations in developing mammalian brain and impair network architecture and synaptic integrity.

2. Materials and methods

2.1. General preparation

All animals were purchased from Charles Rivers, Sulzberg, Germany. The animal experiments were performed following the German Animal Welfare Act. Accordingly, the local authorities obtained informed details about sacrification of animals for scientific purpose. Head of laboratory facilities: Dr. Thilo Spruss.

2.2. Cell culture

Hippocampal cell cultures were prepared as described previously (Sinner et al., 2005, 2006). Briefly, hippocampi of 19 days old embryonic Wistar rats were prepared in ice-cold PBS (Invitrogen Life Technologies, Karlsruhe, Germany), containing 0.05% gentamycin (Sigma Chemicals, Steinheim, Germany). After incubation in 0.02% trypsin (Sigma Chemicals) for 15 min, cells were rinsed 3 times in ice cold MEM (Invitrogen Life Technologies). Neurons were dissociated with a fire-polished pipette in MEM supplemented with 10% horse serum (Invitrogen Life Technologies) and dispersed on cover slips pretreated with 0.01% poly-L-ornithin (Sigma Chemicals). Neurons were cultivated under sterile conditions at 37 °C and 5% CO_2 in MEM containing 10% horse serum.

2.3. Long-term drug exposure

On day 15, cell cultures were incubated with midazolam (Sigma Chemicals) in MEM at concentrations of 100–300 nM. To test the role of the $GABA_A$ receptor, cultures were incubated with muscimol (50 μM, Sigma Chemicals). After 24 h, the medium of the culture dishes was exchanged and cultures were rinsed 5 times with MEM to ensure washout. Control dishes were treated in the same manner without midazolam or muscimol.

2.4. TUNEL (terminal deoxy-uridine triphosphate nick-end labelling) assay

Condensed or fragmented DNA was assayed using the TUNEL AP kit (Roche Diagnostics, Mannheim, Germany) and experiments were performed according to the manufacturer's recommendations. Briefly, dishes were rinsed with PBS and fixed in paraformaldehyde 4%. After rinsing, neurons were permeabilized with Triton X-100 and 0.1% Na citrate (both Sigma Chemicals) for 2 min at 2 °C. TUNEL reaction mixture was applied and dishes were kept at 37 °C for 60 min. The reaction was terminated by transferring the dishes to PBS solution. Converter AP (Roche Diagnostics) was added for 30 min at 37 °C. Substrate solution was applied for 10 min at room temperature. Substrate solution consisted of 200 μl NBT/BCIP (Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) (Roche Diagnostics) stock solution containing TRIS, NaCl (100 mM), Mg Cl_2 (50 mM) adding NaOH to receive a pH of 9.5 (all Sigma Chemicals) was added for 10 min in the dark. Following rinsing with PBS, neurons were examined under a light microscope with a 200× magnification. Violet stained TUNEL positive neurons were counted in 20 different visual fields of each preparation and compared to the total number of neurons.

2.5. Activated Caspase-3 staining

Activated Caspase-3 staining for the detection of apoptosis was performed according to the manufacturer's recommendation (activated Caspase-3 antibody, monoclonal, rabbit, BD PharMingen, San Diego, CA, USA). Dishes were rinsed with PBS and fixed in 4% formaldehyde for 5 min and again rinsed with PBS. Neurons were incubated in 3% H_2O_2 for 10 min and after washout, enzymatic reaction was blocked with Blocking Solution for 10 min. Following rinsing with PBS, neurons were incubated with primary antibody anti-Caspase-3 (1:200) in Tris buffer for 60 min. Subsequently, neurons were incubated with the biotinylated secondary antibody for 10 min. Following rinsing with PBS, strepdavidin horse radish peroxidase (HRP) was added for 25 min. Chromogen solution (AEC) was applied and cultures were controlled under a light microscope until adequate color intensity. Activated Caspase-3 positive neurons were counted in relation to the total number of neurons per visual field.

2.6. Fluorescence imaging

The experiments were performed in standard extracellular solution containing 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM Mg^{2+}, 0.9 mM NaH_2PO_4, 10 mM glucose and 20 mM HEPES. The pH was adjusted to 7.3 using NaOH. For the dye loading procedure, the coverslips were mounted in a perfusion chamber and neurons were loaded with fura-2 AM (10 μM; Molecular Probes, Goettingen, Germany) containing 0.08% Pluronic-127 (Molecular Probes) for 30 min at room temperature. To allow de-esterification of the dye, cells were incubated in MEM for 30 min at 37 °C and 5% CO_2. For the experiments performed at 15 min after washout, the loading procedure was performed in the presence of the respective drug. The experiments were performed at 37 °C using a gas tight measurement chamber. The setup consisted of an inverted microscope (Olympus MT 10, Cell M imaging station, Olympus, Hamburg, Germany) and a CCD camera with a temporal resolution of 0.8 s at 2× binning. A xenon lamp was used for excitation at 340 nm and 380 nm using a filter switch with a cycle time of 5 ms.

2.7. In situ calibration

Ratios were calculated after background subtraction and intracellular calcium concentration was estimated as described by Grynkiewicz et al. (1985). For "in situ" calibration, neurons were permeabilized with the ionophore Br-A-23187 (Molecular Probes, Goettingen, Germany) dissolved in dimethyl sulfoxid (DMSO, Merck, Darmstadt, Germany). The calibration solutions were EGTA (ethylene-glycol-bis[beta-aminoethylether]-N,N,N′,N′,tetraacetic acid, Sigma Chemicals, Steinheim, Germany) buffered solutions containing NaCl 120 mM, KCl 4 mM, Mg acetate 1 mM, HEPES 10 mM, EGTA 10 mM, Sucrose 350 mM and varying levels of Ca ranging from 1 nM to 10 μM free Ca^{2+}.

The frequency and amplitudes of the spontaneous Ca^{2+}-oscillations were evaluated. The intracellular Ca^{2+} concentration was determined after in situ calibration by converting the fluorescence ratio into the free calcium concentration. The amplitude of an oscillation was calculated by subtracting the maximum calcium concentration from the baseline calcium level.

2.8. Confocal microscopy

Confocal imaging was performed 24 h after the washout of midazolam. For this, the culture dishes were mounted in the recording chamber, rinsed three times with ice-cold PBS (Invitrogen) and fixed at −20 °C for 30 min in methanol. After rinsing with PBS three times for 5 min, cells were permeabilized in PBS containing 0.2% Triton-X-100. After 1 h incubation with 5% horse serum in PBS, dishes were rinsed and incubated at room temperature with rabbit anti-synapsin (Invitrogen) (1:500) overnight. Following rinsing with PBS, the dishes were loaded with Alexa 488 anti-rabbit (1:4000; Molecular Probes) secondary antibody in a volume of 750 μl. For negative control, imaging of primary and secondary antibodies alone was performed.

Confocal imaging of the proximal neuronal axon and dendrites up to the second branch was performed with an Olympus Fluoview FV 1000 using a 20× objective and a fourfold zoom setting. XYZ immunofluorescence stacks were taken at 0.2 μm steps and saved as 16-bit tif files for later analysis. Background signal was evaluated from an out-of-focus plane and this threshold used to eliminate background pixel entries on all other sections in this stack. This resulted in background noise reduction and increased delineation of discrete puncta by eliminating sub-threshold pixels.

For data analysis, pixels on each individual neuron were counted at the soma and at the dendrites up to the second branch. Control experiments were performed using primary and secondary antibody alone, respectively.

2.9. Statistical analysis

For Ca^{2+}-measurements, neurons were scanned for 3 min. The intracellular Ca^{2+}-concentration was determined after in situ calibration by converting the fluorescence ratio into the free calcium. The frequency of the spontaneous Ca^{2+}-oscillations was evaluated by counting the number of oscillations within the 3 min period. Oscillations were only counted as oscillation and analyzed for frequency when they exceeded 1.5× hard thresholding over mean noise. The amplitude of an oscillation was calculated by subtracting the maximum calcium concentration from the baseline calcium level, and the average amplitude of each scan was used for the analysis. Statistical significance between treatment groups was determined by analysis of variance (ANOVA). Differences were considered significant for $p < 0.05$. Data are presented as mean ± SEM with n the number of observations.

3. Results

3.1. Midazolam induces neuronal apoptosis

The long-term incubation of the $GABA_A$-receptor agonist muscimol 50 μM did not result in a significant increase of TUNEL or Caspase-3 activated neurons. When neurons were incubated with