

The Toxic Effects of S(+)-Ketamine on Differentiating Neurons In Vitro as a Consequence of Suppressed Neuronal Ca^{2+} Oscillations

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BACKGROUND: In the immature brain, neuronal Ca^{2+} oscillations are present during a time period of high plasticity and regulate neuronal differentiation and synaptogenesis. In this study we examined the long-term blockade of hippocampal Ca^{2+} oscillations, the role of the *N*-methyl-D-aspartate (NMDA) receptors and the effects of S(+)-ketamine on neuronal synapsin expression.

METHODS: Hippocampal neurons were incubated at day 15 in culture with the specific NMDA receptor antagonists dizocilpine (MK 801, 100 μM) or S(+)-ketamine (3 μM to 25 μM) for 24 hours. Terminal-deoxynucleotidyl-transferase (TUNEL) and activated caspase3 were used to detect apoptotic neurons. Ca^{2+} oscillations were detected after loading the neurons with the Ca^{2+} -sensitive dye fura-2AM, and dual wavelength excitation fluorescence microscopy was performed. Ca^{2+} /calmodulin kinase II (CaMKII) was measured using Western blots. Synapsin was identified with confocal antisynapsin immunofluorescence.

RESULTS: Blocking the NMDA receptor with MK 801 or 25 μM S(+)-ketamine resulted in a significant increase in apoptotic neurons. MK 801 led to a significant increase in cytosolic Ca^{2+} concentration and reduction of the amplitude and frequency of the Ca^{2+} oscillations. Similar to MK 801, the long-term application of S(+)-ketamine resulted in a significant increase in cytosolic Ca^{2+} concentration 24 hours after washout. This was associated with a down-regulation of the CaMKII and a reduction of the synapsin 24 hours after washout.

CONCLUSION: Neuronal Ca^{2+} oscillations mediate neuronal differentiation and synaptogenesis via activating CaMKII. By acting via the NMDA receptor, S(+)-ketamine exerts its toxic effect through the suppression of neuronal Ca^{2+} oscillations, down-regulation of the CaMKII, and consecutively reduced synaptic integrity. (Anesth Analg 2011;113:1161–9)

In neonates and infants S(+)-ketamine is used, alone or in combination, for sedation or general anesthesia.¹ In contrast to other sedatives and hypnotics, S(+)-ketamine distinguishes itself by producing unconsciousness and analgesia without major impairment of respiratory function. In clinically relevant concentrations, S(+)-ketamine, a phencyclidine derivative, acts by blocking the *N*-methyl-D-aspartate (NMDA) receptors. The NMDA receptor is a glutamate receptor subtype, which is involved in a variety of processes, including development and differentiation of the central nervous system, learning and memory, and synaptic plasticity.² Pharmacological blockade of the NMDA receptor during the important period of brain development and synaptogenesis leads to widespread apoptosis in immature neurons.^{3,4} Various experiments demonstrate that ketamine concentration-dependently and time-dependently induces apoptosis in the immature brain in rodents and rhesus monkeys.^{5–10}

Besides the effects on neuronal survival, ketamine influences the finely tuned mechanisms responsible for neuronal development and dendritic arborization even at concentrations at which no influence on survival can be detected.^{9,10} These mechanisms could be responsible for the observed neurofunctional deficits in adult mice after being exposed to ketamine during the critical phase of neuronal development.¹¹

In the immature brain, neuronal development is regulated by Ca^{2+} oscillations, which are present during a time period of high neuronal plasticity.¹² Ca^{2+} oscillations are triggering the regulation of gene expression,¹³ neuronal differentiation, and synaptogenesis,^{12,14,15} and are therefore fundamental components of neuronal network development and plasticity.^{14,16,17} Local Ca^{2+} influx via ligand- or voltage-gated Ca^{2+} channels results in an increase in intracellular Ca^{2+} concentration by triggering Ca^{2+} release from the endoplasmic reticulum through inositol triphosphate receptors and ryanodine receptors. This local Ca^{2+} efflux in turn triggers Ca^{2+} release via neighboring inositol triphosphate and ryanodine receptors (Ca^{2+} -induced Ca^{2+} release). Increased intracellular Ca^{2+} concentration is rapidly restored back to baseline levels by reuptake into the endoplasmic reticulum via the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase or into the extracellular space via extrusion. Ca^{2+} oscillations are characterized by periodic increases and decreases of the intracellular Ca^{2+} concentration. Increased intracellular Ca^{2+} differentially binds to numerous proteins, of which calmodulin plays an important role. Calmodulin decodes Ca^{2+} signals on the basis of

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their frequency. The binding of Ca^{2+} to calmodulin induces a conformational change promoting interactions between the Ca^{2+} calmodulin (CaMK) complex and numerous target proteins, e.g., CaMKII, which is involved in neuronal development and plasticity.¹⁵ In the immature brain, neuronal Ca^{2+} oscillations are present during the period of extensive neuronal differentiation, and it has been shown that NMDA receptors play a crucial role in the development and regulation of these Ca^{2+} oscillations.^{16,17} On the other hand, synapsin I is phosphorylated at Ser603, linking neuronal plasticity to Ca^{2+} , among other stimulants.¹⁸ However, the link between Ca^{2+} oscillations and synapsin expression as a prerequisite for brain network development has not been established. Therefore, we examined the role of the NMDA receptor and the impact of long-term applications of the stereoisomer S(+)-ketamine on neuronal Ca^{2+} oscillations and the consequences for synaptogenesis. In particular, we tested the hypothesis that long-term blockade of NMDA-induced Ca^{2+} oscillations in developing mammalian brain would impair network architecture through down-regulation of CAMKII and synapsin. This is confirmed by our results with S(+)-ketamine, which also provide an explanation for potential neurotoxicity in premature and newborn patients in whom the drug is widely used.

METHODS

General Preparation

The experimental procedures and protocols were approved by the local Animal Care Committee.

Cell Culture

Hippocampal cell cultures were prepared as described previously.^{19,20} Briefly, hippocampi of 19-day-old embryonic Wistar rats (E19) were prepared in ice-cold phosphate-balanced solution (PBS, Invitrogen Life Technologies, Karlsruhe, Germany) containing 0.05% gentamycin (Sigma Chemicals, Steinheim, Germany). After incubation in 0.02% trypsin (Sigma Chemicals) for 15 minutes, cells were rinsed 3 times in ice-cold Minimum Eagles Medium (MEM, Invitrogen). Neurons were dissociated with a fire-polished pipette in MEM supplemented with 10% horse serum (Invitrogen) and dispersed on cover slips coated 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.

Long-Term Drug Exposure

For the long-term experiments, drugs were diluted in MEM without horse serum and applied on the hippocampal cell cultures on day 15 in culture. To test the involvement of the NMDA receptor on neurotoxicity, the preparations were incubated for 24 hours with dizocilpine (MK 801, 100 μM , Fluka Taufkirchen, Germany). To evaluate the effects of S(+)-ketamine (Sigma Chemicals), in separate experiments, the drug was applied at concentrations ranging from 3 to 25 μM . Control cultures were incubated in MEM. After 24 hours of incubation, the culture medium was exchanged and cultures were rinsed 5 times with MEM and to ensure washout.

Terminal DeoxyUridine Triphosphate Nick-End Labeling (TUNEL) Assay

Condensed or fragmented DNA was assayed using the TUNEL AP kit (Roche, Grenzach-Wyhlen, Germany), and experiments were performed according to the manufacturer's recommendations. Briefly, dishes were rinsed with PBS and fixed in 4% paraformaldehyde (pH 7.4) for 1 hour at room temperature. After rinsing, neurons were permeabilized with freshly prepared 0.1% Triton X-100 in 0.1% sodium citrate (all, Sigma Chemicals) for 2 minutes at 2°C. TUNEL reaction mixture was applied, and dishes were kept at 37°C and 5% CO_2 for 60 minutes. The reaction was terminated by transferring the dishes to PBS solution. The incorporated fluorescein was detected with an antifluorescein antibody alkaline (AP) conjugate. Antifluorescein AP was added for 30 minutes at 37°C according to the manufacturer's recommendations. Dishes were rinsed and incubated for 30 minutes with the substrate solution to visualize the immunocomplexed AP. Substrate solution consisted of 200 μL Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) stock solution containing TRIS, NaCl (100 mM), and Mg Cl_2 (50 mM) and adding NaOH to receive a pH of 9.5. After a rinse with PBS, neurons were examined under a light microscope with a 200 $\times$ magnification by a person blinded to the treatment. The percentage of TUNEL positive neurons was counted in 10 different visual fields of each preparation and compared with control cultures.

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). Briefly, dishes were rinsed with PBS. After fixation for 5 minutes in 4% formaldehyde, dishes were thoroughly rinsed in PBS. Neurons were incubated in 3% H_2O_2 for 10 minutes and after washout, enzymatic reaction was blocked with Blocking Solution for 10 minutes. After rinsing with PBS, neurons were incubated with primary antibody anticaspase-3 (1:200) in Tris buffer for 60 minutes. Subsequently, neurons were incubated with the biotinylated antibody for 10 minutes. After rinsing with PBS, streptavidin horseradish peroxidase was added for 25 minutes. Chromogen Solution (AEC) was applied and cultures were counted under a light microscope by a person blinded to the treatment. Activated caspase-3-positive neurons were counted in percentage to the total number of neurons per visual field.

Western Blot Analysis

Neurons were removed from the dishes 18 hours after exposure to the treatment with trypsin-EDTA, 1.25% (GIBCO Invitrogen Life Technologies, Karlsruhe, Germany) and lysed with RIPA buffer (10 mM Tris, pH 8.0, 140 mM NaCl, 1% Triton X-100, 1% Na desoxycholate, 1% SDS) and protease inhibitors (all chemicals from GIBCO Invitrogen) with a repeated thawing and freezing procedure in liquid nitrogen. Probes were then centrifuged at 17,000g for 30 minutes at 4°C. The protein concentration of the lysates was determined with the BCA Protein Assay (Thermo Scientific, Rockford, IL) and protein samples (20 μg per lane) were

diluted in Laemmli sample buffer (Tris 0.25M, glycine 1.92M, SDS 1%, pH 8.4 to 8.9, Biorad, Munich, Germany), including β -mercaptoethanol (5%) and boiled for 5 minutes at 98°C, separated in SDS – 10% polyacrylamid gel (buffer: 1.5 M TRIS-HCl pH 8.8, Biorad) and transferred to nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, Freiburg, Germany). The membranes were subsequently incubated with CaMKII (diluted 1:1000, Cell Signaling Technologies, New England Biolabs, Frankfurt, Germany) for 16 hours at 4°C. The membrane was then exposed to a fluorescently labeled secondary antibody (Li-Cor IRDy 680 and 800, Licor Bioscience, Bad Homburg, Germany), and bands were detected using a near-infrared imaging system (Odyssey Infrared Imaging System, Li-Cor Bioscience). β -Actin was used as standard. All bands were densitometrically analyzed, and for each control and treatment sample a ratio to β -actin was determined first individually and then calculated as percentage of control.

Calcium Fluorescence Imaging

The experiments were performed in 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. Fifteen minutes and 24 hours after washout of the tested drug, respectively, 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 20% (Molecular Probes) for 30 minutes at room temperature. To allow deesterification of the dye, we incubated cells in MEM for 30 minutes at 37°C and 5% CO_2 . For the fluorescence experiments performed at the time point “15 minutes after washout,” the loading procedure with fura 2-AM was performed in the presence of MK 801 or S(+)-ketamine, respectively.

The experiments were performed at 37°C using a gas-tight measurement chamber. The setup consisted of an inverted microscope (Olympus MT Cell M imaging station, Olympus, Hamburg, Germany) equipped with a CCD camera with a temporal resolution of 0.8 seconds at 2 $\times$ 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.

In Situ Calibration

Calibration was performed by exposing the neurons to 10 μM of the Ca^{2+} -sensitive ionophore Br-A 23,187 (Molecular Probes) dissolved in dimethyl sulfoxide (Sigma Chemicals) in EGTA (ethylene glycol-bis β -aminoethylether- N,N,N',N' -tetraacetic acid) buffered intracellular solution containing (in mM) HEPES 60, $\text{Mg}(\text{OH})_2$ 5.31, adenosine triphosphate 8.0, creatine phosphate 10.0, EGTA 50.0 (Sigma Chemicals, Steinheim, Germany) at varying levels of Ca^{2+} ranging from 1 nM to 10 μM . By least square Hill fits to the data, the in situ affinity constant K_d of fura-2 could be determined and the free intracellular Ca^{2+} concentration was calculated from the fura-2 fluorescence ratios.

Immunocytochemistry

Immunofluorescence microscopy was performed 24 hours after the washout of S(+)-ketamine. For this, the culture

dishes were rinsed 3 times with ice-cold PBS (Invitrogen) and fixed at -20°C for 30 minutes in methanol. After rinsing with PBS 3 times for 5 minutes, cells were permeabilized in PBS containing 0.2% Triton- $\times$ 100. After 1 hour incubation with 5% horse serum in PBS, dishes were rinsed and incubated at room temperature with rabbit antisynapsin (1:500 dilution, Invitrogen) overnight. After rinsing, the dishes were incubated with Alexa 488 antirabbit secondary antibody (1:4000, Molecular Probes) in a volume of 750 μL . Dishes were rinsed with PBS for confocal microscopy. For negative control, imaging of primary and secondary antibodies alone was performed.

Confocal Microscopy

Confocal imaging of the proximal neuronal axon and dendrites up to the second branch was performed with an Olympus Fluoview FV 1000 (Olympus, Hamburg, Germany) using a 20 $\times$ objective and a 4-fold zoom setting. Fluoview software was used to control a CCD camera, and the Z-stack parameter was set at 0.2 μm . The laser and detector settings were retained for all images collected within experimental groups. Control and experimental groups were always run in parallel within the same immunocytochemical procedure. Stacks of images were taken from the first to last Z-level with immunofluorescence, and saved in 16-bit tagged image file format for later analysis. NIH ImageJ version 1.33 was used to measure synapsin immunofluorescence. Using ImageJ, we converted the stacks to 8-bit gray-scale format, and the Z-section with the lowest amount of light used to determine the threshold. This Z-section was out of the plane of focus of the tissue sections, and the threshold eliminated all pixels in this Z-section. The threshold was used on all other sections in this stack to eliminate background and increase delineation of discrete puncta by eliminating pixels below threshold. Individual pixels above threshold were counted.

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.

Statistical Analysis

For Ca^{2+} measurements, neurons were scanned for 3 minutes. 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-minute period. Oscillations were only counted as oscillation and analyzed for frequency when they exceeded 1.5 $\times$ hard thresholding over mean noise.²⁰ The amplitude of an oscillation was calculated by subtracting the baseline level from the maximum absolute peak value, and the average amplitude of each scan was used for the analysis. Statistical significance between treatment groups was determined with analysis of variance (ANOVA). Differences were considered significant for $P < 0.05$. Data are presented as mean $\pm$ SEM with n being the number of observations.

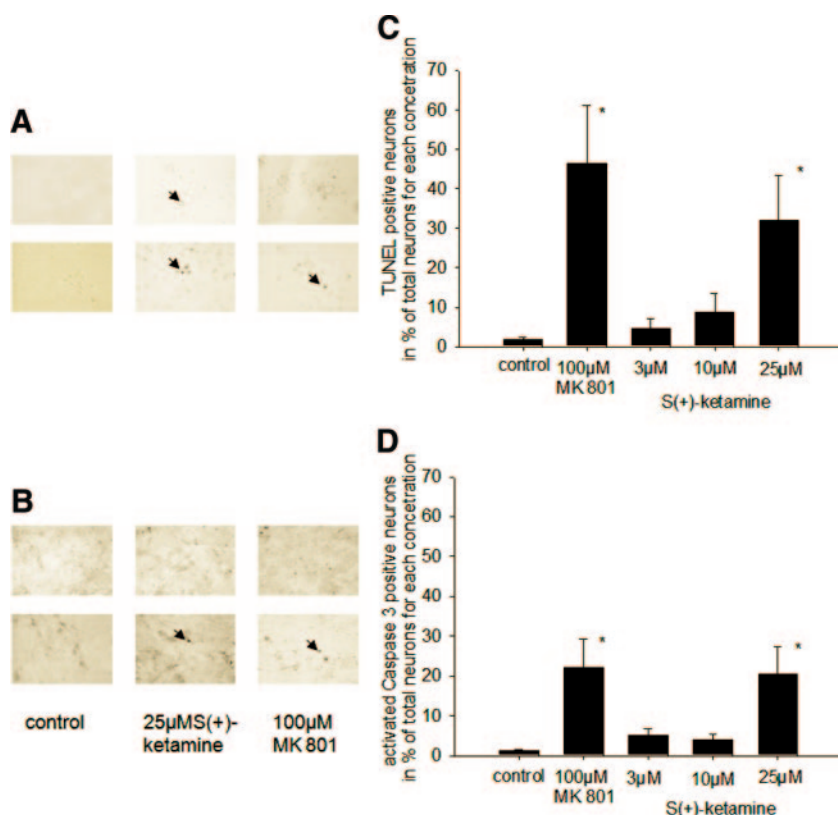


Figure 1. Apoptosis induced by NMDA receptor blockade with S(+)-ketamine and MK-801 in cultured hippocampal rat neurons. A, Shows representative figures for terminal-deoxynucleotidyl-transferase (TUNEL)- staining. B, Shows caspase-3 staining. The upper panels show representative pictures with a 200 $\times$ amplification and the lower panels with 400 $\times$ amplification. Arrows indicate TUNEL and caspase-3 positive neurons. C, The number of apoptotic, TUNEL-positive neurons is vastly increased after exposure to 25 μ M S(+)-ketamine and 100 μ M dizocilpine (MK 801). D, Similarly, the percentage of caspase-3 positive neurons was significantly greater at 25 μ M S(+)-ketamine and 100 μ M MK 801. TUNEL or caspase-3 positive neurons are given as percentage of all hippocampal neurons for each drug and concentration. Three dishes were stained for each preparation and concentration. From each dish the positively stained neurons were counted and compared with the total number of neurons of each field, i.e., at least 200 neurons per visual field. This was repeated for 10 visual fields per dish. TUNEL or caspase-3 positive neurons are given as percentage of all hippocampal neurons for each drug and concentration. Numbers are expressed as mean $\pm$ SEM. * P < 0.05 for drug exposure versus control.

RESULTS

Induction of Apoptosis

Incubation with the NMDA-receptor-antagonist MK 801 over 24 hours resulted in a significant induction of apoptosis as revealed by caspase 3 and TUNEL staining. The long-term application of S(+)-ketamine at concentrations of 3 μ M or 10 μ M did not significantly induce apoptosis. In contrast, the application of 25 μ M S(+)-ketamine significantly induced neuronal cell death (Fig. 1). Importantly, those effects seen at 25 μ M S(+)-ketamine closely resembled the ones seen at 100 μ M MK-801.

Effect of S(+)-Ketamine on Intracellular Ca^{2+}

The calibration of the intracellular Ca^{2+} concentration revealed a K_d of 279 ± 120 nM for fura-2 ($n = 35$ neurons for each Ca^{2+} concentration). The mean basal intracellular Ca^{2+} concentration was 79.8 ± 13.4 nM ($n = 60$). As illustrated in Figure 2 for long-term application of the NMDA receptor antagonist MK 801, intracellular Ca^{2+} concentration was not significantly altered 15 minutes after washout. In contrast, 24 hours after washout of MK 801, the cytosolic Ca^{2+} concentration was significantly increased in comparison with control values (Fig. 2). Similar to the effects of MK 801, application of 3 μ M S(+)-ketamine resulted in an increase in cytosolic Ca^{2+} concentration 24 hours after washout. In contrast, exposure to concentrations of 10 μ M S(+)-ketamine or higher for 24 hours resulted in a significant and concentration-dependent increase of the intracellular Ca^{2+} concentration 15 minutes after washout, which was even more increased 24 hours after removal of the drug ($n = 40$ for each time point and concentration).

Effects of S(+)-Ketamine on Neuronal Ca^{2+} Oscillations

In control neurons the average amplitude of the Ca^{2+} oscillations was 45.7 ± 3.2 nM ($n = 40$). The long-term application of 100 μ M MK 801 led to a significant decrease of the amplitude and frequency of the oscillations immediately after washout. This effect was unchanged even after 24 hours (Fig. 3, A, C, and D).

S(+)-ketamine led to a significant and concentration-dependent attenuation of the amplitude 15 minutes after washout, which was still suppressed 24 hours after washout of the drug (Fig. 3C). After long-term application of 3 μ M S(+)-ketamine the amplitude was reduced to $60.1\% \pm 14.6\%$ of the control amplitude 15 minutes after washout and to $42.9\% \pm 4.0\%$ of the control values 24 hours after washout. Under control conditions the average frequency of the Ca^{2+} oscillations was 0.0375 ± 0.004 per second. After the treatment with S(+)-ketamine for 24 hours, frequency was significantly decreased in a concentration-dependent manner 15 minutes and 24 hours after washout of the specific concentration at both time points and is shown in percentage of control values in Fig. 3D.

CaMKII

Neuronal Ca^{2+} oscillations activate CaMKII. To compare the effect of long-term application of S(+)-ketamine on CaMKII-levels, we performed Western blots in $n = 8$ dishes for each concentration. Blocking the NMDA receptor with the specific antagonist MK 801 resulted in a significant reduction of the CaMKII level. S(+)-ketamine led to a concentration-dependent reduction of the normalized

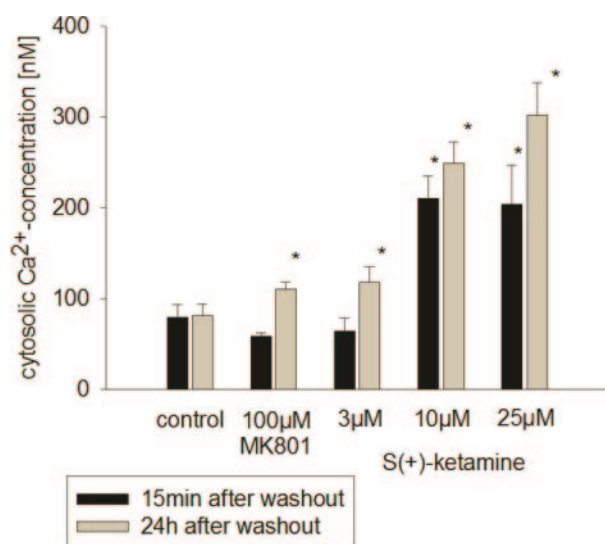


Figure 2. Changes in cytosolic resting Ca^{2+} concentration 15 minutes and 24 hours after washout following 24-hour NMDA receptor blockade with MK-801 and S(+)-ketamine. Cytosolic Ca^{2+} concentration after the treatment with dizocilpine (MK 801) (100 μM) and S(+)-ketamine at given doses were investigated either 15 minutes or 24 hours after a 24-hour long-term incubation with either drug regime. After exposure to the competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist MK 801 (100 μM), the cytosolic Ca^{2+} concentration is unchanged in comparison with control conditions 15 minutes after washout of the drug. In contrast, 24 hours after washout, the cytosolic Ca^{2+} concentration is significantly increased. After exposing neurons to S(+)-ketamine for 24 hours, a concentration-dependent increase in the cytosolic Ca^{2+} concentration can be detected 15 minutes after washout in comparison with control neurons. This effect is not reversible because the Ca^{2+} concentration further increases 24 hours after washout ($n = 40$ per each time point, drug, and concentration). Results are presented as mean $\pm$ SEM. * $P < 0.05$ versus control.

CaMKII concentration volume, which was significant at 10 μM and 25 μM (Fig. 4).

Impact of Long-Term Treatment with S(+)-Ketamine on Synapsin Expression

To examine the effects of the NMDA receptor blockade at various S(+)-ketamine concentrations on synapsin expression of the immature neurons, we performed immunocytochemistry and confocal microscopy in 4 dishes per treatment. The blockade of the NMDA receptor for 24 hours resulted in a significant reduction of synapsin 24 hours after washout of the specific antagonist MK 801. Synapsin was reduced to $28.4\% \pm 12.5\%$ in comparison with control values. In a similar fashion, the application of S(+)-ketamine resulted in a significant and concentration-dependent decrease in synapsin pixel counts of the immature neurons 24 hours after washout (Fig. 5). At 25 μM S(+)-ketamine synapsin expression was reduced to $16.8\% \pm 17.0\%$. At S(+)-ketamine concentrations of 25 μM the effects of S(+)-ketamine on synapsin are comparable to the effects of the NMDA receptor blockade by MK 801.

DISCUSSION

Previous experiments showed that ketamine has neurotoxic properties in developing neurons leading to apoptosis and

disturbed differentiation.^{3–10} In the present study, we demonstrated that 24 hours application of S(+)-ketamine on neuronal cell cultures in vitro has a major impact on intracellular Ca^{2+} homeostasis even at concentrations that do not induce apoptosis. The application of S(+)-ketamine resulted in a concentration-dependent increase in the cytosolic Ca^{2+} concentration, which continued to increase within the first 24 hours after washout of the anesthetic. The amplitude and frequency of the cytosolic Ca^{2+} oscillations, which are involved in neuronal development, are attenuated in a concentration-dependent manner. This reduction is mirrored by a decrease of CaMKII expression and paralleled by a concentration-dependent decline in the expression of synapsin.

NMDA receptors play a crucial role in neuronal development.² The blockade of the NMDA receptor during neuronal development triggers neuronal apoptosis in a dose- and time-dependent manner.^{3–8} In addition, blockade of the NMDA receptors has major impact on the neuronal Ca^{2+} concentration. Turner et al. examined the effect of application of Ca^{2+} -channel blocking substances on the developing brain in an in vivo neonatal rat model.²¹ They proved that blocking the L-type Ca^{2+} channels for 4 hours resulted in apoptosis of the same neuron.^{21,22} Several studies were able to show that the induction of apoptosis was dependent on the developmental stage of the neurons and was significantly increased during synaptogenesis of the specific brain region.^{3,4,6} In our neuronal cell cultures we found a significant increase in apoptotic neurons at concentrations of ≥ 10 μM S(+)-ketamine. As shown by Yon et al. in an in vivo neonatal rat model, anesthetic-induced apoptosis can be induced via the intrinsic and the extrinsic apoptotic pathways, both resulting in the activation of caspase-3.²³ In our experiments, blockade of the NMDA-receptors with MK 801 or 3 μM S(+)-ketamine for 24 hours did not result in a significant change of the intracellular Ca^{2+} concentration 15 minutes after washout. Turner et al. applied nimodipine and MK 801 for only 4 hours on cortical neuronal cell cultures and found a significant decrease in the intracellular Ca^{2+} concentration 45 minutes after washout.²¹ In contrast, we found a significant and irreversible increase in the cytosolic Ca^{2+} concentration after incubation for 24 hours with concentrations of 10 μM S(+)-ketamine or more. Increased cytosolic Ca^{2+} might activate apoptosis by increasing mitochondrial cytochrome C release. Moreover, neuronal apoptosis could also be induced by the suppression of the neuronal Ca^{2+} oscillations. The cytosolic Ca^{2+} oscillations lead to an increase in Ca^{2+} concentration in neuronal nuclei. Ca^{2+} regulates gene transcription by activating nuclear Ca^{2+} -sensitive kinases and phosphatases or by directly affecting Ca^{2+} -binding transcription factors.¹³

In vivo experiments demonstrate that long-term application of racemic ketamine results in an increase in NMDA receptor distribution as verified by determining NR 1 subunit expression.^{5,7} After washout of ketamine, glutamate stimulates neurons via the increased numbers of receptors, resulting in a dangerous “glutamate storm.” Prolonged stimulation of glutamate receptors increases the cytosolic Ca^{2+} concentration.²⁴ This increase might persist after terminating glutamate exposure and might explain

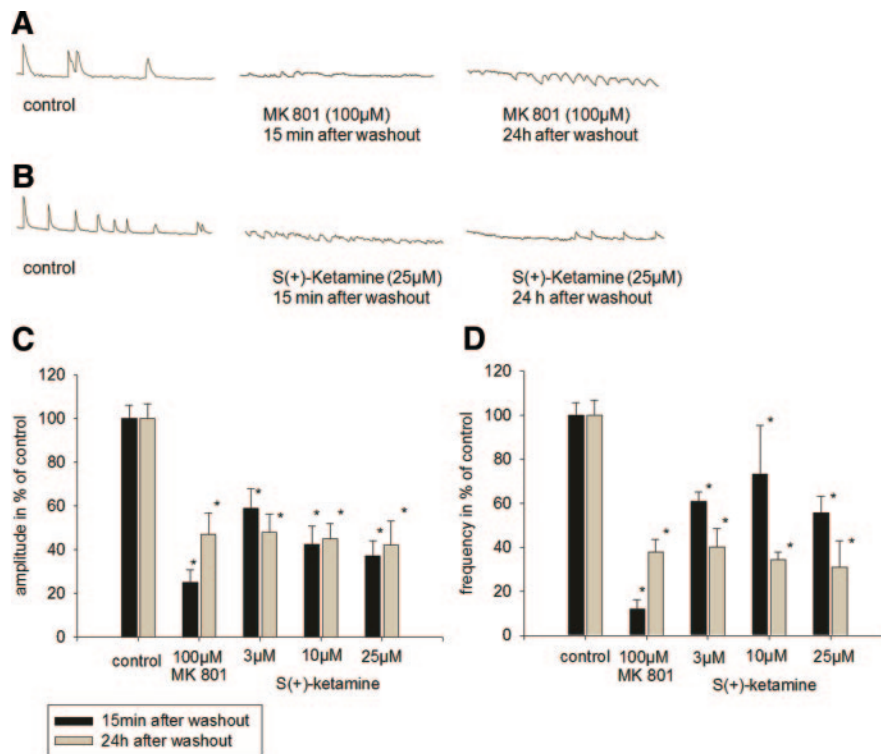


Figure 3. Effect of 24-hour exposure to MK-801 or S(+)-ketamine on amplitude, and frequency of intracellular Ca^{2+} oscillations in hippocampal neurons. A and B, Traces show representative Ca^{2+} oscillations recorded from individual fura 2-AM loaded neurons at either 15 minutes or 24 hours after termination of the 24-hour exposure to (A) dizocilpine (MK 801) (100 μM) or (B) S(+)-ketamine (25 μM). Each trace was recorded over a period of 3 minutes. Note that amplitude and frequency were still reduced 24 hours after S(+)-ketamine withdrawal. C, Amplitude of the neuronal Ca^{2+} oscillations normalized to preexposure control at either 15 minutes or 24 hours after MK-801 or S(+)-ketamine withdrawal from a 24-hour long-term exposure ($n = 40$ per each time point, drug, and concentration). MK 801 as well as all S(+)-ketamine concentrations caused a significant suppression of the amplitude. This depression persisted for 24 hours. S(+)-ketamine suppresses the amplitude in a concentration-dependent manner. Bars indicate mean $\pm$ SEM. *Significantly differs versus control $P < 0.05$. D, Long-term application of MK 801 or S(+)-ketamine results in a reduction in the frequency 15 minutes and 24 hours after washout. The frequency of the neuronal Ca^{2+} oscillations after drug exposure is normalized to control. Blocking the N-methyl-D-aspartate (NMDA) receptor with the specific antagonist MK 801 (100 μM) led to a significant reduction in the frequency 15 minutes and 24 hours after washout. S(+)-ketamine reduced the frequency in a concentration-dependent manner; $n = 40$ per each time point, drug, and concentration. Bars indicate mean $\pm$ SEM. * $P < 0.05$ versus control.

the differences in the Ca^{2+} concentrations after long-term treatment as in our experiments and the results after only 4 hours' treatment.^{21,22}

In the past, neuronal development was largely attributed to gene transcription; however, it is becoming increasingly clear that electrical activity contributes significantly to neuronal differentiation.^{2,12,14,17,25} In this scenario, cytosolic Ca^{2+} ions are an important second messenger. Various studies have ascertained that neuronal Ca^{2+} oscillations play a major role in differentiation, axonal outgrowth, dendritic arborization, and synaptogenesis in the brain and the spinal cord.^{12,14,16,25} As shown for rodents, Ca^{2+} oscillations exhibit a characteristic developmental profile. They can be detected at postnatal day 1 and last up to postnatal day 14.¹⁶ This period is characterized by intensive neuronal differentiation and synaptogenesis that seems to depend on the development of the γ -aminobutyric acid A (GABA_A) receptor. During the early period of neuronal development, the activation of the GABA_A receptor results in a depolarization. With further neuronal development the excitatory effect switches to an inhibitory effect.¹⁷ In rodents, this switch occurs around postnatal day 10 and correlates with the peak of synaptogenesis.^{12,16,17,25} In contrast, in primates

the switch occurs before birth. Khazipov et al. examined the switch of GABA_A receptors in prenatal monkeys in which the medium gestation period is 165 days.²⁶ They observed the first epileptiform events with application of the GABA_A antagonist bicuculline at E105–E109, which coincides with the outgrowth of axonal collaterals of pyramidal cells and the appearance of spines and glutamatergic synaptic currents. This was paralleled by synchronized synaptic activity that increased through gestation in concordance with the development of pyramidal cells and an increase in the number of spines. One month before birth (E134), powerful synaptic activity events were observed in all recorded neurons.²⁶

Recently, our group and others could show that hippocampal neuronal Ca^{2+} oscillations are linked to NMDA receptors.^{12,16,17} The specific and noncompetitive NMDA receptor blocker MK 801 (50 μM) blocked the oscillations completely and reversibly when applied for 3 minutes. In contrast, in the present study long-term application of 100 μM MK 801 (24 hours) caused a significant reduction in the amplitude and frequency of the oscillations after washout of the drug, and this reduction was still present 24 hours after drug withdrawal. This was accompanied by a significant reduction in synapsin. These results are in

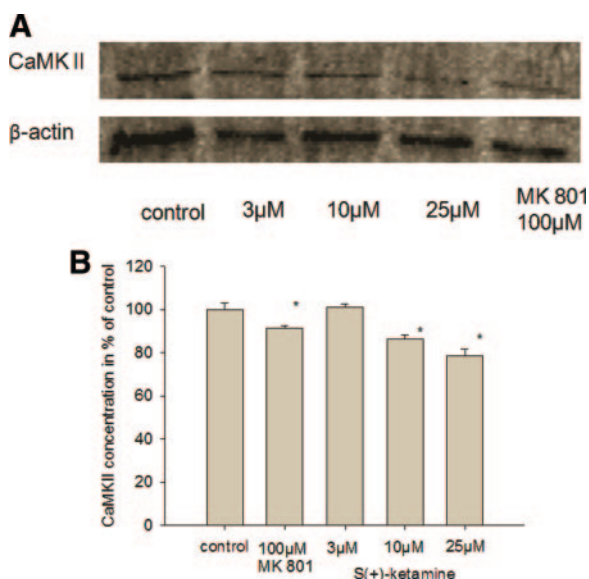


Figure 4. Effect of 24-hour exposure to MK-801 or S(+)-ketamine on CaMKII expression levels. A, Representative Western blot of Ca^{2+} /calmodulin kinase II (CaMKII) for S(+)-ketamine (3 to 25 μM) and dizocilpine (MK 801) (100 μM). β -Actin was used as loading control. B, Western blot analysis of CaMKII levels 18 hours after washout of the respective drug, in relation to control. MK 801 (100 μM) and S(+)-ketamine (10 μM and 25 μM) depressed the level of CaMKII ($n = 8$ dishes per data point and concentration; * $P < 0.05$ versus control).

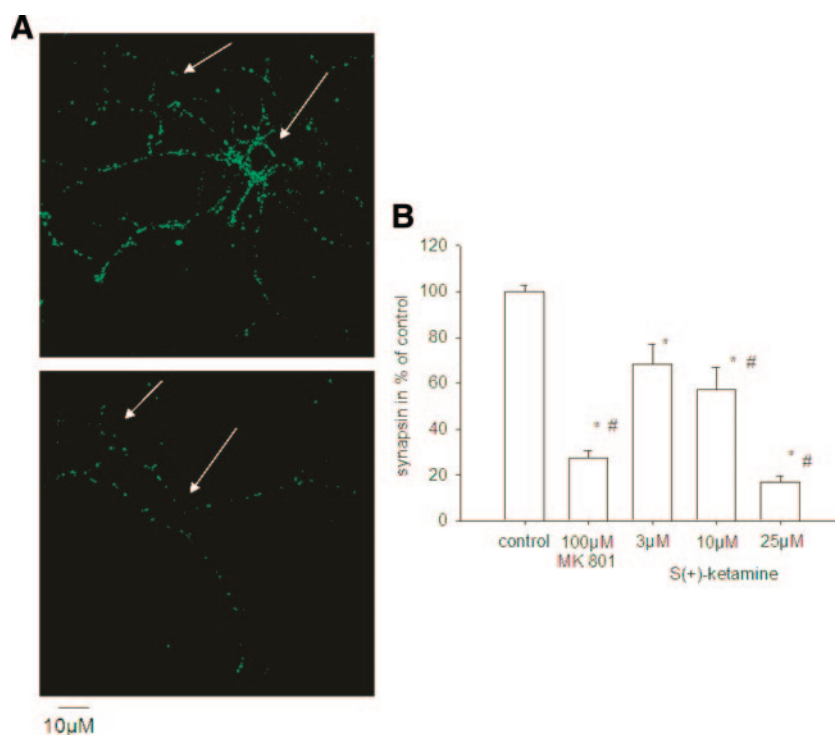
agreement with a previous study in which 100 μM MK 801 applied on GABA-ergic interneurons for 48 hours caused a significant reduction in the dendritic length, primary dendrites, and branching points.^{9,10}

Neuronal Ca^{2+} oscillations activate CaMKII, which in turn alters the phosphorylation state of various transcription factors, including those responsible for neuronal development and

synaptogenesis.^{5,17} Nikizad et al. examined the effect of general anesthesia, i.e., a clinically used combination of midazolam, nitrous oxide, and isoflurane, in 7-day-old rat pups.²⁷ They found a significant reduction in the concentration of CaMKII 18 hours and 42 hours after anesthesia throughout the whole cortex.²⁷ This reduction was associated with a significant decrease in the neuronal density. Follow-up at day 23 after exposure to this anesthetic combination revealed an up-regulation of CaMKII, which might be indicative for compensatory mechanisms. In addition, this group could show a reduction in the synaptic proteins synaptophysin, synaptobrevin, amphiphysin, and SNAP-25, all involved in synaptogenesis, in the first 18 and 42 hours after anesthesia.²⁷ A single injection of ketamine (doses: 5, 10, and 25 mg/kg body weight) resulted in a significant increase in CaMKII and the growth-associated protein 43 in the hippocampus but not in the cortex 24 hours after exposure to the anesthetic on postnatal day 10. The animals exposed to ketamine showed a dose-dependent change in their spontaneous behavior and habituation at the age of 4 months.¹¹ An increase in the CaMKII concentration in comparison with that of controls was also detected when ketamine was applied for 5 hours. In this case, anesthesia with ketamine was accompanied by an increase in the number of dendritic spines.²⁸

S(+)-ketamine, a phencyclidine derivative, is a stereoisomer of ketamine and an IV anesthetic with a well-defined effect on the NMDA receptor in clinical concentrations.²⁹ It blocks the NMDA channel by reducing channel mean open time and by decreasing the frequency of channel opening by an allosteric mechanism in the open state.²⁹ As we know from previous experiments, the short-term application of S(+)-ketamine concentration-dependently and reversibly suppressed amplitude and frequency of the oscillations.¹⁹

Figure 5. Effect of 24-hour exposure to MK-801 or S(+)-ketamine on the synapsin expression in hippocampal neurons. A, Confocal images of antisynapsin-labeled neurons. The upper image shows 2 representative neurons (arrow indicates the somas of the neurons). The lower image shows 2 neurons after 24 hour treatment with 25 μM S(+)-ketamine. B, Synapsin expression 24 hours after exposure to dizocilpine (MK 801) or S(+)-ketamine, normalized to control. Synapsin expression is determined by counting pixels on each individual neuron at the soma and at the dendrites up to the second branch. MK 801 (100 μM) significantly reduced synapsin labeling indicating a major role of the N-methyl-D-aspartate (NMDA) receptor in the differentiation and synaptogenesis of the immature neurons. The application of S(+)-ketamine results in a concentration-dependent reduction in the number of synapses. The depression of synapsin expression by 25 μM S(+)-ketamine is similar to the effect of MK 801 (100 μM) ($n = 40$ per each concentration and drug). Results are presented as mean $\pm$ SEM. *Significant change in synapsin in comparison with control; $P < 0.05$. #Significant difference between 10 and 25 μM S(+)-ketamine and MK 801 (100 μM) versus 3 μM S(+)-ketamine; $P < 0.05$.



Because S(+)-ketamine acts mainly via the NMDA receptor, the application of this anesthetic should induce a similar suppression of amplitude and frequency as did the noncompetitive NMDA receptor blocker MK 801. In the present study, we could demonstrate a concentration-dependent decrease of both properties.

The effects of MK 801 and S(+)-ketamine were similar but of different magnitude: the suppression of amplitude and frequency was more enhanced, and the reduction of synapsin expression was less pronounced with MK 801 than with S(+)-ketamine. This difference indeed could be attributed to a relative nonselectivity of S(+)-ketamine, which also affects other cellular targets. S(+)-ketamine exerts its anesthetic effect mainly via the NMDA receptor but blockade of other glutamate receptors, such as 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) receptors as well as nicotinic and muscarinic acetylcholine or σ receptors have been described, which all could contribute to a mosaic pattern for Ca^{2+} oscillations.^{30,31}

At a concentration of 25 μM , S(+)-ketamine mimics the effect of MK 801 (100 μM) on neuronal oscillations. A major impact of ketamine on neuronal development and survival was demonstrated by Vutskits et al.^{9,10} The authors applied ketamine at different concentrations (0.01 to 40 $\mu\text{g}/\text{mL}$, 0.04 to 146 μM) for various time periods (1 to 96 hours) and revealed a concentration- and time-dependent induction of apoptosis. Even at time periods and concentrations when no apoptosis could be detected, ketamine influenced dendritic arbor development. At ketamine concentrations of 0.01 $\mu\text{g}/\text{mL}$ applied over 24 hours, a significant impairment of dendritic length and number of branching points was found.^{9,10} In neuronal cell cultures, significant synapse formation occurs between days 14 and 21. Our experiments show that after the application of S(+)-ketamine, when applied during a phase of high synaptogenesis, the dendritic arbor development and synaptogenesis are already severely affected at the lowest concentration tested (3 μM).

In conclusion, in developing mammalian neurons, the long-term blockade of the NMDA receptor by the specific antagonist MK 801 or the anesthetic S(+)-ketamine affects neuronal cytosolic Ca^{2+} concentration and Ca^{2+} oscillations. The agents concentration-dependently reduce the amplitude and frequency of the Ca^{2+} oscillations. The long-term application of MK 801 or S(+)-ketamine is associated with a reduction of CaMKII and a decrease in the expression of synapsin even at concentrations that are too low to induce apoptosis. Therefore, our study is the first to link Ca^{2+} oscillations to synapsin expression, for which blocking the former disrupts the latter. This connection establishes a mechanism that may be involved in the neurotoxicity caused by ketamine anesthesia in infants. ■■

DISCLOSURES

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