# Effects of ketamine on neurogenesis, extracellular matrix homeostasis and proliferation in hypoxia-exposed HT22 murine hippocampal neurons

THOMAS PICHL, TITUS KELLER, CHRISTOPH HÜNSELER, BERNHARD ROTH, RUTH JANOSCHEK, SARAH APPEL and EVA HUCKLENBRUCH-ROTHER

Department of Pediatrics and Adolescent Medicine, University of Cologne, Faculty of Medicine and University Hospital, D-50931 Cologne, Germany

**Abstract.** Ketamine is a widely used drug in pediatric anesthesia, and both neurotoxic and neuroprotective effects have been associated with its use. There are only a few studies to date which have examined the effects of ketamine on neurons under hypoxic conditions, which may lead to severe brain damage and poor neurocognitive outcomes in neonates. In the present study, the effects of ketamine on cellular pathways associated with neurogenesis, extracellular matrix homeostasis and proliferation were examined *in vitro* in hypoxia-exposed neurons. Differentiated HT22 murine hippocampal neurons were treated with 1, 10 and 20 $\mu$M ketamine and cultured under hypoxic or normoxic conditions for 24 h followed by quantitative PCR analysis of relevant candidate genes. Ketamine treatment did not exert any notable effects on the mRNA expression levels of markers of neurogenesis (*neuronal growth factor* and *syndecan 1*), extracellular matrix homeostasis (*matrix-metalloproteinase 2* and *9*, *tenascin C* and *tenascin R*) or proliferation markers (*Ki67* and *proliferating cell nuclear antigen*) compared with the respective untreated controls. However, there was a tendency towards downregulation of multiple cellular markers under hypoxic conditions and simultaneous ketamine treatment. No dose-dependent association was found in the ketamine treated groups for genetic markers of neurogenesis, extracellular matrix homeostasis or proliferation. Based on the results, ketamine may have increased the vulnerability of hippocampal neurons *in vitro* to hypoxia, independent of the dose. The results of the present study contribute to the ongoing discussion on the safety concerns around ketamine use in pediatric clinical practice from a laboratory perspective.

*Correspondence to:* Dr Thomas Pichl, Department of Pediatrics and Adolescent Medicine, University of Cologne, Faculty of Medicine and University Hospital, Gebaeude 44a, Forschungspavillon, 16 Robert-Koch Street, D-50931 Cologne, Germany
E-mail: thomas.pichl@nhs.net

## Introduction

Ketamine has been used clinically for >40 years (1). By disconnecting thalamic and limbic brain functions through antagonism of the NMDAR, ketamine induces dissociative anesthesia while preserving spontaneous respiration and cardiovascular stability (2,3). With a low risk profile for severe adverse effects, ketamine is widely used in pediatric anesthesia (4,5). However, the potential neurotoxic effects of ketamine on the developing brain have raised concern amongst healthcare professionals for >20 years (6,7). Numerous animal studies have reported apoptotic neurodegeneration and impaired neurological outcomes due to ketamine treatment, casting doubt on the safety of ketamine use in neonatal and pediatric patients (7-15). Ikonomidou *et al* (7) showed that exposure to ketamine resulted in widespread neuronal apoptosis in rat pups. These findings were supported by subsequent studies on developing rat brains, which linked ketamine exposure to upregulated expression of NMDA receptor subunits and pro-apoptotic genes and proteins, such as p53 and cleaved caspase-3 (10,12). Similarly, increased expression of NMDA receptors and apoptosis was observed in the frontal cortex of ketamine-treated perinatal rhesus monkeys (8). Furthermore, rhesus monkeys underperformed in cognitive behavior tests for several years following exposure to ketamine early in life (9).

In contrast to these findings, neuroprotective effects of ketamine on the developing brain have also been demonstrated. Anand *et al* (16) reported that ketamine may ameliorate pain-induced neurotoxicity in newborn rats, likely, but not solely through the inhibition of inflammatory pathways (6,16).

Perinatal hypoxia-ischemia may lead to a variety of types of severe brain damage in term and preterm neonates, such as hypoxic-ischemic encephalopathy and periventricular leukomalacia (17,18). Several *in vitro* and *in vivo* studies have provided evidence of the neuroprotective effects of ketamine under hypoxic conditions (19-21). In 1987, Rothman *et al* (19) showed that ketamine attenuated the neurotoxic effects of hypoxia on hippocampal neurons *in vitro*. NMDA receptor antagonism is

considered a key factor in the protection of neurons and glial cells from glutamate-induced excitotoxicity, providing improved outcomes in hypoxic-ischemic rat pups (22,23). Chang *et al* (21) showed that ketamine ameliorated the inflammatory response due to hypoxia in the cortex of fetal sheep via a Toll-like receptor mediated pathway. To the best of our knowledge, there are no studies published to date on the interaction between ketamine and hypoxia in primates. Similarly, there are no prospective studies examining the safety of ketamine in paediatric patients to the best of our knowledge; however, retrospective studies on the use of ketamine and other anesthetic drugs in childhood have not found convincing evidence of an association with impaired neurocognitive function in later life (24,25).

In summary, there is conflicting evidence regarding the neurotoxic and neuroprotective effects of ketamine on the developing brain. Ketamine has been shown to protect neurons and glial cells by attenuating glutamate-induced excitotoxicity and inhibiting an inflammatory response (19-21,26). However, the number of studies available on the relevant neurocellular mechanisms are limited (14,21,27-30), and hypoxia-induced neurotoxicity and potential amelioration through ketamine remains poorly understood.

In the present study, it was hypothesized that ketamine attenuated the neurotoxic effects of hypoxia, and this was assessed by measuring the expression of cellular markers of proliferation, neurogenesis and extracellular matrix homeostasis. The results of the present study may contribute to an improved understanding of how hypoxia-induced neurotoxicity is affected by the presence of ketamine.

*In vitro* experiments were used as they allow for identification of relevant cellular pathways under standardized and simplified conditions (31). Experiments were performed on HT22 murine hippocampal cells. Studies investigating pathways in the central nervous system often focus on hippocampal formation, which is known to serve a key role in long-term potentiation and memory consolidation, emotional perception as well as endocrinological responses (32-34). An improved understanding of the hippocampal cellular pathways activated under hypoxic conditions and potential alterations due to ketamine is therefore crucial for the development of potential new anesthetic techniques with the aim of reducing the effects of hypoxia-induced brain damage in neonates.

**Materials and methods**

*Cell culture*. Murine hippocampal HT22 cells were generously provided by Professor Axel Methner, Department of Neurology, Düsseldorf University Hospital. Cells were cultured at 37°C with 5% $CO_2$ volume fraction in DMEM, high glucose (Thermo Scientific Fisher, Inc.) supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA) and 1% penicillin-streptomycin solution (Sigma-Aldrich; Merck KGaA). A total of $1x10^5$-$1x10^6$ HT22 cells were plated in TC Schale 100 cell culture dishes (Sarstedt, Inc.) and cultured for 48 h in supplemented DMEM.

For differentiation, the cells were treated with Neurobasal™ medium (Thermo Fisher Scientific, Inc.) for 24 h, supplemented with 1% 100x $N_2$ supplement (Thermo Fisher Scientific, Inc.) and 10% FBS and 1% penicillin-streptomycin solution.

*Hypoxia and ketamine model*. HT22 cells have long been used as a model cell line for examining oxidative glutamate toxicity (31,35,36), but to the best of our knowledge, have not been previously used to study the effects of hypoxia and ketamine treatment on the developing brain.

After 24 h of differentiation, Neurobasal™ medium was replaced with supplemented DMEM. Ketamine (10 mg/ml; Ketamin-Actavis, Injektionslösung, Actavis Group PTC EHF) was added to culture dishes to final ketamine concentrations of 1, 10 or 20 $\mu$M. Control cell culture dishes were handled in the same manner, but no ketamine or vehicle was added. Ketamine-incubated HT22 cells and controls were cultured for 24 h either under hypoxic (1% $O_2$, 5% $CO_2$) or normoxic (21% $O_2$, 5% $CO_2$) conditions. Ketamine doses of 1, 10 or 20 $\mu$M (equivalent to 0.238, 2.38 or 5.76 $\mu$g/ml, respectively, in culture medium) were chosen in accordance with *in vivo* measurements of brain and plasma ketamine concentration as reported by Liu *et al* (10).

*Reverse transcription-quantitative (q)PCR*. mRNA was isolated from harvested HT22 cells using TriReagent® RNA Isolation Reagent (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. mRNA concentrations were measured using a Nano Quant infinite M200 Pro (Tecan Group, Ltd.). Moloney Murine Leukemia Virus Reverse Transcriptase was used to synthesize first strand cDNA according to the manufacturer's protocol (Promega Corporation).

For TaqMan™ qPCR (7500 Real-Time PCR system, Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.5 $\mu$l each of the forward and reverse primers, and the probe (all purchased from Eurofins Genomics), 2.5 $\mu$l cDNA template and 12.5 $\mu$l Platinum™ Quantitative PCR SuperMix-UDG (Thermo Fisher Scientific, Inc.) were added per well in a 96-well plate and amplified for 40 cycles (annealing temperature, 60°C) according to the manufacturer's protocol. The sequences of the primers used and the respective probes are presented in Table SI. qPCR data of mRNA expression levels were normalized using the $2^{-\Delta\Delta Cq}$ method in Microsoft Excel 2010 (Microsoft Corporation) using GAPDH as the reference gene (37).

*Statistical analysis*. To evaluate the effects of ketamine treatment, comparisons between multiple groups were performed using a Kruskal-Wallis one-way ANOVA with a post-hoc Dunn's test. All statistical tests were performed using GraphPad Prism version 8 (GraphPad Software, Inc.). Results are presented as the means of mRNA expression. Error bars represent the standard deviations.

**Results**

The aim of the present study was to improve our understanding of the effects of ketamine on developing neurons under hypoxic conditions. Differentiated murine HT22 cells were incubated with different concentrations of ketamine (1, 10 or 20 $\mu$M) for 24 h under hypoxic or normoxic conditions. qPCR analysis of cellular markers associated with neurogenesis, proliferation and extracellular matrix homeostasis was performed to determine the effects of ketamine.

*mRNA expression levels of NGF.* To investigate how the mRNA expression levels of *NGF*, an important neurotrophin for migration and maturation of neurons in the developing brain (38), were affected by hypoxia and ketamine treatment, the mRNA expression levels of *NGF* in differentiated HT22 cells were determined after 24 h of treatment with various ketamine concentrations under hypoxic and normoxic conditions.

There was no evidence of differences in the mRNA expression levels of *NGF* following ketamine treatment compared with the hypoxic and normoxic control groups (P>0.99 for all comparisons). However, there was a decrease in *NGF* expression levels in cells cultured under hypoxic conditions compared with the normoxic cultured cells treated with the same concentration of ketamine to 16% (K1; P=0.25), 17% (K10; P=0.38) and 13% (K20; P=0.19). All mRNA expression levels stated are relative to the expression levels in the control (Co NO) group. There was no evidence of differences in the mRNA expression levels in hypoxic and normoxic cultured cells in the control groups (P>0.99; Fig. 1).

*mRNA expression levels of Sdc1.* To investigate how the mRNA expression levels of *Sdc1*, which is expressed by neuronal progenitor cells (39), were affected by hypoxia and ketamine treatment, the mRNA expression levels of *Sdc1* in differentiated HT22 cells after 24 h of treatment with various ketamine concentrations were determined under hypoxic and normoxic conditions.

There was no evidence of differences in the mRNA expression levels of *Sdc1* following ketamine treatment compared with the hypoxic and normoxic control groups (P>0.99 for all comparisons). However, there was a decrease in the expression levels in cells cultured under hypoxic conditions respectively compared with the normoxic cultured cells treated with the same concentration of ketamine to 24% (K10; P=0.11) and 28% (K20; P=0.41). All mRNA expression levels stated are relative to the expression levels in the Co NO. There was no evidence of a difference in mRNA expression levels between hypoxia and normoxia for K1 (P>0.99) or in the control groups (P>0.99; Fig. 1).

*mRNA expression levels of PCNA.* To investigate how the mRNA expression levels of *PCNA*, a widely used proliferation marker (40,41), were affected by hypoxia and ketamine treatment, the mRNA expression levels of *PCNA* were determined in the differentiated HT22 cells after 24 h of treatment with various concentrations of ketamine under hypoxic and normoxic conditions.

There was no evidence of differences in the mRNA expression levels of *PCNA* following ketamine treatment compared with the hypoxic and normoxic control groups (P>0.99 for all comparisons). However, there was a downregulation of *PCNA* expression in cells cultured under hypoxic conditions respectively compared with normoxic cultured cells treated with the same concentration of ketamine to 7% (K1; P=0.09), 11% (K10; P=0.35) and 8% (K20; P=0.25). All mRNA expression levels stated are relative to the expression levels in the Co NO group. There was no evidence of a difference in mRNA expression levels between hypoxic and normoxic cultured cells in the control groups (P>0.99; Fig. 2).

*mRNA expression levels of Ki67.* To investigate how the mRNA expression of *Ki67*, which is expressed in all phases of the cell cycle of proliferating cells (42), was affected by hypoxia and ketamine treatment, the mRNA expression levels of *Ki67* were determined in the differentiated HT22 cells after 24 h of treatment with various concentrations of ketamine under hypoxic and normoxic conditions.

There was no evidence of a difference in the mRNA expression levels of *Ki67* following treatment with ketamine compared with the hypoxic and normoxic control groups (P>0.99 for all comparisons). However, there was a downregulation in the Ki67 expression levels cultured under hypoxic conditions respectively compared with the normoxic cultured cells treated with the same concentration of ketamine to 9% (K1; P=0.04) and 11% (K20; P=0.27). All mRNA expression levels stated are relative to the expression levels in the Co NO group. There was no evidence of a difference in the mRNA expression levels between the hypoxic and normoxic cultured cells in K10 (P=0.63) or in the control group (P>0.99; Fig. 2).

*mRNA expression levels of MMP2.* To investigate how the mRNA expression levels of *MMP2*, an enzyme in the MMP group of proteins which serve important roles in the proteolysis of the extracellular matrix following brain injury (43), were affected by hypoxia and ketamine exposure, the mRNA expression levels of MMP2 in differentiated HT22 cells after 24 h of treatment with various concentrations of ketamine were determined under hypoxic and normoxic conditions. There was no evidence of differences in the mRNA expression levels of *MMP2* following ketamine treatment or hypoxia (P>0.99 for all between-group comparisons) (Fig. 3).

*mRNA expression levels of MMP9.* To investigate how the mRNA expression levels of *MMP9* were affected by hypoxia and ketamine exposure, the mRNA expression levels of *MMP9* in differentiated HT22 cells were determined after 24 h of treatment with various concentrations of ketamine under hypoxic and normoxic conditions.

There was no evidence of differences in the mRNA expression of *MMP9* following ketamine treatment compared with the hypoxic and normoxic control groups (P>0.99 for all comparisons). However, there was a downregulation of *MMP9* expression in cells cultured under hypoxic conditions respectively compared with the normoxic cultured cells treated with the same concentration of ketamine to 28% (K1; P=0.42) and 30% (K10; P=0.23). All mRNA expression levels stated are relative to the expression levels in the Co NO group. There was no significant difference between hypoxia and normoxia for K20 (P>0.99) or in the control groups (P>0.99; Fig. 3).

*mRNA expression levels of TenC.* To investigate how the mRNA expression of *TenC*, which is an extracellular matrix protein expressed following tissue injury (44), was affected by hypoxia and ketamine exposure, the mRNA expression levels of *TenC* in differentiated HT22 cells were determined after 24 h of treatment with various concentrations of ketamine under hypoxic and normoxic conditions.

There was no evidence of differences in the mRNA expression of *TenC* following ketamine treatment or hypoxia (P>0.99 for all between-group comparisons) (Fig. 4).
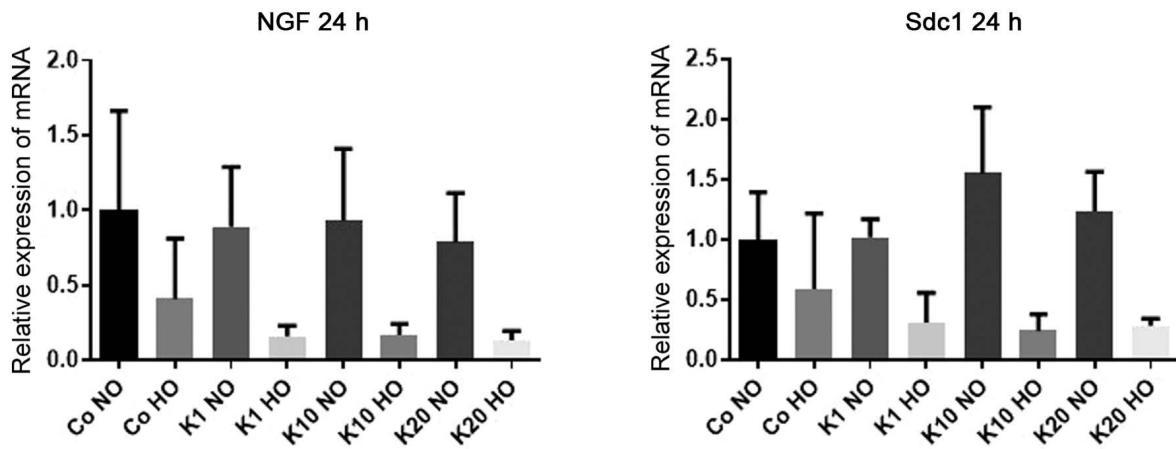
Figure 1. Relative mRNA expression levels of the neurogenesis markers *NGF* and *Sdc1* in differentiated HT22 cells after 24 h of culture under HO or NO conditions. Cells were incubated with various concentrations of ketamine hydrochloride. Results are presented as the mean ± standard deviation of at least three experimental repeats. *NGF, nerve growth factor*; *Sdc, syndecan 1*, HO, hypoxic; NO, normoxic; CO, control; K1, 1 µM ketamine hydrochloride; K10, 10 µM ketamine hydrochloride; K20, 20 µM ketamine hydrochloride.
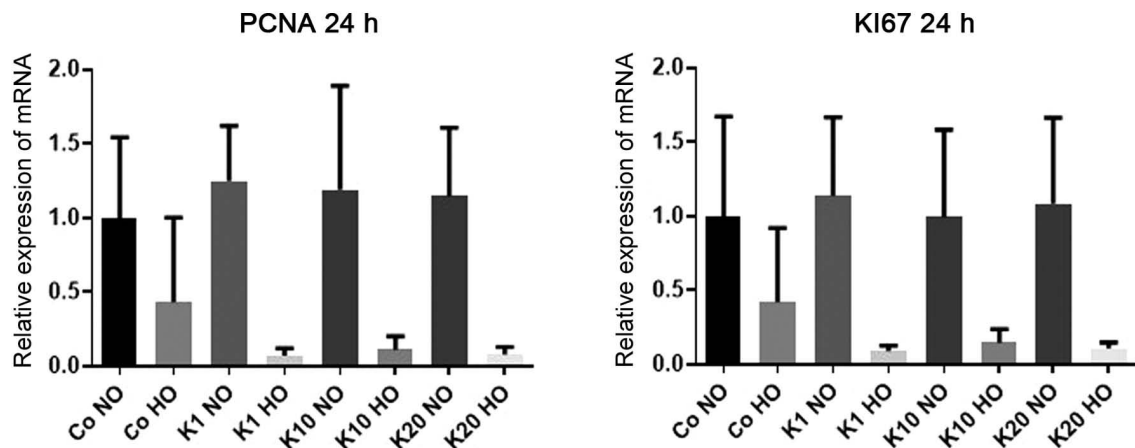


Figure 2. Relative mRNA expression levels of the proliferation markers *PCNA* and *Ki67* in differentiated HT22 cells after 24 h of culture under HO or NO conditions. Cells were incubated with various concentrations of ketamine hydrochloride. Results are presented as the mean ± standard deviation of at least three experimental repeats. *PCNA, proliferating cell nuclear antigen*; HO, hypoxic; NO, normoxic; CO, control; K1, 1 µM ketamine hydrochloride; K10, 10 µM ketamine hydrochloride; K20, 20 µM ketamine hydrochloride.

*mRNA expression levels of TenR*. To investigate how the mRNA expression of *TenR*, an extracellular matrix protein exclusively expressed in the nervous system (45), was affected by hypoxia and ketamine exposure, the mRNA expression levels of *TenR* in differentiated HT22 cells were determined after 24 h of treatment with various concentrations of ketamine under hypoxic and normoxic conditions.

There was no evidence of differences in the mRNA expression levels of *TenR* following ketamine treatment compared with the hypoxic and normoxic control groups. However, there was a downregulation in the expression of *TenR* in cells cultured under hypoxic conditions respectively compared with the normoxic cultured cells treated with the same concentration of ketamine to 12% (K1, P=0.14) and 15% (K10, P=0.25). All mRNA expression levels stated are relative to the expression levels in the Co NO group. There was no significant difference in the expression of *TenR* between cells grown under hypoxic and normoxic conditions for K20 (P=0.89) or between the normoxic and hypoxic controls groups (P>0.99; (Fig. 4).

## Discussion

The aim of the present study was to investigate the effects of ketamine on the expression of markers involved in cellular pathways associated with proliferation, neurogenesis and extracellular matrix homeostasis in hypoxia-exposed neurons.

The results showed that ketamine treatment alone in HT22 cells did not significantly affect the expression of the assessed markers of proliferation, neurogenesis or extracellular matrix homeostasis compared with their respective untreated controls. Furthermore, untreated control groups did not exhibit any significant differences in expression of the markers assessed when compared with hypoxia cultured cells.

However, there was a tendency towards downregulation of markers of proliferation, neurogenesis and, to a lesser extent, extracellular matrix homeostasis under hypoxic conditions combined with ketamine treatment compared with their respective normoxic control group. No dose-dependent association was observed among the ketamine treated groups.
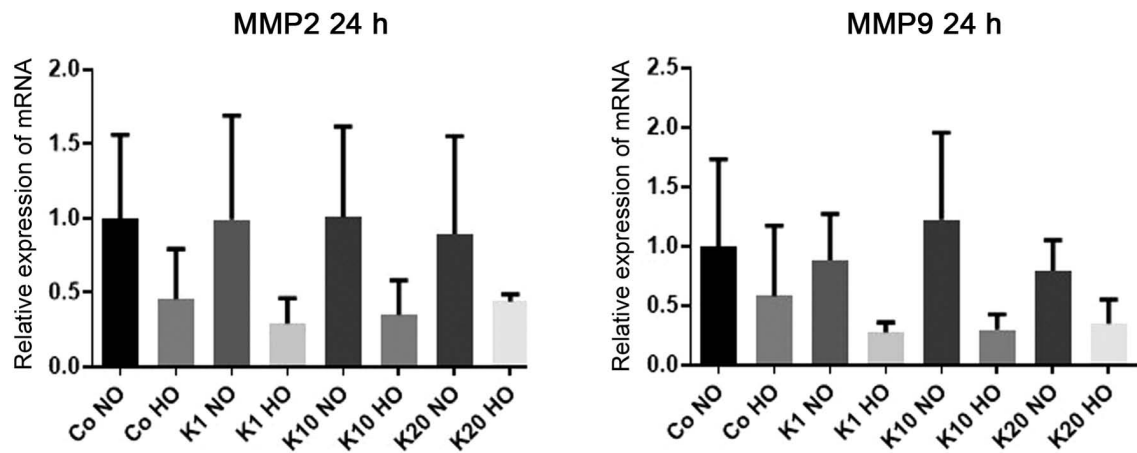
## MMP2 24 h



## MMP9 24 h



Figure 3. Relative mRNA expression levels of markers of extracellular matrix homeostasis, *MMP9* and *MMP9* in differentiated HT22 cells after 24 h of culture under HO or NO conditions. Cells were incubated with various concentrations of ketamine hydrochloride. Results are presented as the mean ± standard deviation of at least three experimental repeats. *MMP, matrix metalloproteinase*; HO, hypoxic; NO, normoxic; CO, control; K1, 1 $\mu$M ketamine hydrochloride; K10, 10 $\mu$M ketamine hydrochloride; K20, 20 $\mu$M ketamine hydrochloride.
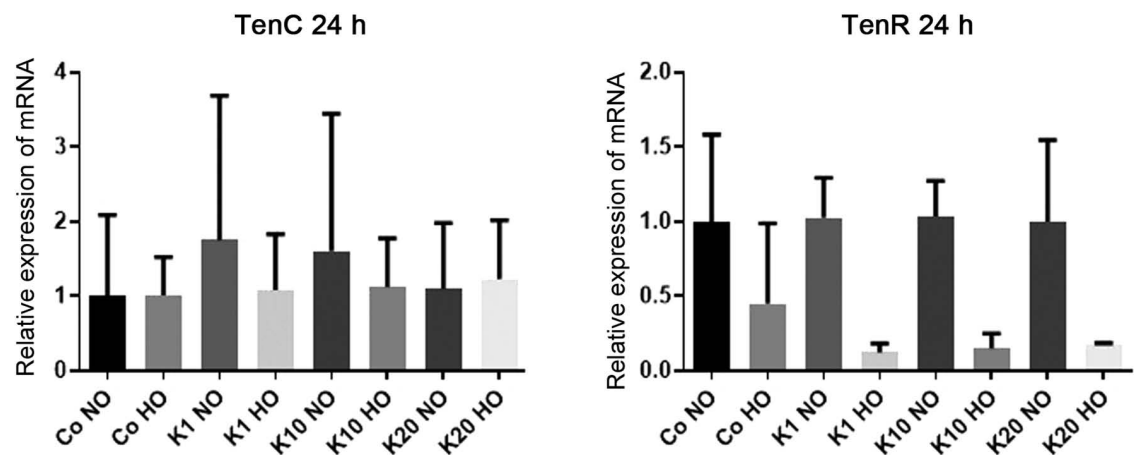
## TenC 24 h



## TenR 24 h



Figure 4. Relative mRNA expression levels of markers of extracellular matrix homeostasis, *TenC* and *TenR* in differentiated HT22 cells after 24 h of culture under HO or NO conditions. Cells were incubated with various concentrations of ketamine hydrochloride. Results are presented as the mean ± standard deviation of at least three experimental repeats. *TenC, tenascin C*; *TenR, tenascin R*; HO, hypoxic; NO, normoxic; CO, control; K1, 1 $\mu$M ketamine hydrochloride; K10, 10 $\mu$M ketamine hydrochloride; K20, 20 $\mu$M ketamine hydrochloride.

Taken together, these results suggest increased vulnerability of hippocampal neurons *in vitro* to hypoxia in the presence of ketamine, independent of the dose of ketamine.

Several tested markers allow for specific interpretations. *Sdc1* is a transmembrane heparan sulfate proteoglycan that is found in abundance in mammalian brains prior to neurogenesis (39). *NGF* stimulates migration and maturation of neurons in the developing brain and has neuroprotective functions in the adult brain (38). *Ki67* and *PCNA* are expressed by replicating cells only and are widely used as markers to evaluate cellular proliferation (40,42). Thus, ketamine treatment may not be neurotoxic itself but may aggravate the effects of hypoxia, which thus results in an earlier onset of neurotoxic effects in the ketamine-treated groups compared with the untreated control groups. This would support findings from previous studies which demonstrated the potential neurotoxicity of ketamine treatment (7-15).

The results of expression of markers of extracellular matrix homeostasis were less conclusive. Only *MMP-9*

and *TenR* exhibited a similar pattern to that described above; whereas there was no evidence of differences in the expression of *MMP2* and *TenC*. Although not significant, *MMP2* mRNA expression levels still exhibited a tendency similar to that of *MMP9*. The downregulation in *MMP2* and *MMP9* expression under hypoxic conditions is a potentially interesting find, as matrix-metalloproteinases degrade the extracellular matrix, activate TNF-α and their expression is known to be elevated in cerebral ischemia (43,46). However, *MMPs* are also known for their dual involvement in cellular processes of early tissue damage and late tissue repair during brain injury (47). Future studies investigating the mRNA expression patterns of *MMPs* following hypoxia exposure for various time periods may provide an improved understanding of this complex matter.

A possible explanation for the observations of the present study lies in the limitation of the cell culture model. Zhao *et al* (31) showed that HT22 cells differentiated using $N_2$ supplement expressed NMDA receptors which were not

expressed by undifferentiated HT22 cells. It is possible that the HT22 cells may have returned to their undifferentiated state as a result of prolonged exposure to hypoxia with a subsequent downregulation of all the examined genes. However, the fact that no significant effects of hypoxia alone were detected in the untreated groups suggests the possibility of a specific ketamine-dependent effect in the present study.

Ketamine concentrations of 1, 10 and 20 $\mu$M were chosen based on a previous study (10). As neurotoxicity due to ketamine was only observed when cells were treated with high doses in various studies (8,10,13), the ketamine concentrations used in the present study were likely too low to produce a direct neurotoxic effect.

Several previous studies have demonstrated the significant effects of ketamine treatment on neuronal tissue when the drug is applied as a bolus (6,10,13-15). In the present study, hippocampal cells were incubated for 24 h to simulate extended ketamine treatment in neonates, which may partly explain the inconsistencies in the data when compared with previous studies. There are certain studies showing significant ketamine neurotoxicity following continuous infusion (8,9,21), thus further investigation that directly compares the effects of short and long term treatment is required to elucidate the effects of the length of treatment with ketamine on neurotoxicity.

Although there are several studies demonstrating the potential neurotoxic and neuroapoptotic effects of ketamine (7-15), comparatively less research has been performed on its effects in hypoxic conditions. Animal studies showed that NMDA receptor blockade and ketamine treatment may result in neuroprotective outcomes in hypoxia (20,22,23). However, Ulbrich *et al* (48) did not observe any significant effects of ketamine incubation in a study performed on a SH-SY5Y neuronal cell line. The latter is consistent with the results of the present study and is in contrast to the findings of Rothman *et al* (19), who described preserved action potentials and higher ATP levels in hypoxic primary hippocampal neurons when treated with ketamine. Similarly, ketamine ameliorated the neuroinflammatory response to transient hypoxia in fetal sheep (21). A possible explanation for these contradictory findings is that previously described neuroprotective findings may not be directly caused by the effects of ketamine on neurons, but rather through more complex interactions between the drug and its metabolites, glial cells and neurons. Glial cells are naturally present in animal studies and in primary neuronal cell cultures, but not in immortalized cell lines consisting of a sole cell type such as HT22 or SH-SY5Y (49). This supports the previously suggested hypothesis of glial cells serving a key role in neuroprotection following acute brain injury (50-52). Furthermore, ketamine is metabolized in complex pathways *in vitro*, which results in the circulation of various ketamine metabolites (53). This may be crucial regarding the neurotoxic and neuroprotective ketamine effects described in animal studies and will be omitted by default in a cell culture model.

Another limitation of the present study is that the experiments were performed on a single cell line only. Further studies, particularly animal and primary cell culture models are required to obtain a more definite answer regarding the potential aggravation of hypoxia-induced damage due to ketamine treatment.

The present study is a modest contribution to the ongoing discussion of the potential neurotoxic and neuroprotective effects of the commonly used anesthetic and analgesic pediatric drug ketamine. Overall, the data obtained from the present study are not conclusive and thus should not be used in clinical decision making. However, the findings do broaden our understanding of neuronal gene expression in hypoxia and may thus serve as a starting point for future investigations.

In conclusion, the results of the present study suggest potential aggravation of hypoxia-induced neurotoxicity in hippocampal neurons in the presence of ketamine, as observed in the patterns of downregulation of genetic markers of proliferation, neurogenesis and extracellular matrix homeostasis. However, there was no dose-response association for ketamine treatment observed, which may be due to the ketamine concentrations used and the limitations of the cell culture model used.

## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

BR, CH, TK and EH-R. participated in the design of the study. Molecular analyses and cell culture experiments were performed by TP and SA. TP, SA, RJ and EH-R. analyzed and interpreted the data. TP and EH-R drafted and revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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