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Midazolam impacts acetyl—And butyrylcholinesterase genes: An epigenetic explanation for postoperative delirium?

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Abstract

Midazolam is a widely used short-acting benzodiazepine. However, midazolam is also criticized for its deliriogenic potential. Since delirium is associated with a malfunction of the neurotransmitter acetylcholine, midazolam appears to interfere with its proper metabolism, which can be triggered by epigenetic modifications. Consequently, we tested the hypothesis that midazolam indeed changes the expression and activity of cholinergic genes by acetylcholinesterase assay and qPCR. Furthermore, we investigated the occurrence of changes in the epigenetic landscape by methylation specific PCR, ChiP-Assay and histone ELISA. In an *in-vitro* model containing SH-SY5Y neuroblastoma cells, U343 glioblastoma cells, and human peripheral blood mononuclear cells, we found that midazolam altered the activity of acetylcholinesterase /buturylcholinesterase (AChE / BChE). Interestingly, the increased expression of the buturylcholinesterase evoked by midazolam was accompanied by a reduced methylation of the BCHE gene and the di-methylation of histone 3 lysine 4 and came along with an increased expression of the lysine specific demethylase KDM1A. Last, inflammatory cytokines were not induced by midazolam. In conclusion, we found a promising mechanistic link between midazolam treatment and delirium, due to a significant disruption in cholinesterase homeostasis. In addition, midazolam seems to provoke profound changes in the epigenetic landscape. Therefore, our results can contribute to a better understanding of the hitherto poorly understood interactions and risk factors of midazolam on delirium.

1. Introduction

Midazolam is the most abundantly used benzodiazepine in anesthesia and emergency medicine [1]. Due to its amnestic and anxiolytic effects, midazolam is considered as a favorable choice for premedication [2, 3]. However, the use of benzodiazepines especially midazolam is associated with postoperative complications such as cognitive impairment and delirium [4].

Currently, it is discussed whether anesthetics cause an alteration of the epigenetic landscape of the cell, which might induce a long-lasting cognitive impairment [5]. One common postoperative complication in elderly critically ill patients is the postoperative delirium (POD) that is also associated with a worse outcome, longer stay on the intensive care unit and higher healthcare related costs [6]. In addition, delirium is also linked to an increased risk of long term cognitive impairments that recover with high inter-individual differences from days to months [7]. Especially the use of benzodiazepine is, in addition to blood transfusion, one of the only modifiable factors with strong evidence for an association with delirium after surgery [8]. Within the group of benzodiazepines midazolam shows highest incidence of POD [9].

Although there are some theories that could explain the positive correlation between midazolam administration and the high incidence of POD, such as the degree of sedation [8] and the function of midazolam as a GABAergic agent [10], the underlying molecular mechanisms and the pathogenesis of POD still remain elusive.

Currently, a pathogenesis is discussed involving a reduced concentration of the neurotransmitter acetylcholine [11], neuroinflammation [12, 13] or decreased antiinflammation [14]. The hydrolysis of acetylcholine is mainly mediated by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) that can be found in the brain, red blood cells, and central nervous system [15]. Especially an altered activity and concentration of BChE seems to impact pathogenesis of POD [16–18] and BChE activity also shows high prognostic capability for POD [19].

Recently, we could demonstrate that the GABAergic agent propofol changes the epigenome [20]. In context with POD and anesthesia, the expression of lysine-specific demethylase (KDM1A) seems of special interest as it is associated with cognitive function [21] and demethylates histone 3 lysine 4 [22]. Hence long-lasting effects on the central nervous system and cognitive abilities caused by the GABAergic midazolam could be caused by changing the epigenetic landscape of the cells [23–25]. Since it is currently unknown whether and how midazolam influences the activity of the *ACHE* or *BCHE* gene. However, we speculate that one possible mechanism is the alteration of the expression of cholinergic genes by changing the epigenetic profile of the cells.

Therefore, in this study we investigated whether the expression, activity and methylation profile of cholinesterases are changed by midazolam. Furthermore, we study whether midazolam changes the epigenetic landscape of the cell by altering KDM1A expression.

2. Materials and methods

2.1. Cell culture

Human neuroblastoma cells SH-SY5Y and the glioblastoma cell line U343 (origin: Cell Lines Service, CLS, Eppelheim Germany, SH-SY5Y item number: 300154 and U343 item number: 300365) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Darmstadt, Germany) at 37˚C and 5% CO_2 with 10% fetal calf serum (FCS; Gibco, Darmstadt, Germany) and 1% penicillin/streptomycin (Penstrep; Gibco, Darmstadt, Germany). Cells were maintained every three to four days by adding 5 ml of Trypsin-EDTA 0.25% (Gibco, Darmstadt, Germany) after medium removal to dissolve adhesive cells. Furthermore, peripheral blood mononuclear cells (PBMCs) were examined, after the Ethics Committee's approval (Ethics Committee of the Ruhr-University Bochum, Bochum, Germany; ref: 17-5964-BR), registration at the German Clinical Trials Register (ref: DRKS00012961, https://www.drks.de/drks_web/navigate.do?navigationId=trial.HTML&TRIAL_ID=DRKS00012961) and written informed consent. 80 ml EDTA blood was taken from eight healthy donors (5 female and 3 male) and PBMCs were isolated, using density gradient centrifugation with Ficoll-Paque (GE Healthcare, Chalfont, UK).

2.2. Quantitative reverse transcription PCR

q-RT-PCR on SH-SY5Y cells, U343 and PBMCs was performed as described previously [26]. Briefly, cells were cultured in 6-well culture plates and incubated with 250 ng/ml, 1 μg/ml or 50 μg/ml midazolam (midazolam hydrochloride injection solution, B. Braun Melsungen) for 2, 4 and 24 h, 10 μg/ml and 50 μg/ml flumazenil or were left untreated (control). Flumazenil incubation was performed two hours after starting midazolam incubation. For incubation, the highest concentration of midazolam (SH-SY5Y 50 μg/ml; U343 250 ng/ml; BV-2 10 μg/ml) and flumazenil was used, which did not reduce cell viability in different cell lines in previous experiments. Cells were incubated at 37˚C and 5% CO_2. After RNA isolation and cDNA synthesis of 1 μg RNA using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany), we utilized 2.5 μl of cDNA together with specific primers (Table 1) and GoTaq qPCR master mix (Promega, Madison, WI, USA) for a standard qPCR reaction protocol.

2.3. Cholinesterase activity after incubation with midazolam

Cholinesterase activity in SH-SY5Y cells was measured after stimulation with midazolam.

For this purpose, 5 x 105 SH-SY5Y cells were seeded in 4 ml of growth medium containing 10% FBS. Cells were incubated for 24 h at 37˚C and incubated for 2, 4 and 24 h with 50 μg/ml midazolam or were left untreated.

The proteins were isolated as previously described [20] after washing the cells with PBS. After the lysates were collected from all experiments, protein quantification was performed using the Rotiquant universal kit (Roth, Karlsruhe, Germany). The lysates were used for detection of cholinesterase activity using an acetylcholinesterase assay kit (fluorometric red) (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Table 1. Primer pairs for PCR.

Primer name	Sequence (5' to 3')	Product size (bp)
BCHE_M1_SE	ATTTAGGTTAAAACGGTGAAATTTC	172
BCHE_M1_AS	AAACTAAAATACCGTAACGCGAT	
BCHE_U1_SE	TTAGGTTAAAATGGTGAAATTTTGG	173
BCHE_U1_AS	CTCAAACTAAAATACCATAACACAAT	
ACHE_M_SE1	AAT TTT ATT AGT TTC GAG CGA GAT C	189
ACHE_M_AS1	GAC CCA AAA ACC TAC AAC GAC	
ACHE_U_SE1	TTT TAT TAG TTT TGA GTG AGA TTG A	188
ACHE_U_AS1	CAA CCC AAA AAC CTA CAA CAA C	
ACTB_SE	CTGGAACGGTGAAGGTGACA	140
ACTB_AS	AAGGGACTTCCTGTAACAATGCA	
KDM1A_RT_SE	GCCCACTTTATGAAGCCAACG	161
KDM1A_RT_AS	GCCAAGGGACACAGGCTTAT	
ACHE_mRNA_SE	GCT TCA GCA AAG ACA ACG AG	115
ACHE_mRNA_AS	GTG TAA TGC AGG ACC ACA GC	
BCHE_mRNA_SE	ATCCTGCATTTCCCCGAAGT	239
BCHE_mRNA_AS	CCGTGCCACCAAAAACTGTC	
BCHE_Prom_SE	GCATGTGCACTGCAAGTTGA	90
	AACTCTCGCGAGCTTTGTCA	
BCHE_Prom_AS	CCCTGCAGGCAGTCATACAT	
	CTGCTGCTCCAGCCTGTAAA	

2.4. Methylation and expression of BCHE gene after incubation with midazolam

The DNA methylation of *BCHE* gene was quantified using methylation-specific PCR after bisulphite conversion in SH-SY5Y cells, before and after incubation. For this purpose, 5 x 105 SH-SY5Y cells per 4 ml were seeded in 6-well culture plates and incubated for 24 h at 37° C and 5% CO_2. The cells were incubated with 50 μg/ml or 250 ng/ml midazolam depending on cell type for 2, 4 and 24h. Subsequently, the DNA was isolated using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Bisulphite conversion was performed with the EZ DNA methylation-gold kit (Zymo Research, Irvine, CA, USA). All DNA samples were diluted to 10 ng /μl qPCR was performed to detect methylation, as previously described [27], with the GoTaq qPCR master mix (Promega, Madison, WI, USA) and specific primers (Table 1).

The percentage of methylation was analyzed as previously described [27, 28].

2.5. Analysis of histone modifications

Furthermore, histone modifications of histone 3 after incubation were analyzed. SH-SY5Y cells and U343 were seeded, as previously described, and incubated with 250 ng / ml of midazolam or left untreated (control) for 24 h exactly as previously described [20].

Histone concentration was quantified by the Rotiquant universal kit (Roth, Karlsruhe, Germany) and histone modification was quantified by ELISA using 50 ng protein for the PathScan® Di-Methyl-Histone H3 (Lys4) Sandwich ELISA kit (Cell Signaling Technology, Cambridge, UK).

2.6. Chromatin immunoprecipitation assay (ChIP assay)

A ChIP assay was used to analyze if the promoter of the cholinergic gene *BCHE* binds to histone H3 lysine K4., 1 x 10^6 SH-SY5Y were used for the Pierce agarose Chip kit (Thermo Fisher Scientific, Waltham, MA, USA). The $H3K4me_2$ polyclonal antibody (EpiGentek, Farmingdale, NY, USA) was used as a specific antibody. As a positive control, an antibody against RNA polymerase II in combination with specific primers against GAPDH was used, while Rabbit IgG in combination with our primers against *BCHE* gene regions was used as a negative control. After DNA isolation, PCR (One Taq Master Mix, New England Biolabs, Frankfurt am Main, Germany) was carried out with, *BCHE*_prom primers (Table 1), and the PCR products were analyzed on agarose gel (Peqlab, Erlangen, Germany).

2.7. LegendPlex assay for the quantification of cytokines (TNFα and IL6)

To measure cytokine release from glial cells, BV2 cells (kind gift from Veselin Grozdanov Department of Neurology, Ulm University, Ulm, Germany) were used. Cell culture supernatant was utilized after midazolam and LPS treatment for quantification of TNFα, IL6 with the Legend Plex InflammationPanel (BioLegend, San Diego, CA), according to manufacturer's recommendations. Briefly, cells were treated with 1 μg/mL midazolam 100 ng/ml LPS or left untreated and incubated for 2, 4 and 24 h in complete growth medium. Cell supernatant was collected and stored at -80°C until use for cytokine quantification. Measurement was performed using FACS Canto II (Becton Dickinson GmbH, Heidelberg, Germany) according to the manufacturer's instructions and analysis was performed using LEGENDplex v8.0 software.

2.8. Statistics

All experiments were performed in duplicate and repeated at least three times. Results are presented as mean ± standard deviation. If not otherwise stated, all datasets were analyzed using
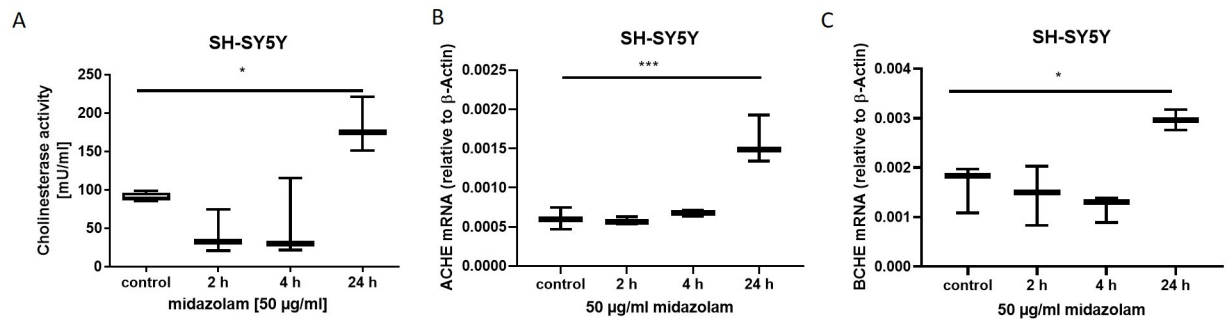
Fig 1. Activity and mRNA expression of AChE and BChE after incubation with midazolam A) the intracellular cholinesterase activity increased 24 h after midazolam exposure (n = 4; p = 0.01) and was measured by fluorometric assay B) *ACHE* mRNA quantified by qPCR expression was increased 24 hours after midazolam exposure in SH-SY5Y cells (n = 3; p<0.01) C) *BCHE* mRNA quantified by qPCR expression was increased 24 hours after midazolam exposure in SH-SY5Y cells (n = 3; p = 0.03). Data are presented as mean ± standard deviation. The reported p-value refers to the Dunnett's post-hoc test, comparing the underlying columns at the ends of each bar.

an unpaired t-Test or one-way ANOVA for multiple comparisons with a Dunnett's multiple comparisons test for specific comparisons. A p-value ≤ 0.05 was considered statistically significant. For multiple comparisons, specific comparisons were only analyzed if the one-way ANOVA showed a statistically significant difference between the groups. All statistical analyses were performed using GraphPad Prism 8 (San Diego, CA, USA).

3. Results

3.1. The activity and expression of AChE and BChE are altered after incubation with midazolam

Cholinesterase activity in SH-SY5Y cells was gradually reduced after incubation with midazolam, without reaching statistical significance (control: mean + SD 90.7 +5.5; 2 hours mean + SD 42.51 + 28.7; p = n.s.; Fig 1A) but the intracellular AChE and BChE activity (p = 0.01; Fig 1A) and *ACHE* (p< 0.01) and *BCHE* (p = 0.03) mRNA expression increased after 24 h by about 80% (Fig 1B and 1C).

3.2. Application of the midazolam antagonist flumazenil reverses midazolam induced effects on BCHE expression

In order to elucidate if midazolam antagonist flumazenil is capable to reduce midazolam induced effects on *ACHE* and *BCHE* expression, SH-SY5Y cells were incubated with flumazenil two hours after midazolam exposure. Here, we show a gradual increasing abolition of the midazolam effect (increased *BCHE* expression) under increasing doses of the antagonist flumazenil (Fig 2B). After the addition of 10μg/mL flumazenil the increased expression associated with midazolam of *BCHE* was reduced (p<0.05; Fig 2B). Interestingly, this effect was not observed on *ACHE* expression (Fig 2A).

3.3. Midazolam induces epigenetic changes in the BCHE gene of neuronal cells

Midazolam induced a decrease in *BCHE* intron 2 DNA methylation (p = 0.01; Fig 3A) and in the di-methylation of H3K4 (p = 0.02; Fig 3B), where *BCHE* promoter binds (Fig 3C). *ACHE* DNA methylation was not altered by incubation with 50 μg/ml midazolam (Fig 3D).
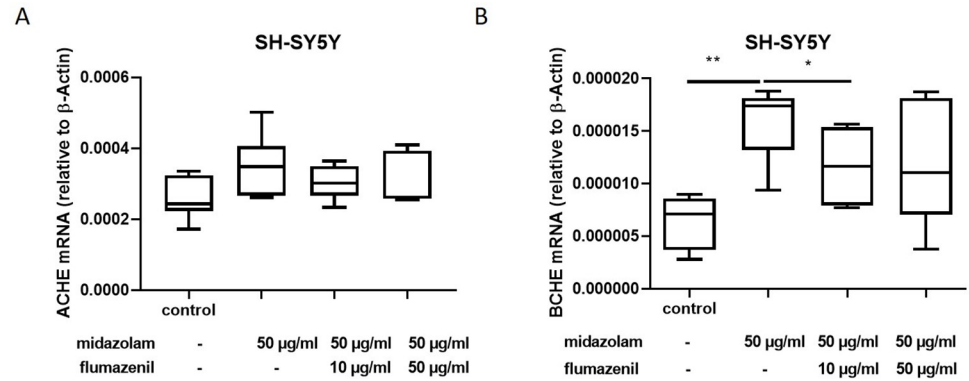
Fig 2. SH-SY5Y cells were incubated with midazolam for 24 h and with flumazenil (starting 2 h after midazolam exposure) for 22 hours. ACHE and BCHE mRNA expression relative to β-Actin mRNA expression were quantified by qPCR. Incubation with flumazenil A) did not alter ACHE expression (n = 6; p = n.s.) and reduced B) BCHE (n = 6; p = 0.046) expression. Data are presented as mean ± standard deviation. The reported p-value refers to the Dunnett's post-hoc test, comparing the underlying columns at the ends of each bar.
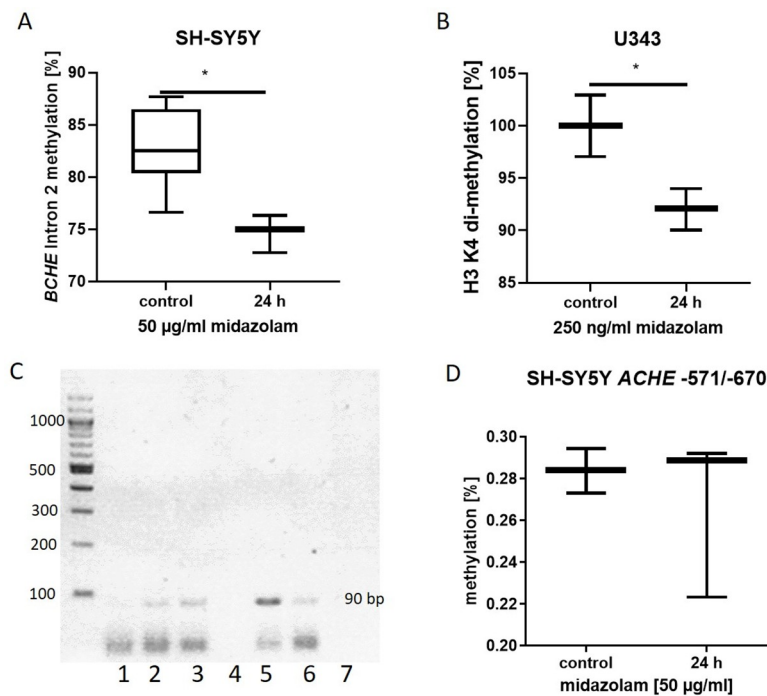
Fig 3. Methylation of *BCHE* in neuronal SH-SY5Y and U343 cells after midazolam incubation A) *BCHE* intron 2 methylation reduced after midazolam (50 μg/ml) exposure of SH-SY5Y cells (n = 3; p = 0.01) analyzed by methylation specific PCR. B) ELISA showed that histone H3 lysine 4 di-methylation (H3K4me2) decreased in U343 cells after incubation with 250 ng/ml midazolam (n = 3; p = 0.02) C) Chip-Assay confirmed binding of *BCHE* promoter region (90 bp) to H3K4me2; a 100 bp DNA Ladder was utilized; lanes 1, 7 show incubation with H3K27 antibody; lanes 2 and 6 show incubation with H3K4 antibody; lane 4 shows negative control without antibody and lane 5 shows positive control with RNA-polymerase II antibody (two experiments out of three are shown; n = 3). D) ACHE -571-/-670 promoter methylation was not affected by midazolam (50 μg/ml) exposure of SH-SY5Y cells (n = 3; p = n.s.) analyzed by methylation specific PCR. Data are presented as mean ± standard deviation.
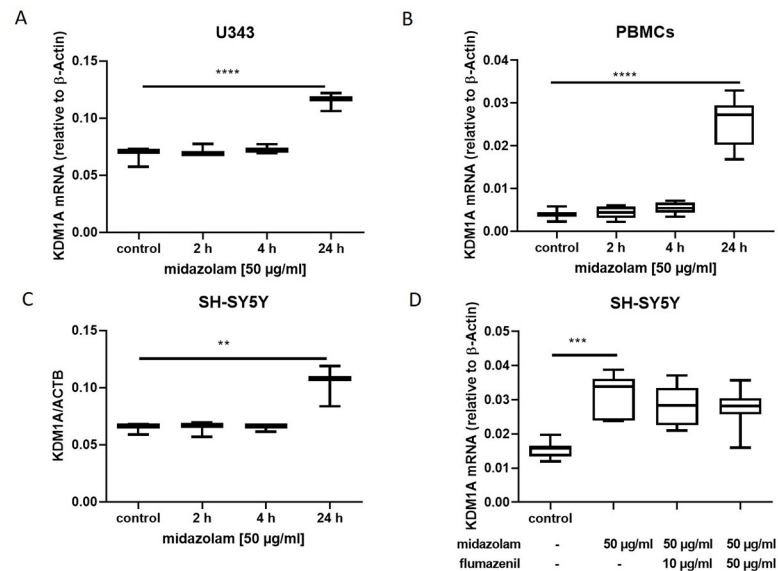
Fig 4. KDM1A mRNA expression was quantified relative to β-Actin mRNA expression by qPCR. Increased expression of lysine specific demethylase (*KDM1A*) in different cells after midazolam [50 μg/ml] exposure for 24 hours analyzed by qPCR. *KDM1A* expression increased in U343 (n = 3; A, in peripheral blood mononuclear cells (PBMCs) (n = 8; B) and in SH-SY5Y (n = 3; C). Flumazenil did not reduce KDM1A expression (n = 6, D). Data are presented as mean ± standard deviation. The reported p-value refers to the Dunnett's post-hoc test, comparing the underlying columns at the ends of each bar.

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## 3.4. Midazolam increases the expression of lysine specific demethylase KDM1A

To explore the underlying mechanisms for the decrease in H3K4me2, we analyzed the expression of lysine specific demethylase *KDM1A* after exposure to midazolam. *KDM1A* mRNA expression was increased in, in U343 by about 50% (p <0.01; Fig 4A), in PBMCs by more than 100% (p<0.01; Fig 4B) and in SH-SY5Y by about 50% (p = 0.0038; Fig 4C). Incubation with flumazenil reduced midazolam induced effects in a visible dose dependent manner in SH-SY5Y cells, while incubation with midazolam alone led to increased expression of KDM1A (p < 0.001; Fig 4D) expression.

3.5. Midazolam does not induce the release of cytokines from BV-2 glial cells

Since postoperative delirium is strongly associated with neuroinflammation, we finally investigated whether midazolam itself evoked cytokine secretion in neural glial cells (BV-2, RRID: CVCL_0182). Midazolam did not induce any change in cytokine secretion in BV-2 cells (p = ns), compared to untreated cells. Cells incubated with lipopolysaccharide (LPS) as positive control had higher TNF-α cytokine levels (p = 0.01; Fig 5A) and higher IL-6 levels (p = 0.02; Fig 5B) compared to cells incubated with midazolam for 24 hours.

4. Discussion

Midazolam is a widely used benzodiazepine although its application is associated with the occurrence of POD [9]. A potential mechanism for the development of delirium is impaired cholinergic transmission based on the deficiency of acetylcholine in the brain [29]. However, as the causal relationship between midazolam and the cholinergic system is unknown, we systematically analyzed the expression and epigenetic regulation of cholinergic genes in neuronal
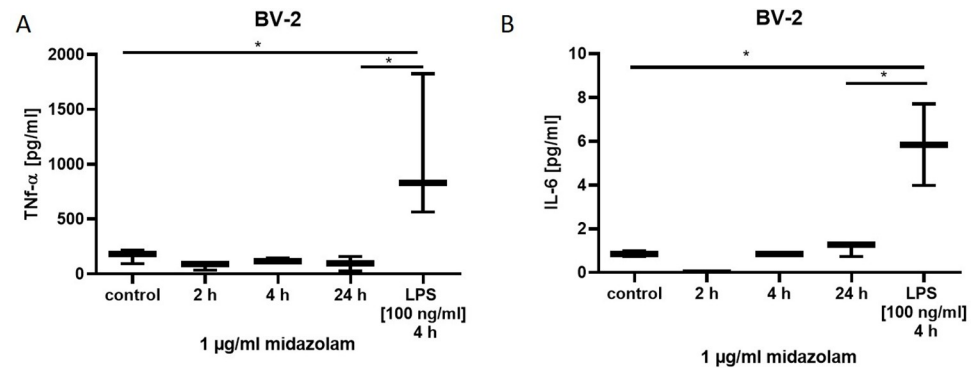
Fig 5. Cytokine secretion in BV-2 glial cells after midazolam (1µg/ml) for 24 hours and LPS (100 ng/ml) for 4 hours (n = 3). BV-2 cells were incubated with midazolam (1µg/ml) or lipopolysaccharide LPS (100 ng/ml) or left untreated. Cytokine expression was quantified using a bead-based immunoassay. Data are presented as mean ± standard deviation. The reported p-value refers to the Dunnett's post-hoc test, comparing the underlying columns at the ends of each bar.

cells after midazolam exposure. As a different postoperative activity of the proteins AChE and BChE in patients is already described [16, 30, 31] it seems of special interest, how their gene expression is regulated after midazolam exposure.

First, we could detect a visibly early decrease in cholinesterase activity and a slight decrease in the expression of *BCHE* mRNA, but a late increase in the activity and the expression of *ACHE* and *BCHE* mRNA. Our results regarding AChE and BChE activity and expression are in line with other studies analyzing AChE and BChE activities in peripheral blood from preoperative and postoperative patients [16, 19, 30]. AChE and BChE concentrations in blood and cerebrospinal fluid were altered in patients undergoing total hip/knee replacement, and BChE concentration showed the highest prognostic value for the development of POD [19]. Thus, increased gene expression, especially BChE, could represent an important mechanism, as it could be found in the brain of patients with Alzheimer's disease [32] and several studies explored the therapeutic implication of cholinesterase inhibitors in alleviating postoperative delirium [33].

Second, we tested the methylation of a *BCHE* gene region and a histon, with *BCHE* binding affinity. Methylation of the *BCHE* gene region (Intron 2) and the H3K4 di-methylation decreased after midazolam incubation. Thus, it seems appropriate to suggest that the region of the *BCHE* gene we investigated has activating effects on the transcription of this gene. However, it must be mentioned that the reduction in methylation was only about 10%. This seems to be questionable for a more than doubled amount of mRNA expression. In fact, other studies have already shown that a small change in DNA methylation of approximately 5% can have a great impact on gene expression [34]. Therefore, it seems possible that this small change in methylation state may cause this effect on mRNA expression.

Third: Since midazolam changed the di-methylation of H3K4, and we could detect binding of *BCHE* to this histone, it seems appropriate that midazolam might change the epigenome of the cell by influencing histon-modifying enzymes. H3K4me2 has been shown to mark actively transcribing genes [35]. In our analyzed neuronal cell line di-methylation was nearly 100 percent and midazolam could decrease the methylation slightly. A reduction of the di-methylation of H3K4 could therefore mean an overall increase in BChE expression. The demethylation of H3K4 is facilitated by KDM1A and is a well-established mechanism underlying transcriptional gene repression, but recently its role in gene activation could be shown [36]. The KDM1A demethylation of H3K4me2 in GR-targeted enhancers was shown to be important for GC-

mediated gene transcription, facilitating a molecular mechanism for the demethylation of H3K4me2 in gene activation [36]. Since changes in the methylation of histone 3 is facilitated by KDM1A, we analyzed the expression of *KDM1A* in our cell lines, and because POD is also associated with an altered cholinesterase activity in blood samples [16], we additionally investigated the expression of these enzymes in PBMCs. Strikingly, *KDM1A* showed a significant increased expression after midazolam treatment in all investigated cell lines (including PBMCs). Thus, our results provide first evidence that midazolam indeed rewrites the epigenetic landscape of the cell. Interestingly, the application of KDM1A inhibitors is associated with positive effects on memory. Recently it could be demonstrated that inhibition of KDM1A corrects memory deficit and behavior alterations in a mouse model of Alzheimer's Disease [21]. Another KDM1A inhibitor T-448 improved learning function in mice suffering from neuronal glutamate receptor hypofunction [37]. Thus, it seems tempting to speculate that KDM1A inhibitors might represent a therapeutic approach against POD. However, this crude thesis needs to be evaluated in upcoming studies.

Fourth: As increased expression of *BCHE* seems to be critical mechanisms after midazolam exposure. In this context, we analyzed if the midazolam antagonist flumazenil could inhibit midazolam induced effects. Indeed, we could show that flumazenil application reduced midazolam-induced expression in a dose-dependent manner. Regarding the effects of flumazenil application after midazolam anesthesia on brain function, we can only speculate. However, it is known that cognitive abnormalities can significantly be ameliorated after benzodiazepine use by slow subcutaneous infusion of flumazenil [38] and that flumazenil administration attenuates cognitive impairment [38]. Therefore, flumazenil use might be effective in reducing POD.

Lastly, since POD is related to neuroinflammation [39], we analyzed if there is a link between midazolam treatment for neuroinflammation. We could demonstrate that in our positive control, the incubation of glial cells with LPS TNF-alpha and IL-6 were significantly upregulated. However, midazolam treatment had no influence on the expression of these cytokines. IL-6 seems to be of particular interest as it seems to be a consistent predictor of delirium in surgical samples [40]. Therefore, we can conclude that midazolam does not strongly contribute to pro-inflammatory signaling, being discussed as additional factors in the development of POD [12–14].

We have to discuss the limitations of our study. Direct transfer to the bedside is inappropriate because we worked with cell lines as a model for the human brain. However, for instance we chose the neuronal cell line SH-SY5Y, because these represent an established cell line used to study brain disorders such as Alzheimer's disease or Parkinson [41, 42]. In addition, the extraction of neuronal cells from healthy volunteers or patients with POD is ethically not feasible [43]. Despite great efforts made to achieve the highest possible degree of standardization, variance in effect sizes or observed effects can occur within the individual experiments, which limits the statistical or mathematical accuracy of our experiments. However, this had no or only a negligible effect on the interpretation of our data. Therefore, considering the limitations of immortalized cell lines, we are confident that it is appropriate to perform our investigations in our selected cell lines. In addition, direct measurement of acetylcholine would be interesting but is not suitable as it is extremely unstable [44]. Thus, we mainly refer to the central effectors and regulators of acetylcholine concentration.

5. Conclusions

In summary, we found that midazolam upregulates intracellular BCHE expression. This upregulation in expression might be caused by demethylation of BCHE gene and H3K4 me2

demethylation and be facilitated by KDM1A. Thus, our results underpin the thesis, that over-expression of BCHE might aggravate postoperative delirium, due to an increased hydrolysis of acetyl-choline. Although POD is closely related to neuroinflammation, midazolam appears to be a separate trigger, independent of inflammation. Further studies should validate our promising results and mechanistic implications in the clinical context regarding feasibility and transferability.

Supporting information

S1 Raw images.
(JPG)

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Supervision: Lars Bergmann, Hartmuth Nowak, Michael Adamzik, Tim Rahmel.

Writing – original draft: Katharina Rump.

Writing – review & editing: Caroline Holtkamp, Lars Bergmann, Hartmuth Nowak, Matthias Unterberg, Michael Adamzik, Björn Koos, Tim Rahmel.

References

1. Halbeck E, Dumps C, Bolkenius D. [Drugs for intravenous induction of anesthesia: ketamine, midazolam and synopsis of current hypnotics]. Anaesthesist. 2018; 67(8):617–34. https://doi.org/10.1007/s00101-018-0469-7 PMID: 30069734

2. Khanderia U, Pandit SK. Use of midazolam hydrochloride in anesthesia. Clin Pharm. 1987; 6(7):533–47. PMID: 3319363

3. Alwardt CM, Redford D, Larson DF. General anesthesia in cardiac surgery: a review of drugs and practices. J Extra Corpor Technol. 2005; 37(2):227–35. PMID: 16117465

4. Li WX, Luo RY, Chen C, Li X, Ao JS, Liu Y, et al. Effects of propofol, dexmedetomidine, and midazolam on postoperative cognitive dysfunction in elderly patients: a randomized controlled preliminary trial. Chin Med J (Engl). 2019; 132(4):437–45.

5. Ji M, Dong L, Jia M, Liu W, Zhang M, Ju L, et al. Epigenetic enhancement of brain-derived neurotrophic factor signaling pathway improves cognitive impairments induced by isoflurane exposure in aged rats. Mol Neurobiol. 2014; 50(3):937–44. https://doi.org/10.1007/s12035-014-8659-z PMID: 24553857

6. Titsworth WL, Hester J, Correia T, Reed R, Guin P, Archibald L, et al. The effect of increased mobility on morbidity in the neurointensive care unit. J Neurosurg. 2012; 116(6):1379–88. https://doi.org/10.3171/2012.2.JNS111881 PMID: 22462507

7. Silverstein JH, Timberger M, Reich DL, Uysal S. Central nervous system dysfunction after noncardiac surgery and anesthesia in the elderly. Anesthesiology. 2007; 106(3):622–8. https://doi.org/10.1097/00000542-200703000-00026 PMID: 17325520

8. Devlin JW, Skrobik Y, Gélinas C, Needham DM, Slooter AJC, Pandharipande PP, et al. Clinical Practice Guidelines for the Prevention and Management of Pain, Agitation/Sedation, Delirium, Immobility, and

Sleep Disruption in Adult Patients in the ICU. Crit Care Med. 2018; 46(9):e825–e73. https://doi.org/10.1097/CCM.0000000000003299 PMID: 30113379

9. Cui Y, Li G, Cao R, Luan L, Kla KM. The effect of perioperative anesthetics for prevention of postoperative delirium on general anesthesia: A network meta-analysis. J Clin Anesth. 2020; 59:89–98. https://doi.org/10.1016/j.jclinane.2019.06.028 PMID: 31284222

10. Zhou Y, Li Y, Wang K. Bispectral Index Monitoring During Anesthesia Promotes Early Postoperative Recovery of Cognitive Function and Reduces Acute Delirium in Elderly Patients with Colon Carcinoma: A Prospective Controlled Study using the Attention Network Test. Med Sci Monit. 2018; 24:7785–93. https://doi.org/10.12659/MSM.910124 PMID: 30378591

11. Zujalovic B, Barth E. Delirium Accompanied by Cholinergic Deficiency and Organ Failure in a 73-Year-Old Critically Ill Patient: Physostigmine as a Therapeutic Option. Case Rep Crit Care. 2015; 2015:793015. https://doi.org/10.1155/2015/793015 PMID: 26550498

12. Cascella M, Muzio MR, Bimonte S, Cuomo A, Jakobsson JG. Postoperative delirium and postoperative cognitive dysfunction: updates in pathophysiology, potential translational approaches to clinical practice and further research perspectives. Minerva Anestesiol. 2018; 84(2):246–60. https://doi.org/10.23736/S0375-9393.17.12146-2 PMID: 28984099

13. Umholtz M, Nader ND. Anesthetic Immunomodulation of the Neuroinflammation in Postoperative Cognitive Dysfunction. Immunol Invest. 2017; 46(8):805–15. https://doi.org/10.1080/08820139.2017.1373898 PMID: 29058541

14. Maldonado JR. Neuropathogenesis of delirium: review of current etiologic theories and common pathways. Am J Geriatr Psychiatry. 2013; 21(12):1190–222. https://doi.org/10.1016/j.jagp.2013.09.005 PMID: 24206937

15. Das UN. Acetylcholinesterase and butyrylcholinesterase as markers of low-grade systemic inflammation. Ann Hepatol. 2012; 11(3):409–11. PMID: 22481463

16. Müller A, Olbert M, Heymann A, Zahn PK, Plaschke K, von Dossow V, et al. Relevance of peripheral cholinesterase activity on postoperative delirium in adult surgical patients (CESARO): A prospective observational cohort study. Eur J Anaesthesiol. 2019; 36(2):114–22. https://doi.org/10.1097/EJA.0000000000000888 PMID: 30431498

17. John M, Ely EW, Halfkann D, Schoen J, Sedemund-Adib B, Klotz S, et al. Acetylcholinesterase and butyrylcholinesterase in cardiosurgical patients with postoperative delirium. J Intensive Care. 2017; 5:29. https://doi.org/10.1186/s40560-017-0224-1 PMID: 28560042

18. Adam EH, Haas V, Lindau S, Zacharowski K, Scheller B. Cholinesterase alterations in delirium after cardiosurgery: a German monocentric prospective study. BMJ Open. 2020; 10(1):e031212. https://doi.org/10.1136/bmjopen-2019-031212 PMID: 31941763

19. Lin X, Tang J, Liu C, Li X, Cao X, Wang B, et al. Cerebrospinal fluid cholinergic biomarkers are associated with postoperative delirium in elderly patients undergoing Total hip/knee replacement: a prospective cohort study. BMC Anesthesiol. 2020; 20(1):246. https://doi.org/10.1186/s12871-020-01166-9 PMID: 32988381

20. Holtkamp C, Koos B, Unterberg M, Rahmel T, Bergmann L, Bazzi Z, et al. A novel understanding of postoperative complications: In vitro study of the impact of propofol on epigenetic modifications in cholinergic genes. PLoS One. 2019; 14(5):e0217269. https://doi.org/10.1371/journal.pone.0217269 PMID: 31141559

21. Maes T, Mascaró C, Rotllant D, Lufino MMP, Estiarte A, Guibourt N, et al. Modulation of KDM1A with vafidemstat rescues memory deficit and behavioral alterations. PLoS One. 2020; 15(5):e0233468. https://doi.org/10.1371/journal.pone.0233468 PMID: 32469975

22. Rudolph T, Beuch S, Reuter G. Lysine-specific histone demethylase LSD1 and the dynamic control of chromatin. Biol Chem. 2013; 394(8):1019–28. https://doi.org/10.1515/hsz-2013-0119 PMID: 23612539

23. Culley DJ, Baxter MG, Yukhananov R, Crosby G. Long-term impairment of acquisition of a spatial memory task following isoflurane-nitrous oxide anesthesia in rats. Anesthesiology. 2004; 100(2):309–14. https://doi.org/10.1097/00000542-200402000-00020 PMID: 14739805

24. Culley DJ, Baxter M, Yukhananov R, Crosby G. The memory effects of general anesthesia persist for weeks in young and aged rats. Anesth Analg. 2003; 96(4):1004–9. https://doi.org/10.1213/01.ANE.0000052712.67573.12 PMID: 12651650

25. Mori K, Iijima N, Higo S, Aikawa S, Matsuo I, Takumi K, et al. Epigenetic suppression of mouse Per2 expression in the suprachiasmatic nucleus by the inhalational anesthetic, sevoflurane. PLoS One. 2014; 9(1):e87319. https://doi.org/10.1371/journal.pone.0087319 PMID: 24498074

26. Rump K, Brendt P, Frey UH, Schafer ST, Siffert W, Peters J, et al. Aquaporin 1 and 5 expression evoked by the beta2 adrenoreceptor agonist terbutaline and lipopolysaccharide in mice and in the human monocytic cell line THP-1 is differentially regulated. Shock. 2013; 40(5):430–6. https://doi.org/10.1097/SHK.0000000000000035 PMID: 24088990

27. Unterberg M, Kreuzer MJ, Schafer ST, Bazzi Z, Adamzik M, Rump K. NFKB1 Promoter DNA from nt +402 to nt+99 Is Hypomethylated in Different Human Immune Cells. PLoS One. 2016; 11(6):e0156702. https://doi.org/10.1371/journal.pone.0156702 PMID: 27249028

28. Agrawal S, Unterberg M, Koschmieder S, zur Stadt U, Brunnberg U, Verbeek W, et al. DNA methylation of tumor suppressor genes in clinical remission predicts the relapse risk in acute myeloid leukemia. Cancer Res. 2007; 67(3):1370–7. https://doi.org/10.1158/0008-5472.CAN-06-1681 PMID: 17283175

29. Hshieh TT, Fong TG, Marcantonio ER, Inouye SK. Cholinergic deficiency hypothesis in delirium: a synthesis of current evidence. J Gerontol A Biol Sci Med Sci. 2008; 63(7):764–72. https://doi.org/10.1093/gerona/63.7.764 PMID: 18693233

30. Cerejeira J, Batista P, Nogueira V, Firmino H, Vaz-Serra A, Mukaetova-Ladinska EB. Low preoperative plasma cholinesterase activity as a risk marker of postoperative delirium in elderly patients. Age Ageing. 2011; 40(5):621–6. https://doi.org/10.1093/ageing/afr053 PMID: 21576115

31. Cerejeira J, Nogueira V, Luís P, Vaz-Serra A, Mukaetova-Ladinska EB. The cholinergic system and inflammation: common pathways in delirium pathophysiology. J Am Geriatr Soc. 2012; 60(4):669–75. https://doi.org/10.1111/j.1532-5415.2011.03883.x PMID: 22316182

32. Bono GF, Simão-Silva DP, Batistela MS, Josviak ND, Dias PF, Nascimento GA, et al. Butyrylcholinesterase: K variant, plasma activity, molecular forms and rivastigmine treatment in Alzheimer's disease in a Southern Brazilian population. Neurochem Int. 2015; 81:57–62. https://doi.org/10.1016/j.neuint.2014.12.009 PMID: 25624079

33. Yu A, Wu S, Zhang Z, Dening T, Zhao S, Pinner G, et al. Cholinesterase inhibitors for the treatment of delirium in non-ICU settings. Cochrane Database Syst Rev. 2018; 6(6):Cd012494. https://doi.org/10.1002/14651858.CD012494.pub2 PMID: 29952000

34. Butts B, Butler J, Dunbar SB, Corwin E, Gary RA. Effects of Exercise on ASC Methylation and IL-1 Cytokines in Heart Failure. Med Sci Sports Exerc. 2018; 50(9):1757–66. https://doi.org/10.1249/MSS.0000000000001641 PMID: 29683921

35. Hyun K, Jeon J, Park K, Kim J. Writing, erasing and reading histone lysine methylations. Exp Mol Med. 2017; 49(4):e324. https://doi.org/10.1038/emm.2017.11 PMID: 28450737

36. Clark EA, Wu F, Chen Y, Kang P, Kaiser UB, Fang R, et al. GR and LSD1/KDM1A-Targeted Gene Activation Requires Selective H3K4me2 Demethylation at Enhancers. Cell Rep. 2019; 27(12):3522–32.e3. https://doi.org/10.1016/j.celrep.2019.05.062 PMID: 31216473

37. Matsuda S, Baba R, Oki H, Morimoto S, Toyofuku M, Igaki S, et al. T-448, a specific inhibitor of LSD1 enzyme activity, improves learning function without causing thrombocytopenia in mice. Neuropsychopharmacology. 2019; 44(8):1505–12. https://doi.org/10.1038/s41386-018-0300-9 PMID: 30580376

38. Federico A, Lugoboni F, Mantovani E, Martini A, Morbioli L, Casari R, et al. Detoxification Improves Multidomain Cognitive Dysfunction in High-Dose Benzodiazepine Abusers. Front Neurosci. 2020; 14:747. https://doi.org/10.3389/fnins.2020.00747 PMID: 32848544

39. Yang T, Velagapudi R, Terrando N. Neuroinflammation after surgery: from mechanisms to therapeutic targets. Nat Immunol. 2020; 21(11):1319–26. https://doi.org/10.1038/s41590-020-00812-1 PMID: 33077953

40. Adamis D, van Gool WA, Eikelenboom P. Consistent patterns in the inconsistent associations of Insulin-like growth factor 1 (IGF-1), C-Reactive Protein (C-RP) and Interleukin 6 (IL-6) levels with delirium in surgical populations. A systematic review and meta-analysis. Arch Gerontol Geriatr. 2021; 97:104518. https://doi.org/10.1016/j.archger.2021.104518 PMID: 34536657

41. Inoue N, Ogura S, Kasai A, Nakazawa T, Ikeda K, Higashi S, et al. Knockdown of the mitochondria-localized protein p13 protects against experimental parkinsonism. EMBO Rep. 2018; 19(3). https://doi.org/10.15252/embr.201744860 PMID: 29371327

42. Lauzon MA, Faucheux N. A small peptide derived from BMP-9 can increase the effect of bFGF and NGF on SH-SY5Y cells differentiation. Mol Cell Neurosci. 2018; 88:83–92. https://doi.org/10.1016/j.mcn.2018.01.003 PMID: 29341901

43. Koskderelioglu A, Onder O, Gucuyener M, Altay T, Kayali C, Gedizlioglu M. Screening for postoperative delirium in patients with acute hip fracture: Assessment of predictive factors. Geriatr Gerontol Int. 2017; 17(6):919–24. https://doi.org/10.1111/ggi.12806 PMID: 27283273

44. Soreq H, Seidman S. Acetylcholinesterase—new roles for an old actor. Nat Rev Neurosci. 2001; 2 (4):294–302. https://doi.org/10.1038/35067589 PMID: 11283752