Aus der Klinik für Anästhesiologie mit Schwerpunkt operative Intensivmedizin
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DISSERTATION

Untersuchung der Wirkungsweise von Dexmedetomidin auf das adulte Rattenhirn

zur Erlangung des akademischen Grades

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Inhaltsverzeichnis

Abkürzungsverzeichnis

α7nAChR	α7 nikotinerger Acetylcholin Rezeptor
ACh	Acetylcholin
AChE	Acetylcholinesterase
AChE-I	Acetylcholinesterase-Inhibitoren
BDNF	Brain-derived neurotrophic factor
BHS	Blut-Hirn-Schranke
CBA	Cytometrischer Bead Array
DEX	Dexmedetomidin
DNA	Desoxyribonukleinsäure
GSH	reduziertes Glutathion
GSSG	oxidiertes Glutathion
i.p.	intraperitoneal
IL1-beta	Interleukin1-beta
LPS	Lipopolysaccharid
LTP	Langzeitpotenzierung
MAPK	Mitogen-Activated Protein Kinase
MCI	Mild Cognitive Impairment
MDA	Malondialdehyd
miR	microRNA
mRNA	messenger Ribonukleinsäure
MyD88	Myeloid Differentiation Primary Response Gene 88
Neo	Neostigmin
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B-cells
Phy	Physostigmin
PKS	postoperative kognitive Störungen
qPCR	Real Time PCR
RNA	Ribonukleinsäure
SEM	Standardfehler des Mittelwerts
SIRT1	Sirtuin1
TLR4	Toll Like Rezeptor 4
TNF-alpha	Tumornekrosefaktor-alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
ZNS	zentrales Nervensystem

1 Zusammenfassung

1.1. Abstract deutsch

Das Gehirn weist eine hohe Vulnerabilität gegenüber Inflammation und oxidativem Stress auf. Beide Stressoren sind mit einer vermehrten Neurodegeneration in unterschiedlichen Bereichen des Gehirns assoziiert. Für das Medikament Dexmedetomidin (DEX) sind neben antiinflammatorischen und antioxidativen auch neuroprotektive Eigenschaften beschrieben, die komplette Wirkungsweise ist bisher jedoch nicht vollständig geklärt.

In der vorliegenden Arbeit galt es, an adulten Wistar Ratten den Einfluss von DEX auf eine durch systemische Applikation von Lipopolysaccharid (LPS) induzierte Neuroinflammation zu untersuchen. Speziell wurden dazu die Zytokine *IL1-beta* und *TNF-alpha* sowie die microRNAs (miR) 124, 132, 134 und 155 zu verschiedenen Zeitpunkten im Hippocampus und Cortex mittels Real Time PCR (qPCR) analysiert. Weiterhin fand eine Analyse dieser miRNAs im Plasma statt. Es zeigte sich, dass DEX die durch LPS induzierte Expression von *IL1-beta* und *TNF-alpha* signifikant reduziert und die Expression der LPS-induzierten miR 124, 132, 134 und 155 in beiden untersuchten Hirnregionen signifikant verringert. Zusätzlich wiesen miR 132 und 134 im Plasma während der Neuroinflammation ein verändertes Expressionsmuster auf.

In einem zweiten Tiermodel wurden nach Schädigung des neonatalen Rattengehirns durch Hyperoxie verschiedene DEX-Konzentrationen bezüglich ihrer möglichen Protektion untersucht. Speziell stand dabei die Begutachtung des apoptotischen Zelltods in unterschiedlichen Hirnarealen im Vordergrund. Zusätzlich wurde oxidativer Stress durch die Analyse von Glutathion und Malondialdehyd (MDA) bestimmt sowie das Zytokin IL1-beta molekularbiologisch analysiert. DEX entfaltete im Modell protektive Effekte durch Verminderung der Neurodegeneration und IL1-beta Expression sowie durch Beeinflussung der untersuchten oxidativen Stressparameter.

In einer weiteren Arbeit wurde an adulten Wistar Ratten durch eine Laparotomie sowie nachfolgender LPS-Applikation eine Neuroinflammation induziert und dabei die Wirkung der Acetylcholinesterase-Inhibitoren (AChE-I) Physostigmin (Phy) und Neostigmin (Neo) untersucht. Es wurden verschiedene Zytokine im Gehirn, im Plasma und in der Milz mittels qPCR, Western Blot und Cytometrischem Bead Array (CBA) untersucht sowie der Grad an Neurodegeneration und die Aktivität der Acetylcholinesterase (AChE) im Hippocampus und Cortex bestimmt. Die Applikation von Phy und Neo reduzierte eine LPS-induzierte Neuroinflammation und - degeneration sowie die LPS-vermittelte Erhöhung der AChE-Aktivität. Ferner wurden peripher antiinflammatorische Effekte in der Milz sowie im Plasma detektiert.

Zusammenfassend ergibt sich, dass unterschiedliche medikamentöse Interventionen protektive Eigenschaften entfalten, um potentiellen Hyperoxie-Schäden bzw. LPS-induzierten Schäden entgegen zu wirken. Besonders die durch DEX vermittelte Beeinflussung der miRNA Expression eröffnet neue Erkenntnisse, um die komplexe Wirkungsweise dieses Medikaments besser verstehen zu können.

1.2. Abstract englisch

The brain is highly vulnerable to inflammation and oxidative stress. Both stressors are associated with increased neurodegeneration in different areas of the brain. The drug dexmedetomidine (DEX) exerts neuroprotective as well as anti-inflammatory and antioxidative properties. However, the complete molecular mechanisms are not fully understood.

In the present work, adult Wistar rats were used to investigate the influence of DEX on neuroinflammation induced by the systemic application of lipopolysaccharide (LPS). The cytokines *IL1-beta* and *TNF-alpha*, as well as the microRNAs (miR) 124, 132, 134 and 155 were analyzed in the hippocampus and cortex using real-time PCR (qPCR). Furthermore, these miRNAs were studied in plasma. It was found that DEX significantly reduced the LPS-induced expression of *IL1-beta* and *TNF-alpha* and significantly decreased the expression of LPS-induced miR 124, 132, 134, and 155 in both brain regions. In addition, miR 132 and 134 in plasma showed an altered expression pattern during neuroinflammation.

In a second animal model various DEX concentrations were tested in the neonatal rat brain exposed to hyperoxia. In particular, the focus was on the evaluation of apoptotic cell death in different areas of the brain. Moreover, oxidative stress was determined by the analysis of glutathione and malondialdehyde (MDA) and the cytokine IL1-beta was investigated. DEX showed protective effects in the neonatal brain by reducing hypoxia-induced neurodegeneration and IL1-beta expression. Furthermore, the drug influenced the oxidative stress parameters in a positive way.

In a further work, neuroinflammation was induced in adult Wistar rats by laparotomy and subsequent LPS administration to investigate the effect of the acetylcholinesterase-inhibitors (AChE-I) physostigmine (Phy) and neostigmine (Neo). Different cytokines in the brain, plasma, and spleen were investigated using qPCR, western blot, and cytometric bead array (CBA). Furthermore, the degree of neurodegeneration and the activity of acetylcholinesterase (AChE) were evaluated in the hippocampus and cortex. The application of Phy and Neo reduced LPS-induced neuroinflammation and -degeneration as well as the LPS-mediated increase of AChE activity in both investigated brain regions. Peripheral anti-inflammatory effects were also observed in the spleen and plasma.

In summary, different drug interventions show protective properties in order to reduce potential hyperoxic- or LPS-induced damage. The influence of DEX on miRNA expression, in particular, opens up new insights into the complex mode of action of this drug.

1.3. Einleitung und Zielsetzung

Trotz medizinischem Fortschritt stellen neurologische Defizite ein großes Problem der modernen Gesellschaft dar. Aufgrund der steigenden Lebenserwartung erkranken immer mehr Menschen an verschiedensten neurodegenerativen Erkrankungen. Auch Frühgeborene, welche häufig aufgrund der Unreife ihrer Organe hohen Sauerstoffkonzentrationen ausgesetzt werden müssen, zeigen neurodegenerative Veränderungen und eine gestörte neurologische Entwicklung.

Einen potentiellen Auslöser dieser negativen Effekte stellt die Neuroinflammation dar. Zahlreiche Studien zeigen, dass eine periphere Infektion in direktem Zusammenhang mit einer Inflammation im Gehirn steht. Während neuroinflammatorischer Ereignisse kann die Funktion des zentralen Nervensystems (ZNS) negativ beeinflusst und die Kognition verschlechtert werden. Dabei können Mikroglia, welche aus myeloiden Vorläuferzellen entstehen und das angeborene Immunsystem im ZNS repräsentieren, im Gehirn proinflammatorische Mediatoren wie IL1-beta und TNF-alpha sezernieren. Während einer systemisch verursachten Neuroinflammation wurden in unterschiedlichen Modellen neurodegenerative Prozesse beobachtet [1-6]. Zusätzlich waren beeinträchtigte Lern-, und Erinnerungsprozesse detektierbar [5,6]. Während einer Operation lässt sich vor allem bei älteren Patienten zunehmend ein Zusammenhang zwischen einer Aktivierung des Immunsystems und einer Neuroinflammation erkennen. Als Folge leiden Patienten an langanhaltenden Bewusstseinsstörungen, den so genannten postoperativen kognitiven Störungen (PKS), welche mit einem höheren Risiko der Entwicklung postoperativer Komplikationen, wie z. B. einer Sepsis, einer verlängerten Beatmungs- und Verweildauer im Krankenhaus sowie einer erhöhten Mortalitätsrate verbunden sind [7]. Die Detektion dieser multifaktoriellen Krankheit ist aufwendig und wird häufig im Krankenhausalltag vernachlässigt. Daher stellt die Identifizierung geeigneter Biomarker einen interessanten Forschungsansatz dar, um negative Folgen für Patienten zu vermeiden.

Trotz der Tatsache, dass die Überlebensrate von Frühgeborenen in den letzten Jahrzehnten dramatisch angestiegen ist, ist die Prävalenz von neurologischen und kognitiven Beeinträchtigungen weiterhin hoch und häufig die Ursache für Langzeitmorbiditäten bei Frühgeborenen [8-10]. Ein Grund dafür ist oxidativer Stress, da die Frühgeborenen postnatal unphysiologisch hohen Sauerstoffkonzentrationen ausgesetzt sind. Aufgrund des nicht vollständig entwickelten antioxidativen Systems erzeugen Sauerstoffradikale eine Störung des intrazellulären Redoxgleichgewichtes. Dabei können beispielsweise Modifizierungen an DNA, Proteinen und

Lipiden entstehen, was eine Induktion proinflammatorischer Zytokine und eine hauptsächlich apoptotische Neurodegeneration zur Folge hat.

Das Medikament Dexmedetomidin (DEX) gehört zur Gruppe der alpha-2-Agonisten und zeigt neben sedativen, anxiolytischen und analgesierenden Effekten auch antiinflammatorische, antioxidative und neuroprotektive Eigenschaften in unterschiedlichen Studien. Die genauen molekularen Wirkungsmechanismen dieses Medikaments sind jedoch noch nicht vollständig aufgeklärt. Klinische Studien zeigen, dass DEX die Prävalenz, Schwere und Dauer von PKS deutlich vermindert [11,12]. Weiherhin zeigt sich eine Verkürzung der künstlichen Beatmung in Erwachsenen sowie Frühgeborenen, wodurch negative Langzeitfolgen vermindert werden können [13,14].

Da microRNAs (miRNAs) nachgewiesenermaßen an verschiedensten Prozessen im Körper beteiligt sind und in kognitiven Prozessen eine wichtige Rolle spielen, stellen sie einen interessanten Untersuchungsparameter dar. MiRNAs sind ca. 22 bp lange RNAs, die die Genexpression posttranskriptionell regulieren. Es wird angenommen, dass ca. 60 % aller Gene durch miRNAs beeinflusst werden [15]. Die Mehrheit der bisher bekannten miRNAs wird im Gehirn exprimiert, wobei einige exklusiv in diesem Organ lokalisiert sind [16,17]. Abhängig von der Komplementarität wird eine mRNA (messenger RNA) entweder degradiert oder die Translation wird inhibiert.

Ziel der Arbeit war es, die Wirkungsweise von DEX im Gehirn der Ratte genauer zu untersuchen, um den protektiven Charakter dieses Medikamentes besser verstehen zu können. Der Fokus lag dabei zum einen auf der Untersuchung der Wechselwirkung zwischen DEX und verschiedenen miRNAs im Hippocampus und Cortex adulter Ratten während einer systemisch induzierten Neuroinflammation. Zusätzlich fand die Untersuchung von miRNAs im Plasma statt, um potentielle zirkulierende Biomarker einer Neuroinflammation zu identifizieren. Zum anderen sollten die Wirkeffekte verschiedener DEX-Konzentrationen in einem neonatalen Hyperoxie-Schädigungsmodell bezüglich ihrer Wirkung auf Inflammation, Apoptose und oxidative Stressparameter im sich entwickelnden Gehirn untersucht werden, um mögliche neuroprotektive Effekte unter oxidativem Stress zu klären. Weiterhin sollte in einem LPS-Schädigungsmodell der adulten Ratte der Einfluss der AChE-I Phy und Neo auf verschiedene inflammatorische Parameter sowie die Neurodegeneration untersucht werden.

1.4. Methodik

Die im Detail beschriebenen verwendeten Arbeitsmethoden sind den entsprechenden Veröffentlichungen zu entnehmen. Adulten Wistar Ratten (250-300 g) wurde 1 mg/kg Lipopolysaccharid (LPS) i.p. appliziert, um eine Neuroinflammation zu induzieren (Paeschke et

al. 2017). Die Tiere der DEX-Gruppe wurden 10 Minuten vor LPS-Applikation mit 5 µg/kg DEX i.p. vorbehandelt. Zu unterschiedlichen Zeitpunkten (6 h, 24 h und 7 d) wurden die Ratten nach tiefer Isofluran-Narkose durch eine transkardiale Herzpunktion getötet. Nach RNA-Isolierung wurde die Expression der proinflammatorischen Zytokine *IL1-beta* und *TNF-alpha* auf Genebene sowie der miR 124, 132, 134 und 155 im Hippocampus und Cortex mittels Real Time PCR (qPCR) untersucht. Zusätzlich fand eine Untersuchung der miRNAs im Plasma statt, um potentielle zirkulierende Biomarker zur Detektion einer Neuroinflammation zu identifizieren.

Zur Untersuchung der Wirkung von DEX in einem neonatalen Hyperoxie-Schädigungsmodell wurden an sechs Tage alten Wistar Ratten drei unterschiedliche DEX-Konzentrationen (1, 5, 10 µg/kg) getestet (Sifringer et al. 2015). Das Medikament wurde 15 Minuten vor der 80 %-igen Sauerstoffexposition bzw. Raumluftexposition i.p. appliziert. Nach 24 h wurden die Ratten mit Ketamin (50 mg/kg), Xylazin (10 mg/kg) und Acepromazin (2 mg/kg) anästhesiert und transkardial perfundiert. Zur Untersuchung der Apoptose im frontalen Cortex, retrosplenialen Cortex, Hypothalamus und Thalamus wurde ein DNA-Fragmentierungsassay (TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling)) an 5 µm dicken Paraffinschnitten verwendet. Neben der Bestimmung von Malondialdehyd (MDA) als Marker der Lipidperoxidation fand auch die Bestimmung von reduziertem (GSH) und oxidiertem Glutathion (GSSG) sowie deren Ratio mittels verschiedener spektrometrischer Messungen statt. Zusätzlich wurde das proinflammatorische Zytokin IL1-beta im Gehirn molekularbiologisch untersucht.

Zur Überprüfung der Wirksamkeit der reversiblen Acetylcholinesterase-Inhibitoren (AChE-I) Physostigmin (Phy) und Neostigmin (Neo) erhielten männliche adulte Wistar Ratten (250-300 g) eine Anästhesie mit Isofluran sowie eine Meloxicam-Applikation (0,2 mg/kg) (Kalb et al. 2013). Zur Simulation einer humanen abdominalen Operation wurde eine Laparotomie durchgeführt. Nach Eröffnung des Bauchraumes wurde den Tieren der LPS-Gruppen 1 mg/kg LPS appliziert. Je nach Gruppe erhielten die Ratten zusätzlich 100 µg/kg Phy oder Neo vor LPS-Gabe. Nach dem Eingriff wurden die Ratten dreimal mit 100 µg/kg der Medikamente subkutan nachbehandelt. Zur Schmerzreduktion wurde erneut 0,2 mg/kg Meloxicam appliziert. Zu verschiedenen Zeitpunkten (1 h, 24 h und 3 d) wurden die Tiere tief mit Isofluran anästhesiert und nach Thorakotomie durch Herzpunktion getötet. Die Analyse des Zytokins IL1-beta fand auf Genebene mittels qPCR und auf Proteinebene mittels Western Blot im Hippocampus und Cortex statt. Zusätzlich wurden verschiedene Zytokine in der Milz und im Plasma analysiert. Dabei kam u. a. auch der Cytometrische Bead Array (CBA) zum Einsatz. Die Neurodegeneration wurde anhand von 12 µm dicken Fluoro-Jade B gefärbten Kryoschnitten analysiert. Des Weiteren wurde die Aktivität der Acetylcholinesterase (AChE) mit Hilfe eines spektrometrischen Assays bestimmt.

1.5. Ergebnisse

1.5.1. Dexmedetomidin beeinflusst die Expression verschiedener microRNAs während einer Neuroinflammation im Gehirn adulter Ratten (Paeschke et al. 2017)

Eine i.p. Applikation von 1 mg/kg LPS resultierte nach 6 h und 24 h in einer im Vergleich zur Kontrollgruppe signifikant vermehrten *IL1-beta* Expression im Hippocampus und Cortex adulter Ratten (Abb. 1 A, B). Zusätzlich zeigte sich *TNF-alpha* deutlich erhöht (Abb. 1 C, D). Die Applikation von 5 µg/kg DEX verminderte die LPS-induzierte Expression beider Zytokine nach 24 h signifikant, wobei sich *TNF-alpha* zu diesem Zeitpunkt auf Kontrollniveau befand, *IL1-beta* im Vergleich zur Kontrolle jedoch erhöht blieb. Nach 7 d war DEX in der Lage die durch LPS-gesteigerte *IL1-beta* Expression im Cortex signifikant zu verringern.

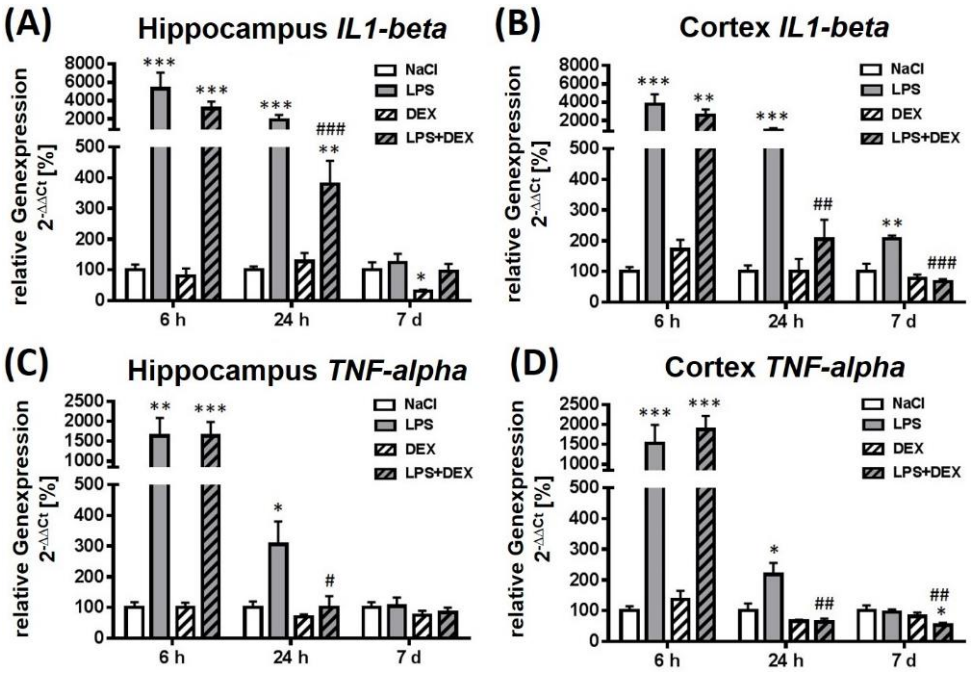

Abbildung 1: Darstellung der *IL1-beta* (A, B) und *TNF-alpha* (C, D) Genexpression nach systemischer LPS- und DEX-Applikation zu verschiedenen Zeitpunkten im adulten Hippocampus und Cortex der Ratte. Modifiziert nach Paeschke et al. 2017. Dargestellt sind die Mittelwerte der relativen *IL1-beta* und *TNF-alpha* Genexpressionsänderungen sowie deren Standardabweichungen (±SEM) nach qPCR Analyse. Signifikante Unterschiede wurden mit dem Mann-Whitney *U*-Test berechnet und sind mit * $p<0,05$, ** $p<0,01$, *** $p<0,001$ (versus Kontrolle) und # $p<0,05$, ## $p<0,01$, ### $p<0,001$ (versus LPS) gekennzeichnet. Jede Gruppe enthielt 6-8 Tiere (n=6-8).

Die in der vorliegenden Arbeit untersuchten miRNAs miR 124, 132, 134 und 155 spielen eine wichtige Rolle bei inflammatorischen und kognitiven Prozessen. Es stellte sich heraus, dass 1 mg/kg LPS i.p. alle untersuchten miRNAs sowohl im Hippocampus als auch im Cortex spätestens nach 24 h signifikant ansteigen lässt (Abb. 2-5). Im Allgemeinen ist die miRNA-Expression im Cortex stärker ausgeprägt als im Hippocampus. Die Applikation von DEX zeigte sich als protektiv und verminderte die LPS-induzierten Expressionen der untersuchten miRNAs in beiden untersuchten Hirnregionen, hauptsächlich nach 24 h, signifikant. Im Plasma beeinflusste DEX die Expression

der untersuchten miRNAs nicht so stark wie im Gehirn (Abb. 3-5). MiR 132 wurde im Plasma 24 h und 7 d nach LPS-Gabe signifikant vermehrt exprimiert, jedoch entfaltete das Medikament hier keine Wirkung (Abb. 3). Bei der Untersuchung von miR 134 stellte sich heraus, dass sich diese miRNA nach LPS-Applikation zu allen untersuchten Zeitpunkten im Plasma signifikant vermindert zeigte (Abb. 4). Die durch LPS induzierte miR 155 wurde nach 6 h im Plasma durch DEX verringert, nach 24 h hatte das Medikament jedoch keinen Einfluss mehr auf die vermehrte Expression (Abb. 5). In den folgenden Abbildungen sind die Ergebnisse detailliert gezeigt.

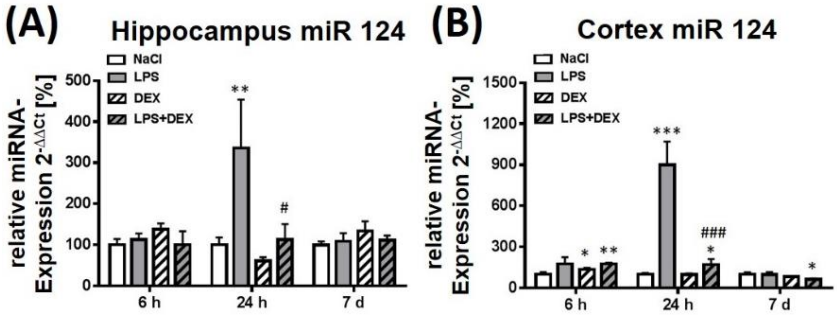

Abbildung 2: Darstellung der Expression von miR 124 im Hippocampus (A) und Cortex (B) 6 h, 24 h und 7 d nach LPS-induzierter Neuroinflammation und DEX-Applikation. Modifiziert nach Paeschke et al. 2017. LPS führt zu einer signifikant erhöhten miR 124 Expression nach 24 h im Hippocampus und Cortex, welche durch das Medikament DEX deutlich herabgesetzt werden kann. Die alleinige Applikation des Medikaments sowie in Kombination mit LPS steigert miR 124 nach 6 h im Cortex im Vergleich zur Kontrollgruppe. Im Plasma konnte miR 124 nicht detektiert werden. Dargestellt sind die Mittelwerte der relativen miR 124 Expressionsänderungen sowie deren Standardabweichungen (±SEM). Signifikante Unterschiede wurden mit dem Mann-Whitney *U*-Test berechnet und sind mit * p<0,05, ** p<0,01, *** p<0,001 (versus Kontrolle) und # p<0,05, ### p<0,001 (versus LPS) gekennzeichnet. Jede Gruppe enthielt 6-8 Tiere (n=6-8).

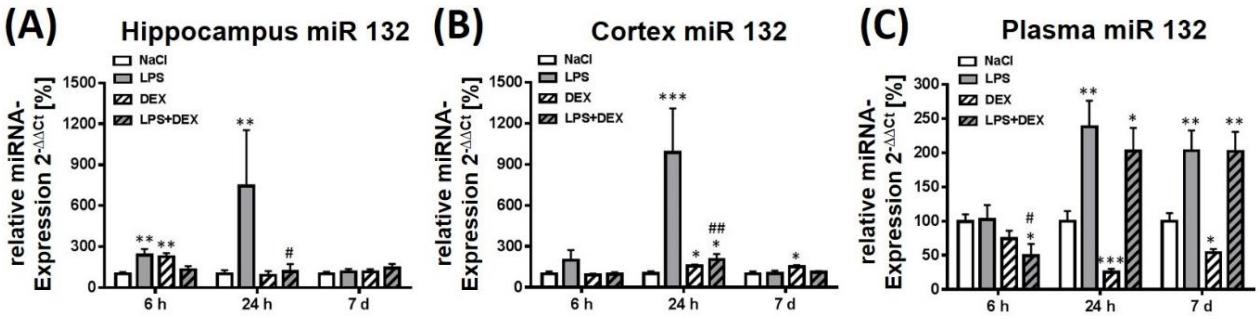

Abbildung 3: Darstellung der Expression von miR 132 im Hippocampus (A), Cortex (B) und Plasma (C) 6 h, 24 h und 7 d nach LPS-induzierter Neuroinflammation und DEX-Applikation. Modifiziert nach Paeschke et al. 2017. LPS führt zu einer signifikant vermehrten miR 132 Expression im Hippocampus und Cortex nach 24 h. Diese kann durch DEX deutlich vermindert werden. Im Hippocampus zeigt sich miR 132 bereits 6 h nach LPS-Gabe erhöht. Die alleinige Applikation von 5 µg/kg DEX steigert miR 132 nach 6 h im Hippocampus und nach 24 h und 7 d im Cortex. Im Plasma induziert LPS miR 132 nach 24 h und 7 d signifikant, wobei DEX diese Expression nicht beeinflusst. Die alleinige DEX-Applikation führt im Plasma zu einer verringerten Expression der hier untersuchten miRNA nach 24 h und 7 d. Dargestellt sind die Mittelwerte der relativen miR 132 Expressionsänderungen sowie deren Standardabweichungen (±SEM). Signifikante Unterschiede wurden mit dem Mann-Whitney *U*-Test berechnet und sind mit * p<0,05, ** p<0,01, *** p<0,001 (versus Kontrolle) und # p<0,05, ## p<0,01, (versus LPS) gekennzeichnet. Jede Gruppe enthielt 6-8 Tiere (n=6-8).

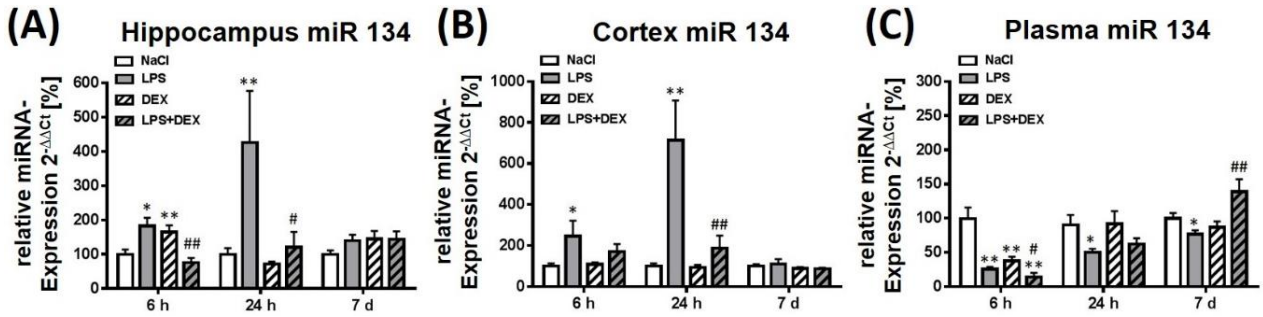

Abbildung 4: Darstellung der Expression von miR 134 im Hippocampus (A), Cortex (B) und Plasma (C) 6 h, 24 h und 7 d nach LPS-induzierter Neuroinflammation und DEX-Applikation. Modifiziert nach Paeschke et al. 2017. Nach 6 h sowie 24 h resultiert eine einmalige systemische LPS-Applikation in einer verglichen zur Kontrollgruppe gesteigerten miR 134 Expression im Hippocampus und Cortex adulter Ratten. DEX ist in der Lage diese Expression in beiden Hirnregionen nach 24 h signifikant zu verringern, im Hippocampus findet bereits nach 6 h eine Reduktion statt. Das Medikament selbst steigert die miR 134 Expression nach 6 h im Hippocampus. Im Plasma zeigt sich die Expression von miR 134 nach LPS-Gabe zu allen untersuchten Zeitpunkten signifikant im Vergleich zur Kontrollgruppe verringert. Dargestellt sind die Mittelwerte der relativen miR 134 Expressionsänderungen sowie deren Standardabweichungen (±SEM). Signifikante Unterschiede wurden mit dem Mann-Whitney *U*-Test berechnet und sind mit * p<0,05, ** p<0,01 (versus Kontrolle) und # p<0,05, ## p<0,01 (versus LPS) gekennzeichnet. Jede Gruppe enthielt 6-8 Tiere (n=6-8).

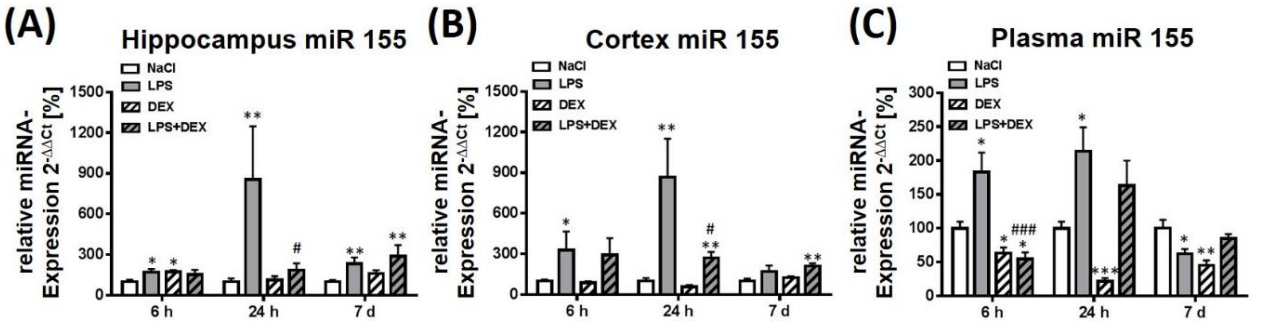

Abbildung 5: Darstellung der Expression von miR 155 im Hippocampus (A), Cortex (B) und Plasma (C) 6 h, 24 h und 7 d nach LPS-induzierter Neuroinflammation und DEX-Applikation. Modifiziert nach Paeschke et al. 2017. LPS induziert miR 155 nach 6 h und 24 h in beiden Hirnregionen sowie im Plasma signifikant im Vergleich zur jeweiligen Kontrollgruppe. Die Applikation von DEX senkt die LPS-induzierte Expression dieser miRNA nach 24 h im Hippocampus und Cortex sowie nach 6 h im Plasma. Sieben Tage nach LPS-Exposition zeigt sich miR 155 im Hippocampus noch erhöht, wobei das Medikament zu diesem Zeitpunkt keinen weiteren Einfluss auf die Expression hat. Im Plasma verursacht die alleinige DEX-Applikation eine reduzierte miR 155 Expression zu allen untersuchten Zeitpunkten. Dargestellt sind die Mittelwerte der relativen miR 155 Expressionsänderungen sowie deren Standardabweichungen (±SEM). Signifikante Unterschiede wurden mit dem Mann-Whitney *U*-Test berechnet und sind mit * p<0,05, ** p<0,01, *** p<0,001 (versus Kontrolle) und # p<0,05, ### p<0,001 (versus LPS) gekennzeichnet. Jede Gruppe enthielt 6-8 Tiere (n=6-8).

1.5.2. Dexmedetomidin reduziert Hyperoxie-induzierte Schädigungen im neonatalen Rattengehirn (Sifringer et al. 2015)

Die Untersuchungen der Gen-und Proteinexpression wiesen darauf hin, dass Hyperoxie im Gehirn von sechs Tage alten Ratten IL1-beta signifikant im Vergleich zur Kontrollgruppe ansteigen lässt (Abb. 6). Die Vorbehandlung mit 5 oder 10 µg/kg DEX verringerte diese Expression signifikant im Vergleich zur Hyperoxie-Gruppe, jedoch zeigte sie sich verglichen mit der Kontrollgruppe weiterhin erhöht. Die alleinige Applikation des Medikaments verursachte keine Regulation.

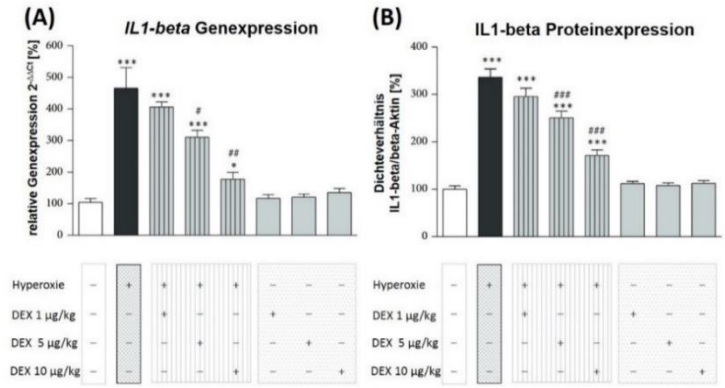

Abbildung 6: Darstellung der IL1-beta Gen- (A) und Proteinexpression (B) im Gehirn 24 h nach Hyperoxie und DEX-Applikation. Modifiziert nach Sifringer et al. 2015. Dargestellt sind die Mittelwerte der relativen *IL1-beta* Genexpressionsänderungen bzw. die Mittelwerte des Dichteverhältnisses von IL1-beta normalisiert zu beta-Aktin sowie deren Standardabweichungen (±SEM). Der Mittelwert der jeweiligen Normoxie-Kontrollgruppe wurde auf 100 % gesetzt. Signifikante Unterschiede wurden mit dem ungepaarten *t*-Test nach Bonferroni Korrektur berechnet und sind mit * p<0,05, *** p<0,001 (versus Kontrolle) und # p<0,05, ## p<0,01, ### p<0,001 (versus Hyperoxie) gekennzeichnet. Jede Gruppe enthielt 6 Tiere (n=6).

Hyperoxie führte im neonatalen Gehirn zu einer Induktion von oxidiertem Glutathion (Abb. 7 B) und einer Verringerung von reduziertem Glutathion (Abb. 7 A) sowie deren Ratio im Vergleich zur Kontrollgruppe (Abb. 7 C). Die Applikation von 5 und 10 µg/kg DEX kann dem signifikant entgegenwirken. Bei der Untersuchung von MDA als Marker der Lipidoxidation entfalteten 5 und 10 µg/kg DEX während einer Hyperoxie protektive Wirkungen (Abb. 7 D). Die Konzentration von 1 µg/kg DEX hat keinen Einfluss auf die untersuchten Parameter.

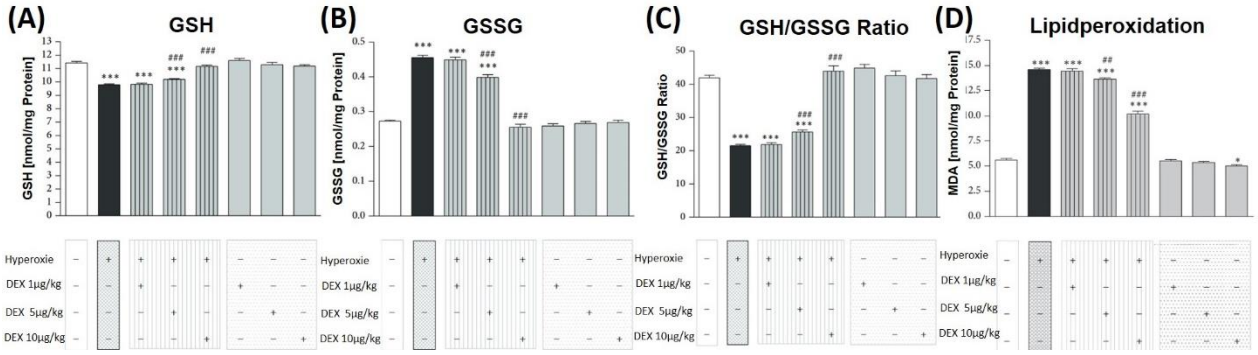

Abbildung 7: Darstellung des DEX-vermittelten Effektes auf die durch Hyperoxie veränderte Expression von GSH (A), GSSG (B), deren Ratio (C) sowie auf MDA (D) im sich entwickelnden Gehirn der Ratte. Modifiziert nach Sifringer et al. 2015. Dargestellt sind die Mittelwerte von GSH, GSSG, deren Ratio und MDA bezogen auf mg Gesamtprotein sowie deren Standardabweichungen (±SEM). Signifikante Unterschiede wurden mit dem ungepaarten *t*-Test nach Bonferroni Korrektur berechnet und sind mit *** p<0,001 (versus Kontrolle) und ## p<0,01, ### p<0,001 (versus LPS) gekennzeichnet. Jede Gruppe enthielt 6 Tiere (n=6).

Hyperoxie induzierte in sechs Tage alten Wistar Ratten im frontalen Cortex, retrosplenialen Cortex, Hypothalamus und Thalamus des sich entwickelnden Gehirns eine um ca. den Faktor 5 gesteigerte Apoptose. Die Applikation von 1, 5 und 10 µg/kg DEX vor Hyperoxie-Exposition

verringerte in den untersuchten Hirnregionen signifikant die apoptotische Zelltodrate. Bei der alleinigen Applikation von 10 µg/kg DEX zeigte sich im Vergleich zur Kontrollgruppe ein induzierter Zelltod im frontalen und retrosplenialen Cortex. Im Thalamus führten alle alleinig applizierten DEX-Konzentrationen zu einer im Vergleich zur Kontrolle gesteigerten Apoptose (Abb. 8).

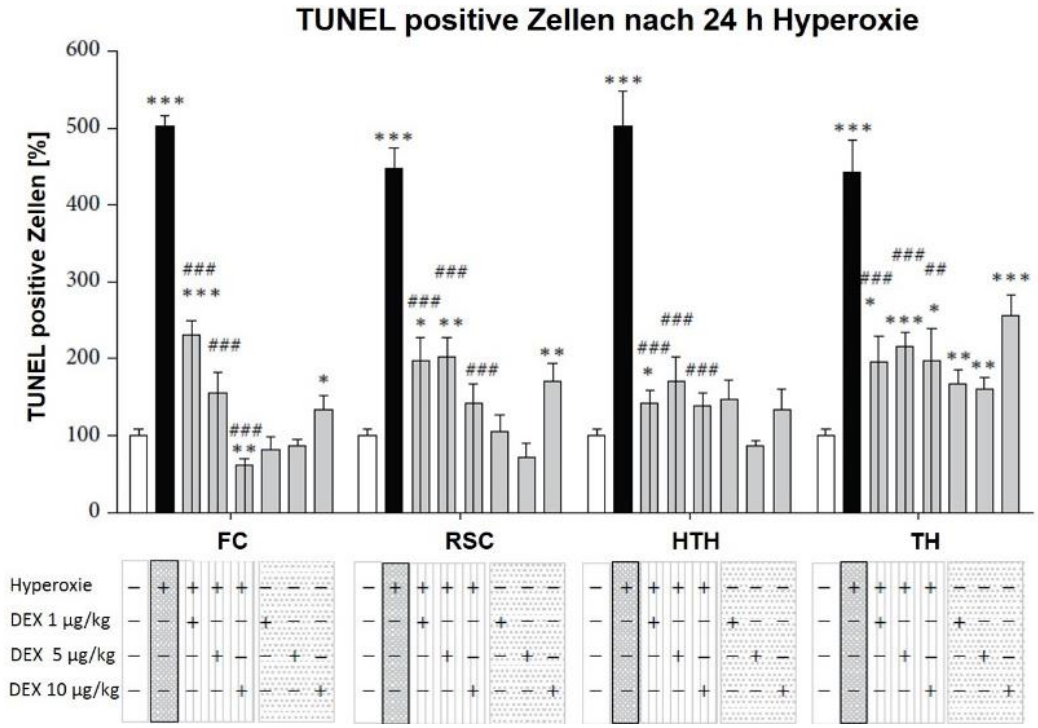

Abbildung 8: Darstellung von quantifizierten TUNEL positiven Zellen nach Hyperoxie und Applikation verschiedener DEX-Konzentrationen im frontalen Cortex (FC), retrosplenialen Cortex (RSC), Hypothalamus (HTH) und Thalamus (TH) aus 5 µm dicken Paraffinschnitten nach 24 h relativ zur Kontrolle. Modifiziert nach Sifringer et al. 2015. Dargestellt sind die Mittelwerte der TUNEL positiven Zellen relativ zu den Kontrolltieren sowie deren Standardabweichungen (±SEM). Signifikante Unterschiede wurden mit dem ungepaarten *t*-Test nach Bonferroni Korrektur berechnet und sind mit * p<0,05, ** p<0,01, *** p<0,001 (versus Kontrolle) und ## p<0,01, ### p<0,001 (versus Hyperoxie) gekennzeichnet. Jede Gruppe enthielt 6 Tiere (n=6).

1.5.3. Die Applikation von Physostigmin und Neostigmin verringert eine LPS-induzierte Neuroinflammation und -degeneration im Hippocampus und Cortex adulter Ratten (Kalb et al. 2013)

Eine Laparotomie in Kombination mit 1 mg/kg LPS führte im Hippocampus (Abb. 9 A, B) und im Cortex (Abb. 9 E, F) zu einer signifikant erhöhten *IL1-beta* Genexpression nach 1 h und 24 h, welche durch die reversiblen AChE-I Phy und Neo signifikant vermindert werden konnte. Drei Tage nach LPS-Applikation waren keine signifikanten Unterschiede zu beobachten (Abb. 9 C, G). Auf Proteinebene bestätigen sich die Ergebnisse: LPS-induziertes IL1-beta konnte im Hippocampus nach 24 h und im Cortex nach 1 h durch Applikation von Phy und Neo signifikant reduziert werden (Abb. 9 D, H).

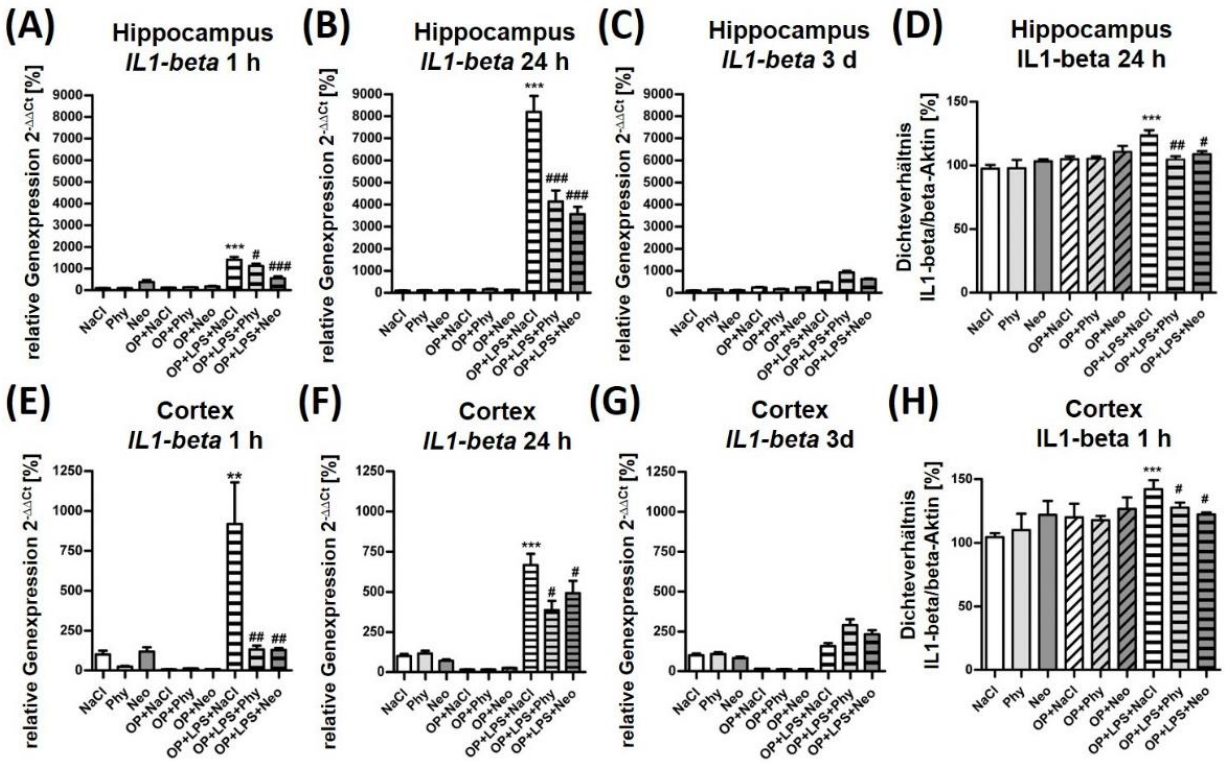

Abbildung 9: Darstellung der IL1-beta Gen- und Proteinexpression 1 h, 24 h und 3 d nach Laparotomie in Kombination mit LPS sowie Applikation von Phy oder Neo im Hippocampus (A-D) und Cortex (E-H). Modifiziert nach Kalb et al. 2013. Dargestellt sind die Mittelwerte der relativen *IL1-beta* Genexpressionsänderungen sowie die Ergebnisse der densitometrischen Auswertung der Ratio von IL1-beta zu beta-Aktin und deren Standardabweichungen (±SEM). Signifikante Unterschiede wurden mit dem ungepaarten *t*-Test nach Bonferroni Korrektur berechnet und sind mit ** p<0,01, *** p<0,001 (versus Kontrolle) und # p<0,05, ## p<0,01, ### p<0,001 (versus OP+LPS) gekennzeichnet. Jede Gruppe enthielt 8 Tiere (n=8).

In der Milz vermittelten Phy und Neo peripher antiinflammatorische Wirkungen, indem sie die LPS-induzierte *IL1-beta* und *TNF-alpha* Genexpression signifikant reduzierten (Abb. 10 A, B). Das antiinflammatorische *IL-10*, welches durch LPS induziert wurde, konnte durch beide AChE- I in seiner Genexpression noch gesteigert werden (Abb. 10 C), war jedoch auf Proteinebene nach 1 h nicht detektierbar. Zur Proteinbestimmung wurde in der Milz und im Plasma ein CBA Kit der Firma BD Biosciences verwendet. Da kein Ratten IL1-beta Antikörper erhältlich war, wurde als Alternative IL1-alpha bestimmt. Auf Proteinebene zeigten sich IL1-alpha und TNF-alpha in der Milz nach Laparotomie und LPS-Applikation erhöht (Abb. 10 D, E). Beide AChE- I verminderten diese gesteigerte Expression. Die IL1-alpha Proteinergebnisse konnten im Western Blot für IL1-beta bestätigt werden (Daten nicht gezeigt). Im Plasma stiegen TNF-alpha und IL-10 nach LPS-Gabe signifikant im Vergleich zur Kontrolle an, IL1-alpha blieb unbeeinflusst (Abb. 10 G-I). Die Applikation von Phy und Neo resultierte auch im Plasma in einer verringerten TNF-alpha Expression im Vergleich zur OP+LPS Gruppe.

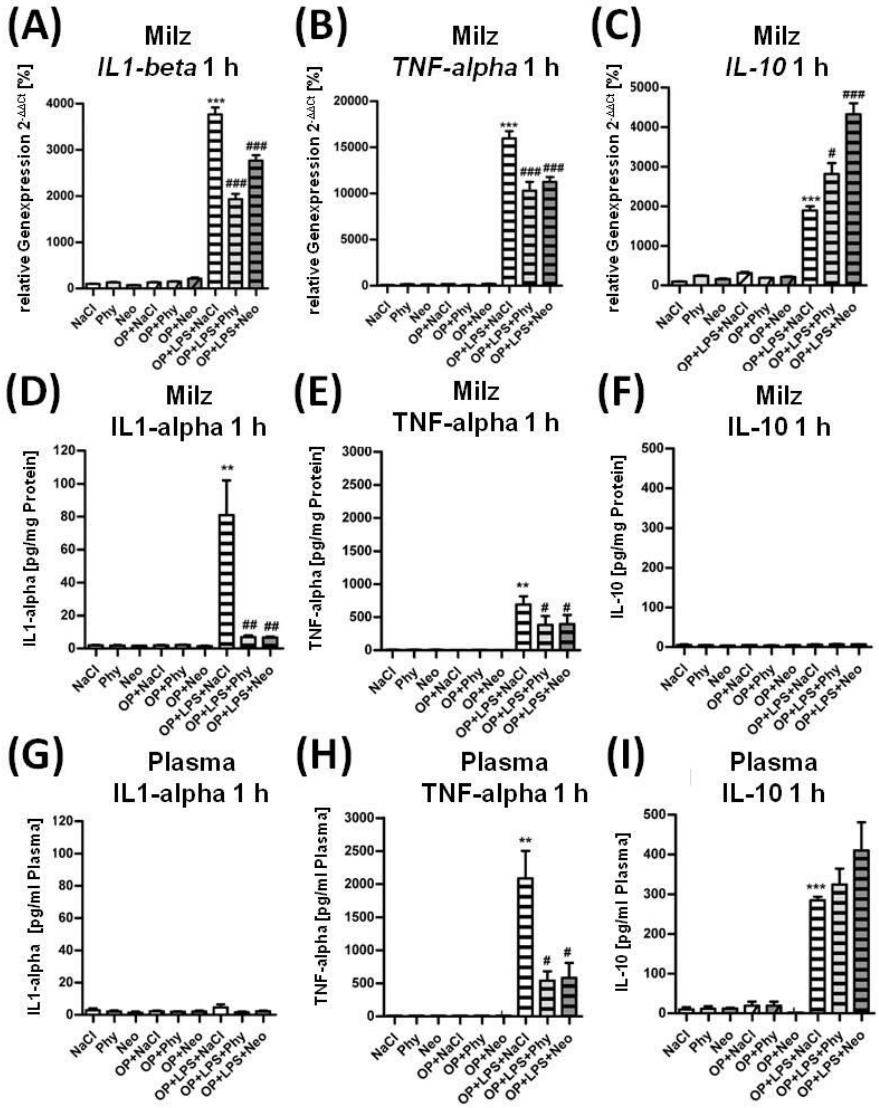

Abbildung 10: Darstellung der Genexpression von *IL1-beta* (A), *TNF-alpha* (B) und *IL-10* (C) 1 h nach Laparotomie sowie LPS- und Phy- oder Neo-Applikation in der Milz. Zusätzlich sind die Proteinexpression von IL1-alpha, TNF-alpha und IL-10 in der Milz und im Plasma 1 h nach Versuchsstart aufgeführt (D-I). Modifiziert nach Kalb et al. 2013. Dargestellt sind die Mittelwerte der relativen *IL1-beta*, *TNF-alpha* und *IL-10* Genexpressionsänderungen und deren Standardabweichungen (±SEM). Die Proteinexpression von IL1-alpha, TNF-alpha und IL-10 in der Milz und im Plasma wurden mittels CBA-Messung bestimmt und sind auf mg Gesamtprotein in der Milz bzw. pro ml Plasma bezogen. Die Ergebnisse sind als Mittelwerte ±SEM dargestellt. Signifikante Unterschiede wurden mit dem ungepaarten *t*-Test nach Bonferroni Korrektur berechnet und sind mit ** $p<0,01$, *** $p<0,001$ (versus Kontrolle) und # $p<0,05$, ## $p<0,01$, ### $p<0,001$ (versus OP+LPS) gekennzeichnet. Jede Gruppe enthielt 8 Tiere (n=8).

Mittels Fluoro-Jade B Färbung zeigte sich, dass die Kombination aus OP und LPS die Anzahl der toten Neuronen sowohl im Hippocampus (Abb. 11 B) als auch im Cortex (Abb. 11 A, C) signifikant ansteigen lässt. Sowohl das nur peripher wirkende Neo als auch das zusätzlich zentralgängige Phy waren in der Lage, die Neurodegeneration in beiden Hirnregionen zu vermindern. Bei der Untersuchung der AChE-Aktivität stellte sich heraus, dass beide getesteten AChE-I die durch LPS-gesteigerte Aktivität signifikant verringern (Abb. 11 D, E).

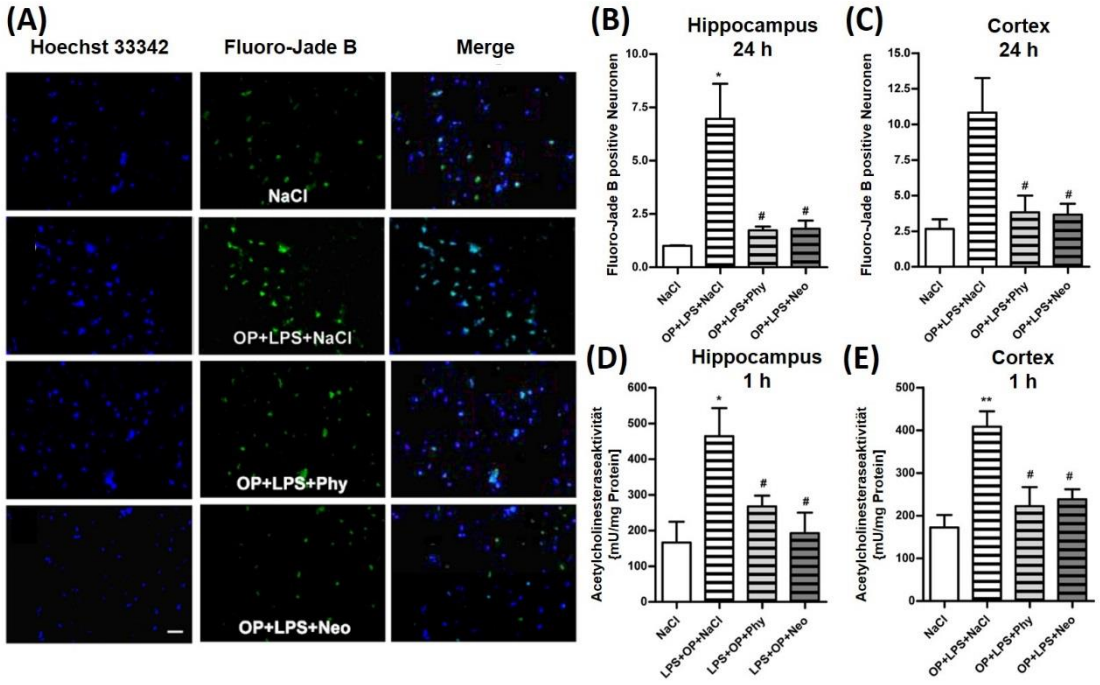

Abbildung 11: Darstellung einer repräsentativen 200-fach vergrößerten mikroskopischen Aufnahme Fluoro-Jade B positiver Zellen im Cortex (A) sowie der quantitativen Auswertung dieser Färbung im Hippocampus (B) und Cortex (C) 24 h nach Laparotomie sowie LPS-, Phy- und Neo-Applikation. Zusätzlich ist die Aktivität der AChE nach 1 h im Hippocampus (D) und Cortex (E) gezeigt. Modifiziert nach Kalb et al. 2013. Dargestellt sind die Mittelwerte der Anzahl an Fluoro-Jade B positiven Neuronen und der AChE-Aktivität, sowie deren Standardabweichungen (±SEM). Signifikante Unterschiede wurden mit dem ungepaarten *t*-Test nach Bonferroni Korrektur berechnet und sind mit * $p<0,05$, ** $p<0,01$ (versus Kontrolle) und # $p<0,05$ (versus OP+LPS) gekennzeichnet. Jede Gruppe enthielt 8 Tiere (n=8).

1.6. Diskussion

Da eine Neuroinflammation nachgewiesenermaßen hauptsächlich an der Pathogenese verschiedener Krankheiten beteiligt ist, stellt die Untersuchung medikamentöser Interventionen einen wichtigen Forschungsschwerpunkt dar. Durch die systemische Applikation von LPS wurde in der vorliegenden Arbeit eine Neuroinflammation in adulten Ratten induziert (Paeschke et al. 2017). Zahlreiche Studien bestätigen den Zusammenhang zwischen peripheren Infektionen und einer Inflammation im ZNS [2-4]. Speziell *IL1-beta* und *TNF-alpha* zeigten sich signifikant im Hippocampus und Cortex erhöht. Hohe IL1-beta Spiegel stehen in direktem Zusammenhang mit einem Zelluntergang in diesem Organ. Barrientos und Kollegen veröffentlichten, dass die Gedächtnisfunktion nach IL1-beta Injektion im Hippocampus gestört ist [18]. Unphysiologisch hohe IL1-beta Konzentrationen können im Gyrus Dentatus des Hippocampus inhibitorische Effekte auf die Langzeitpotenzierung (LTP), welche hauptsächlich am Lernen und Erinnern beteiligt ist, verursachen [19]. IL1-beta induziert neben kognitiven Beeinträchtigungen auch in verschiedenen Modellen Verhaltensänderungen. Die Vorbehandlung mit DEX verringerte die Expression von *IL1-beta* und *TNF-alpha* in beiden untersuchten Hirnregionen nach 24 h

signifikant. Durch Reduzierung der gesteigerten *IL1-beta* Expression nach 7 d im Cortex scheint DEX langanhaltend protektiv zu wirken. Zahlreiche weitere experimentelle und klinische Studien bestätigen die antiinflammatorischen Wirkungen des Medikaments. In einem Tiermodell mit septischen Ratten wurde beschrieben, dass DEX den Toll Like Rezeptor 4 (TLR4), den Myeloid Differentiation Primary Response Gene 88 **(**MyD88) und den Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B-cells (NFκB) Signalweg inhibiert [20]. Des Weiteren bestehen Hinweise auf eine Beeinflussung des cholinergen antiinflammatorischen Signalwegs in verschiedenen Tierstudien [21]. Auch Ning et al. publizierten kürzlich, dass DEX im Gehirn der Maus IL1-beta und TNF-alpha nach systemischer Applikation von LPS senkt [2]. Zusätzlich reduziert DEX die Expression der u.a. mit einer verschlechterten synaptischen Plastizität sowie inflammatorischen Ereignissen in Zusammenhang stehenden miR 124, 132, 134 und 155, welche durch LPS-Exposition verstärkt exprimiert werden. Die höchsten Expressionen der untersuchten miRNAs sind in Folge hoher Zytokinkonzentrationen nach 24 h detektierbar. Demnach scheinen hohe Mengen an Zytokinen die Bildung von miRNAs zu induzieren. Die Applikation von DEX verringert die LPS-induzierte Expressionen der untersuchten miRNAs nahezu auf Kontrollniveau. Die oben beschriebenen antiinflammatorischen Wirkungen stehen wahrscheinlich in direktem Zusammenhang mit der DEX-vermittelten miRNA Reduktion. Durch Unterdrückung des NFκB und MAPK (Mitogen-Activated Protein Kinase) Signalweges könnte DEX möglicherweise die Produktion von miR 155 vermindern, da NFκB und MAPK bekannte Stimulatoren dieser miRNA sind [22]. Ob die durch DEX verringerte miRNA Expression direkt im Gehirn vermittelt wird oder eine Konsequenz einer verminderten peripheren Inflammation ist, gilt es in Folgeexperimenten zu klären. Es wird vermutet, dass eine peripher verminderte Inflammation den Vagus Nerv-Signalweg vermindert und somit auch die Neuroinflammation und miRNA Expression.

Hohe Expressionen von miR 132 und 134, wie nach LPS-Applikation zu beobachten, sind im Gehirn u. a. mit einer verschlechterten Kognition verknüpft. Durch Beeinflussung von BDNF (Brain-derived neurotrophic factor) und SIRT1 (Sirtuin1), zwei wichtige Mediatoren beim Lernen und Erinnern, ist miR 132 aktiv an der Kognition beteiligt. Eine moderate Überexpression von miR 132 kann die kognitiven Fähigkeiten verbessern, jedoch zeigen sich bei zu hohen Expressionen Verschlechterungen der Kognition [23,24]. Da miR 132 knockout Tiere Defizite beim Erinnern aufweisen, muss die Expression dieser miRNA präzise kontrolliert werden, um physiologisch wirken zu können [25]. SIRT1 stellt auch experimentell ein validiertes Target von miR 134 dar. Zusätzlich beeinflusst diese miRNA die synaptische Plastizität, indem sie das synaptische Spine Volumen und die Spine Stärke vermindert, was sich in einer verschlechterten Kognition äußert [26]. Die Reduzierung von miR 132 und 134 während der Neuroinflammation

nach DEX-Applikation trägt unter Umständen dazu bei, dass sich das Medikament positiv auf das Verhalten und die Kognition, wie in verschiedenen Tiermodellen und klinischen Studien zu beobachten, auswirkt [11,12,27,28].

Auch miR 155 wird durch Vorbehandlung mit DEX im Vergleich zur LPS Gruppe vermindert exprimiert. In Folge einer LPS-Exposition können hohe Mengen miR 155 eine verminderte Neurogenese im Hippocampus bedingen [29]. Weiterhin wird diese miRNA als negativer Regulator der Blut-Hirn-Schranke (BHS) während einer Neuroinflammation beschrieben [30]. Ning et al. zeigten in ihrer 2017 veröffentlichten Studie, dass DEX in einem LPS-Model die Zerstörung der BHS vermindert [2]. Der Grund dafür könnte in der DEX-vermittelten Verminderung der miR 155 Expression liegen. Dieser Zusammenhang sollte zukünftig noch genauer untersucht werden.

Die durch DEX regulierten miRNAs haben zusätzlich einen großen Einfluss auf inflammatorische Prozesse. So sind miR 124 und 132 beispielsweise als „neuroimmiR“ beschrieben, da sie sowohl das Immunsystem als auch das ZNS beeinflussen. Beide miRNAs werden durch LPS induziert und binden die AChE, sodass die Konzentration von Acetylcholin (ACh) aufrechterhalten wird und antiinflammatorisch wirken kann [31,32]. MiR 155 trägt ebenfalls wesentlich zum Inflammationsgeschehen bei, da diese miRNA sowohl pro- als auch antiinflammatorische Kaskaden partizipiert.

Da miRNAs aus Geweben in die Blutzirkulation sezerniert und stabil exprimiert werden, eignen sie sich hervorragend als Biomarker. Im Plasma verursacht LPS eine signifikante Induktion von miR 132 nach 24 h und 7 d. Eine Veröffentlichung von 2015 zeigt, dass miR 132 als Biomarker für die Detektion von einem Mild Cognitive Impairment (MCI), eine kognitive Beeinträchtigung, die mit einem erhöhten Risiko von Alzheimer Demenz assoziiert ist, geeignet sein könnte [33]. Die vorliegende Arbeit unterstützt diese These und lässt zusätzlich aufgrund der zeitlichen Expression auf eine Sezernierung dieser miRNA aus dem Gehirn ins Plasma schließen. Die Zunahme von miR 155 im Plasma durch LPS ist wahrscheinlich eher als Konsequenz der peripheren Inflammation zu deuten als ein Indikator für eine Neuroinflammation. Jedoch veröffentlichten Wu et al., dass miR 155 im Serum als prognostischer Marker für eine postoperative kognitive Dysfunktion vor laparoskopischen Operationen zur Entfernung von Darmkrebs anzusehen ist [34]. Die Expression von miR 134 wird im Plasma zu allen Zeitpunkten signifikant, im Vergleich zur Kontrollgruppe, verringert. Avansini et al. veröffentlichten kürzlich, dass miR 134 im Plasma von Patienten mit mesialer Temporallappen Epilepsie vermindert exprimiert wird, obwohl sich diese miRNA im Hippocampus der Patienten hochreguliert zeigt [35]. Demnach könnte die Detektion von miR 134 im Plasma Hinweise auf funktionelle

Änderungen im Gehirn geben. Ob miR 132 und 134 zukünftig als Biomarker einer Neuroinflammation eingesetzt werden können, muss in größeren Studien validiert werden. Die Applikation von DEX beeinflusst die Expression der im Plasma gemessenen miRNAs kaum. Es verringert die Expression von miR 132, 134 und 155 in Kombination mit LPS nach 6 h verglichen mit der jeweiligen Kontrollgruppe, wobei miR 155 die einzige durch LPS-induzierte miRNA darstellt. Die Reduktion von miR 155 im Plasma nach Medikamenten-Applikation kann als Zeichen einer reduzierten peripheren Inflammation angesehen werden.

Im neonatalen Schädigungsmodell entfaltete DEX ebenfalls positive Wirkungen indem es Hyperoxie-induzierte Schädigungen entgegenwirkt (Sifringer et al. 2015). Auch hier zeigte sich die antiinflammatorische Eigenschaft des Medikamentes deutlich. Zusätzlich konnten in mit DEX vorbehandelten neonatalen Ratten antioxidative Aktivitäten beobachtet werden. Das Medikament reduzierte Hyperoxie-induziertes MDA als Marker der Lipidperoxidation und steigerte die durch oxidativen Stress verminderte Ratio von GSH/GSSG signifikant. Oxidative Veränderungen an Proteinen, Lipiden oder DNA können neuroinflammatorische und -degenerative Prozesse im Gehirn auslösen. Unterschiedliche Studien bestätigen die antioxidativen Wirkungen von DEX im Gehirn [2,36]. Zusätzlich verringerte DEX den Anstieg TUNEL positiver Zellen im frontalen Cortex, retrosplenialen Cortex, Hypothalamus und Thalamus nach 24-stündiger Hyperoxie. Dies ist konform mit zahlreichen Studien, die eine DEX-vermittelte Neuroprotektion darlegen [2,28,37,38]. Im Vergleich zu anderen Studien konnte bei der alleinigen Applikation von 10 µg/mg DEX im neonatalen Gehirn ein Anstieg von apoptotischen Neuronen im Vergleich zu den Kontrolltieren beobachtet werden. Auch Liu et al. konnten in ihrer Arbeit bei hohen DEX Konzentrationen einen Anstieg der Neuroapoptose beobachten. Die Autoren vermuten, dass bei hohen Konzentrationen des Medikaments „off-target“ Effekte über eine verstärkte alpha-1-Rezeptor Aktivierung vermittelt werden, da sich die Blockade dieses Rezeptors als protektiv erweist [39].

Die Kombination aus einer Laparotomie und LPS Gabe führt zu einer deutlichen Neuroinflammation im adulten Hippocampus und Cortex, welche durch Applikation der reversen AChE-I Phy und Neo signifikant vermindert werden kann (Kalb et al. 2013). Zusätzlich verringern beide medikamentösen Interventionen eine LPS-induzierte Neurodegeneration in den untersuchten Hirnregionen. Durch Inhibierung der AChE steigern Phy und Neo das ACh-Niveau, wodurch antiinflammatorische Eigenschaften über den α7nAChR (α7 nikotinerger Acetylcholin Rezeptor) entfaltet werden. Veröffentlichungen zeigten bereits, dass zentralgängige AChE-I proinflammatorische Zytokine im ZNS und in der Peripherie nach Schädigung signifikant verringern [40,41]. Beide in dieser Arbeit getesteten AChE-I verringern zusätzlich die LPS-

induzierte AChE-Aktivität im Hippocampus und Cortex, was den protektiven Charakter der Medikamente unterstützt, da hohe Aktivitäten dieses Enzyms mit einem vermehrten Zelluntergang im Gehirn assoziiert sind. Obwohl Neo im Gegensatz zu Phy aufgrund der quartären Ammoniumstruktur nicht in der Lage ist, die BHS zu überqueren, entfaltet es im Gehirn mit Phy vergleichbare positive Effekte. Dies lässt darauf schließen, dass die Kontrolle des peripheren Immungeschehens ausreicht, um negative Folgen für das ZNS zu vermeiden. Eine verminderte Aktivierung des Immunsystems, die nach Applikation beider getesteten AChE-I bezüglich der untersuchten Inflammationsparameter in Milz und Plasma im Modell nachzuweisen war, führt wahrscheinlich zu einer verminderten Vagus-Stimulation und folglich zu einer verringerten Neuroinflammation sowie -degeneration.

In der vorliegenden Arbeit konnte gezeigt werden, dass sowohl die Applikation von DEX als auch der AChE-I Phy und Neo in den untersuchten Modellen positive Wirkungen entfalten kann. Speziell die Regulierung der mit einer verschlechterten Kognition assoziierten miR 132 und 134 sowie der in Zusammenhang mit einer Inflammation stehenden miR 124, 132 und 155 durch DEX öffnet neue Möglichkeiten, um die komplexe Wirkungsweise dieses Medikamentes besser zu verstehen. Zukünftig gilt es in Verhaltenstests zu klären, ob die DEX-vermittelte Reduktion von miR 132 und 134 im Gehirn, wie vermutet, die kognitiven Fähigkeiten verbessert. Zusätzlich stehen Untersuchungen von Targets der verändert exprimierten miRNAs im Vordergrund. Den potentiellen Einsatz von miR 132 und 134 als Biomarker für eine Neuroinflammation gilt es in größeren Studien zu validieren. Dazu könnten beispielsweise Versuche mit humanem Plasma stattfinden. Auch der Zusammenhang zwischen DEX und ACh stellt einen interessanten Untersuchungsparameter dar, denn das ACh Level ist, wie in der der Arbeit gezeigt, ein wichtiger Regulator der Inflammation und Neurodegeneration.

Zusammenfassend zeigt sich, dass DEX auf unterschiedlichen Ebenen im adulten und neonatalen Modell positive Wirkungen entfaltet. Dies deckt sich mit den Beobachtungen aus klinischen Studien. Aufgrund seiner vielfältigen protektiven Wirkungen eignet sich dieses Medikament hervorragend für eine individuelle Patientenversorgung. So sollte DEX bei Patienten, die ein hohes Risiko für die Entwicklung von PKS tragen, gängige Sedativa perspektivisch ersetzen. Zahlreiche klinische Studien beweisen, dass DEX die Kognition von Patienten nachweislich verbessert und inflammatorische Prozesse vermindert. Durch eine flache Sedierungstiefe sind Patienten zusätzlich leichter erweckbar und kooperativer, woraus eine effektivere Schmerzbehandlung resultiert. Auch in der Neonatologie ist ein grundsätzlicher Einsatz von DEX aufgrund seiner positiven Eigenschaften durchaus denkbar. Hierfür sind jedoch weiterführende klinische Studien unumgänglich. Trotz des hohen Preises des Medikaments sollten die positiven Eigenschaften und

die verringerten Folgekosten bedacht werden. Im Vergleich zu den getesteten AChE-I bringt der Einsatz von DEX in Bezug auf PKS deutlich mehr Vorteile mit sich. Trotz positiver Wirkung in Tiermodellen, zeigte sich der Einsatz von AChE-I in klinisch Studien bezüglich der PKS Reduktion nur wenig vorteilhaft [42].

1.7. Literaturverzeichnis

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2 Eidesstattliche Versicherung

„Ich, Nadine Paeschke, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Untersuchung der Wirkungsweise von Dexmedetomidin auf das adulte Rattenhirn“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.
Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Datum, 18. Dezember 2017 Unterschrift

Anteilserklärung an den erfolgten Publikationen

Nadine Paeschke hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1 (Paeschke et al. 2017):

Paeschke, N.; von Haefen, C.; Endesfelder, S.; Sifringer, M.; Spies, C.D. Dexmedetomidine prevents lipopolysaccharide-induced microRNA expression in the adult rat brain. *Int J Mol Sci* 2017*, 18*

Beitrag im Einzelnen:
- Beteiligung an Tierexperimenten, Probengewinnung
- Isolierung von RNA aus Gewebe und Plasma
- Durchführung von Genexpressionsanalysen
- Etablierung und Optimierung von miRNA Analysen
- Durchführung von miRNA Expressionsanalysen im Hippocampus, Cortex und Plasma
- Auswertung und Darstellung der Daten
- Literaturrecherche
- Erstellung des Manuskriptentwurfes sowie Abstimmung mit den Koautoren; korrespondierender Autor

Publikation 2 (Sifringer et al. 2015):

Sifringer, M.; von Haefen, C.; Krain, M.; Paeschke, N.; Bendix, I.; Buhrer, C.; Spies, C.D.; Endesfelder, S. Neuroprotective effect of dexmedetomidine on hyperoxia-induced toxicity in the neonatal rat brain. *Oxid Med Cell Longev* 2015*, 2015*, 530371

Beitrag im Einzelnen:
- Beteiligung an Tierexperimenten, Probengewinnung
- Durchführung der Western Blot Analysen
- Literaturrecherche
- Beteiligung bei der Erstellung des Manuskriptes

Publikation 3 (Kalb et al. 2013):

Kalb, A.; von Haefen, C.; Sifringer, M.; Tegethoff, A.; Paeschke, N.; Kostova, M.; Feldheiser, A.; Spies, C.D. Acetylcholinesterase inhibitors reduce neuroinflammation and -degeneration in the cortex and hippocampus of a surgery stress rat model. *PLoS One* 2013, *8*, e62679

Beitrag im Einzelnen:
- Isolierung von RNA aus Milzgewebe und Durchführung von Gen-expressionsanalysen
- Isolierung von Proteinen
- Beteiligung an CBA Messungen aus Milz und Plasma
- Beteiligung an der Anfertigung, Färbung und Mikroskopie von Kryoschnitten des Gehirns
- Literaturrecherche
- Beteiligung bei der Erstellung des Manuskriptes

_____

Unterschrift des Doktoranden/der Doktorandin

3 Druckexemplare der ausgewählten Publikationen

1. **Paeschke, N.**; von Haefen, C.; Endesfelder, S.; Sifringer, M.; Spies, C.D. Dexmedetomidine prevents lipopolysaccharide-induced microRNA expression in the adult rat brain. *Int J Mol Sci* **2017**, *18*.

2. Sifringer, M.; von Haefen, C.; Krain, M.; **Paeschke, N.**; Bendix, I.; Buhrer, C.; Spies, C.D.; Endesfelder, S. Neuroprotective effect of dexmedetomidine on hyperoxia-induced toxicity in the neonatal rat brain. *Oxid Med Cell Longev* **2015**, *2015*, 530371.

3. Kalb, A.; von Haefen, C.; Sifringer, M.; Tegethoff, A.; **Paeschke, N.**; Kostova, M.; Feldheiser, A.; Spies, C.D. Acetylcholinesterase inhibitors reduce neuroinflammation and -degeneration in the cortex and hippocampus of a surgery stress rat model. *PLoS One* **2013**, *8*, e62679.

3.1. Publikation 1: Paeschke et al. (2017)

Paeschke, N.; von Haefen, C.; Endesfelder, S.; Sifringer, M.; Spies, C.D.

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Dexmedetomidine Prevents Lipopolysaccharide-Induced MicroRNA Expression in the Adult Rat Brain

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Abstract: During surgery or infection, peripheral inflammation can lead to neuroinflammation, which is associated with cognitive impairment, neurodegeneration, and several neurodegenerative diseases. Dexmedetomidine, an α-2-adrenoceptor agonist, is known to exert anti-inflammatory and neuroprotective properties and reduces the incidence of postoperative cognitive impairments. However, on the whole the molecular mechanisms are poorly understood. This study aims to explore whether dexmedetomidine influences microRNAs (miRNAs) in a rat model of lipopolysaccharide (LPS)-induced neuroinflammation. Adult Wistar rats were injected with 1 mg/kg LPS intraperitoneal (i.p.) in the presence or absence of 5 µg/kg dexmedetomidine. After 6 h, 24 h, and 7 days, gene expressions of interleukin 1-β (*IL1-β*), tumor necrosis factor-α (*TNF-α*), and microRNA expressions of miR 124, 132, 134, and 155 were measured in the hippocampus, cortex, and plasma. Dexmedetomidine decreased the LPS-induced neuroinflammation in the hippocampus and cortex via significant reduction of the *IL1-β* and *TNF-α* gene expressions after 24 h. Moreover, the LPS-mediated increased expressions of miR 124, 132, 134, and 155 were significantly decreased after dexmedetomidine treatment in both brain regions. In plasma, dexmedetomidine significantly reduced LPS-induced miR 155 after 6 h. Furthermore, there is evidence that miR 132 and 134 may be suitable as potential biomarkers for the detection of neuroinflammation.

Keywords: neuroinflammation; dexmedetomidine; miRNA; hippocampus; cortex

1. Introduction

Neuroinflammation plays a major role in the pathogenesis of several neurodegenerative diseases. A clinical and experimental correlation between elevated peripheral proinflammatory cytokines and neuroinflammation has been largely described [1–3]. Lipopolysaccharide (LPS) or surgical trauma activate the immune system and are capable of causing memory impairment [3–5]. Studies indicate that high levels of the proinflammatory cytokines interleukin 1-β (*IL1-β*) and tumor necrosis factor-α (*TNF-α*) are associated with postoperative cognitive impairments [6,7]. These are characterized by neuronal dysfunction and/or neuronal death, which can induce long-lasting cognitive decline and aggravation in learning and memory [2,3,8]. Surgery, especially in the elderly, can lead to

neuroinflammation, cause long-lasting postoperative cognitive impairments, and permanently affect the patient's life [9,10]. Patients with those impairments have a higher risk of developing postoperative complications such as sepsis, and show a higher mortality rate [11,12].

Dexmedetomidine, a highly selective α-2-adrenergic agonist, exerts sedative, anxiolytic, and analgesic properties and is therefore used as perioperative sedative agent [13]. Several studies indicate that dexmedetomidine has protective properties regarding postoperative cognitive impairments in clinical trials [14–17]. Compared to other anesthetics, dexmedetomidine shortens the duration of artificial ventilation, the extubating time, and the occurrence of postoperative cognitive impairments [18–21]. Dexmedetomidine shows anti-inflammatory as well as neuroprotective properties in several cell and rodent studies [8,22–29]. In different rodent behavior experiments, dexmedetomidine also prevents the occurrence of cognitive impairments [30,31]. However, the exact molecular mechanisms of the positive and protective character of dexmedetomidine are not entirely understood.

MicroRNAs (miRNAs) are approximately 22 base pairs long, non-coding RNAs which play a significant role in post-transcriptional gene regulation. It is currently assumed that about 60% of all genes are regulated by miRNAs [32]. The majority of known miRNAs are found in the brain, whereas some of them are exclusive to neuronal tissue [33,34]. Depending on the complementarity of the binding between miRNA and mRNA, the mRNA is degraded or its translation repressed [35]. Studies have shown that the expression of certain miRNAs is altered in different neurodegenerative processes [36].

The aim of this study was to determine whether dexmedetomidine has an impact on the expression of *IL1-β*, *TNF-α*, and several miRNAs in the hippocampus and cortex in a neuroinflammation model of adult rats. Additionally, miRNAs that showed an altered expression in the brain were measured also in plasma to study whether dexmedetomidine influences LPS-induced changes and whether those miRNAs may be suitable biomarkers for the detection of neuroinflammation.

2. Results

2.1. Dexmedetomidine-Attenuated, LPS-Induced IL1-β and TNF-α Gene Expression in the Hippocampus and Cortex

As shown in Figure 1, the administration of 1 mg/kg LPS caused a significant upregulation of *IL1-β* (Figure 1A,B) and *TNF-α* (Figure 1C,D) gene expression after 6 and 24 h in the hippocampus and cortex compared to the saline-treated rats. Seven days post LPS injection, the *IL1-β* expression was still significantly elevated in the cortex compared to the control. Dexmedetomidine significantly reduced the LPS-induced rise of both cytokines after 24 h, indicating an anti-inflammatory effect of this drug. Despite the dexmedetomidine-mediated reduction of LPS-induced *IL1-β* expression in the hippocampus, the expression was significantly elevated compared to the saline-treated animals. Twenty-four hours after LPS injection, the dexmedetomidine-treated animals showed *TNF-α* levels similar to the control group in both brain regions. Seven days after LPS injection dexmedetomidine was still able to reduce the LPS-mediated rise of *IL1-β* in the cortex. The drug alone significantly decreased hippocampal *IL1-β* expression after 7 days.

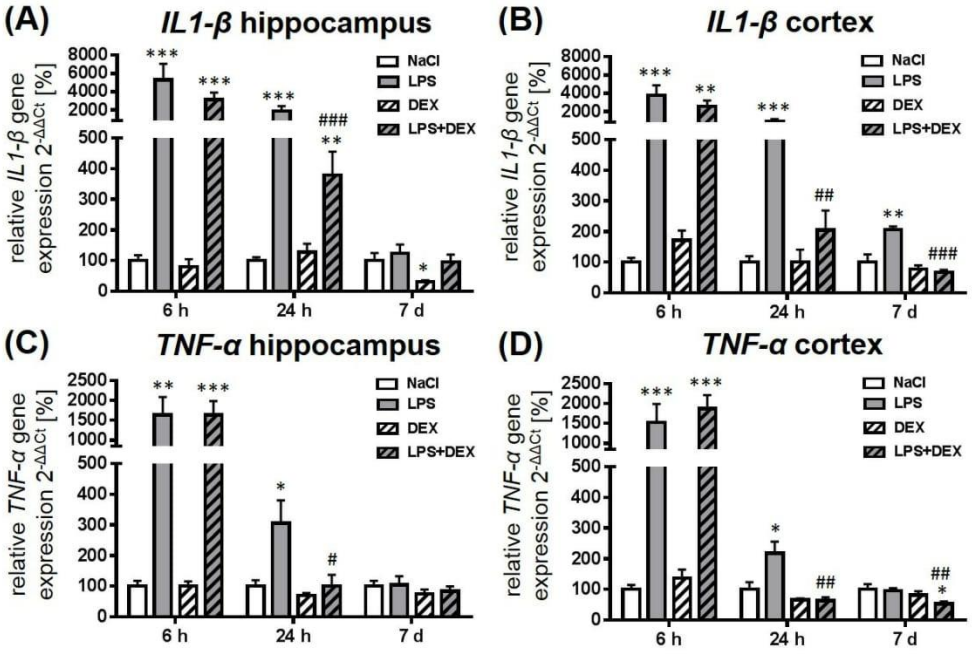

Figure 1. Representation of interleukin 1-β *(IL1-β)* (**A**,**B**) and tumor necrosis factor-α (*TNF-α)* (**C**,**D**) gene expression after LPS (lipopolysaccharide) and dexmedetomidine treatment. Results are shown as mean ± standard error of the mean (SEM) (n = 6–8 per group). Data are normalized to levels of saline-treated rats (control = 100%). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ represent the difference compared to saline-treated groups. $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$ represent the difference between LPS and LPS in combination with dexmedetomidine-treated groups.

2.2. Dexmedetomidine Modulated the miRNA Expression in the Hippocampus, Cortex, and Plasma of LPS Treated Rats

The aim of this study was to determine whether dexmedetomidine influences the expression of different miRNAs in the brain and the plasma of adult rats in a neuroinflammation model.

2.2.1. Expression of MicroRNA 124

As shown in Figure 2, the administration of LPS mediated a significant increase of miR 124 expression after 24 h in the hippocampus (Figure 2A) and the cortex (Figure 2B) compared to the respective control groups. LPS-induced expression of miR 124 was more pronounced in the cortex than in the hippocampus. After 7 days the endotoxin had no influence on the expression of this miRNA. LPS in combination with dexmedetomidine prevented LPS-induced miR 124 expression after 24 h in both investigated brain regions significantly. Six hours after LPS and dexmedetomidine administration miR 124 was significantly enhanced in the cortex, whereas it was significantly reduced after 7 days compared to the respective control. Moreover, the drug alone caused a significant, enhanced miR 124 expression after 6 h in the cortex when compared to the control. In plasma, miR 124 expression was not detectable at any time point.

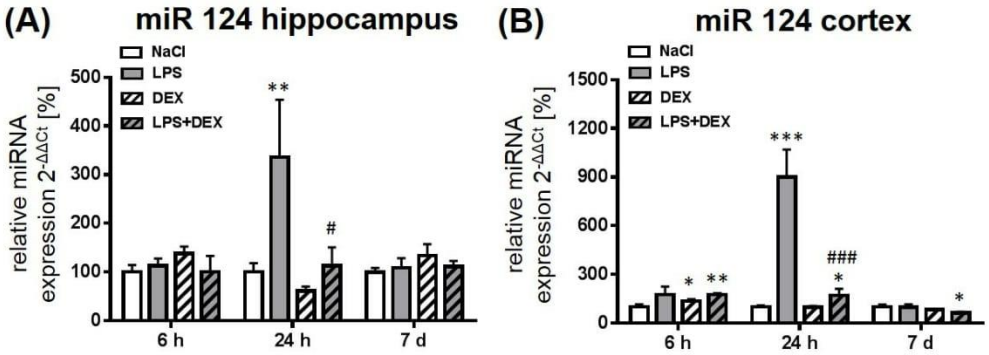

Figure 2. Representation of miR 124 expression after LPS and dexmedetomidine treatment in the hippocampus (**A**) and cortex (**B**). Results are shown as mean ± SEM (n = 6–8 per group). Data are normalized to levels of saline-treated rats (control = 100%). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ represent the difference compared to saline-treated groups. # $p < 0.05$ and ### $p < 0.001$ represent the difference between LPS and LPS in combination with dexmedetomidine-treated groups.

2.2.2. Expression of MicroRNA 132

Rats treated with LPS showed a significant upregulation of miR 132 expression in the hippocampus after 6 and 24 h when compared to the control, whereas the endotoxin had no influence on the expression after 7 days (Figure 3A). Dexmedetomidine in combination with LPS significantly prevented the LPS-mediated upregulation of miR 132 in this brain region after 24 h. The drug alone caused a significant rise of miR 132 expression after 6 h in the hippocampus that is comparable with the LPS-induced expression.

In the cortex, treatment with LPS significantly enhanced the expression of miR 132 after 24 h, which was significantly reduced by administration of dexmedetomidine (Figure 3B). The drug alone rose miR 132 in the cortex after 24 h and 7 days in a significant way when compared to saline-treated rats.

The miR 132 expression in plasma significantly increased 24 h and 7 days post LPS administration, what was not influenced by dexmedetomidine (Figure 3C). After 6 h, the combination of dexmedetomidine and LPS significantly reduced the miR 132 expression in plasma compared to the control and the LPS group. The drug alone reduced plasma miR 132 after 24 h and 7 days significantly.

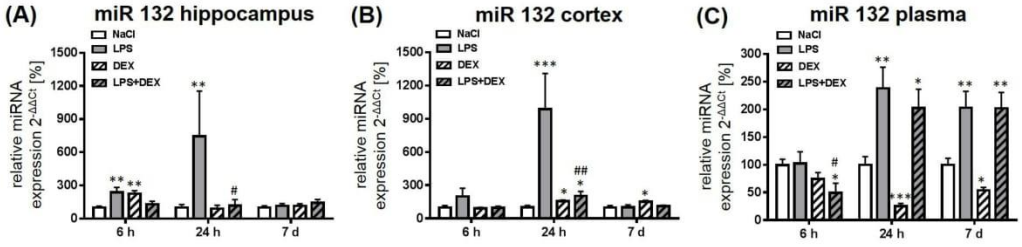

Figure 3. Representation of miR 132 expression after LPS and dexmedetomidine treatment in the hippocampus (**A**), cortex (**B**), and plasma (**C**). Results are shown as mean ± SEM (n = 6–8 per group). Data are normalized to levels of saline-treated rats (control = 100%). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ represent the difference compared to saline-treated groups. # $p < 0.05$ and ## $p < 0.01$ represent the difference between LPS and LPS in combination with dexmedetomidine-treated groups.

2.2.3. Expression of MicroRNA 134

In comparison to saline-treated rats, expression of miR 134 was significantly upregulated 6 and 24 h after LPS treatment in the hippocampus of adult rats (Figure 4A). Dexmedetomidine significantly attenuated LPS-induced miR 134 expression in this brain region. The drug alone caused a significant increase of miR 134 after 6 h in the hippocampus compared to control.

In the cortex, treatment with LPS also significantly increased miR 132 expression after 6 and 24 h (Figure 4B) compared to the control, what was significantly reduced by the administration of dexmedetomidine after 24 h.

In comparison to brain tissue, plasma miR 134 significantly decreased at all time points in LPS-treated animals (Figure 4C). Dexmedetomidine alone and in combination with LPS significantly reduced the expression of miR 134 after 6 h compared to saline-treated rats. The combination of dexmedetomidine and LPS enhanced the expression of miR 134 after 7 days significantly compared to the LPS group.

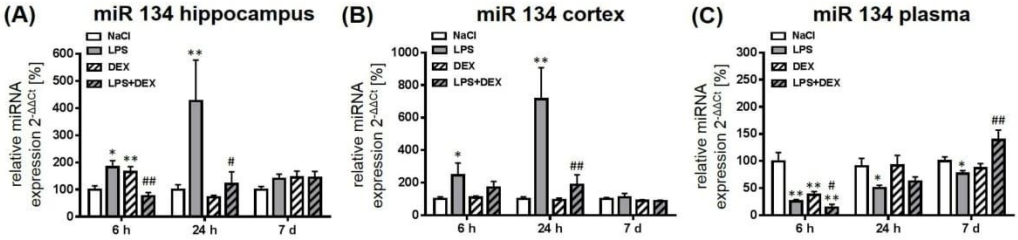

Figure 4. Representation of miR 134 expression after LPS and dexmedetomidine treatment in the hippocampus (**A**), cortex (**B**), and plasma (**C**). Results are shown as mean ± SEM (n = 6–8 per group). Data are normalized to levels of saline-treated rats (control = 100%). * $p < 0.05$ and ** $p < 0.01$ represent the difference compared to saline-treated groups. $^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ represent the difference between LPS and LPS in combination with dexmedetomidine-treated groups.

2.2.4. Expression of MicroRNA 155

LPS significantly increased the expression of miR 155 at all investigated time points in the hippocampus (Figure 5A) compared to the saline-treated rats. Application of dexmedetomidine significantly reduced LPS-mediated, enhanced miR 155 expression after 24 h, whereas it had no influence on the expression after 6 h and 7 days. The drug alone led to a significant increase of miR 155 after 6 h in the hippocampus that is comparable with the LPS-induced expression of this miRNA.

When compared to respective control groups, miR 155 was significantly enhanced in the cortex 6 and 24 h following LPS exposition (Figure 5B). Dexmedetomidine significantly inhibited the LPS-induced expression after 24 h but compared to control group the expression was still significantly increased. LPS, in combination with dexmedetomidine, significantly elevated miR 155 after 7 days in the cortex compared to the control.

In the plasma, LPS mediated a significant enhancement of miR 155 after 6 and 24 h and a significant reduction after 7 days (Figure 5C). Dexmedetomidine, in combination with LPS, significantly decreased the LPS-induced miR 155 level after 6 h in the plasma in a way that it was even significantly downregulated compared to the saline-treated animals. Moreover, the drug alone caused a decrease of miR 155 at all time points in the plasma when compared to the control animals.

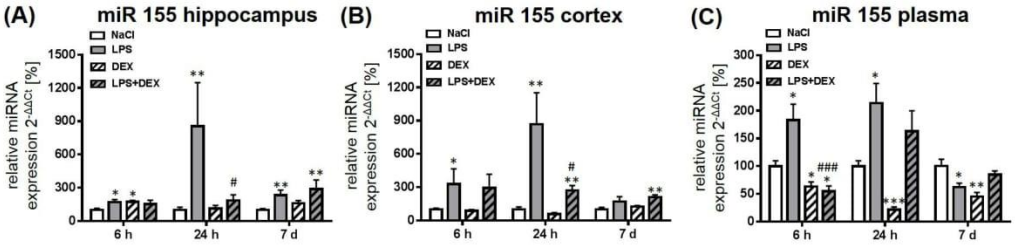

Figure 5. Representation of miR 155 after LPS and dexmedetomidine treatment in the hippocampus (**A**), cortex (**B**), and plasma (**C**). Results are shown as mean ± SEM (n = 6–8 per group). Data are normalized to levels of saline-treated rats (control = 100%). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ represent the difference compared to saline-treated groups. $^{\#}$ $p < 0.05$ and $^{\#\#\#}$ $p < 0.001$ represent the difference between LPS and LPS in combination with dexmedetomidine-treated groups.

3. Discussion

The aim of this study was to investigate the effect of dexmedetomidine on LPS-induced neuroinflammation, and miRNA expression in the hippocampus and cortex of the adult rat brain, as those brain regions play a major role in cognition and memory. Our study demonstrates that treatment with dexmedetomidine reduced LPS-mediated neuroinflammation and prevented LPS-induced enhancement of miR 124, 132, 134, and 155.

Many studies have already shown in rodents that systemic administration of LPS activates the innate immune system, which leads to neuroinflammation, memory impairment, and neuronal cell death, particularly in the cortical and hippocampal regions of the rodent brain [1–4]. High concentrations of *IL1-β* and *TNF-α* in the brain are associated with cognitive impairments [5–7]. Dexmedetomidine, a selective α-2-agonist, reduces the occurrence of cognitive impairments in animal and human studies, but the molecular mechanisms are not entirely understood. In the work at hand, dexmedetomidine was applied shortly before LPS treatment. The drug is known to exert protective properties when applied before intervention in several animal studies [23,30,31]. A study from Yamanaka showed that only early dexmedetomidine (applied directly after intervention), but not late dexmedetomidine (applied 24 h after intervention), exerts protective effects [31]. Some studies also show protective properties after drug treatment 30 min post-damage induction [37,38]. Nevertheless, we chose a dexmedetomidine treatment prior to LPS application to reduce potential LPS-induced damage as fast as possible. Dexmedetomidine treatment decreased LPS-induced *IL1-β* and *TNF-α* mRNA expression in the hippocampus and cortex after 24 h in our model. At this point, the *TNF-α* concentration returned to control levels, whereas *IL1-β* was still elevated in both brain regions when compared to the control. Via reducing LPS-induced *IL1-β* expression after 7 days in the cortex, dexmedetomidine seems to have long-lasting anti-inflammatory properties. Ning et al. previously published that dexmedetomidine reduced *IL1-β*, *TNF-α*, and oxidative stress in mice brain after systemic LPS administration [8]. In the lungs of septic rats, dexmedetomidine inhibits inflammation via the toll like receptor 4/myeloid differentiation factor 88/ nuclear factor kappa-light-chain-enhancer of activated B cells (TLR4/MyD88/NF-κB) signaling pathway [39]. Moreover, an involvement of the cholinergic anti-inflammatory pathway is described in dexmedetomidine´s anti-inflammatory action in different rodent studies [22,23,40].

miRNAs regulate about 60% of genes on the posttranscriptional level and play an important role in different cell processes [32]. In our work, we investigated four miRNAs that are believed to play a role in inflammation and cognition: miR 124, 132, 134, and 155. A study from 2013 proposed members of the miR-132 and miR-134 families as biomarkers for the detection of mild cognitive impairment (MCI) [41]. As the anti-inflammatory cholinergic system plays an important role in the inflammation response, we investigated miR-124 and 132, because these miRNAs are known to influence this pathway [42].

Moreover, serum expression of miRNA-155 was shown by multiple logistic regression analysis to be an independent predictive indicator for postoperative cognitive impairment after surgery [43].

LPS administration increased the expression of all investigated miRNAs at several time points significantly in the hippocampus and cortex of adult rats. The measured miRNA level was higher in the cortex than in the hippocampus, indicating a stronger involvement of the cortex in the LPS-mediated miRNA machinery. After 7 days miR 124, 132, and 134 expressions were restored to a normal level, whereas miR 155 was still significantly upregulated in the hippocampus. Expression of miR 155 is known to be high in the central nervous system (CNS) of amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) patients, two inflammation-related disorders [44]. High expressions of miR 155 could be a potential trigger for long lasting CNS changes in our model after administration of 1 mg/kg LPS, but this hypothesis needs further research. Even if 6 h following LPS administration some miRNAs were slightly elevated in the brain, we suggest that the high cytokine expressions after 6 h are inducing the miRNAs after 24 h. It is described that cytokines induce individual miRNAs. A study from Liu et al. indicates that in LPS-stimulated alveolar macrophages, miR 132 is upregulated after measurement of high concentrations of *IL1-β* and *TNF-α* [45]. In a human monocyte cell line, miR 132 and 155 are upregulated after *IL1-β* or *TNF-α* exposure [46]. Moreover, murine macrophages exposed to *TNF-α* show an increased miR 155 expression [47]. We suspect that the level of proinflammatory cytokines is crucial for the activation of miRNAs in our model. Induction of miRNAs after LPS treatment can be explained as a potential counter reaction and/or protection against LPS-induced damage to participate in attenuating inflammation via indirect suppression of cytokines.

miR 124 is the most abundant miRNA expressed in the CNS and is an essential factor for neuronal differentiation. It is described as "neurimmiR", indicating an involvement in the nervous and immune systems [42]. In our model, LPS treatment significantly increased miR 124 expression after 24 h in the hippocampus and the cortex. An upregulation of miR 124 could also be detected in LPS-treated mouse macrophages and mice, where it was reported to be a critical mediator of the cholinergic anti-inflammatory action [48,49].

miR 132, that is, enhanced after LPS treatment in our model in the hippocampus and the cortex, also acts as "neurimmiR" [42]. This miRNA is induced in primary human macrophages, rat alveolar macrophages, and several organs of mice after LPS treatment [45,50]. miR 132 functions as a negative regulator of the inflammatory response in alveolar macrophages by potentiating the cholinergic anti-inflammatory pathway [45]. Acetylcholinesterase, an enzyme that cleaves acetylcholine in choline and acetic acid, is a validated target of miR 132 [45,50,51]. Inhibition of this esterase leads to higher acetylcholine amounts, which can mediate anti-inflammatory properties via binding on α-7-nAChR on macrophages. Moderate overexpression of miR 132 was shown to improve cognition, whereas high overexpression of this miRNA in rat cortex and mouse forebrain is associated with cognitive impairments [52–54]. Moreover, miR 132 knockout animals exert deficits in memory [55]. Therefore, miR 132 expression needs to be precisely controlled in brain tissue.

miR 134 was significantly upregulated after LPS treatment in the hippocampus and the cortex in our model. This brain-specific miRNA is activity-regulated and associated with the control of dendritic spine morphology. Elevated miR 134 levels are linked to impaired synaptic plasticity [56,57], whereas overexpression of this miRNA is associated with a reduced spine volume and reduced synaptic strength. Gao et al. proved that high miR 134 expressions result in impaired long-term potentiation in the hippocampus [57].

In our study, LPS elevates the expression of miR 155 in the hippocampus the and cortex, which is in agreement with other reports. miR 155 is one of the best-described miRNAs concerning inflammation. This miRNA is described to participate in pro- and anti-inflammatory mechanisms. Quinn et al. published that LPS-induced expression of transcription factor E26 transformation-specific 2 (Ets2) is responsible for induction of miR 155 expression, but also the NF-κB and mitogen-activated protein kinase (MAPK) pathway, which is activated during LPS infection, stimulates the expression of miR 155 [58]. miR 155 can bind to several modulators of toll like receptor (TLR)/IL-1 signaling

to attenuate expression of cytokines in mouse monocyte-derived dendritic cells, macrophages, and mice [59,60]. Via targeting phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1), a negative regulator of TNF-α, and suppressor of cytokine signaling 1 (SOCS1), a negative regulator of cytokines, miR 155 also mediates an increased inflammatory response. Treating mice with LPS decreases neurogenesis in the hippocampus, which might be a consequence of miR 155-mediated IL-6 production in microglia [44].

The investigated miRNAs were not just only measured in brain, but also in plasma in our study. Because of their good stability and tissue specificity, these small non-coding RNAs are suitable as circulating biomarkers. The usage of miRNAs as biomarkers has several advantages compared to other biomarkers: miRNAs are stably expressed in plasma and even low expressions can be detected by qPCR (semiquantitative Real Time PCR) [61]. Moreover, miRNAs do not have any posttranslational modifications, which can influence their measurement [61]. Identifying miRNAs as suitable biomarkers for the fast and reliable detection of neuroinflammation is important, for instance, for the fast recognition of postoperative cognitive impairments after surgery, since rapid recognition and initiation of a proper countermeasure is essential to improving the outcome of patients. In our study, plasma miR 132 increased significantly 24 h and 7 days after LPS stimulation. A study from 2015 suggests miR 132 as a circulating biomarker for the detection of MCI, a cognitive decline that is associated with a high risk of developing Alzheimer Dementia [62]. Our study supports that miR 132 might be a potential marker for the detection of neuroinflammation, because it is enhanced in plasma after massive upregulation in the brain, indicating a release of miR 132 from the brain into the plasma. To prove if miR 132 is a suitable target for the detection of neuroinflammation in plasma, further research will be necessary. Expression of miR 134 was significantly reduced at all time points after LPS treatment in our study. Avansini et al. recently published that miR 134 is downregulated in the plasma of patients with mesial temporal lobe epilepsy, whereas it is upregulated in the hippocampus of the patients [63]. Therefore, miR 134 might be a potential circulating biomarker for the detection of functional changes in the brain. Because of its downregulation after 6 h, 24 h, and 7 days, miR 134 may be used as an early and late marker of neuroinflammation. This point needs to be further investigated and validated. LPS-induced enhancement of miR 155 expression in plasma in the study at hand is, in our opinion, more a consequence of peripheral inflammation rather than an indicator of neuroinflammation, as miR 155 is unspecific and upregulated during inflammation.

To our knowledge, this study explores for the first time that dexmedetomidine significantly suppressed the expression of miR 124, 132, 134, and 155 after LPS-induced neuroinflammation in the hippocampus and cortex. Dexmedetomidine mediated suppression of the cytokines *IL1-β* and *TNF-α* may prevent massive upregulation of all investigated miRNAs, because of the lower level of inflammation, for which there is no trigger for miRNA induction. Application of the drug alone does not cause any effects, as there is no trigger to counteract any intervention. Via suppressing the LPS-induced TLR4/MyD88/NF-κB and MAPK pathway, dexmedetomidine may avert upregulation of miR 155, as NF-κB and MAPK are known stimulators of this miRNA [58]. Whether dexmedetomidine's attenuation of miRNA expression is directly regulated in the brain or a consequence of reduced peripheral inflammation cannot be determined. This point must be further investigated, but we hypothesize that dexmedetomidine-induced anti-inflammatory peripheral actions probably reduce vagus nerve signaling to the brain, which results in reduced neuroinflammation and therefore reduced miRNA expression. Ning et al. published that LPS-induced disturbance of the blood-brain barrier can be attenuated by dexmedetomidine [8]. We speculate that this might be a consequence of the dexmedetomidine-mediated reduction of miR 155, as miR 155 is described as a negative regulator of the blood-brain barrier during neuroinflammation. As the drug partly triggers the same pathways like LPS-induced miRNAs in our model, dexmedetomidine in parts mimics the endogen miRNA function to attenuate inflammation without negative side effects. Because high amounts of miR 132 and 134 are known to participate in impaired memory function and impaired synaptic plasticity, we speculate that dexmedetomidine-mediated preventive enhancement of those miRNAs can be

a potential reason for the better outcome of animals and patients profiting from dexmedetomidine in different cognition studies.

Dexmedetomidine is more likely to influence the miRNA expression in the brain than in plasma after LPS administration. In plasma, the drug downregulated miR 132, 134, and 155, in combination with LPS after 6 h compared to the control and LPS-treated groups, whereas miR 155 was the only LPS-induced miRNA in plasma. Reduction of miR 155 after 6 h can be interpreted as a sign of reduced peripheral inflammation. After 24 h and 7 days, dexmedetomidine had no influence on the LPS-induced miR 132 and 155 expressions in plasma. miR 132 and 134 are both significantly enhanced 7 days after dexmedetomidine administration in combination with LPS, indicating long-lasting miRNA changes in plasma after endotoxin in combination with drug administration.

There are several limitations in our study. First, we only measured cytokine expressions on an mRNA level, to prove our model. Second, no behavior tests were performed, so we do not know whether high concentrations of miR 132 and 134 in our model are associated with cognitive impairments and whether dexmedetomidine can reduce these. Further research is needed to clarify how dexmedetomidine exactly influences the miRNA expression to get a full understanding of its protective properties. Therefore, analyses with specific miRNA mimics and inhibitors should be performed in cell culture experiments. Moreover, dexmedetomidine was applied only once before LPS treatment, while in the clinical setting it is infused over a longer period. To prove the usage of miR 132 and 134 as potential biomarkers, a validation step is essential. This should be performed in the plasma of patients suffering from neuroinflammation.

Taken together, our data indicate for the first time that dexmedetomidine does not just only reduce LPS-mediated, enhanced cytokine expression, but also prevents LPS-induced expression of miR 124, 123, 134, and 155 in the adult hippocampus and cortex during neuroinflammation. That opens new approaches to understand the molecular mechanism of dexmedetomidine-mediated protection. Moreover, there are hints that miR 132 and 134 may be suitable plasma biomarkers for neuroinflammation.

4. Materials and Methods

4.1. Animal Model

Adult male Wistar rats (250–300 g) underwent intraperitoneal (i.p.) LPS injection in the presence or absence of the α-2-receptor agonist dexmedetomidine (DEX). Rats were treated with i.p. LPS (1 mg/kg body weight), i.p. DEX (dexdor®, Orion Pharma, Espoo, Finland; 5 µg/kg body weight), or control vehicle NaCl (0.9%) after a short anesthesia in isoflurane-oxygen narcosis. Animals were divided into four groups: (1) NaCl, (2) LPS, (3) DEX, and (4) LPS+DEX. DEX was administrated 10 min before LPS treatment. Rats were hosted in groups at room temperature (22 ± 2 °C) under a standard 12–12 h light-dark cycle. Food and water were available ad libitum. All animal experiments were approved and performed in accordance with the guidelines of the Charité-Universitätsmedizin Berlin, Germany and the national ethic principles (registration no. G 0145/13, 1 July 2013).

4.2. Tissue Preparation

After 6 h, 24 h, 7 days, the animals were sacrificed in deep isoflurane-oxygen narcosis. The rats were transcardially perfused with normal saline (pH 7.4) and then decapitated. The brain was immediately removed and divided into two hemispheres. The whole cortical and hippocampal tissue was microdissected with a stereo magnifying glass from one hemisphere and directly snap frozen in liquid nitrogen. To obtain plasma, EDTA whole blood was centrifuged at 2500× *g* for 10 min. Afterward, the plasma was centrifuged again at 1000× *g* for 15 min and stored at −80 °C.

4.3. RNA Extraction and Semiquantitative Real Time PCR

Total RNA containing miRNA was isolated from snap frozen hippocampus and cortex by acidic phenol/chloroform extraction (peqGOLD RNAPure; PEQLAB Biotechnologie, Erlangen, Germany) according to the manufacturer's instructions. RNAPure FL (PEQLAB) was used for the isolation of miRNA from plasma. The RNA-precipitation from plasma was performed overnight using glycogen as a carrier.

For the mRNA analyses, 2 µg of RNA underwent DNase treatment (Ambion, Austin, TX, USA) and were reverse transcribed with 2 µM oligo d(T) 16 primer (Promega, Mannheim, Germany) and 200 U M-MLV reverse transcriptase (Promega) at 42 °C. The cDNA was quantified in real time with dye-labeled probes and primers (metabion, Planegg/Steinkirchen, Germany) (sequences shown in Table 1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) functioned as the endogen control gene. The concentration of cDNA used as input for semiquantitative Real Time PCR (qPCR) differed from 50–100 ng. The qPCR volume was 13 µL, whereas 6.5 µL 2× mastermix (Applied Biosystems, Foster City, CA, USA), 2.5 µL primer mix (1.25 µM) and 0.5 µL probe (0.5 µM).

miRNA analyses were performed after a method published by Balcells et al. [64]. Briefly, miRNA is first polyadenylated and then reverse transcribed with a special primer (RT-primer). For qPCR analyses, two specific primers for each miRNA were designed using a software tool from Busk [65]. For miRNA analysis from tissue, an amount of 500 ng, for plasma of 50 ng total RNA containing miRNA, was reverse transcribed with 1 µM RT-primer (sequence shown in Table 1), 0.1 mM dNTP mix, 1 mM ATP, 100 U M-MLV reverse transcriptase (Promega), 1 U Poly-A-Polymerase (New England Biolabs, Frankfurt am Main, Germany), and 1 µL of 10× poly(A)polymerase buffer (New England Biolabs). The volume was filled up to 10 µL and incubated for 60 min at 42 °C. 10 ng cDNA from tissue and 1 ng cDNA from plasma were used as input for qPCR with 2× Bright Green (Promega, Mannheim, Germany). SnU6RNA functioned as the endogen control for all miRNA analyses performed in brain tissue. In plasma, miR 103 was used as reference miRNA. The mRNA and miRNA expressions were analyzed with the ABI Prism® 7500 detection system (Applied Biosystems, Foster City, CA, USA) and QuantStudio5® (Thermo Fisher, Darmstadt, Germany) according to the $2^{-\Delta\Delta CT}$ method [66].

Table 1. Primer and probe sequences for messenger RNA (mRNA) and microRNA (miRNA) analyses. All primers and probes were synthesized from metabion. F: forward primer, R: reverse primer, P: probe, RT: reverse transcription.

IL1-β NM_031512	F	aacaaaaatgcctcgtgctgtct	*TNF-α* NM_012675	F	tcgagtgacaagcccgtagc
	R	tgttggcttatgttctgtccattg		R	ctcagccactccagctgctc
	P	6-fam-acccatgtgagctga aagctctccacc-tamra		P	6-fam-cgtcgtagcaaacca ccaagcaga-tamra
GAPDH NM_017008	F	gatgctggtgctgagtatgtcgt	RT-primer		caggtccagttttttttttttttt
	R	tcaggtgagccccagcct			
	P	6-fam-tctactggcgtcttc accaccatggaga-tamra			
miR 103 MIMAT0000824	F	gcagagcagcattgtacag	miR 134 MIMAT0000840	F	gcagtgtgactggttgac
	R	ggtccagttttttttttttttttcatag		R	cagttttttttttttttccccctct
miR 124 MIMAT0004728	F	gcagcgtgttcacagc	miR 155 MIMAT0030409	F	cgcagttaatgctaattgtgatag
	R	tccagttttttttttttttttcaaggt		R	aggtccagttttttttttttttttacc
miR 132 MIMAT0008381	F	gcagtaacagtctacagcca	snU6RNA NR_004394	F	atacagagaagattagcatggcc
	R	gtccagttttttttttttttttcgac		R	cgaatttgcgtgtcatccttg

4.4. Statistical Analyses

Experiments were performed in seven or eight animals per group (n = 7–8). Data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Values are presented as means ± standard error of the mean (SEM). Comparisons among groups were made using the Mann-Whitney *U*-test. p Values of <0.05 was considered to be significant.

5. Conclusions

Dexmedetomidine attenuated LPS-induced neuroinflammation via reducing *IL1-β* and *TNF-α* expression in the hippocampus and cortex of adult rats. Moreover, the drug prevented upregulation of miR 124, 132, 134 and 155 after LPS application in both investigated brain regions.

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Author Contributions: Clarissa von Haefen, Marco Sifringer, and Stefanie Endesfelder conceived and designed the experiments; Clarissa von Haefen, Marco Sifringer, Nadine Paeschke, and Stefanie Endesfelder performed the experiments; Nadine Paeschke analyzed the data; Claudia D. Spies contributed reagents/materials/analysis tools; Nadine Paeschke wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

α-7-nAChR	α-7 Nicotinic acetylcholine receptor
ALS	Amyotrophic Lateral Sclerosis
cDNA	Complementary DNA
CNS	Central Nervous System
DEX	Dexmedetomidine
EDTA	Ethylene-Diamine-Tetra-Acetic acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
i.p.	Intraperitoneal
IL1	Interleukin 1
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MCI	Mild Cognitive Impairment
miRNA	MicroRNA
M-MLV	Moloney Murine Leukemia Virus
mRNA	messengerRNA
MS	Multiple Sclerosis
MyD88	Myeloid Differentiation Factor 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
SHIP1	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1
SOCS1	Suppressor of cytokine signaling 1
TLR	Toll Like Receptor
TNF-α	Tumor necrosis factor-α

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Research Article

Neuroprotective Effect of Dexmedetomidine on Hyperoxia-Induced Toxicity in the Neonatal Rat Brain

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Dexmedetomidine is a highly selective agonist of α2-receptors with sedative, anxiolytic, analgesic, and anesthetic properties. Neuroprotective effects of dexmedetomidine have been reported in various brain injury models. In the present study, we investigated the effects of dexmedetomidine on neurodegeneration, oxidative stress markers, and inflammation following the induction of hyperoxia in neonatal rats. Six-day-old Wistar rats received different concentrations of dexmedetomidine (1, 5, or 10 μg/kg bodyweight) and were exposed to 80% oxygen for 24 h. Sex-matched littermates kept in room air and injected with normal saline or dexmedetomidine served as controls. Dexmedetomidine pretreatment significantly reduced hyperoxia-induced neurodegeneration in different brain regions of the neonatal rat. In addition, dexmedetomidine restored the reduced/oxidized glutathione ratio and attenuated the levels of malondialdehyde, a marker of lipid peroxidation, after exposure to high oxygen concentration. Moreover, administration of dexmedetomidine induced downregulation of IL-1β on mRNA and protein level in the developing rat brain. Dexmedetomidine provides protections against toxic oxygen induced neonatal brain injury which is likely associated with oxidative stress signaling and inflammatory cytokines. Our results suggest that dexmedetomidine may have a therapeutic potential since oxygen administration to neonates is sometimes inevitable.

1. Introduction

Premature birth is the leading cause of child mortality and morbidity in preterm infants because most organs and also the antioxidant enzyme system are not fully developed structurally and functionally [1, 2]. Despite advances in perinatal medicine, whereby the chances of survival of premature infants could be significantly increased, in preterm infants with low birth weight impairment of brain development is often observed [3, 4].

A general anesthesia is sometimes essential for premature infants with medical indication. In pediatric anesthesia usually the same anesthetics and adjuvants are used as in adults. NMDA antagonists like ketamine and/or $GABA_A$ receptor agonists, such as benzodiazepines, barbiturates, isoflurane, or propofol, are employed [5, 6]. In particular, premature and newborn infants demonstrate a significantly higher risk of anesthesia [7] and perioperative morbidity and mortality is increased due to the immature organ systems [8]. In addition to required medical interventions under sedation also high oxygen concentrations are a major problem. In recent years, experimental studies and clinical observations showed that oxygen, which is widely used in neonatal intensive care for treatment of respiratory distress, triggers a disruption of intracellular redox homeostasis and disturbed neurological development of preterm infants [9–11]. As we demonstrated recently in experimental models, this disturbance can induce oxidative stress by modulation of the glutathione ratio and an increasing lipid peroxidation [12–15], inflammation by increased levels of proinflammatory cytokines [13, 15, 16], leading to increased neurodegeneration [12, 16, 17], and

inhibition of neuronal maturation [17, 18] in the developing brain.

Dexmedetomidine is a potent and highly selective agonist of α2-receptors with sedative, anxiolytic, analgesic, and anesthetic properties [19–21]. In addition, it is generally reported that dexmedetomidine has neuroprotective effects in different animal models [22–27] and minimal side effects on the respiratory tract and the gastrointestinal function that minimizes the exposure to other narcotics and benzodiazepines [23, 28–30]. Recent studies suggest the α2-adrenoceptor agonist dexmedetomidine to attenuate anesthetic agent induced neuroapoptosis [26, 31, 32] and it appears for long term sedation as an alternative to midazolam and propofol [33, 34]. Advantages of dexmedetomidine are shorter ventilation and recovery times and lower hypertension and tachycardia. In particular, the shortening of the duration of ventilation seems to be relevant in connection with the higher oxygen toxicity by prolonged ventilation and the associated development of chronic lung disease and the consequent motor and cognitive deficits in preterm born infants. First results of clinical trials in preterm infants revealed a decrease in the duration of mechanical ventilation by half and no need for additional sedative administration [35, 36].

The aim of this study was to investigate the effect of different concentrations of dexmedetomidine to the immature brain in a neonatal rat model of oxygen toxicity on neurodegeneration, oxidative stress markers, and the expression of the proinflammatory cytokine IL-1β.

2. Materials and Methods

2.1. Animals and Drug Administration. All procedures were approved by the State Animal Welfare Authorities (LAGeSo G0145/13) and followed institutional guidelines. Six-day-old Wistar rats from time-pregnant mothers were obtained from Charité-Universitätsmedizin Berlin (Germany) and randomly assigned to cages and treatment.

Dexmedetomidine (DEX; dexdor, Orion Pharma, Espoo, Finland) was dissolved in phosphate buffered saline. Three doses of the drug (1, 5, and 10 μg/kg body weight) were used and all injections were given intraperitoneally (i.p.) as a fixed proportion of body weight (100 μL/10 g). The rat pups are divided into different biological groups (description with the relevant experimental abbreviations): (1) control group (CON; 21% O_2, room air) with 0,9% saline, (2) verum group (21% O_2) with 1 μg/kg DEX (DEX1), (3) verum group with 5 μg/kg DEX (DEX5), (4) verum group with 10 μg/kg DEX (DEX10), (5) hyperoxia group (HY; 80% O_2, OxyCycler BioSpherix, Lacona, NY) with 0,9% saline, (6) hyperoxia with 1 μg/kg DEX (HYDEX1), (7) hyperoxia with 5 μg/kg DEX (HYDEX5), and (8) hyperoxia with 10 μg/kg DEX (HYDEX10), each with a number of six animals per group and different gender. For hyperoxia or normoxia exposure, pups were kept together with their dams. Saline or DEX was administrated once 15 min before the start of oxygen exposure.

2.2. Tissue Preparation. At 24 h of exposure the animals were anaesthetized with an i.p. injection of ketamine (50 mg/kg), xylazine (10 mg/kg), and acepromazine (2 mg/kg) 5 min before being perfused. For molecular analysis, pups were transcardially perfused with normal saline (pH 7.4) and then decapitated, the olfactory bulb and cerebellum were removed, and brain hemispheres were snap-frozen in liquid nitrogen and stored at −80°C. For immunohistochemical analysis, animals were perfused with PBS followed by perfusion with 4% paraformaldehyde at pH 7.4 and the brains were postfixed at 4°C for 3 days, embedded in paraffin, and processed for histological staining.

2.3. DNA Fragmentation Assay. Paraffin-embedded sections were cut (5 μm), deparaffinized in Roti-Histol (Carl Roth, Karlsruhe, Germany) twice for 10 min each, rehydrated in descending ethanol series, and rinsed in phosphate buffered saline for 3 min each at room temperature. After deparaffinization of sections an *in situ* detection of cells with DNA-strand breaks was performed by the TUNEL labeling method using a TdT-FragEL DNA fragmentation detection kit (Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Negative controls were performed by substituting Tris-buffered saline for the TdT enzyme.

The TUNEL positive cells were analyzed in frontal cortex (FC), retrosplenial cortex (RSC), hypothalamus (HTH), thalamus (TH), and the hippocampus. Sections were viewed by light microscopy while blinded using a LEICA DM 2000 microscope equipped with a 200x magnification. TUNEL positive cells were counted in the anatomical regions of the brain in up to twelve different sections per animal and region.

2.4. Determination of Total Glutathione (GSH and GSSG). Total glutathione (GSH and GSSG) was measured in brain homogenates using the thiol reagent 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) as shown previously [14]. In brief, for the determination of reduced glutathione (GSH) and oxidized glutathione (GSSG), the brains were homogenized and the homogenates were treated with a mixture of metaphosphoric acid, EDTA, and NaCl. After centrifugation, aliquots were taken for neutralization with disodium hydrogen phosphate followed by addition of DTNB. GSH was determined after reaction with DTNB in a spectrophotometer at 412 nm. For the determination of GSSG 4-vinylpyridine was added and then incubated for 1 hour at room temperature. 4-Vinylpyridine is able to mask the GSH content without interfering with the spectrophotometrical determination of GSSG at 412 nm. GSH and GSSG levels are reported as nmol/mg protein.

2.5. Measurement of Lipid Peroxidation. Lipid peroxidation was determined by the reaction of thiobarbituric acid with malondialdehyde (MDA), a product of lipid breakdown caused by oxidative stress as previously described [14]. A SUPELCOSIL LC-18-DB HPLC reversed-phase column (Sigma-Aldrich, Munich, Germany; 5 μm particle size, 250 × 10 mm I.D.) was utilized for the detection of MDA levels in brain homogenates. The MDA level was determined by fluorescence (525/550 nm) with a 50 mM potassium phosphate buffer (pH 6.8) and 40% methanol mobile phase at 1.5 mL/min flow rate.

2.6. RNA Extraction and Semiquantitative Real-Time PCR. Total RNA was isolated from snap-frozen tissue by acidic phenol/chloroform extraction (peqGOLD RNAPure; PEQLAB Biotechnologie, Erlangen, Germany) and 2 μg of RNA was reverse-transcribed. The PCR products of *IL-1β* and *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*, as internal standard) were quantified in real time, using dye-labeled fluorogenic reporter oligonucleotide probes and primers (Metabion, Munich, Germany) with the following sequences and corresponding GenBank accession numbers: *IL-1β* (NM_031512) sense 5′-AACAAAAATGCCTCGTGCTGTCT-3′, antisense 5′-TGTTGGCTTATGTTGTGTCCATTG-3′, probe 5′-ACCCATGTGAGCTGAAAGCTCTCC-3′; *HPRT* (NM_012583) sense 5′-GGAAAGAACGTCTTGATTGTTGAA-3′, antisense 5′-CCAACACCTTGAGAGGTCCTTTT-3′, and probe 5′-CTTTCCCTTGGTCAAGCAGTACAGCCCC-3′. All probes were labeled at their 5′ ends with the reporter dye 6-carboxyfluoresceine (FAM) and at their 3′ ends with the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA). Real-time PCR and detection were performed in triplicate and repeated 3 times for each sample using a total reactive volume of 13 μL which contained 6,5 μL of 2x TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2.5 μL of 1.25 μM oligonucleotide mix, 0,5 μL (0,5 μM) of probe, and 50 ng of cDNA template. The PCR amplification was performed in 96-well optical reaction plates for 40 cycles with each cycle at 94°C for 15 s and 60°C for 1 min. The expression of *IL-1β* and *HPRT* was analyzed with the real-time PCR ABI Prism 7500 sequence detection system (Applied Biosystems) according to the $2^{-\Delta\Delta CT}$ method [37].

2.7. Immunoblotting. Snap-frozen brain tissue was homogenized in RIPA buffer solution for protein extraction. The homogenate was centrifuged at 1,050 g (4°C) for 10 min, and the microsomal fraction was subsequently centrifuged at 17,000 g (4°C) for 20 min. After collecting the supernatant, protein concentrations were determined using the Pierce BCA kit (Pierce, Rockford, IL, USA) with a 30 min incubation at 37°C prior to spectrophotometry at 562 nm. Protein extracts (30 μg per sample) were denatured in Laemmli sample loading buffer at 95°C, separated by 15% of sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrotransferred in transfer buffer to a nitrocellulose membrane (0.2 μm pore, Protran; Schleicher & Schüll, Dassel, Germany). Nonspecific protein binding was prevented by treating the membrane with 5% nonfat dry milk in Tris-buffered saline/0.1% Tween 20 for 1 h at room temperature. Equal loading and transfer of proteins was confirmed by staining the membranes with Ponceau S solution (Fluka, Buchs, Switzerland). The membranes were incubated overnight at 4°C with rabbit polyclonal anti-IL-1β (17 kDa; 1 : 1000; PromoKine, Heidelberg, Germany). Horseradish peroxidase-conjugated secondary anti-rabbit antibody was diluted 1 : 2000 (Amersham Biosciences, Bucks, United Kingdom). Positive signals were visualized using the SuperSignal West Pico kit (Pierce) according to the manufacturer's directions and quantified using a ChemiDoc XRS+ system and the software Image Lab (Bio-Rad, Munich, Germany). Membranes were stripped and then washed, blocked, and reprobed overnight at 4°C with mouse anti-β-actin monoclonal antibody (42 kDa; 1 : 10.000; Sigma-Aldrich). Each experiment was repeated three times.

2.8. Statistical Analyses. All data are expressed as mean ± standard error of the mean (SEM). Groups were compared using a one-way analysis of variance (ANOVA), and significance was determined using Bonferroni's correction for multiple comparisons with independent sample *t*-test. A two-sided P value < 0.05 was considered to be significant. All graphics and statistical analyses were performed using the GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA).

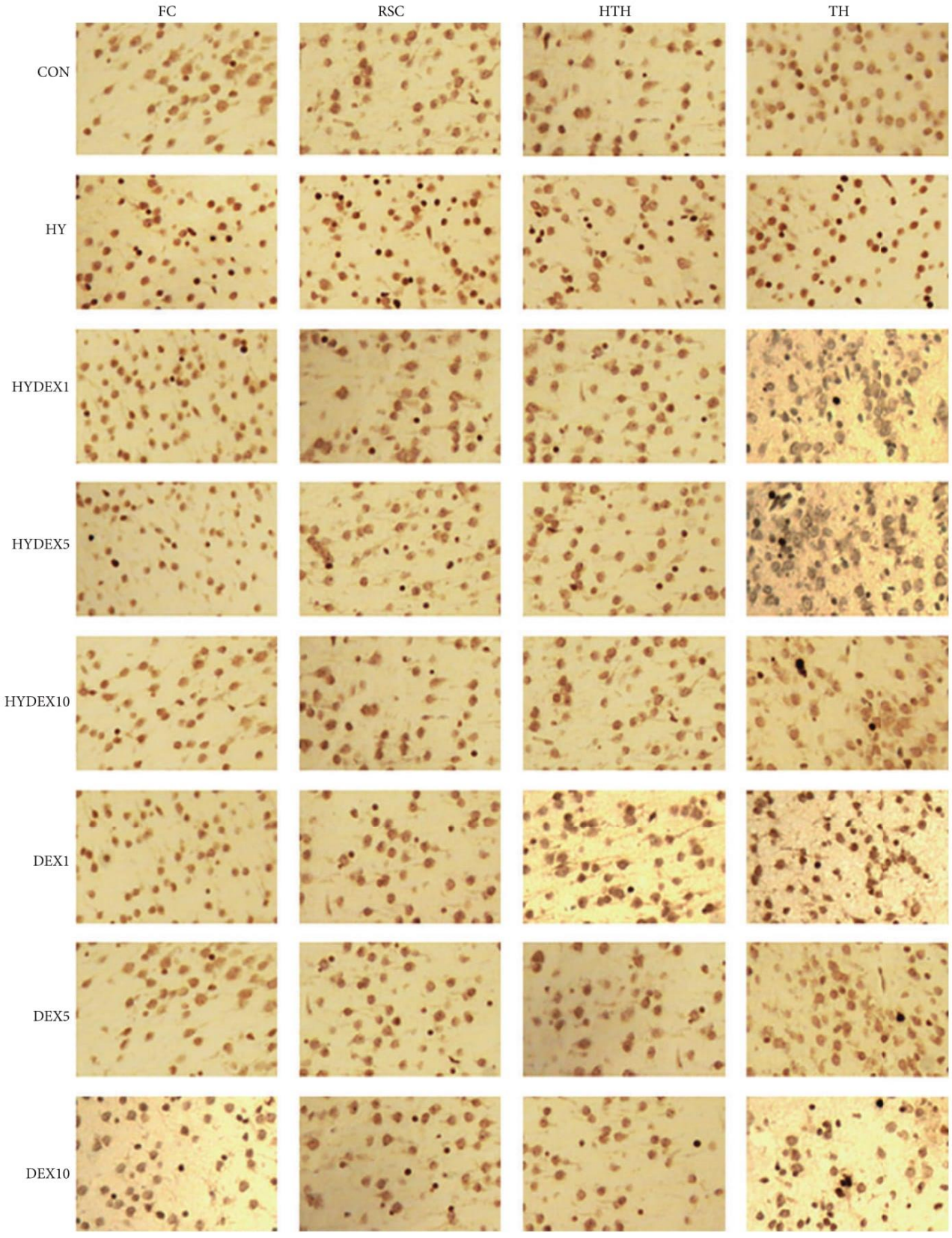
3. Results

3.1. Dexmedetomidine Ameliorates Hyperoxia-Induced Neurodegeneration in the Infant Brain. That oxidative stress is a trigger of cell death is well known. We have investigated the changes in oxidative stress on the apoptotic cell death by TUNEL assay. These investigations were carried out in the frontal cortex (FC) and retrosplenial cortex (RSC), in the deep gray matter (hypothalamus (HTH), thalamus (TH)), and in the hippocampus.

In detail, exposure to 24 h of hyperoxia (HY) from P6 to P7 resulted in a large increase of TUNEL positive cells and the antiapoptotic ability of dexmedetomidine was able to be detected by a significant decrease in the investigated different concentrations (FC: HY 502.9 + 13.0% versus HYDEX1 230.7 + 19.5%, HYDEX5 155.0 + 27.2%, and HYDEX10 62.1 + 7.2%; RSC: HY 448.3 + 25.3% versus HYDEX1 196.8 + 31.2%, HYDEX5 203.4 + 24.6%, and HYDEX10 142.2 + 25.9%; HTH: HY 502.9 + 45.0% versus HYDEX1 142.6 + 16.9%, HYDEX5 169.9 + 32.5%, and HYDEX10 138.7 + 16.2%; TH: HY 442.4 + 42.3% versus HYDEX1 195.5 + 33.2%, HYDEX5 215.6 + 18.7%, and HYDEX10 197.5 + 41.9%) compared to control animals (CON; Figures 1(a) and 1(b)). Particularly DEX10 under hyperoxic conditions can reduce apoptotic cell rate in the cortex and hypothalamus to control level and significantly among them in the frontal cortex. In the thalamus, a significant reduction by DEX under oxygen exposure was demonstrated but did not reach the controls. DEX under normoxic conditions showed a significant increase of TUNEL positive cells in the cortices with DEX10 and in the thalamus with DEX all over (Figure 1(b)). The results of the dentate gyrus show a similar antiapoptotic effect of DEX (data not shown). A statistical evaluation of the dentate gyrus was not possible due to low cell counts.

3.2. Treatment with Dexmedetomidine Modifies Hyperoxia-Affected Levels of Reduced (GSH) and Oxidized Glutathione (GSSG) in the Developing Brain. As we showed previously, neonatal oxygen toxicity causes an imbalance of the glutathione-redox-system correlating with an increase of neurodegeneration in the immature brain [12–15].

Analysis of GSH and GSSG levels by reaction with the classical thiol reagent DTNB and spectrophotometrical measurement at 412 nm was performed in samples from total brain extracts of 7-day-old rats ($n = 6$ per group) exposed



(a)

Figure 1: Continued.

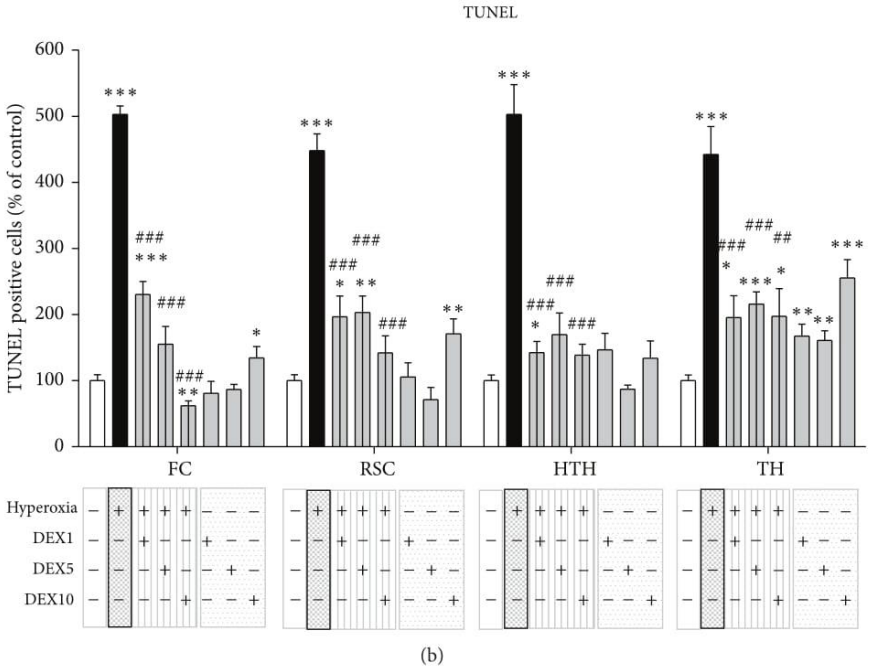


Figure 1: Apoptosis caused by hyperoxia is prevented by dexmedetomidine. (a) Representative TUNEL staining images (original magnification ×400) of rat brain frontal cortices (FC), retrosplenial cortices (RSC), hypothalamus (HTH), and thalamus (TH) of P7 control pups in room air without (CON) and with dexmedetomidine administration (DEX1, DEX5, and DEX10, corresponding to the concentrations of 1, 5, and 10 μg/kg) and after 24 h of hyperoxia from P6 to P7 without (HY) and with dexmedetomidine administration (HYDEX1, HYDEX5, and HYDEX10). (b) Quantitation of TUNEL positive cells in the rat brain frontal cortices (FC), retrosplenial cortices (RSC), hypothalamus (HTH), and thalamus (TH) showed that relative to the control (white bars) hyperoxia at 24 h significantly increased these cell counts in cortex and deep grey matter (black bars). These levels were significantly decreased through systemic dexmedetomidine pretreatment (hatched grey bars; DEX 1, 5, and 10 μg/kg). However, dexmedetomidine administration resulted in increased TUNEL positive cells in control rats most profound in TH (grey bars; DEX 1, 5, and 10 μg/kg). Data are expressed relative to the normoxia-exposed control group (white bars, 100%). Bars represent mean + SEM; $n = 6$ per group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus normoxia/control; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus hyperoxia.

to (i) normoxia and saline injections (CON), (ii) hyperoxia and saline injections (HY), (iii) hyperoxia and DEX (HYDEX; 1, 5, or 10 μg/kg), and (iv) normoxia and DEX (DEX; 1, 5, or 10 μg/kg). 24 h of hyperoxia triggered the decrease of GSH levels in brain extracts (Figure 2(a): CON: 11.42 + 0.11 nmol/mg protein and HY: 9.79 + 0.06 nmol/mg protein). When DEX was administered together with hyperoxia, it significantly increased the levels of GSH at a concentration of 5 and 10 μg/kg DEX (HYDEX5: 10.19 + 0.06 nmol/mg protein and HYDEX10: 11.16 + 0.09 nmol/mg protein).

Figure 2(b) shows the increased level of GSSG in the developing brain after 24 h of hyperoxia (CON: 0.273 + 0.003 nmol/mg protein and HY: 0.046 + 0.007 nmol/mg protein). DEX coapplication reduced the hyperoxia-induced increase of GSSG levels significantly at 5 and 10 μg/kg DEX (HYDEX5: 0.398 + 0.008 nmol/mg protein and HYDEX10: 0.255 + 0.008 nmol/mg protein).

In total, hyperoxia triggered the decrease of the GSH/GSSG ratio in the developing rat brain (Figure 2(c): CON: 41.94 + 0.78 and HY: 21.52 + 0.40). When DEX was administered at the start of hyperoxia, the levels of GSH/GSSG ratio significantly increased at 5 and 10 μg/kg of DEX (HYDEX5: 25.63 + 0.60 and HYDEX10: 43.95 + 1.62).

3.3. Induction of Lipid Peroxidation by Hyperoxia in the Neonatal Brain Is Attenuated by Dexmedetomidine Treatment. To demonstrate that the changes obtained in the level of GSH and GSSG may be connected with an altered lipid peroxidation, we examined immature rat brains after treatment of hyperoxia, normoxia, dexmedetomidine, and/or normal saline treatment for the concentration of malondialdehyde (MDA). Increased MDA levels, as a sign of lipid breakdown, were evident in rat brains at 24 h of hyperoxia when compared to normoxic animals, whereas a single DEX administration before beginning hyperoxia exposure reduced these levels significantly at 5 and 10 μg/kg DEX (Figure 3: CON: 5.63 + 0.14 nmol/mg protein and HY: 14.57 + 0.16 nmol/mg protein; HYDEX5: 13.62 + 0.19 nmol/mg protein and HYDEX10: 10.18 + 0.31 nmol/mg protein).

3.4. Dexmedetomidine Treatment Reduces IL-1β Expression under Hyperoxic Conditions in the Infant Brain. As previously shown by our group hyperoxic conditions lead to an increase in IL-1β expression in the immature brain [13, 15, 16].

Hyperoxia triggered an increase of *IL-1β* mRNA in brain homogenates of neonatal rats as shown by semiquantitative real-time PCR (Figure 4(a): HY: 465.99 + 64.69%). Control

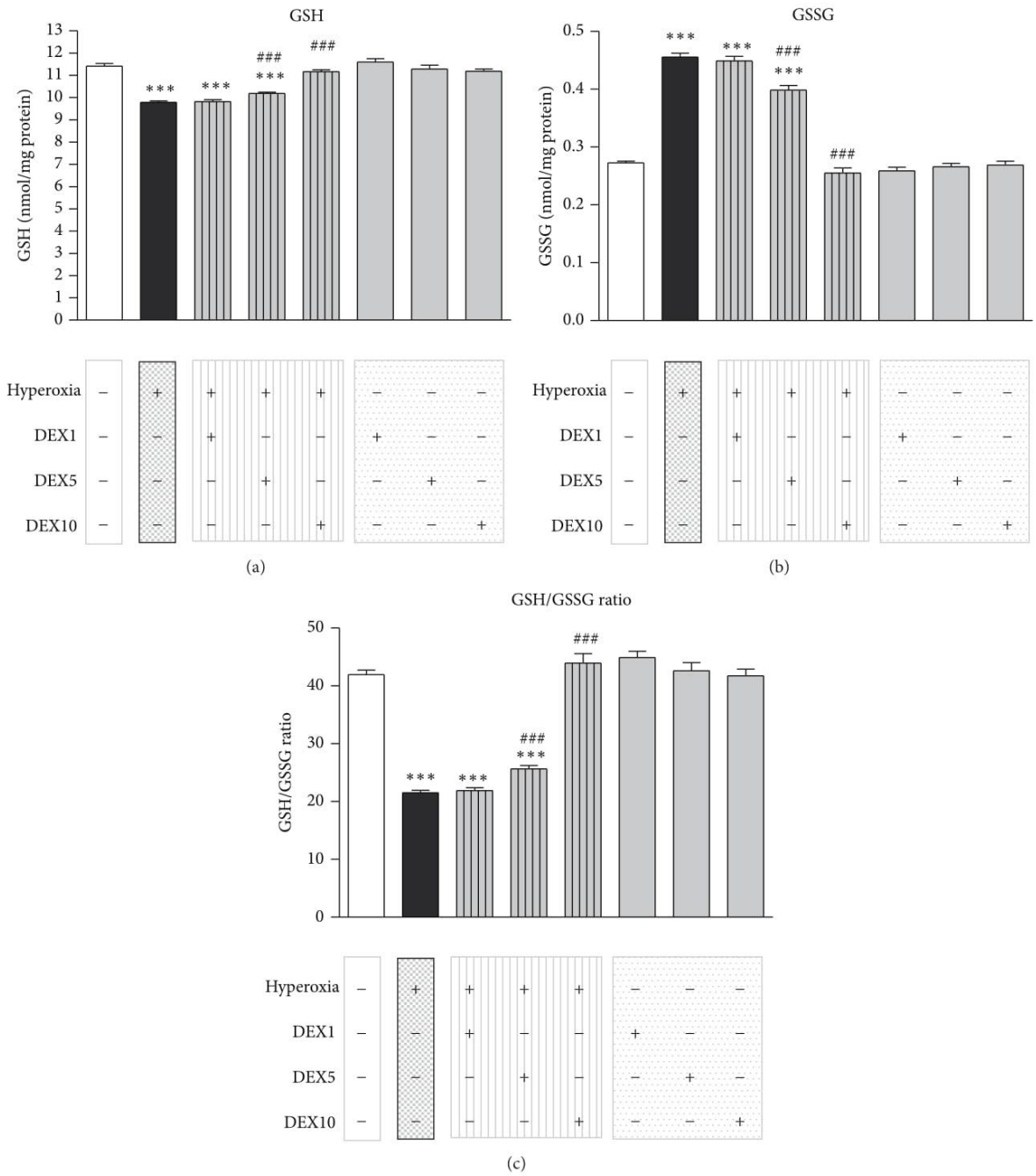


Figure 2: Effect of dexmedetomidine on hyperoxia-modified GSH and GSSG levels in the developing brain. (a) Reduced GSH levels were evident in total rat brain extracts 24 h after the initiation of hyperoxia (black bar) when compared to normoxic animals (white bar). These levels were increased through dexmedetomidine (DEX) pretreatment in a concentration dependent manner (hatched grey bars: 1, 5, and 10 μg/kg). (b) Increased levels of oxidized GSSG were obvious in total brain extracts at 24 h of hyperoxia (black bar) when compared with normoxic control animals (white bar). These levels were decreased through pretreatment with DEX (hatched grey bars: 1, 5, and 10 μg/kg). (c) Reduced GSH/GSSG ratio levels were evident in rat brain extracts at 24 h of hyperoxia (black bar) when compared to normoxic control animals (white bar). These levels were upregulated through DEX pretreatment (hatched grey bars: 1, 5, and 10 μg/kg). Application of dexmedetomidine under room air (grey bars) showed no effect on GSH or GSSG levels. Bars represent mean + SEM; $n = 6$ per group; $^{***}P < 0.001$ versus normoxia/control; $^{###}P < 0.001$ versus hyperoxia.

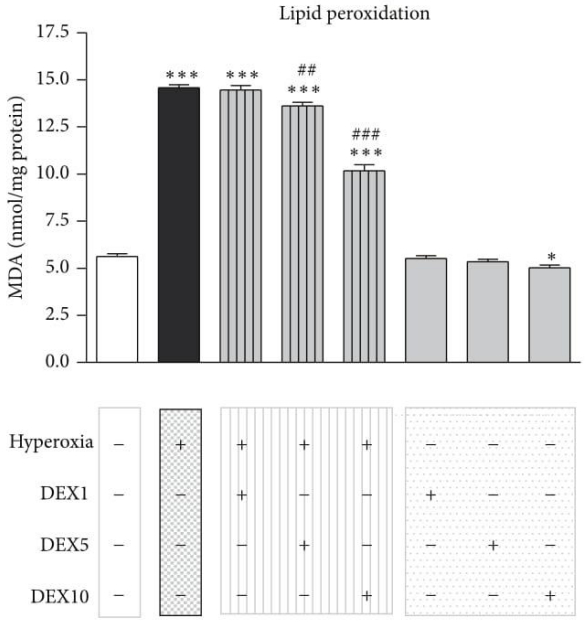


FIGURE 3: Alteration of lipid peroxidation by hyperoxia in the immature brain. Hyperoxia lead to a significant increase of MDA levels after 24 h of oxygen exposure (black bar), whereas a single DEX application of 5 or 10 μg/kg (hatched grey bars) before hyperoxia exposure reduced these levels significantly. Bars represent mean + SEM, $n = 6$ per group, $^{*}P < 0.05$ and $^{***}P < 0.001$ versus normoxia/control; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus hyperoxia.

animals showed low mRNA levels of *IL-1β* (CON: 100.36 + 11.41%). When dexmedetomidine was administered together with hyperoxia, it significantly ameliorated the expression of proinflammatory *IL-1β* depending on the DEX concentration (HYDEX5: 310.29 + 21.77% and HYDEX10: 177.66 + 22.33%) on mRNA levels in rat pups. Pretreatment with DEX in animals kept under normoxic conditions had no effect on *IL-1β* gene expression (DEX1: 117.18 + 11.58%, DEX5: 121.41 + 9.14%, and DEX10: 135.31 + 12.85%).

Western blotting demonstrated that protein expression of IL-1β is significantly increased following oxygen treatment for 24 h (Figure 4(b): HY: 336.66 + 17.25%) compared to control animals (CON: 100.00 + 7.31%). A significant effect on IL-1β protein levels was seen upon a single dexmedetomidine application of 5 or 10 μg/kg DEX in hyperoxia-exposed animals (HYDEX5: 250.96 + 13.55% and HYDEX10: 171.05 + 12.27%). Animals under normoxic conditions and DEX pretreatment showed low protein levels of IL-1β (DEX1: 111.95 + 4.43%, DEX5: 107.86 + 5.15%, and DEX10: 112.44 + 5.43%).

4. Discussion

In the present study, we demonstrate that the α2-adrenoceptor agonist dexmedetomidine leads to a decrease of neurodegeneration and affects the level of oxidative stress parameters and the proinflammatory cytokine IL-1β in a model of neonatal hyperoxia-induced brain injury.

As shown previously oxygen toxicity leads to an increased neurodegeneration in the immature brain [12, 16, 17, 38]. In line with these studies here we indicate that an exposure of six-day-old rats to a high oxygen concentration (FiO_2 80%) over 24 h resulted in an increase of neurodegeneration to the neonatal brain (Figures 1(a) and 1(b)). Moreover, we point out a significant decrease of cell death in the developing rat brain when infant rats were treated intraperitoneally with 1, 5, or 10 μg/kg dexmedetomidine before exposure to high oxygen conditions. These dosages are at/under the lower levels of dexmedetomidine concentrations used in common adult rat models of neuronal damage [39, 40] and the present data are not intended to demonstrate the evidence of an effective dose. However, our findings conform with different other animal studies which examined the neuroprotective capacity of dexmedetomidine [22–27]. Hyperoxia-exposed rats revealed an up to 5-fold increase in cell degeneration within the cortex and deep gray matter (Figure 1(b)), which has been demonstrated in previous studies [17, 41]. Interestingly, dexmedetomidine treatment showed a profound decrease of degenerating cells in all analysed brain regions of rats exposed to hyperoxia (Figure 1(b)). Dexmedetomidine alone induced no oxidative stress, but at a relatively low concentration of dexmedetomidine under normoxic conditions, a significant increase in apoptosis in the cortex and the thalamus has been shown. This result is controversial to other inventions [31, 32, 42] and to the description of only protective and no toxic effects [24, 25, 43]. *In vitro* it was shown by Kuhmonen et al. [22] that dexmedetomidine is more effective at lower doses and protects against delayed cell death. In an ischemia-reperfusion model [39] antiapoptotic proteins were upregulated and proapoptotic proteins are suppressed by dexmedetomidine treatment. In the study by Li et al. there are no changes in the expression of proapoptotic mediators in an isoflurane-induced model of neuroapoptosis [40]. A proven high blood pressure after the administration of high-dose dexmedetomidine correlates with cerebral hypoperfusion may be due to alpha-2-induced cerebral vasoconstriction [44], so that this could contribute to apoptotic cell death triggered by other cellular mechanisms. However, in our study dexmedetomidine also led to negative effects in control animals notably in the thalamus, and potential side effects should be further considered in studies on protection of the immature brain using dexmedetomidine.

The potency of dexmedetomidine in this model of oxygen toxicity indicates that the α2-adrenoceptor agonist has antioxidant activities. Of note, our hyperoxia model is predominantly based on free radical generation, as we previously found an induction of different oxidative stress parameters [12–15]. Therefore, we evaluated the effects on biochemical markers of oxidative stress in the developing brain. The concentrations of GSH and GSSG, the GSH/GSSG ratio, and the MDA level were used as indicators of oxidative stress and lipid breakdown. Hyperoxia-exposed P6 rats showed significant decreased GSH and increased GSSG levels at 24 h of hyperoxia. In addition, a decrease in GSH/GSSG ratio and an increase in MDA level in the developing brain indicate neuronal damage due to oxidative stress, but there was a modulation of the levels of all parameters

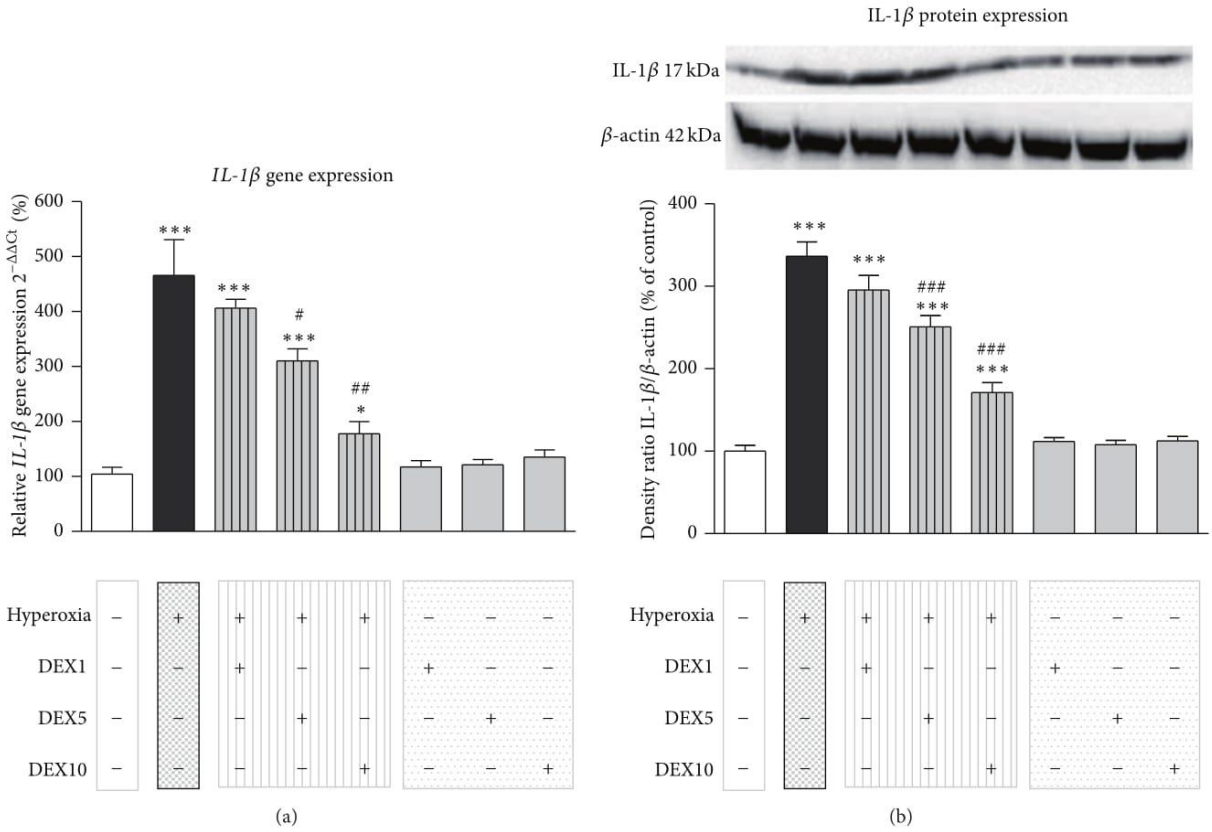


FIGURE 4: (a) Quantitative analysis of mRNA expression by real-time PCR showed a marked increase of *IL-1β* mRNA expression in the brain of P6 rat pups that were kept for 24 h under hyperoxia (black bar), whereas dexmedetomidine treatment restores *IL-1β* upon control level (hatched grey bars) depending on the dexmedetomidine concentration. Application of dexmedetomidine under room air (grey bars) showed no significant regulation on *IL-1β* mRNA expression. (b) The analysis of IL-1β protein expression by western blot showed a similar expression pattern. The protein expression of IL-1β is significantly increased after 24 h of hyperoxia and a single application of 5 or 10 μg/kg dexmedetomidine could restore the IL-1β protein expression almost up to control level. The densitometric data represent the ratio of the pixel intensity of the IL-1β band to the corresponding β-actin band. Blots are representative of a series of three blots. Data are normalized to levels of rat pups exposed to normoxia (CON = 100%, white bars). Bars represent mean + SEM; $n = 6$ per group; $^{***}P < 0.001$ versus normoxia/control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus hyperoxia.

up to normoxia levels in hyperoxia-exposed neonatal rats that received dexmedetomidine (Figures 2 and 3). These observations suggest that dexmedetomidine seemingly has an antioxidant activity against hyperoxia-induced oxidative stress in the developing brain. In line with our findings there are several studies that suggest an increased antioxidative capacity of dexmedetomidine in the brain [24] and further organs (reviewed by Tse et al. [45]).

This study indicates that dexmedetomidine significantly decreases hyperoxia-induced IL-1β upregulation in the developing rat brain. These results illustrate the protective effect of dexmedetomidine on neuroinflammation as shown before in clinical and experimental studies [46, 47]. Dexmedetomidine itself did not affect IL-1β levels, indicating that the α2-adrenoceptor agonist only acts to normalize the induced proinflammatory cytokine (Figure 4). Here, dexmedetomidine appears to inhibit the production of inflammatory mediators from multiple cell types, for example, astrocytes and microglia [48, 49].

There are several limitations of this study pointing to areas of future investigations. Dexmedetomidine was given only once to the animals, while preterm infants receive an initial loading dose and subsequent infusions [50] and the high doses of dexmedetomidine studied here exceed those doses recommended for clinical use; short term hyperoxia was examined and preterm infants are often exposed to a longer period of supraphysiological oxygen concentrations [51], and we have not determined the plasma levels of dexmedetomidine. Further details of dexmedetomidine-induced side effects remain to be elucidated.

To our knowledge this is the first report demonstrating that dexmedetomidine mediates a decrease of neurodegeneration and oxidative stress and affects the expression of the proinflammatory cytokine IL-1β in the hyperoxia-exposed neonatal brain. Since oxidative stress and inflammation are involved in dysfunction of the immature brain under high oxygen conditions, α2-adrenoceptor activation seems to be a potential neuroprotective treatment.

5. Conclusion

The essential core of this work is the finding that dexmedetomidine prevents oxidative stress, inflammation, and cell death in the neonatal brain under hyperoxic conditions. Exposure to high oxygen in the developing brain of six-day-old rat pups leads to increased oxidative stress-induced and inflammatory DNA damage, as demonstrated by an increased apoptotic rate, a reduction of the GSH/GSSG ratio, increased lipid peroxidation, and increased IL-1β levels. Remarkably, a single dose of dexmedetomidine has weakened or abolished all these detrimental effects under hyperoxic conditions. However, in this study dexmedetomidine also shows negative effects in the control animals, and these potential side effects should be taken into consideration in further studies on the protection of the immature brain with dexmedetomidine.

Based on these data the hyperoxic model indicates dexmedetomidine as a sedative in pediatric anesthesia and a possible promising agent for neuroprotective strategies in preterm infants.

Conflict of Interests

The authors declare that there is no conflict of interests with any financial organization regarding the commercial identities mentioned in the paper.

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Acetylcholinesterase Inhibitors Reduce Neuroinflammation and -Degeneration in the Cortex and Hippocampus of a Surgery Stress Rat Model

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Abstract

Exogenous stress like tissue damage and pathogen invasion during surgical trauma could lead to a peripheral inflammatory response and induce neuroinflammation, which can result in postoperative cognitive dysfunction (POCD). The cholinergic anti-inflammatory pathway is a neurohumoral mechanism that plays a prominent role by suppressing the inflammatory response. Treatments with acetylcholinesterase inhibitors enhance cholinergic transmission and may therefore act as a potential approach to prevent neuroinflammation. In the presence or absence of acetylcholinesterase inhibitors, adult Wistar rats underwent surgery alone or were additionally treated with lipopolysaccharide (LPS). Physostigmine, which can overcome the blood-brain barrier or neostigmine acting only peripheral, served as acetylcholinesterase inhibitors. The expression of pro- and anti-inflammatory cytokines in the cortex, hippocampus, spleen and plasma was measured after 1 h, 24 h, 3 d and 7 d using Real-Time PCR, western blot analysis or cytometric bead array (CBA). Fluoro-Jade B staining of brain slices was employed to elucidate neurodegeneration. The activity of acetylcholinesterase was estimated using a spectrofluorometric method. Surgery accompanied by LPS-treatment led to increased IL-1beta gene and protein upregulation in the cortex and hippocampus but was significantly reduced by physostigmine and neostigmine. Furthermore, surgery in combination with LPS-treatment caused increased protein expression of IL-1, TNF-alpha and IL-10 in the spleen and plasma. Physostigmine and neostigmine significantly decreased the protein expression of IL-1 and TNF-alpha. Neuronal degeneration and the activity of acetylcholinesterase were elevated after surgery with LPS-treatment and reduced by physostigmine and neostigmine. Along with LPS-treatment, acetylcholinesterase inhibitors reduce the pro-inflammatory response as well as neurodegeneration after surgery in the cortex and hippocampus. This combination may represent a tool to break the pathogenesis of POCD.

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Introduction

Peripheral inflammation in response to major surgery or infection can affect the function of the central nervous system (CNS), including memory and cognition [1,2]. The activation of the immune system, by either lipopolysaccharide (LPS) administration or surgical trauma, has been shown to be capable of affecting hippocampal function thereby causing memory impairment [3,4]. Postoperative cognitive dysfunction (POCD) is a threatening complication after major surgery and is independently associated with increased mortality [5–7]. POCD seems to be a heterogeneous and multifactorial disorder with known risk factors including advanced age, duration of surgery and postoperative infection [7–11]. Major surgery does appear to be one principle culprit. Yet, increased inflammatory activity triggered by surgery may play a mean role in the pathogenesis of POCD [12–14].

Neural reflex circuits regulate cytokine release to prevent potentially inflammation and maintain homeostasis. Sensory input elicited by infection or injury moves through the afferent vagus nerve to the brainstem. Incoming signals generate action potentials that travel via efferent nerves from the brainstem to the spleen and other organs [15–22]. Neurotransmitters from the peripheral autonomic nerves subsequently promote the release of acetylcholine from a subset of CD4-positive T cells which activates alpha7 nicotinic acetylcholine receptors (α7 nAChR) on macrophages [23–25]. Acetylcholine attenuates the production of TNF-alpha, IL-1beta, IL-6 and IL-18 by macrophages at the posttranscriptional stage [16,26,27]. This mechanism is called the *inflammatory reflex* and triggers the cholinergic anti-inflammatory pathway, which in turn is a physiological neuroimmune mechanism that regulates innate immune function and controls inflammation [15,16,21,22]. Animal studies indicate that stimulation of the vagus nerve or administration of α7 nAChR agonists reduces pro-inflammatory cytokine production by 50–75% but does not eliminate their activity [23,28,29]. Target therapies that increase the activity of the *inflammatory reflex* as well as the anti-inflammatory pathway normalize innate immune responses without abolishing them or causing immunosuppression [15]. The levels of acetyl-

choline are continuously regulated by the hydrolytic enzyme acetylcholinesterase (AChE), which rapidly degrades acetylcholine in the periphery and the brain. AChE is expressed in cholinergic neurons and neuromuscular junctions as well as tissues that are not innervated by cholinergic neurons [30,31]. Acetylcholinesterase inhibitors prevents the Hacetylcholinesterase enzymeH from breaking down acetylcholine, thereby increasing both, the level and action time of the Hneurotransmitter acetylcholine [32]. It has been shown that pharmacologic cholinesterase inhibition improves survival in experimental sepsis [33]. The aim of this study was to investigate if AChE inhibitors reduce stress-induced neuroinflammation by maintaining acetylcholine. Furthermore, we investigated whether there is a difference between physostigmine which can cross the blood-brain barrier and neostigmine which does not enter the CNS. Here we show how surgery alone or combined with LPS-treatment with or without application of the AChE inhibitors physostigmine and neostigmine affects the expression of pro- and anti-inflammatory genes and proteins in different brain regions, spleen and plasma of adult rats. We demonstrate that surgery accompanied by LPS-treatment enhanced pro-inflammatory cytokines in the brain, spleen and plasma, and were reduced by physostigmine and neostigmine. Additionally, we provide evidence that surgery combined with LPS-treatment triggers neurodegeneration which is also affected by physostigmine and neostigmine.

Materials and Methods

Animal Model

Adult male Wistar rats (age: 10 weeks, weight: 250–300 g) underwent laparotomy alone to mimic human abdominal surgery or laparotomy combined with LPS-treatment in the presence or absence of physostigmine or neostigmine. The animals were kept at room temperature (22±2°C) under standard 12–12 h light–dark cycle. Food and water were available ad libitum except for the times of experiments.

Ethics Statement

All animal experiments were approved and performed in accordance with the guidelines of the Charité - Universitätsmedizin Berlin, Germany and the local ethics committee (Landesamt für Gesundheit und Soziales (LAGeSO), Berlin, Germany), ethical permit number: G 0253/09.

Surgical Procedure and Drug Treatment

Rats are spontaneously breathing and deeply anesthetized using isoflurane (2,3 expirative volume % and 100% oxygen) and meloxicam (Metacam®, Boehringer Ingelheim Pharma GmbH, Ingelheim, Germany), 0,2 mg/kg body weight (BW). After midline section LPS (1 mg/kg BW) was applied into the peritoneal cave and above the omentum majus. The animals received 100 µg/kg BW of physostigmine (Anticholium®, Dr. Franz Köhler Chemie, Alsbach-Hähnlein, Germany), or the same amount of neostigmine (Neostig®, Carinopharm GmbH, Gronau, Germany), after opening the abdominal wall and five minutes before the LPS-application. After surgical treatment the animals got three times (in the morning and in the evening) the same doses of subcutaneous physostigmine or neostigmine. The vehicle-treated animals received the equivalent dose NaCl 0.9% at the same time-points. The surgical procedure was performed by the same person and lasted approximately 15 minutes and while being operated the animals lay under an infra-red heating light.

Meloxicam (0.2 mg/kg BW) was administered intramuscularly before opening the abdominal wall. This medication was given intracutaneously once daily untill the third postoperative day. Under this analgesic treatment the animals showed mild signs of distress and recovered soon.

Tissue Sampling

After 1 h, 24 h, 3 d and 7 d the animals were sacrificed in deep isoflurane-oxygen-mixture narcosis. After thoracotomy blood was taken direct from the heart and the animals were dying via exsanguination. The organs were jetted with ice-cold phosphate-buffered saline and the brain was immediately removed. For Real-Time PCR and western blotting the spleen, hippocampal and cortical tissue of a half of the brain were snap-frozen in liquid nitrogen. The other brain hemisphere was deep frozen on dry ice for immunohistochemical methods. All samples were kept at −80°C until further processing.

Semiquantitative Real-Time PCR

Total cellular RNA from the hippocampus, cortex and spleen was isolated from snap-frozen tissue by acidic phenol/chloroform extraction and DNase I treatment (Roche Diagnostics, Mannheim, Germany). 500 ng of RNA was reverse transcribed at 42°C (1 h) with 200 U of Moloney murine leukemia virus reverse transcriptase and 2 µM oligo d(T) 16 primer (Promega, Madison, WI, USA) in 25 µl total reaction mixture. The resulting cDNA was amplified by Real-Time PCR. The oligonucleotide primers used for the PCR reactions of *TNF-alpha*, *IL-1beta* and *IL-10* and the internal standard *hypoxanthine–guanine phosphoribosyltransferase* (*HPRT*) are summarized in table 1. The expression of the target genes and the housekeeping gene *HPRT* were analyzed by Real-Time PCR using the ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) according to the $2^{-\Delta\Delta Ct}$ method [34].

Immunoblotting

The hippocampus, cortex and spleen were homogenized on ice in cold lysis buffer containing 10 mM Tris/HCl, pH 7.5, 300 mM NaCl, 1% Triton X-100, 2 mM $MgCl_2$, 5 µM EDTA, and the protease inhibitor cocktail, Complete Mini (Roche Diagnostics). The lysates were centrifuged at 13,000 g for 15 min at 4°C to remove debris. Protein concentration was determined using the bicinchoninic acid assay from Pierce (Rockford, IL, USA). Samples with equal amounts of protein were then separated by 10% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. After blocking in 5% low fat milk solution, the membranes were incubated over night (4°C) with a primary polyclonal rabbit anti-rat IL-1beta antibody (0.2 µg/ml, PromoKine, Heidelberg, Germany) or monoclonal mouse anti-rat β-actin (1:5.000, BD Biosciences, Heidelberg, Germany) in 5% low fat milk in PBST. Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse, Southern Biotechnology Associates, Birmingham, AL, USA) were diluted 1:25000 in PBST. Chemiluminescent detection was performed using an ECL west pico detection kit (Pierce).

Cytometric Bead Array

A panel of pro- and anti-inflammatory cytokines including IL-1alpha, TNF-alpha and IL-10 were simultaneously quantified by a multiplex bead array system (BD™ CBA Flex Sets, BD Biosciences) on a FACS Canto II flow cytometer (BD Biosciences). This assay provided micro-bead populations with

Table 1. Sequences of oligonucleotides and gene locus.

Gene	Oligonucleotide sequences 5′-3′	Gene Bank Accession No.
IL-10 forward reverse probe	gaa gac cct ctg gat aca gct gc tgc tcc act gcc ttg ctt tt cgc tgt cat cga ttt ctc ccc tgt ga	NM_012854
TNF-alpha forward reverse probe	tcg agt gac aag ccc gta gc ctc agc cac tcc agc tgc tc cgt cgt agc aaa cca cca agc aga	NM_012675
IL-1beta forward reverse probe	aac aaa aat gcc tcg tgc tgt ct tgt tgg ctt atg ttg tgt cca ttg acc cat gtg agc tga aag ctc tcc acc	NM_031512
HPRT forward reverse probe	gga aag aac gtc ttg att gtt gaa cca aca ctt cga gag gtc ctt tt ctt tcc ttc gtc aag cag tac agc ccc	NM_012583

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distinct fluorescent intensities and was precoated with capture antibodies specific for the cytokines. When the beads were incubated with the corresponding phycoerythrin conjugated detection antibodies and the test sample, sandwich complexes were formed. For our analysis, 50 µl of spleen tissue homogenate, plasma or the provided standard cytokines were added to the pre-mix micro-beads and incubated for 1 h in the dark at room temperature. After addition of 50 µl detecting reagent, the mixture was incubated for two hours in the dark at room temperature. This mixture was washed and centrifuged at 200 *g* for five minutes and the pellet was resuspended in 300 µl washing buffer. The FACS Canto II flow cytometer was calibrated with the setup beads, and 1500 events were acquired for each sample. The quantities of individual cytokines as indicated by their fluorescent intensities were computed using the standard reference curve of the CBA Software (BD Biosciences).

Immunohistochemistry

Brain sections (12 µm) were cut on a cryostat and the slices were mounted on a cover slide. The slides were incubated in a solution of 0.06% potassium permanganate for 20 min, rinsed in distilled water for 1 min, and transferred into Fluoro-Jade B (FJB) (Chemicon, Jefferson, AR, USA) staining solution (0.001% FJB/0.1% acetic acid) for 20 min. The slides were thereafter rinsed three times in distilled water and transferred into Hoechst 33342 staining solution (Sigma, Taufkirchen, Germany) for 5 min. The slides were rinsed three times in distilled water and air dried, then coverslipped with mounting media. The slides were visualized by fluorescent microscopy (Olympus, Hamburg, Germany) and digitally photographed. Because the Fluoro-Jade B staining was obvious on digital imaging, the number of Fluoro-Jade B-positive cells per section was quantified with ImageJ (Analysis program developed at the National Institutes of Health (NIH), Bethesda, MD, USA). For each measurement, two blinded independent investigators counted 6–8 brains per group, 3 sections per brain.

4BAcetylcholinesterase Assay

The Amplex Red Acetylcholine/Acetylcholinesterase assay kit from Molecular probes Inc. (Invitrogen, Karlsruhe, Germany) was employed to estimate acetylcholinesterase activity using a fluorescence microplate reader. A working solution of 400 µM Amplex Red reagent containing 2 U/mL horseradish peroxidase, 0.2 U/mL choline oxidase and 100 µM acetylcholine [33] was prepared from the stock solutions. The reaction began when 100 µl of the working solution was added to each well containing the samples and controls diluted 20×. Samples and controls were tested in duplicates. Fluorescence emitted by the individual samples was measured in a fluorescence microplate reader (Infinite® 200, Tecan, Crailsheim, Germany) at an excitation of 560 nm and emission detection at 590 nm. Background fluorescence was eliminated by subtracting values derived from the negative control. Using the standard curve, concentrations of acetylcholinesterase from the samples of different groups were calculated.

Statistical Evaluation

Experiments were performed in eight animals per group (n = 8). Values are presented as means ± standard error of the mean (SEM). Group effects were assessed by ANOVA followed by post hoc independent-sample t-test multiple comparison. p values are presented after Bonferroni correction. $P<0.05$ was considered as statistically significant.

Results

Surgery Combined with LPS-treatment is Associated with Increased IL-1beta Expression in the Cortex and Hippocampus which is Reduced by Physostigmine and Neostigmine

As shown in Fig. 1, surgery combined with LPS-treatment led to increase *IL-1beta* gene expression in the cortex after 1 h (Fig. 1A) and 24 h (Fig. 1B) and was significantly reduced by physostigmine and neostigmine application. After 3 days (Fig. 1C) and 7 days (data not shown) no comparable expression of *IL-1beta* was detectable. Furthermore, the upregulation of IL-1beta in the cortex was confirmed after 1 h at the protein level by western blot analysis (Fig. 1D–E). Surgery in combination with LPS-treatment caused elevated *IL-1beta* gene expression in the cortex. The cholinergic activation by physostigmine and neostigmine minimized levels of IL-1beta protein expression. Similar results are shown for the hippocampus (Fig. 2). Surgery and treatment with LPS enhanced *IL-1beta* gene expression after 1 h (Fig. 2A) and 24 h (Fig. 2B) and was significantly reduced by physostigmine and neostigmine. After 3 days (Fig. 2C) and 7 days (data not shown) no comparable expression of *IL-1beta* was detectable. Western blot analysis in samples from the hippocampus extracted 24 h postintervention confirmed the upregulation of IL-1beta protein expression after surgery combined with LPS-treatment and its inhibition by physostigmine and neostigmine (Fig. 2D–E). No changes in the expression of the cytokines TNF-alpha and IL-10 were detected in the cortex or in the hippocampus (data not shown).

Surgery Combined with LPS-treatment is Associated with Increased Gene Expression of Pro-inflammatory Cytokines in the Spleen which are Reduced by Physostigmine and Neostigmine

As shown in Fig. 3, surgery accompanied by LPS-treatment augmented *IL-1beta* (Fig. 3A) and *TNF-alpha* (Fig. 3B) gene expression in the spleen after 1 h. The cholinergic activation by physostigmine and neostigmine lowered levels of *IL-1beta* and *TNF-alpha* in the spleen. In comparison, after 24 h, 3 days and 7 days (data not shown) no increase of *IL-1beta* or *TNF-alpha* was detectable.

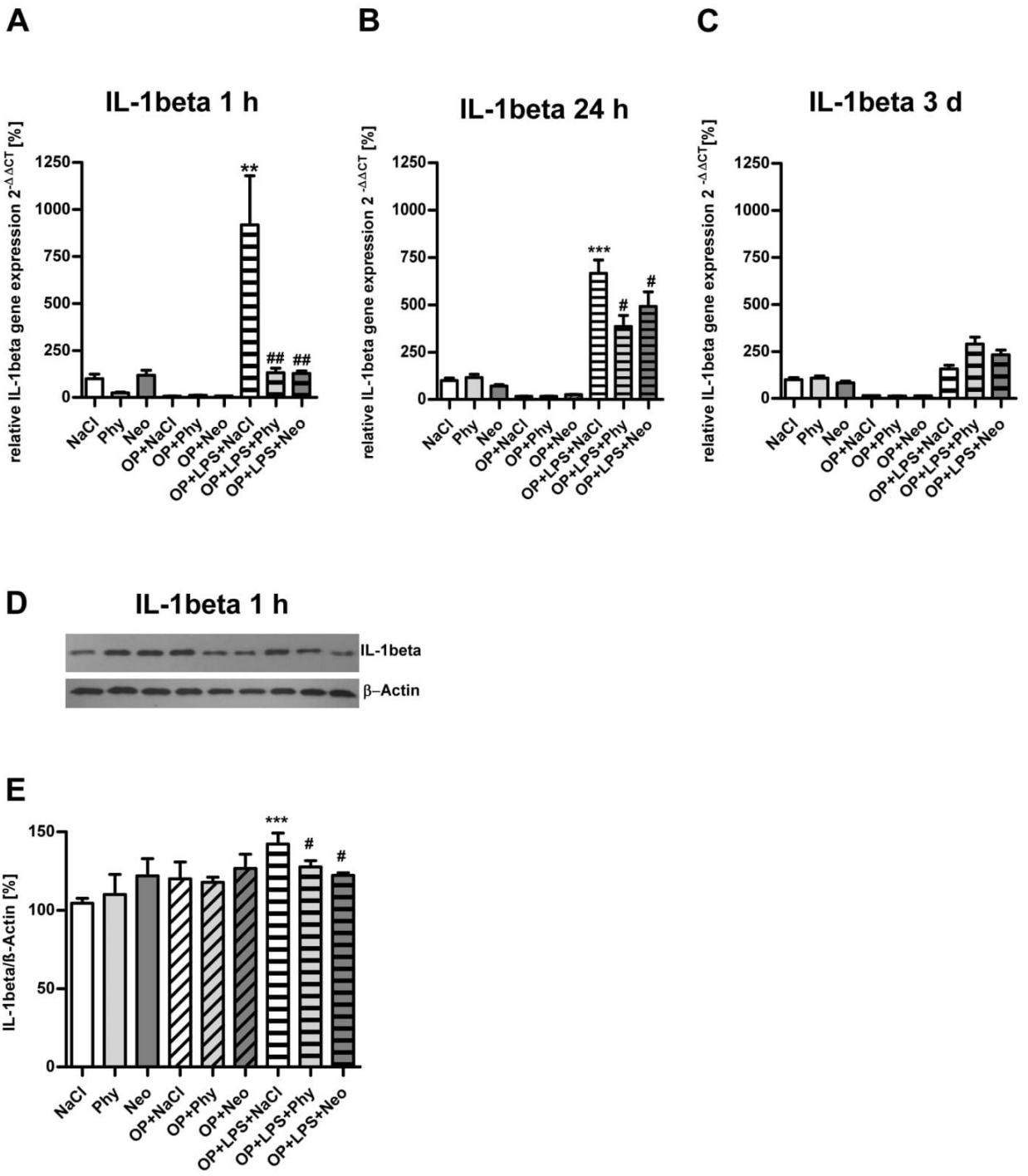

Figure 1. Physostigmine and neostigmine reduce surgery combined with LPS-induced *IL*-1beta expression in the cortex. IL-1beta expression was measured by quantitative Real-Time PCR in cortex samples extracted 1, 24, and 72 h postintervention (A–C). Also, IL-1beta expression was detected by western blot analysis in cortex samples extracted 1 h postintervention (D–E). Surgery combined with LPS-treatment resulted in an increased gene expression of *IL-1beta* after 1 and 24 h, which was decreased by physostigmine and neostigmine administration. Results of Real-Time PCR quantification are shown as mean ± SEM (n = 8 per group). Data are normalized to levels of saline treated rats (Control = 100%; bars represent mean ± SEM, n = 8 per group). Blots are representative of a series of three blots. The densitometric data represent the ratio of the IL-1beta band to the corresponding β-actin band density. ***$P<0.001$ and **$P<0.01$ represent the difference between LPS and saline treated groups. $^{\#\#}P<0.01$ and $^{\#}P<0.05$ represent the difference between LPS and physostigmine or neostigmine treated groups.
doi:10.1371/journal.pone.0062679.g001

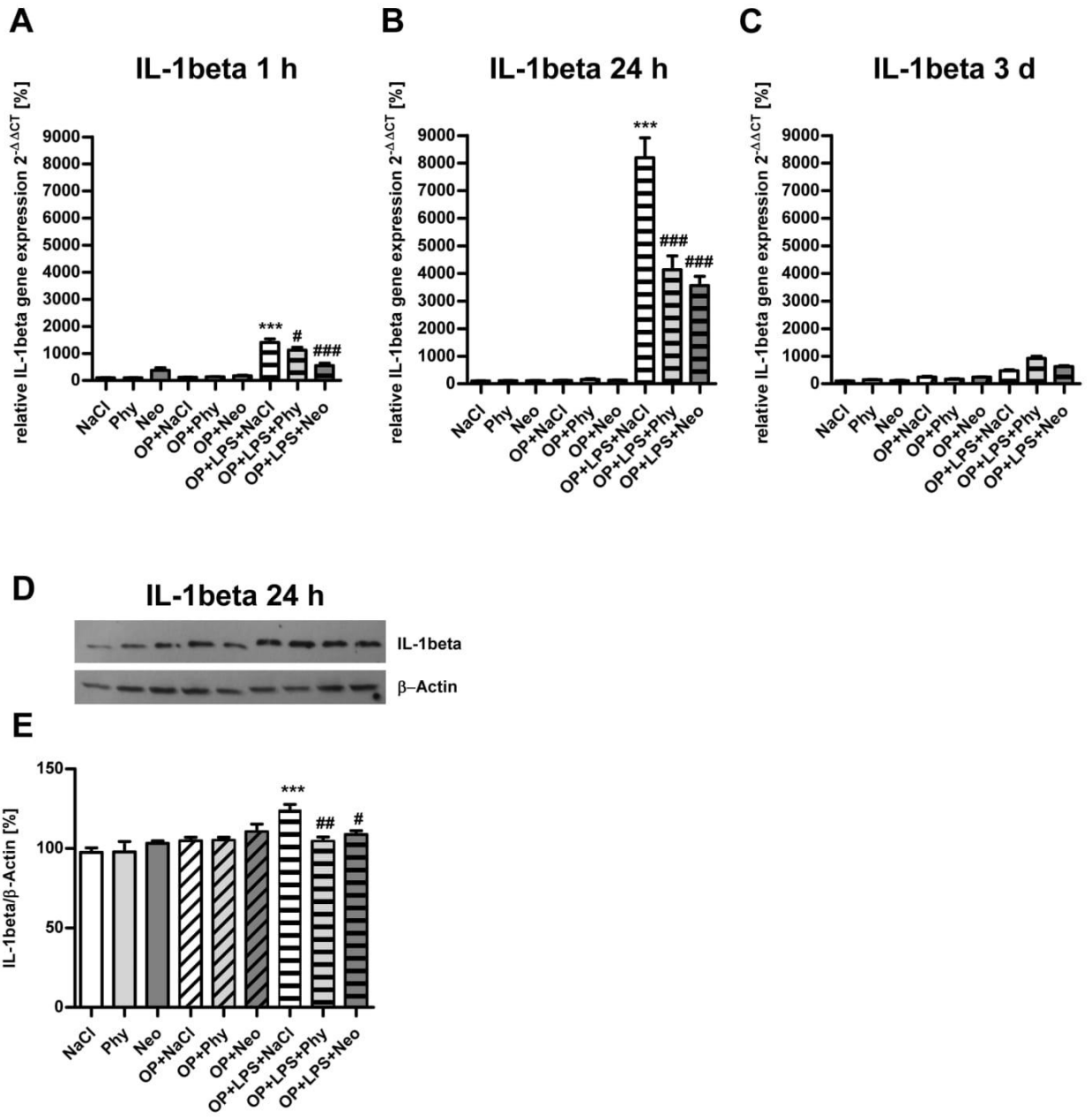

Figure 2. Physostigmine and neostigmine reduce surgery combined with LPS-induced IL-1beta expression in the hippocampus. IL-1beta expression was measured by quantitative Real-Time PCR in hippocampal samples extracted 1, 24, and 72 h postintervention (A–C). Additionally, IL-1beta expression was quantified by western blot analysis in hippocampal samples extracted 24 h postintervention (D–E). Surgery combined with LPS-treatment resulted in an increased expression of IL-1beta after 1 and 24 h and was reduced by physostigmine and neostigmine administration. Results of Real-Time PCR quantification are shown as mean ± SEM (n = 8 per group). Data are normalized to levels of saline treated rats (Control = 100%; bars represent mean ± SEM, n = 8 per group). Blots are representative of a series of three blots. The densitometric data represent the ratio of the IL-1beta band to the corresponding β-actin band density. ***$P<0.001$ represents the difference between LPS and saline treated groups. ###$P<0.001$, ##$P<0.01$, #$P<0.05$ represent the difference between LPS and physostigmine or neostigmine treated groups.
doi:10.1371/journal.pone.0062679.g002

The expression of the anti-inflammatory gene *IL-10* was upregulated after 1 h (Fig. 3C) and still strong after 24 h, however less pronounced (Fig. 3D) following surgery combined with LPS-treatment. In contrast to the downregulation of the pro-inflammatory cytokines, physostigmine and neostigmine significantly enhanced the gene expression of *IL-10* in the spleen but only after 1 h. Both AChE inhibitors have no effect on the expression of *IL-10* after 24 hours (Fig. 3D). After 3 and 7 days (data not shown) no increase of *IL-10* was detectable.

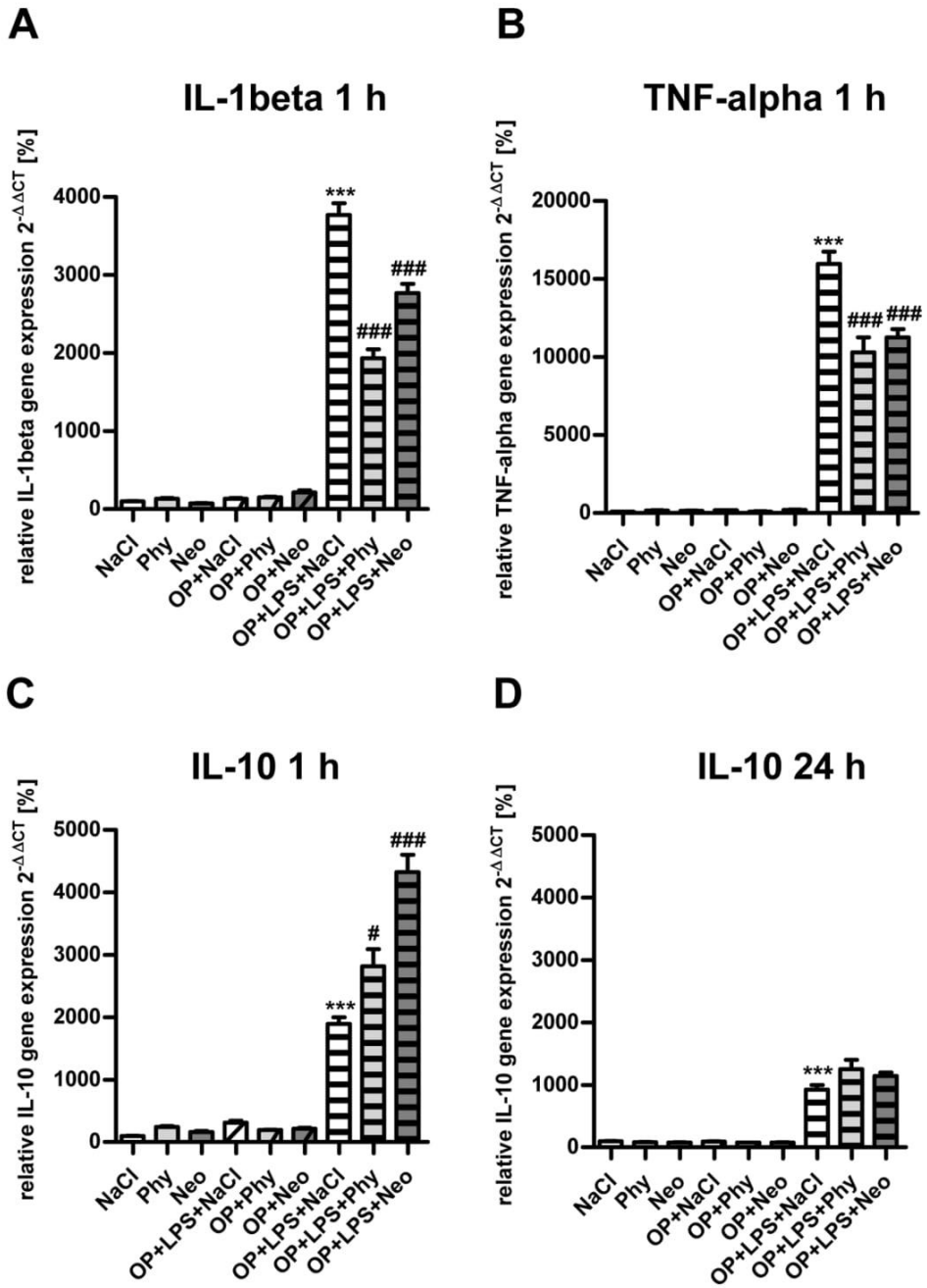

Figure 3. Physostigmine and neostigmine reduce surgery combined with LPS-induced *IL-1beta* and *TNF-alpha* gene expression in the spleen. *IL-1beta* (A) and *TNF-alpha* (B) expression was measured by quantitative Real-Time PCR in spleen samples extracted 1 h postintervention. Surgery combined with LPS-treatment resulted in an increased gene expression of *IL-1beta* and *TNF-alpha* after 1 h and was reduced by physostigmine and neostigmine treatment. *IL-10* expression was measured by quantitative Real-Time PCR in spleen samples extracted 1 h (C) and 24 h (D) postintervention. Surgery in combination with LPS-treatment led to an increased gene expression of *IL-10* after 1 and 24 h and is enhanced by physostigmine and neostigmine application after 1 h. Results of Real-Time PCR quantification are shown as mean ± SEM and normalized to levels of saline treated rats (Control = 100%, n = 8 per group). ***$P<0.001$ represents the difference between LPS and saline treated groups. $^{###}P<0.001$, $^{#}P<0.05$ represents the difference between LPS and physostigmine or neostigmine treated groups.
doi:10.1371/journal.pone.0062679.g003

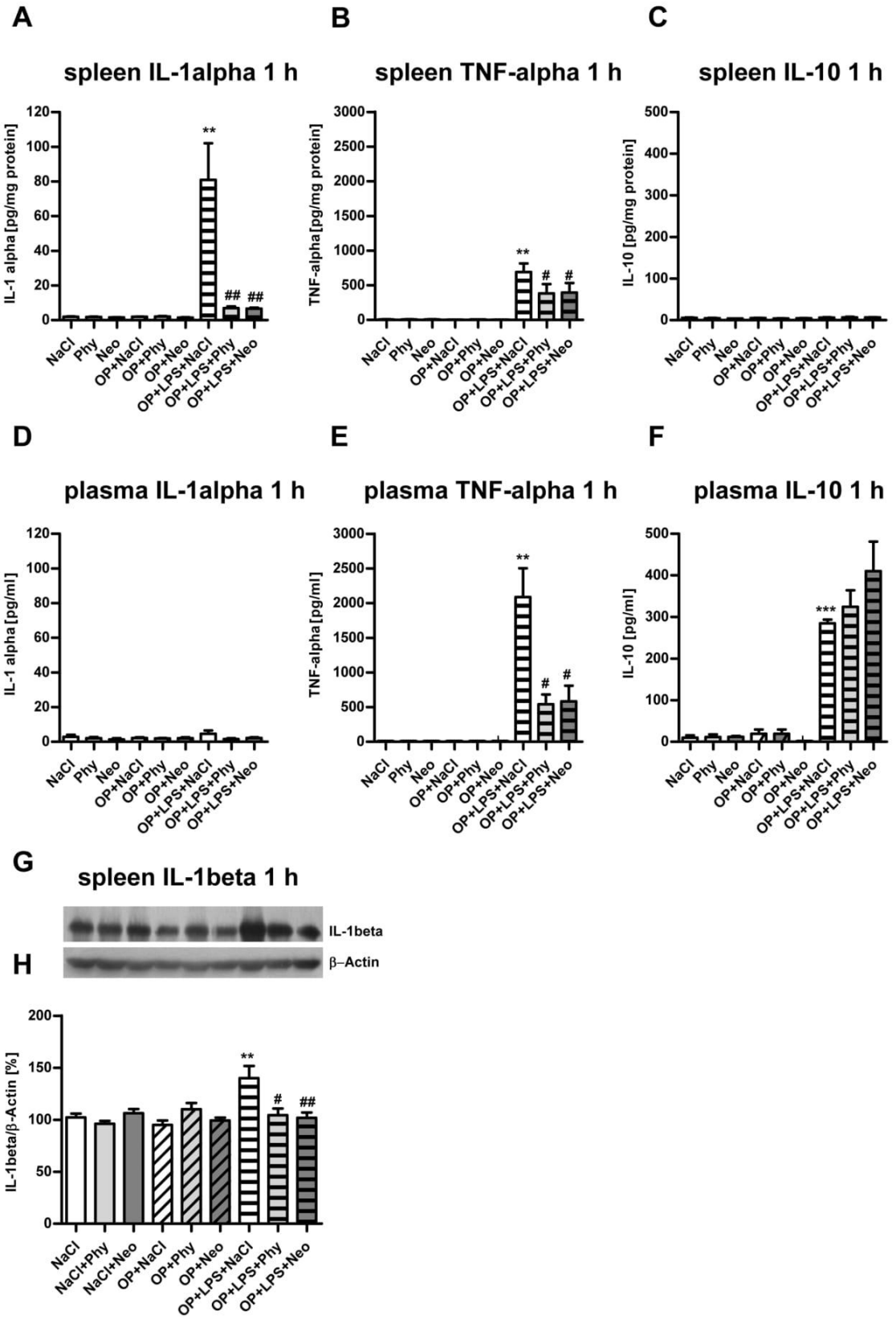
A
spleen IL-1alpha 1 h
IL-1 alpha [pg/mg protein]
B
spleen TNF-alpha 1 h
TNF-alpha [pg/mg protein]
C
spleen IL-10 1 h
IL-10 [pg/mg protein]
D
plasma IL-1alpha 1 h
IL-1 alpha [pg/ml]
E
plasma TNF-alpha 1 h
TNF-alpha [pg/ml]
F
plasma IL-10 1 h
IL-10 [pg/ml]
NaCl
Phy
Neo
OP+NaCl
OP+Phy
OP+Neo
OP+LPS+NaCl
OP+LPS+Phy
OP+LPS+Neo
G
spleen IL-1beta 1 h
IL-1beta
H
β–Actin
IL-1beta/β-Actin [%]
NaCl+Phy
NaCl+Neo

Figure 4. Physostigmine and neostigmine reduce surgery combined with LPS-induced IL-1beta and TNF-alpha protein expression in spleen and plasma. IL-1-alpha, TNF-alpha and IL-10 were measured by Cytometric bead array analysis in spleen (A–C) and plasma (D–F) samples 1 h postintervention. Surgery and LPS-treatment resulted in an increased protein expression of IL-1alpha and TNF-alpha in the spleen after 1 h. Treatment with the AChE inhibitors physostigmine and neostigmine significantly reduced the expression of IL-1alpha in the spleen. In plasma, surgery accompanied by LPS-treatment caused an increased protein expression of TNF-alpha and IL-10. TNF-alpha concentration was significantly diminished by physostigmine and neostigmine application, whereas the expression of IL-10 did not change significantly. Additionally, IL-1beta expression was quantified by western blot analysis in spleen samples extracted 1 h postintervention (G–H). Surgery and LPS-treatment resulted in an increased protein expression of IL-1beta in the spleen after 1 h. Treatment with the AChE inhibitors physostigmine and neostigmine significantly reduced the expression of IL-1beta in the spleen. Results of Cytometric bead array and western blot analysis are shown as mean ± SEM (n = 8 per group). ***P<0.001, **P<0.01 represent the difference between LPS and saline treated groups. #P<0.05, ##P<0.01 represent the difference between LPS and physostigmine or neostigmine treated groups.
doi:10.1371/journal.pone.0062679.g004

Surgery Combined with LPS-treatment is Associated with Increased Protein Levels of Pro-inflammatory Cytokines in the Spleen and Plasma which are Reduced by Physostigmine and Neostigmine

Cytometric bead array analysis in samples from the spleen extracted 1 h postintervention confirmed the upregulation of pro-inflammatory cytokines IL-1alpha (Fig. 4A) and TNF-alpha protein expression after 1 h (Fig. 4B) as well as the inhibition of IL-1alpha by physostigmine and neostigmine. No upregulation of IL-10 protein expression 1 h following surgery treatment with LPS was detectable in the spleen (Fig. 4C). As shown in Fig. 4D, surgery accompanied by LPS-treatment did not trigger an increased IL-1alpha protein expression in the plasma. However, TNF-alpha protein expression was elevated after 1 h (Fig. 4E). The enhanced expression of TNF-alpha after 1 h was significantly decreased by physostigmine and neostigmine. Following surgery and LPS-treatment, IL-10 protein expression in the plasma was detectable after 1 h postintervention and did not change significantly using physostigmine and neostigmine (Fig. 4F). After 24, 72 h or 7 days no increase of pro- or anti-inflammatory protein expression was detectable (data not shown). Additionally, IL-1beta expression was quantified by western blot analysis in spleen samples extracted 1 h postintervention (Fig. 4G–H). Surgery and LPS-treatment resulted in an increased protein expression of IL-1beta in the spleen after 1 h. Treatment with the AChE inhibitors physostigmine and neostigmine significantly reduced the expression of IL-1beta in the spleen.

Surgery Combined with LPS-treatment is Associated with Increased Neurodegeneration and Activity of Acetylcholinesterase in the Cortex and Hippocampus which are Reduced by Physostigmine and Neostigmine

To examine if neuroinflammation is associated with neurodegeneration, brain sections were stained with Fluoro-Jade B, a marker for neurons that undergo cell death and additionally with Hoechst 33342, a marker for DNA. Compared to the control group (Fig. 5A) surgery in combination with LPS-treatment resulted in an increased number of neuronal damage in the cortex after 24 h (Fig. 5B). Treatment with physostigmine (Fig. 5C) and neostigmine (Fig. 5D) led to significantly reduced neuronal damage. The comparison of the number of Fluoro-Jade B-positive cells in the cortex is shown in Fig. 5E. Compared to the control group surgery together with LPS-treatment caused an increased activity of acetylcholinesterase in the cortex after 24 h, which could be diminished by physostigmine and neostigmine (Fig. 5F).

Compared to the control group (Fig. 6A) surgery in combination with LPS-treatment resulted in an increased number of neuronal damage after 24 h (Fig. 6B) in the dentate gyrus of the hippocampus. Treatment with physostigmine (Fig. 6C) and neostigmine (Fig. 6D) led to significantly reduced neuronal damage. The comparison of the number of Fluoro-Jade B-positive cells from the dentate gyrus granule cell layer of the hippocampus is shown in Fig. 6E. Compared to the control group surgery together with LPS-treatment caused an increased activity of acetylcholinesterase in the hippocampus after 24 h, which could be diminished by physostigmine and neostigmine (Fig. 6F).

Discussion

The study at hand could show that surgery combined with LPS-treatment leads to a significant upregulation of the pro-inflammatory cytokine IL-1beta in the cortex and hippocampus after 1 and 24 h. These findings are in line with several studies, which report that the production of IL-1beta in the brain in general and in the hippocampus in particular is induced by LPS [35,36]. Figaldo et al. reported that sub-clinical infection may sensitise the immune system augmenting the severity of post-operative cognitive dysfunction [3].

IL-1beta mediates an inflammatory process in the hippocampus that underlies memory impairment [37]. IL-1 is a cytokine, produced mainly by monocytes and macrophages, as well as by glia cells and neurons within the brain. Various studies have shown that IL-1beta plays a potential role in the pathophysiology of neurodegenerative diseases [38]. One consequence of an increased IL-1beta concentration is the inhibitory effect on long-term potentiation in dentate gyrus of the hippocampus [39,40]. Long-term potentiation is widely considered one of the major cellular mechanisms that underlie learning and memory [41,42].

The present data show, that in our model of adult rats (3 months) surgery (laparotomy) alone does not induce a neuroinflammatory response. These findings confirm the studies done by Barrrientos et al., showing an increase in IL-1beta, which correlates with hippocampal-dependent memory impairment in old rats (24 months) after laparotomy but not in adult rats (3 months). The authors demonstrated that aged animals are more vulnerable to cognitive decline after a peripheral immune challenge [43]. The expression of IL-1beta and IL-1 type 1 receptors is particularly high in the hippocampus. The concentration of IL-1beta is elevated in the hippocampus of old rats [44]. The aging brain seems to be more susceptive to peripheral bacterial infection and surgical stress and reacts with specifically exaggerated and persistent IL-1beta response [41,45]. This could be a reason why especially elderly patients suffer from POCD [8,9,46–48].

Cibelli and colleagues demonstrated that a peripheral orthopedic surgery-induced innate immune response triggers an IL-1beta-mediated inflammatory process in the hippocampus underlying memory impairment [49]. Therefore, we concluded that laparotomy alone is not sufficient to induce IL-1beta in the brain of adult rats. However, in combination with LPS, it serves as a model for neuroinflammation to examine the effectiveness of cholinergic stimulation.

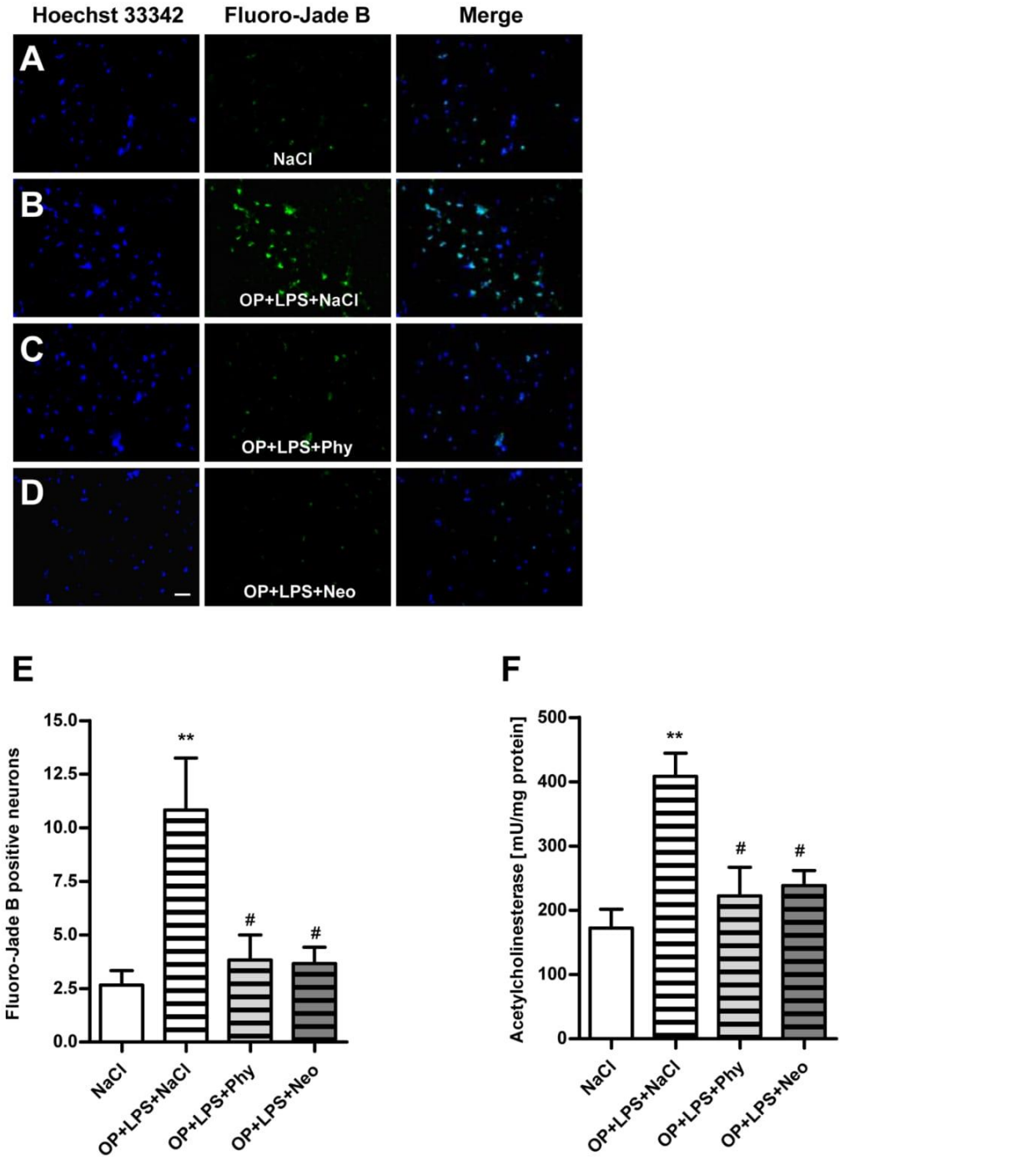

Figure 5. Physostigmine and neostigmine reduce surgery combined with LPS-induced neurodegeneration and activity of acetylcholinesterase in the cortex. Representative photomicrographs (original magnification X200) of Hoechst 33342 (left panel) and Fluoro-Jade B (right panel) staining from the cortex (Scale bar = 20 μm) of adult rats which were treated with saline (A), surgery combined with LPS-treatment+saline (B), surgery combined with LPS-treatment+physostigmine (C) or surgery combined with LPS-treatment+neostigmine (D). (E) Comparison of the number of Fluoro-Jade B-positive cells from the cortex of adult rats. (F) Activity of acetylcholinesterase in the cortex after 1 h. Surgery combined with LPS-treatment triggered the number of degenerated neurons and the activity of acetylcholinesterase in the cortex after 1 h. Physostigmine and neostigmine led to significantly reduced neuronal damage and activity of acetylcholinesterase. Number of Fluoro-Jade B-positive cells are shown as mean ± SEM (n = 5–6 per group). **$P<0.01$ represents the difference between surgery combined with LPS-treatment and saline treated groups. #$P<0.05$ represents the difference between surgery together with LPS-treatment and in combination with physostigmine or neostigmine treated groups.
doi:10.1371/journal.pone.0062679.g005

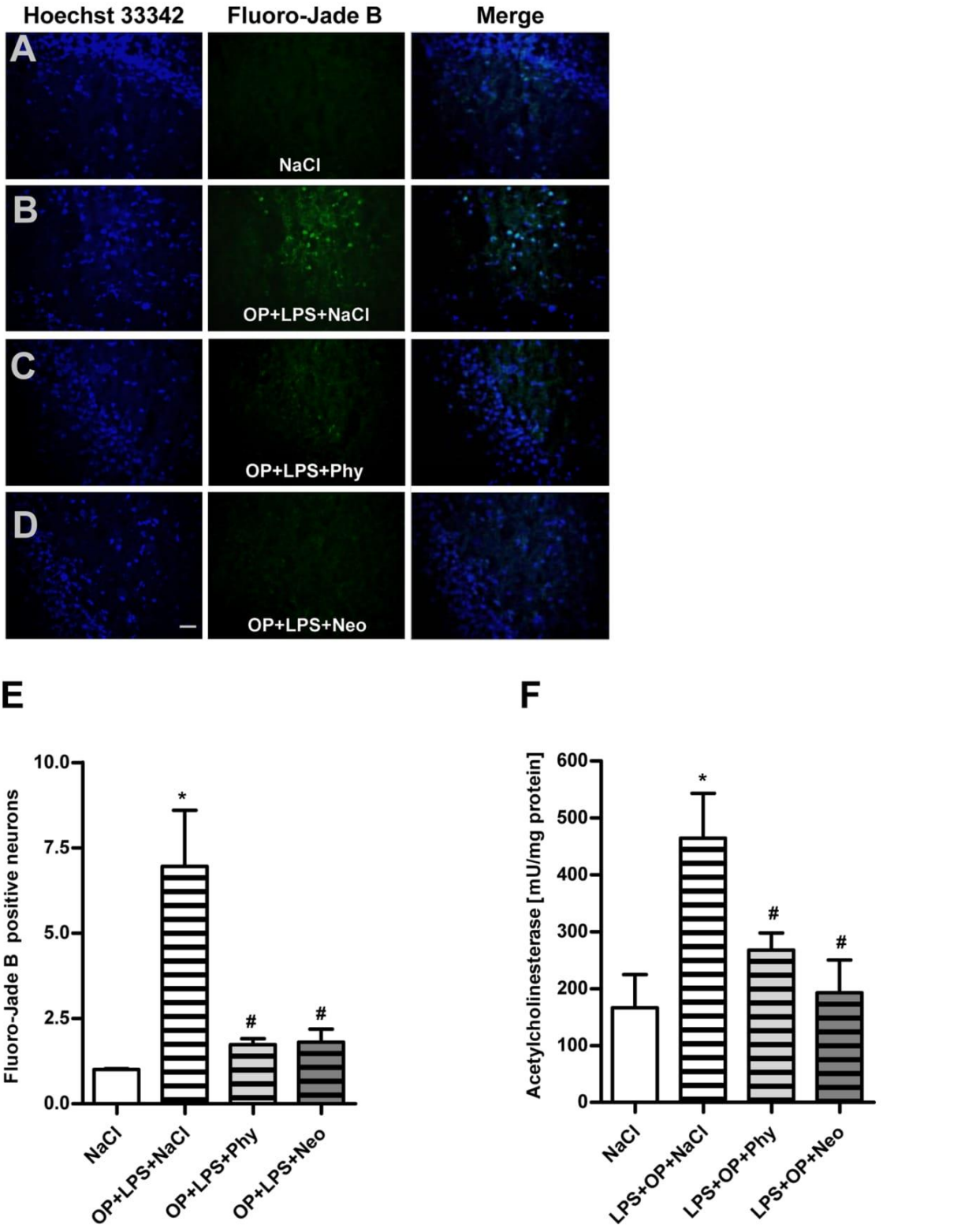

Figure 6. Physostigmine and neostigmine reduce surgery combined with LPS-induced neurodegeneration and activity of acetylcholinesterase in the hippocampus. Representative photomicrographs (original magnification X200) of Hoechst 33342 (left panel) and Fluoro-Jade B (right panel) staining from the hippocampal dentate gyrus granule cell layer (Scale bar = 20 μm) of adult rats which were treated with saline (A), surgery combined with LPS-treatment+saline (B), surgery combined with LPS-treatment+physostigmine (C) or surgery combined with LPS-

treatment+neostigmine (D). (E) Comparison of the number of Fluoro-Jade B-positive cells from the hippocampal dentate gyrus granule cell layer of adult rats. (F) Activity of acetylcholinesterase in the hippocampus after 24 h. Surgery combined with LPS-treatment triggered the number of degenerated neurons and the activity of acetylcholinesterase in the hippocampus after 24 h. Physostigmine and neostigmine led to significantly reduced neuronal damage and activity of acetylcholinesterase. Number of Fluoro-Jade B-positive cells are shown as mean ± SEM (n = 5–6 per group). *P<0.05 represents the difference between surgery combined with LPS-treatment and saline treated groups. #P<0.05 represents the difference between surgery together with LPS-treatment and in combination with physostigmine or neostigmine treated groups.
doi:10.1371/journal.pone.0062679.g006

The result of this study demonstrates that the raise of IL-1beta in the hippocampus and cortex was significantly reduced by intraperitoneal administration of physostigmine and neostigmine, before surgery following LPS-treatment. It has been shown earlier that the administration of CNS-active acetylcholinesterase inhibitors depletes systemic pro-inflammatory cytokines and ameliorates both central and peripheral inflammation [50,51].

In contrast to the centrally-acting acetylcholinesterase inhibitor physostigmine, neostigmine does not cross the blood-brain barrier, and thus has no effect on AChE within the brain. Interestingly, in our study neostigmine inhibited the production of IL-1beta within the cortex and hippocampus. It could be demonstrated recently, that neostigmine has no anti-inflammatory effect in neuroinflammation induced by an intracerebroventricular injection of LPS [52]. A possible explanation for our results would be the reduction of the peripheral expression of pro-inflammatory cytokines like IL-1beta and TNF-alpha, which decreases the subsequent signal of the vagus nerve to the brain and leads to a reduced neuroinflammation. This confirmed the possibility to control postoperative neuroinflammation in the brain by suppressing the peripheral immune response during surgical treatment. We suspect that neostigmine has not the same effect in patients with decreased levels of cholinergic neurotransmission or increased expression of IL-1beta in the brain that has been related to progressing memory deficits with aging. Recently, it has been demonstrated that a single intracisternal but not peripheral administration of an interleukin-1 receptor antagonist throughout surgery was sufficient to block both the neuroinflammatory response and the behavioral deficit [43]. Therefore, we deduce that the peripheral downregulation of the IL-1beta pathway is not sufficient since other proinflammatory cytokines like TNF-alpha send signals from the vagus nerve to the brain.

To verify the peripheral cytokine expression after surgery alone or in combination with LPS-treatment, pro- and anti-inflammatory cytokines were measured in plasma and spleen. As might have been expected, surgery combined with LPS-treatment led to a significant upregulation of the pro-inflammatory cytokines IL-1beta, TNF-alpha as well as the anti-inflammatory cytokine IL-10 in spleen and plasma. In the present study it was observed that the gene expression of the pro-inflammatory cytokines *IL-1beta* and *TNF-alpha* increased in spleen tissue 1 h after surgery and LPS-treatment. The protein IL-1alpha remains in the spleen as it could not be detected in plasma, while protein expression of TNF-alpha was mainly detected in plasma. Physostigmine and neostigmine significantly decreased the gene expression of *IL-1beta* and *TNF-alpha* as well as the protein expression of IL-1 alpha and TNF-alpha. Unfortunately, the measurement of IL-1beta protein expression was not available for the BD™ CBA Flex Set, so instead IL-1alpha was determined. To see if IL-1beta is similarly regulated in the spleen we additionally quantified IL-1beta expression by western blot analysis in spleen samples extracted 1 h postintervention. Surgery and LPS-treatment resulted in an increased protein expression of IL-1beta in the spleen after 1 h. Treatment with the AChE inhibitors physostigmine and neostigmine significantly reduced the expression of IL-1beta in the spleen.

The expression of the anti-inflammatory gene *IL-10* in the spleen was upregulated after 1 h and to a lesser extent after 24 h following surgery together with LPS-treatment. In contrast to the downregulation of the pro-inflammatory cytokines, physostigmine and neostigmine significantly but only briefly augmented the gene expression of *IL-10* in spleen tissue after 1 h. The protein expression of IL-10 was detectable in plasma, and here, physostigmine and neostigmine did not alter the expression of IL-10 significantly. Our data are consistent with the results of Tracey et al. [15]. They describe the *inflammatory reflex* that controls the innate immune responses by a mechanism that targets pro-inflammatory cytokines but do not alter the production of the anti-inflammatory cytokine IL-10. IL-10 signaling suppresses nuclear localization and activation of NF-kB, leading to the inhibition of pro-inflammatory cytokine gene transcription in human monocytes [53]. Li and colleagues reported that IL-10 upregulates the expression of α7 nAChR in RAW264.7 cells [54]. Furthermore, Lynch et al. reported that LPS leads to an increased expression of IL-beta and cell death in the hippocampus, which can be reduced by IL-10 [55].

In order to demonstrate whether the enhanced level of IL-1beta induces cell death in the cortex and hippocampus, we stained brain sections with Fluoro-Jade B. It has been proposed that the impairment in long-term potentiation, the cellular mechanism that underlies learning and memory, is a consequence of the increase in IL-1beta concentration and IL-1beta-induced cell death [56]. Our results demonstrated that IL-1beta is associated with enlarged neurodegeneration in the cortex and the dentate gyrus of the hippocampus and this neurodegeneration is minimized by the AChE inhibitors physostigmine and neostigmine. Furthermore, it could be shown that the activity of AChE is enhanced after surgery in combination with LPS and diminished after treatment with physostigmine and neostigmine. Our finding is supported by other groups, who found increased number of cell deaths that strongly correlated to AChE activity [31,52,57,58]. Interestingly, even neostigmine inhibits the activity of acetylcholinesterase in the hippocampus although it cannot pass the blood-brain barrier, thus having no effect of AChE within the brain. This confirms once again that neostigmine acts indirectly on the cortex and hippocampus by peripherally reducing the expression of pro-inflammatory cytokines. The results provide evidence for the relationship between the immune system and the CNS and suggest a link between IL-1beta, AChE activity and dysfunction in the cortex and hippocampus. Therefore, our finding is of important clinical value. The stimulation of the cholinergic anti-inflammatory pathway through AChE inhibitors can reduce the production of IL-1beta in the brain which in turn protects against neurodegeneration.

Conclusion

Our study showed for the first time, that physostigmine and neostigmine can decrease the expression of IL-1beta and cortical/hippocampal neurodegeneration after surgery and subsequent LPS-treatment. Since neuroinflammation is involved in brain dysfunction, cholinergic activation seems to be a potential neuroprotective treatment.

Limitations

Further studies are required whose aim should be the examination of memory impairment in this animal model. There is compelling evidence that relative expression of IL-1beta is a key element in the development of postoperative cognitive dysfunction. IL-1beta is mediating inflammation and causing hippocampal-dependent memory impairment [37,49,59]. More investigations are necessary to learn about the protective effect of cholinergic stimulation and its cellular and molecular mechanisms.

Author Contributions

Conceived and designed the experiments: AK CvH AF CDS. Performed the experiments: AK CvH MS AT NP MK. Analyzed the data: AK CvH MS AT NP MK. Contributed reagents/materials/analysis tools: CDS. Wrote the paper: AK CvH CDS.

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4 Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

5 Komplette Publikationsliste

5.1. Originalarbeiten

- **Paeschke, N.**; von Haefen, C.; Endesfelder, S.; Sifringer, M.; Spies, C.D. Dexmedetomidine prevents lipopolysaccharide-induced microRNA expression in the adult rat brain. *Int J Mol Sci* **2017**, *18*.

- Sifringer, M.; von Haefen, C.; Krain, M.; **Paeschke, N.**; Bendix, I.; Buhrer, C.; Spies, C.D.; Endesfelder, S. Neuroprotective effect of dexmedetomidine on hyperoxia-induced toxicity in the neonatal rat brain. *Oxid Med Cell Longev* **2015**, *2015*, 530371.

- Kalb, A.; von Haefen, C.; Sifringer, M.; Tegethoff, A.; **Paeschke, N.**; Kostova, M.; Feldheiser, A.; Spies, C.D. Acetylcholinesterase inhibitors reduce neuroinflammation and -degeneration in the cortex and hippocampus of a surgery stress rat model. *PLoS One* **2013**, *8*, e62679.

5.2. Poster

- Hauptkongress für Anästhesiologie und Intensivtherapie, September 2011
- International Symposium on Neuroprotection and Neurorepair, Mai 2012

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