ORIGINAL ARTICLE

Transvenous Diaphragm Neurostimulation Mitigates Ventilation-associated Brain Injury

Thiago G. Bassi[1,2], Elizabeth C. Rohrs[1,3], Karl C. Fernandez[1,3], Marlena Ornowska[1], Michelle Nicholas[1,3], Matt Gani[2], Doug Evans[2], and Steven C. Reynolds[1,3]

[1]Simon Fraser University, Burnaby, British Columbia, Canada; [2]Lungpacer Medical Inc., Vancouver, British Columbia, Canada; and [3]Fraser Health Authority, Royal Columbian Hospital, New Westminster, British Columbia, Canada

ORCID ID: 0000-0001-5570-5057 (T.G.B.).

Abstract

Rationale: Mechanical ventilation (MV) is associated with hippocampal apoptosis and inflammation, and it is important to study strategies to mitigate them.

Objectives: To explore whether temporary transvenous diaphragm neurostimulation (TTDN) in association with MV mitigates hippocampal apoptosis and inflammation after 50 hours of MV.

Methods: Normal-lung porcine study comparing apoptotic index, inflammatory markers, and neurological-damage serum markers between never-ventilated subjects, subjects undergoing 50 hours of MV plus either TTDN every other breath or every breath, and subjects undergoing 50 hours of MV (MV group). MV settings in volume control were V_T of 8 ml/kg, and positive end-expiratory pressure of 5 cm H_2O.

Measurements and Main Results: Apoptotic indices, microglia percentages, and reactive astrocyte percentages were greater in the MV group in comparison with the other groups ($P < 0.05$). Transpulmonary pressure at baseline and at study end were both lower in the group receiving TTDN every breath, but lung injury scores and systemic inflammatory markers were not different between the groups. Serum concentrations of four neurological-damage markers were lower in the group receiving TTDN every breath than in the MV group ($P < 0.05$). Heart rate variability declined significantly in the MV group and increased significantly in both TTDN groups over the course of the experiments.

Conclusions: Our study found that mechanical ventilation is associated with hippocampal apoptosis and inflammation, independent of lung injury and systemic inflammation. Also, in a porcine model, TTDN results in neuroprotection after 50 hours, and the degree of neuroprotection increases with greater exposure to TTDN.

Keywords: mechanical ventilators; brain injuries; post-ICU syndrome; apoptosis; ICU

At a Glance Commentary

Scientific Knowledge on the Subject: Mechanical ventilation is associated with hippocampal apoptosis and inflammation.

What This Study Adds to the Field: In a porcine model, mechanical ventilation is associated with hippocampal apoptosis and inflammation, independent of lung injury and systemic inflammation. Also, diaphragm neurostimulation results in neuroprotection after 50 hours, and the degree of neuroprotection increases with greater exposure to diaphragm neurostimulation.

(Received in original form January 11, 2021; accepted in final form September 1, 2021)

Supported by Lungpacer Medical Inc., Royal Columbian Hospital Foundation, TB Vets Charitable Foundation, and Mitacs.

Author Contributions: T.G.B. and D.E. were responsible for hypothesis generation. T.G.B., S.C.R., and E.C.R. were responsible for the conception of this study. T.G.B., S.C.R., E.C.R., and M.G. contributed to study design and data interpretation. T.G.B., E.C.R., S.C.R., and M.G. were responsible for writing the article. T.G.B., E.C.R., K.C.F., M.O., S.C.R., and M.N. performed data acquisition. T.G.B., E.C.R., M.O., S.C.R. and M.N. conducted data analysis. All authors have agreed with the final version of the manuscript before submission.

Correspondence and requests for reprints should be addressed to Thiago G. Bassi, M.D., Ph.D., 130-610 West Cordova Street, Vancouver, BC V6B 1G1, Canada. E-mail: thiagobassi@yahoo.com.

This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Crit Care Med Vol 204, Iss 12, pp 1391–1402, Dec 15, 2021

Originally Published in Press as DOI: 10.1164/rccm.202101-0076OC on September 7, 2021
Internet address: www.atsjournals.org

Mechanical ventilation (MV) is a lifesaving technology that is the foundation of the modern intensive care unit (1, 2). However, many drawbacks of MV have been identified, such as ventilator-induced lung injury and ventilator-induced diaphragm dysfunction (1, 2). Recently a novel concept, ventilation-associated brain injury (VABI), has been proposed and studied (3–6).

VABI in neonatology has been associated with either the use of intermittent positive-pressure ventilation or hyperoxia (7). According to the proposed pathophysiology for VABI in neonates, intermittent positive-pressure ventilation or hyperoxia could lead to lung injury and consequently to systemic inflammation, triggering neuroinflammation and neuronal apoptosis (7). In adults, VABI has not been conclusively demonstrated yet. However, preclinical studies investigating VABI in fully grown subjects have shown that mechanically ventilated subjects have greater numbers of microglia, greater numbers of reactive astrocytes, and a higher incidence of cellular apoptosis compared with never-ventilated subjects (3, 5, 6, 8). Two recently published systematic reviews on MV associated with brain injury reported 13 preclinical papers that describe hippocampal apoptosis and neuroinflammation as experimental findings linked to VABI (9, 10). In a porcine model, our group showed that even lung-protective MV settings were associated with brain injury after 50 hours of MV (5); moreover, in the same study, our group showed that serum concentrations of GFAP (glial fibrillary acid protein) and UCHL1 (ubiquitin carboxy-terminal hydrolase L1) were greater in the MV subjects than in the never-ventilated subjects, revealing an opportunity for the use of these markers for VABI (5, 6).

It has been hypothesized that either an inflammatory or a neural pathway might lead to the development of VABI (3, 4, 8, 11, 12). According to the systemic inflammatory pathway theory, ventilation-induced lung injury triggers systemic inflammation, which in turn could lead to brain injury (8, 11). Conversely, the neural pathway postulates that the cyclical alveolar stretch during MV alters the vagal pulmonary afferent signal, triggering VABI (3, 4). Although there is uncertainty as to the exact mechanism, preclinical studies have consistently reported hippocampal inflammation and apoptosis as a result of MV (3, 8, 11).

Temporary transvenous diaphragm neurostimulation (TTDN) is a hybrid ventilation strategy that combines bilateral phrenic nerve stimulation in synchrony with MV, aiming initially to rescue the diaphragm from atrophy secondary to MV (13, 14). In a preclinical study, our group has shown that TTDN also reduced atelectasis and preserved lung homogeneity during MV (15, 16). We hypothesize that by preserving lung homogeneity during MV, TTDN could either dampen the inflammatory process associated with MV or change the pulmonary afferent signal during MV. Consequently, reduced inflammation or a change in the vagal signal during MV could mitigate cellular apoptosis in the hippocampus. Some of the results of these studies have been previously reported in the form of abstracts (6, 14, 15).

Methods

Animals

Juvenile Yorkshire pigs (4–5 mo old) were procured, housed, maintained, and studied following the local animal care committee guidelines after UBC Ethics Committee and Animal Care Committee approvals.

Experimental Protocol

Subjects were assigned to four groups: lung-protective mechanical ventilation only (MV group), temporary transvenous diaphragm neurostimulation either every other breath (TTDN50% + MV) or every breath (TTDN100% + MV) in synchrony with lung-protective MV, and those who were never ventilated (NV group). Temperature, mean arterial pressure, heart rate, glucose levels, and Pa_{CO_2} were monitored during the experiment to ensure values stayed within normal ranges.

Ventilation Settings

Subjects were ventilated with Dräger Evita XL ventilators in volume control mode set to 5 cm H_2O positive end-expiratory pressure, 8 ml/kg V_T, and plateau pressure less than 30 cm H_2O. V_T values slightly over 6 ml/kg were necessary owing to greater dead space in our subjects but remained in the range of 6–8 ml/kg from the initial ARDSnet study (17). An airway sensor, placed between the ventilator "Y-piece" and the endotracheal tube, captured air flow and airway pressure. The sensor was connected to the neurostimulation system, which recorded the data during the experiments for later analysis. Esophageal pressure was measured at the end of inspiration. Transpulmonary plateau pressure was measured during the end-inspiratory plateau. Driving pressure was calculated as end-inspiratory plateau pressure − end-expiratory pressure.

Diaphragm Contraction and Diaphragm Contribution During Lung-Protective MV

A central venous catheter embedded with electrodes (LIVE Catheter; Lungpacer Medical Inc.) was used to stimulate the phrenic nerves bilaterally for diaphragm contractions, targeting a ventilator pressure–time product reduction of 15–20%, as previously described (2, 13, 14, 18). Ventilator pressure–time product was obtained by respiratory monitoring (FluxMed GrT; MBMed). Esophageal pressure, plateau pressure, driving pressure, and transpulmonary plateau pressure were measured at the beginning and at the end of the experiment.

Hippocampal Sampling and Preparation

Molecular, granular, and subgranular layers were randomly sampled in the dentate gyrus, and the pyramidal layer was randomly sampled in the CA1 and CA3 hippocampal areas (all in the same slide section). Equal-sized sample areas (200 μm by 200 μm) and equal numbers of areas (21 per subject per immunochemistry marker) were analyzed in all subjects. An independent laboratory (Wax-it Histology Services Inc.), blinded to sample group, performed immunochemistry preparation and processing for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, GFAP, and IBA-1 (ionizing calcium-binding adaptor molecule-1) markers.

Cell Counting

Hippocampal cells were classified (positive-stained, negative-stained, and extracellular matrix) and counted by machine-learning software (ImageJ).

Serum Samples

All subjects had blood samples taken to determine serum concentration of biomarkers at the end of the experiment. Brain injury biomarkers were: S100α, NSE (neuron specific enolase), GFAP, and UCHL1. Inflammatory biomarkers were: TNFα, IL-1α, IL-1β, IL-6, IL-8, and IL-10. An independent laboratory, Eve

Technologies Corporation, blinded to study group allocation, analyzed the samples.

Pa_{O_2}/FI_{O_2} Ratio

Arterial blood gas samples were used to calculate the Pa_{O_2}/FI_{O_2} ratio. Mechanically ventilated subjects had samples taken at baseline, every six hours or when appropriate, and NV subjects had one sample taken before euthanasia.

Lung Histology

Lungs were harvested, and an independent laboratory, Wax-it Histology Services Inc., blinded to study group allocation, stained the lung slides with hematoxylin and eosin. Samples were then scored for lung injury by examiners who were blinded to the study groups, following a method adapted from Matute-Bello (19).

Heart Rate Variability Analysis

Root mean square of the standard deviation (RMSSD) of R–R intervals, a surrogate measure of autonomic nervous system activity, was calculated for the first 6 hours after experiment initiation and the last 6 hours before subject euthanasia.

Statistical Analysis

Statistical analyses used GraphPad Prism 8.4.2 software. Nonparametric tests were used and included either the Kruskal-Wallis test and Dunn's multiple comparison test or the Wilcoxon signed-rank test, when appropriate. Data are expressed as median and interquartile range (IQR), unless otherwise stated. *P* values ≤ 0.05 were considered statistically significant.

Results

Thirty-one female subjects were studied, with weights of 57 kg (43–66) in the MV group ($n = 10$), 55 kg (48–63) in the TTDN50% + MV group ($n = 8$), 69 kg (65–71) in the TTDN100% + MV group ($n = 7$), and 54 kg (50–56) in the NV group ($n = 6$), $P = 0.0016$. When appropriate, results were normalized to weight. Temperature, mean arterial pressure, heart rate, glucose levels, and Pa_{CO_2} were within normal ranges for all subjects from all groups. All subjects that received TTDN had bilateral phrenic-nerve capture.

Fluid Balance and Spontaneous Breathing Efforts

Fluid balance to the ventilated subjects was within the target range of 0.1–2.0 ml/kg/hr (20). Spontaneous breathing activity during 50 hours of lung-protective MV was detected by direct examination of air flow and airway pressure recordings. The average number of breaths per subject per experiment was 36,917. The average number of episodes of spontaneous breathing activity (defined as subject-triggered breaths) was 8 per subject.

Sedation and Drug Consumption

Differences in propofol, midazolam, fentanyl, and ketamine usage were not statistically significant between the groups when normalized to weight.

Hippocampal Histological Markers

Apoptotic indices (Figure 1), microglia percentages (Figure 2), and reactive astrocyte percentages (Figure 3) were significantly greater in the MV group in comparison to the other groups ($P < 0.05$), as shown in Table 1.

Inflammatory Serum Markers

TNFα, IL-1α, IL-1β, IL-6, IL-8, and IL-10 levels were not significantly different between the groups, as shown in Table 2.

Systemic Markers for Neuronal Injury

GFAP (Figure 4), UCHL1 (Figure 5), S100β, and NSE concentrations were significantly lower in the TTDN100%+MV group than the other groups ($P < 0.05$), as shown in Table 1.

Lung Physiology

Pa_{O_2}/FI_{O_2} ratios, esophageal pressures, plateau pressures, driving pressures, and transpulmonary plateau pressures are shown in Tables 3 and 4.

Lung Histology Data

Differences in lung injury scores were not statistically significant between groups, as shown in Table 3.

Heart Rate Variability

RMSSD for the MV group was 0.90 ms (0.75–1.03) at the start, declining to 0.81 ms (0.66–0.84) at the end of the study, $P = 0.0078$. For the TTDN50% + MV group RMSSD was 0.81 ms (0.60–0.98) at the start, increasing to 0.91 ms (0.76–0.96) at the end of the study, $P = 0.0078$. For the TTDN100% + MV group, RMSSD was 1.00 ms (0.86–1.38) at the start, increasing to 1.03 ms (0.88–1.09) at the end of the study, $P = 0.0156$.

Discussion

Our study found that mechanical ventilation is associated with hippocampal apoptosis and inflammation, independent of lung injury and systemic inflammation. Moreover, our study also found that temporary transvenous diaphragm neurostimulation in synchrony with lung-protective MV considerably mitigates hippocampal apoptosis and neuroinflammation with lower microglia percentages and reactive astrocyte percentages after 50 hours of MV.

The association between MV and hippocampal apoptosis has been reported previously (3, 5, 12). The work presented in this study contributes to the growing body of evidence indicating that MV is associated with hippocampal apoptosis (3, 5, 12). Moreover, the brain insult is independent of lung injury and systemic inflammation (3, 5, 12). Previous experiments that investigated MV and its association with hippocampal apoptosis have not studied therapies to mitigate the brain injury associated with MV (3, 5, 12). Our study showed that TTDN mitigates hippocampal apoptosis associated with MV. Furthermore, the extent of hippocampal apoptosis mitigation increased with greater exposure to TTDN. Greater TTDN exposure resulted in lower hippocampal cell death, which is evidence that this intervention directly impacted hippocampal apoptosis. The TTDN100% + MV group showed degrees of hippocampal apoptosis after 50 hours that were statistically indistinguishable from the NV group, whereas the TTDN50% + MV group showed significant mitigation of apoptosis, but less so than the TTDN100% + MV group. Although our study did not evaluate the clinical impact of mitigating hippocampal apoptosis after MV, it establishes a foundation and biological plausibility that can be used as the basis of future studies.

Microglia and astrocytes are the primary cells that trigger and control the apoptotic process in the brain (21). This is important clinically as greater percentages of microglia and reactive astrocytes have been associated with acute cognitive dysfunction (22). For example, an analysis of hippocampal tissue harvested from deceased

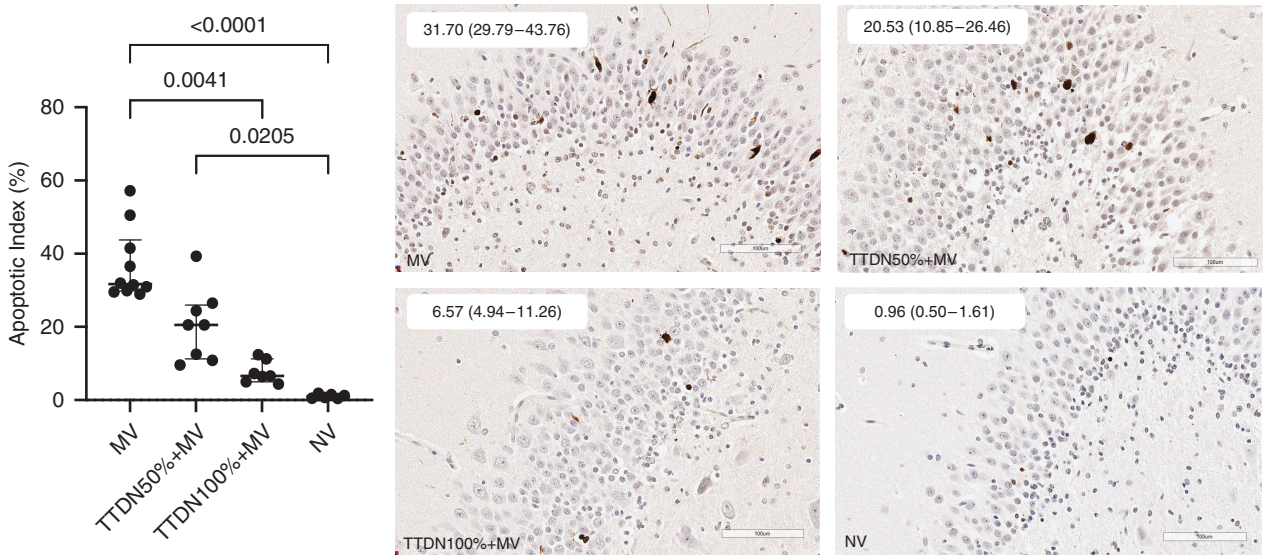

Figure 1. Left: Dot plot of the hippocampal apoptotic indices (%) for all groups. Apoptotic indices found were 31.70 (29.79–43.76) for the mechanical ventilation (MV) group, 20.53 (10.85–26.46) for the TTDN50% + MV group, 6.57 (4.94–11.26) for the TTDN100% + MV group, and 0.96 (0.50–1.61) for the never-ventilated (NV) group. *Post hoc* analysis using Dunn's multiple comparison test showed statistically significant differences between the MV and NV groups (31.70 vs. 0.96, $P < 0.0001$), between the MV and TTDN100% + MV groups (31.70 vs. 6.57, $P = 0.0041$), and between the TTDN50% + MV and NV groups (20.53 vs. 0.96, $P = 0.0205$). Center and right: Examples of hippocampus slides for all groups, showing terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling–positive cells (brown). Scale bars, 100 μm. TTDN50% = temporary transvenous diaphragm neurostimulation every other breath; TTDN100% = temporary transvenous diaphragm neurostimulation every breath.

patients with acute respiratory distress syndrome (ARDS) and acute cognitive impairment before death showed increased numbers of activated microglia and reactive astrocytes when compared with patients with ARDS without acute cognitive impairment (22). In our study the microglia percentages in the hippocampus were considerably greater in the MV group than the other groups. Conversely, the TTDN100% + MV group showed microglia percentages and astrocyte percentages similar to the NV

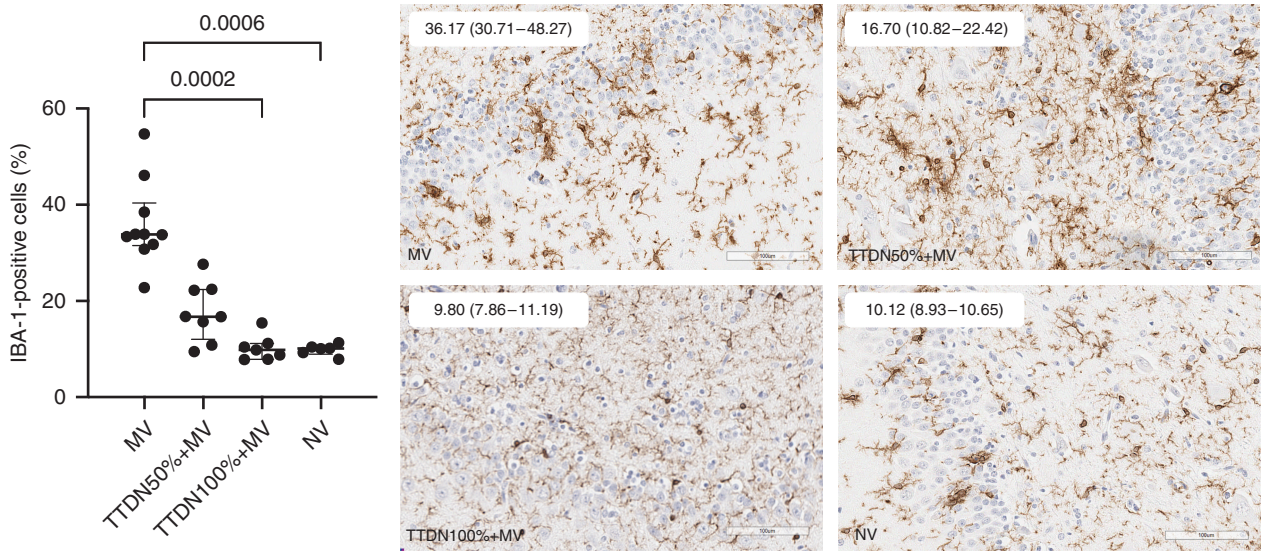

Figure 2. Left: Dot plot of percentages of IBA-1 (ionizing calcium-binding adaptor molecule-1)–positive hippocampal cells (%) for all groups. IBA-1–positive cell percentages found were 36.17 (30.71–48.27) for the mechanical ventilation (MV) group, 16.70 (10.82–22.42) for the TTDN50% + MV group, 9.80 (7.86–11.19) for the TTDN100% + MV group, and 10.12 (8.93–10.65) for the never-ventilated (NV) group. *Post hoc* analysis using Dunn's multiple comparison test showed statistically significant differences between the MV and NV groups (36.17 vs. 10.12, $P = 0.0006$), and between the MV and TTDN100% + MV groups (36.17 vs. 9.80, $P = 0.0002$). Center and right: Examples of hippocampus slides for all groups, showing IBA-1–positive cells (brown). Scale bars, 100 μm. TTDN50% = temporary transvenous diaphragm neurostimulation every other breath; TTDN100% = temporary transvenous diaphragm neurostimulation every breath.

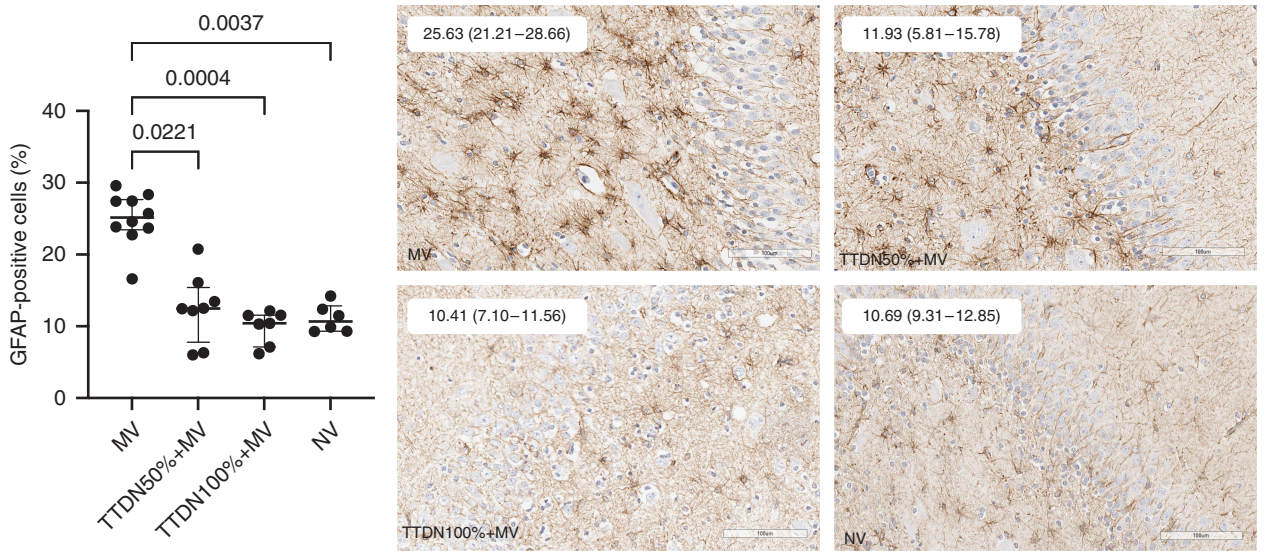

Figure 3. Left: Dot plot of percentages of GFAP (glial fibrillary acid protein)–positive cells (%). GFAP-positive cell percentages found were 25.63 (21.21–28.66) for the mechanical ventilation (MV) group, 11.93 (5.81–15.78) for the TTDN50% + MV group, 10.41 (7.10–11.56) for the TTDN100 + MV group, and 10.69 (9.31–12.85) for the never-ventilated (NV) group. *Post hoc* analysis using Dunn's multiple comparison test showed statistically significant differences between the MV and NV groups (25.63 vs. 10.69, $P = 0.0037$), between the MV and TTDN100% + MV groups (25.63 vs. 10.41, $P = 0.0004$), and between the MV and TTDN50% + MV group (25.63 vs. 11.93, $P = 0.0221$). Center and right: Examples of hippocampus slides for all groups, showing GFAP-positive cells (brown). Scale bars, 100 μm. TTDN50% = temporary transvenous diaphragm neurostimulation every other breath; TTDN100% = temporary transvenous diaphragm neurostimulation every breath.

group. Furthermore, TTDN in synchrony with MV had a greater effect on microglia percentages when delivered every breath than when delivered every other breath; the TTDN100% + MV group had the lowest microglia percentages among the mechanically ventilated groups, and the TTDN50% + MV group had lower microglia percentages, but to a lesser degree than the TTDN100% + MV group. Not only are the lower proportions of microglia in the total cellular populations important, but groups receiving TTDN demonstrated a shift in the microglia cellular characteristics toward antiinflammatory predominance.

When found in the serum, GFAP is a protein known to correlate with astrocyte injury (23, 24). UCHL1 is another systemic marker commonly used to identify neuronal injury (24). Our data showed that the TTDN100% + MV group had statistically significantly lower serum concentrations of GFAP and UCHL1 than both the MV and the TTDN50% + MV groups. Greater exposure to TTDN resulted in lower GFAP serum concentrations, with the TTDN100% + MV group showing the lowest concentrations compared with the other groups, and the TTDN50% + MV group also showing lower concentrations than the MV group, but to a lesser degree than the TTDN100% + MV group. The TTDN100% + MV group also showed lower serum concentrations of S100β and NSE with statistical significance compared with the other groups (25–28). From our findings this would be expected when compared with the MV group, which consistently demonstrated the highest degrees of cellular apoptosis, but it was surprising that the MV group showed lower concentrations of S100β and NSE than the NV group. Although S100β is used as a marker for blood–brain barrier integrity, it also reflects muscle activity (25–28). NSE is used as a marker for neuronal injury; however, NSE is also affected by the level of neuronal metabolism, because NSE is a protein responsible for neuronal glycolysis (25–28). The greater serum concentrations of S100β and NSE in the NV group than in the mechanically ventilated groups could be because of the shorter time between initiation of sedation and study termination (30 min vs. 50 h), reflecting more recent neuronal and metabolic activity in the NV group. Conclusions about the blood–brain barrier integrity between the groups are therefore difficult to draw (25–28). Nevertheless, elevated GFAP and UCHL1 serum concentrations are consistent with our histological findings of hippocampal apoptosis in the MV group. Similarly, there was both histological and serological evidence of mitigation of hippocampal injury in the subjects receiving TTDN.

It may be argued that the hippocampal insult observed was a consequence of sedation and/or physical immobility in the supine position (3, 5, 29, 30). However, sedative drug usage and time of immobility in the supine position were indistinguishable between mechanically ventilated groups, and therefore these variables could not account for the changes observed. We found two variables that were significantly different between the groups at the beginning of the study: the subject weights and the transpulmonary plateau pressures. Although the TTDN100% + MV group had a statistically significant difference in weights compared with the other groups, after normalizing for weight, the differences in drug doses between the groups were not statistically significant. Transpulmonary plateau pressures at the beginning of the study were also different between the groups, with the lowest pressures observed in the TTDN100% + MV group. This difference might be owing to differences in the

Table 1. Hippocampal Apoptotic Index, Microglia Percentage, Reactive Astrocyte Percentage, and Serum Biomarkers for Brain Injury Results for All Groups

Brain Injury and Neuroinflammation Outcomes	Median (IQR)				*P* Value (Kruskal-Wallis Test)	*P* Value (Dunn's Multiple Comparison Test)	
	MV Group (*n* = 10)	TTDN50% + MV Group (*n* = 8)	TTDN100% + MV Group (*n* = 7)	NV Group (*n* = 6)			
Hippocampal apoptotic index	31.70 (29.79–43.76)	20.53 (10.85–26.46)	6.57 (4.94–11.26)	0.96 (0.50–1.61)	<0.0001	MV vs. TTDN50% + MV MV vs. TTDN100% + MV MV vs. NV TTDN50% + MV vs. TTDN100% + MV TTDN50% + MV vs. NV TTDN100% + MV vs. NV	ns 0.0041 <0.0001 ns 0.0205 ns
IBA-1–positive hippocampal cells*, %	36.17 (30.71–48.27)	16.70 (10.82–22.42)	9.80 (7.86–11.19)	10.12 (8.93–10.65)	0.0002	MV vs. TTDN50% + MV MV vs. TTDN100% + MV MV vs. NV TTDN50% + MV vs. TTDN100% + MV TTDN50% + MV vs. NV TTDN100% + MV vs. NV	ns 0.0002 0.0006 ns ns ns
IBA-1–positive hippocampal cells with proinflammatory characteristics, %	8.11 (6.74–9.69)	2.50 (2.02–2.88)	1.60 (1.10–1.77)	1.63 (1.32–2.06)	0.0004	MV vs. TTDN50% + MV MV vs. TTDN100% + MV MV vs. NV TTDN50% + MV vs. TTDN100% + MV TTDN50% + MV vs. NV TTDN100% + MV vs. NV	ns 0.0025 0.0011 ns ns ns
IBA-1–positive hippocampal cells with antiinflammatory characteristics, %	27.82 (23.63–32.00)	12.23 (7.36–19.54)	8.37 (6.86–9.41)	8.20 (7.68–8.50)	0.0004	MV vs. TTDN50% + MV MV vs. TTDN100% + MV MV vs. NV TTDN50% + MV vs. TTDN100% + MV TTDN50% + MV vs. NV TTDN100% + MV vs. NV	ns 0.0022 0.0018 ns ns ns
GFAP-positive hippocampal cells, %	25.63 (21.21–28.66)	11.93 (5.81–15.78)	10.41 (7.10–11.56)	10.69 (9.31–12.85)	0.0009	MV vs. TTDN50% + MV MV vs. TTDN100% + MV MV vs. NV TTDN50% + MV vs. TTDN100% + MV TTDN50% + MV vs. NV TTDN100% + MV vs. NV	0.0221 0.0004 0.0037 ns ns ns
GFAP serum concentration, ng/ml	0.40 (0.28–0.57)	0.29 (0.25–0.32)	0.04 (0.02–0.06)	0.15 (0.07–0.23)	<0.0001	MV vs. TTDN50% + MV MV vs. TTDN100% + MV MV vs. NV TTDN50% + MV vs. TTDN100% + MV TTDN50% + MV vs. NV TTDN100% + MV vs. NV	ns <0.0001 0.0043 0.0015 ns ns

(*Continued*)

Table 1. (*Continued*)

Brain Injury and Neuroinflammation Outcomes	Median (IQR)				*P* Value (Kruskal-Wallis Test)	*P* Value (Dunn's Multiple Comparison Test)	
	MV Group (*n* = 10)	TTDN50% + MV Group (*n* = 8)	TTDN100% + MV Group (*n* = 7)	NV Group (*n* = 6)			
UCHL1 serum concentration, pg/ml	96.96 (80.65–109.60)	110.00 (97.59–200.40)	44.68 (36.56–58.34)	76.57 (42.48–90.26)	0.0013	MV vs. TTDN50% + MV	ns
						MV vs. TTDN100% + MV	0.0325
						MV vs. NV	ns
						TTDN50% + MV vs. TTDN100% + MV	0.0015
						TTDN50% + MV vs. NV	0.0348
						TTDN100% + MV vs. NV	ns
S100β serum concentration, pg/ml	193.10 (129.20–223.30)	230.50 (157.90–361.80)	150.30 (110.30–200.10)	360.90 (252.10–803.90)	<0.0001	MV vs. TTDN50% + MV	ns
						MV vs. TTDN100% + MV	ns
						MV vs. NV	0.0012
						TTDN50% + MV vs. TTDN100% + MV	ns
						TTDN50% + MV vs. NV	ns
						TTDN100% + MV vs. NV	0.0002
NSE serum concentration, ng/ml	16.84 (5.66–24.28)	16.72 (8.29–27.74)	4.17 (3.71–4.51)	32.23 (6.86–38.25)	0.0004	MV vs. TTDN50% + MV	ns
						MV vs. TTDN100% + MV	ns
						MV vs. NV	ns
						TTDN50% + MV vs. TTDN100%+MV	0.0185
						TTDN50% + MV vs. NV	ns
						TTDN100% + MV vs. NV	0.0001

Definition of abbreviations: GFAP = glial fibrillary acid protein; IBA = ionizing calcium-binding adaptor molecule; IQR = interquartile range; MV = mechanical ventilation; ns = not significant; NSE = neuron specific enolase; NV = never ventilated; TTDN50% = temporary transvenous diaphragm neurostimulation every other breath; TTDN100% = temporary transvenous diaphragm neurostimulation every breath; UCHL1 = ubiquitin carboxy-terminal hydrolase L1.
*Includes hippocampal cells with proinflammatory and antiinflammatory characteristics.

pulmonary mechanics secondary to a change in diaphragm tone induced by TTDN. In addition to its primary function to draw air into the lungs during inspiration, the diaphragm also serves as a "brake" during expiration, where residual diaphragm tone preserves functional residual capacity, preventing lung collapse (31). It is difficult to isolate how much this maintenance of functional residual capacity might have contributed to our neuroprotection findings. However, there is reasonable biological plausibility that the differences in transpulmonary plateau pressures were owing to TTDN on every breath as opposed to an unrecognized systematic difference between the groups.

The hippocampal inflammation observed in the mechanically ventilated groups might have been triggered by the activation of the neuroimmunological reflex (32, 33). This reflex is directly dependent on the balance between vagal and sympathetic system activity (32, 33). Increased vagal tone has been shown to reduce the production of inflammatory markers and the release of inflammatory cells (32). Conversely, increased sympathetic activity increases the production of proinflammatory proteins along with the release of proinflammatory cells into the bloodstream (32, 33). Heart rate variability was used to investigate the balance between sympathetic and parasympathetic activity in the mechanically ventilated subjects during our study (34). Heart rate variability had considerably different behavior in the MV group compared with the TTDN groups. The RMSSD of the R–R intervals in the MV group declined significantly over the 50 hours of the experiment, whereas conversely the RMSSD increased considerably in the TTDN groups. This means that the autonomic system in the MV group had predominantly sympathetic activity, whereas the TTDN groups had predominantly parasympathetic activity. Many factors can affect heart rate variability, such as the use of sedation, systemic inflammation, the use of vasoactive drugs, fluid balance, immobility, respiratory phase (inspiration or expiration) and breathing rate (34–36). Our analysis showed that these variables were nonstatistically different among the groups. Thus, the different behavior of the RMSSD between the MV group and the TTDN groups indicates that there was a vagal, or parasympathetic, predominance in the TTDN groups. It is unclear the extent to which the

Table 2. Serum Inflammatory Marker Results for the Mechanically Ventilated Groups

Serum Inflammatory Markers (End of Study)	Concentration (*pg/ml*) [*Median (IQR)*]			*P* Value (Kruskal-Wallis Test)
	MV (*n* = *10*)	TTDN50% + MV (*n* = *8*)	TTDN100% + MV (*n* = *7*)	
IL-1α	12.89 (5.10–45.74)	9.15 (5.10–18.33)	13.78 (2.21–48.26)	0.7832
IL-1β	153.70 (66.61–458.00)	100.30 (18.23–136.10)	182.30 (115.40–263.60)	0.1378
IL-6	45.27 (24.29–215.90)	17.90 (0.33–215.00)	126.80 (77.52–179.70)	0.1056
IL-8	12.99 (0.00–55.06)	9.48 (6.35–15.83)	3.67 (0.00–9.41)	0.4473
IL-10	195.00 (115.60–937.20)	83.64 (52.38–256.20)	169.10 (63.74–5501.00)	0.3155
TNFα	3.58 (0.00–25.82)	7.17 (0.20–19.48)	0.00 (0.00–0.00)	0.2896

Definition of abbreviations: IQR = interquartile range; MV = mechanical ventilation; TTDN50% = temporary transvenous diaphragm neurostimulation every other breath; TTDN100% = temporary transvenous diaphragm neurostimulation every breath.
Blood samples were taken at the end of the experiment (50 h).

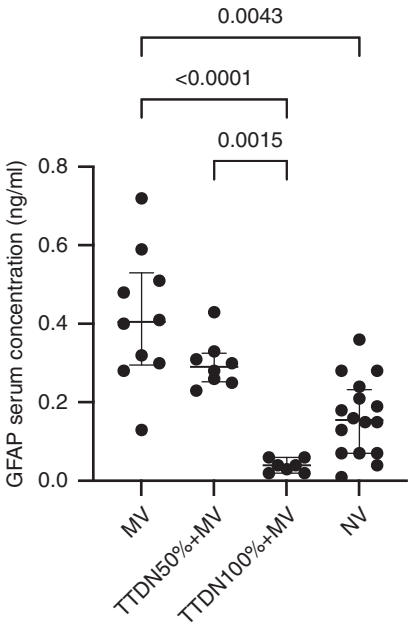

Figure 4. Dot plot showing GFAP (glial fibrillary acid protein) concentrations in the serum: 0.40 ng/ml (0.28–0.57) for the mechanical ventilation (MV) group, 0.29 ng/ml (0.25–0.32) for the TTDN50% + MV group, 0.04 ng/ml (0.02–0.06) for the TTDN100% + MV group, and 0.15 ng/ml (0.07–0.23) for the never-ventilated (NV) group. *Post hoc* analysis using Dunn's multiple comparison test showed statistically significant differences in GFAP serum concentrations between the MV and NV groups (0.40 vs. 0.15, $P = 0.0043$), between the MV and TTDN100% + MV groups (0.40 vs. 0.04, $P < 0.0001$), and between the TTDN50% + MV and TTDN100% + MV groups (0.29 vs. 0.04, $P = 0.0015$). TTDN50% = temporary transvenous diaphragm neurostimulation every other breath; TTDN100% = temporary transvenous diaphragm neurostimulation every breath.

parasympathetic predominance contributed to the observed neuroprotective effect, nor the mechanism of this increased parasympathetic tone. This will be important to elucidate in future studies.

Two pathways for VABI have been discussed previously in the literature: the inflammatory and the neural pathways (3, 4, 8, 12, 37). Several preclinical studies have shown that the inflammatory pathway may trigger VABI (8, 11). According to this hypothesis, inflammatory proteins and inflammatory cells are released into the bloodstream because of ventilation-induced lung injury (30, 38). These inflammatory proteins and inflammatory cells reach the hippocampus through the circumventricular organs, such as the plexus choroid, without having to cross the blood–brain barrier, thereby triggering VABI (30, 38). For instance, one preclinical study showed that when inflammation was blocked in mechanically ventilated subjects, brain injury was mitigated (8); when the authors knocked out Toll-like receptor 4 (an inflammatory sensing protein) to block inflammation, mice showed less hippocampal injury than wild-type subjects after MV (8). However, in our experiment, the systemic inflammatory markers and lung injury scores were similar between the groups, indicating that the experimental conditions did not result in appreciably different degrees of inflammation and lung injury. The similar lung injury scores and the absence of significant differences in systemic inflammation between the mechanically ventilated groups provide evidence against the inflammatory pathway triggering VABI. Another preclinical study provided supporting evidence against the inflammatory pathway triggering VABI, demonstrating that the brain insult associated with MV was similar between pigs with healthy lungs mechanically ventilated for 12 hours and pigs whose lungs were injured by oleic acid mechanically ventilated for 12 hours (12). The authors concluded that the MV itself triggered the brain injury and not the lung injury induced by oleic acid injection (12).

In other preclinical studies, the neural pathway has been shown to trigger hippocampal apoptosis by abnormal activation of pulmonary stretch receptors, such as pulmonary TRPV4 (transient receptor potential vanilloid channel 4) (3, 4). Pulmonary TRPV4 is a cation-selective protein acting as a polymodal signal integrator that responds to pulmonary stretch in addition to a variety of other stimuli, such as mechanical force, products of lipid peroxidation, and prostaglandins (4). When TRPV4 is activated, it releases adenosine triphosphate, stimulating the purinergic receptors, which in turn contributes to the pulmonary vagal afferent signal (4). The vagal afferent signal reaches the hippocampus through the nucleus tractus solitarius–locus coeruleus–hippocampus pathway, releasing dopamine in the hippocampus (39). It has been shown that injurious MV settings (20–30 ml/kg) result in a hyperdopaminergic state, initiating hippocampal apoptosis by the dephosphorylation of the protein kinase B/glycogen synthase kinase-3β (3). To confirm that the vagus nerve played an important role in VABI, one preclinical study showed that either chemical or surgical vagotomy resulted in mitigation

Table 3. $Pa_{O_2}/F_{I_{O_2}}$ Ratios at the Start and the End of the Study, and Lung Injury Scores for All Groups

Gas Exchange Measures and Lung Injury Score	Median (IQR)				*P* Value (Kruskal-Wallis Test)
	MV Group (*n* = *10*)	TTDN50% + MV Group (*n* = *8*)	TTDN100% + MV Group (*n* = *7*)	NV Group (*n* = *6*)	
$Pa_{O_2}/F_{I_{O_2}}$ ratio, study start, mm Hg	521 (454–552)	461 (438–498)	492 (468–509)	568 (546–571)	>0.9999
$Pa_{O_2}/F_{I_{O_2}}$ ratio, study end, mm Hg	403 (357–444)	374 (335–413)	433 (430–470)	—	0.0440
Lung injury score	0.19 (0.16–0.21)	0.21 (0.16–0.44)	0.24 (0.21–0.26)	0.19 (0.17–0.21)	0.4036

Definition of abbreviations: IQR = interquartile range; MV = mechanical ventilation; NV = never ventilated; TTDN50% = temporary transvenous diaphragm neurostimulation every other breath; TTDN100% = temporary transvenous diaphragm neurostimulation every breath.
Subjects from the NV group had arterial blood samples taken once only, at the beginning of the study. Lung injury score ranges from 0.00 to 1.00.

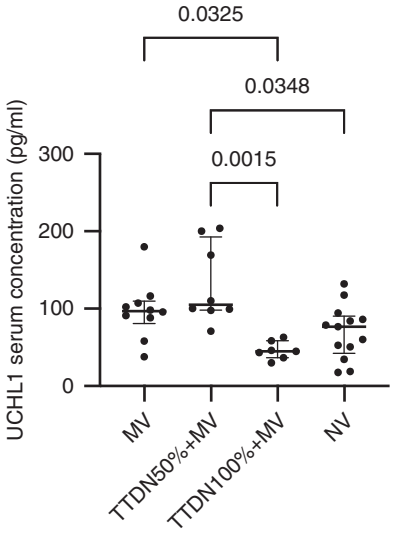

Figure 5. Dot plot showing UCHL1 (ubiquitin carboxy-terminal hydrolase L1) concentrations in the serum: 96.96 pg/ml (80.65–109.60) for the mechanical ventilation (MV) group, 110.00 pg/ml (97.59–200.40) for the TTDN50% + MV group, 44.68 pg/ml (36.56–58.34) for the TTDN100% + MV group, and 76.57 pg/ml (42.48–90.26) for the never-ventilated (NV) group. *Post hoc* analysis using Dunn's multiple comparison test showed statistically significant differences between the MV and TTDN100% + MV groups (96.96 vs. 44.68, $P = 0.0325$), between the TTDN50% + MV and TTDN100% + MV groups (110.00 vs. 44.68, $P = 0.0015$), and between the TTDN50% + MV and NV groups (110.00 vs. 76.57, $P = 0.0348$). TTDN50% = temporary transvenous diaphragm neurostimulation every other breath; TTDN100% = temporary transvenous diaphragm neurostimulation every breath.

of VABI (3). It is unknown whether lung-protective MV also results in an injurious hippocampal hyperdopaminergic state, although it is reasonable to postulate that a hyperdopaminergic state may have triggered the hippocampal injury observed in our mechanically ventilated groups. As the primary effect of TTDN is pulmonary insufflation from diaphragm activation, we postulate that TTDN modulates pulmonary stretch receptor activation, including but not necessarily limited to TRPV4, by invoking a stretch response similar to that seen during spontaneous breathing. This in turn could regulate the release of dopamine in the hippocampus, consequently providing neuroprotection. Although we postulate that the neural pathway triggered VABI in our study, our experiment was not designed to elucidate mechanisms of VABI mitigation by TTDN. Further work is needed to better characterize the pathways responsible for the neuroprotective effect of TTDN.

Our study has some limitations. It may have missed identifying some differences between groups because of its small sample size, although it is adequately powered to detect significant differences between the groups for the variables analyzed. Despite the fact that it may be considered a long-term preclinical experiment, our study lasted only 50 hours, which might be considered a short-term experiment for clinical studies. Our model used subjects with healthy lungs, therefore limiting its generalizability. However, using normal lungs allowed us to isolate the variables of interest. Additionally, we did not directly record the vagus nerve signal. Measuring heart rate variability via RMSSD does, however, provide a validated, noninvasive method to quantify autonomic system activity (34). Our study did not analyze the clinical outcomes of the neuroprotection observed, which should be considered in future studies. Also, our study only used female subjects, so our results may not be extrapolated to male subjects if there is a sex-specific aspect to neuroprotection. Additionally, our study did not analyze potential implications of hippocampal plasticity in VABI, as hippocampal plasticity could act as a mechanism of adaptation to neural insult (21). Our study did not collect cardiac output and systemic vascular resistance for all subjects owing to equipment limitations. It has been shown that diaphragm stimulation during MV might assist in increasing venous return by instituting a more negative pressure gradient in the thorax (40). This has a relatively minor hemodynamic impact in stable animals, and it is unlikely to be the basis of our findings, but it should be studied in future experiments. Finally, although we only observed rare dyssynchronous events, reverse triggering episodes cannot be entirely ruled out.

Conclusions

Mechanical ventilation is associated with hippocampal apoptosis and inflammation, independent of lung injury and systemic inflammation. In a porcine model, temporary transvenous diaphragm neurostimulation results in neuroprotection when applied in synchrony with lung-protective MV for 50 hours. The

Table 4. Lung Physiology Results for the Mechanically Ventilated Groups

Time	Measurement	Median (IQR)			*P* Value (Kruskal-Wallis Test)	*P* Value (Dunn's Multiple Comparison Test)	
		MV Group (*n* = 10)	TTDN50% + MV Group (*n* = 8)	TTDN100% + MV Group (*n* = 7)			
Baseline	Esophageal pressure, cm H_2O	9 (6–14)	9 (6–13)	10 (8–12)	ns	MV vs. TTDN50% + MV MV vs. TTDN100% + MV TTDN50% + MV vs. TTDN100% + MV	— — —
	Plateau pressure, cm H_2O	16 (14–16)	16 (15–18)	16 (15–17)	ns	MV vs. TTDN50% + MV MV vs. TTDN100% + MV TTDN50% + MV vs. TTDN100% + MV	— — —
	Driving pressure, cm H_2O	11 (9–11)	11 (11–13)	12 (11–12)	ns	MV vs. TTDN50% + MV MV vs. TTDN100% + MV TTDN50% + MV vs. TTDN100% + MV	— — —
	Transpulmonary plateau pressure, cm H_2O	9 (8–10)	10 (8–13)	6 (5–7)	0.0015	MV vs. TTDN50% + MV MV vs. TTDN100% + MV TTDN50% + MV vs. TTDN100% + MV	ns 0.0013 0.0079
Study end	Esophageal pressure, cm H_2O	7 (6–10)	7 (6–10)	10 (6–12)	ns	MV vs. TTDN50% + MV MV vs. TTDN100% + MV TTDN50% + MV vs. TTDN100% + MV	— — —
	Plateau pressure, cm H_2O	19 (18–21)	20 (18–22)	18 (16–18)	0.0258	MV vs. TTDN50% + MV MV vs. TTDN100% + MV TTDN50% + MV vs. TTDN100% + MV	ns ns 0.0431
	Driving pressure, cm H_2O	14 (13–16)	15 (13–17)	13 (12–14)	0.0258	MV vs. TTDN50% + MV MV vs. TTDN100% + MV TTDN50% + MV vs. TTDN100% + MV	ns ns 0.0431
	Transpulmonary plateau pressure, cm H_2O	14 (13–16)	14 (12–16)	7 (5–13)	0.0018	MV vs. TTDN50% + MV MV vs. TTDN100% + MV TTDN50% + MV vs. TTDN100% + MV	ns 0.0232 0.0367

Definition of abbreviations: IQR = interquartile range; MV = mechanical ventilation; ns = not significant; TTDN50% = temporary transvenous diaphragm neurostimulation every other breath; TTDN100% = temporary transvenous diaphragm neurostimulation every breath.

Esophageal pressure, plateau pressure, driving pressure, and transpulmonary plateau pressure were measured at the beginning and the end of the study. Esophageal pressure was measured at the end of inspiration. Transpulmonary plateau pressure was measured during the end-inspiratory plateau. Driving pressure was calculated as end-inspiratory plateau pressure − end-expiratory pressure.

neuroprotection observed was characterized by levels of hippocampal apoptosis, hippocampal inflammation, and neurological injury markers in the serum similar to those in the never-ventilated group. In addition, the degree of neuroprotection increases with greater exposure to TTDN. This is an important finding that supports further translational research. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the following people for their efforts and assistance in completing this body of work: Suzette Willems, Jessica Wittmann, Kate Orchard, Pamela Zurek, Stephanie Smith, Dr. Ian Welch, Dawn Bitz, and Viral Thakkar.

References

1. Goligher EC, Ferguson ND, Brochard LJ. Clinical challenges in mechanical ventilation. *Lancet* 2016;387:1856–1866.
2. Reynolds SC, Meyyappan R, Thakkar V, Tran BD, Nolette MA, Sadarangani G, *et al.* Mitigation of ventilator-induced diaphragm atrophy by transvenous phrenic nerve stimulation. *Am J Respir Crit Care Med* 2017;195:339–348.
3. González-López A, López-Alonso I, Aguirre A, Amado-Rodríguez L, Batalla-Solís E, Astudillo A, *et al.* Mechanical ventilation triggers hippocampal apoptosis by vagal and dopaminergic pathways. *Am J Respir Crit Care Med* 2013;188:693–702.
4. González-López A, López-Alonso I, Pickerodt PA, von Haefen C, Amado-Rodríguez L, Reimann H, *et al.* Lung purinoceptor activation triggers ventilator-induced brain injury. *Crit Care Med* 2019;47:e911–e918.
5. Bassi TG, Rohrs EC, Fernandez KC, Ornowska M, Nicholas M, Gani M, *et al.* Brain injury after 50 h of lung-protective mechanical ventilation in a preclinical model. *Sci Rep* 2021;11:5105.
6. Bassi T, Fernandez K, Rohrs E, Ornowska M, Nicholas M, Gani M, *et al.* Diaphragm neurostimulation reduces percentages of hippocampal reactive astrocytes and GFAP serum concentrations in pigs mechanically ventilated for 50 hours [abstract]. *Am J Respir Crit Care Med* 2021;203:A2813.
7. Barton SK, Tolcos M, Miller SL, Christoph-Roehr C, Schmölzer GM, Moss TJ, *et al.* Ventilation-induced brain injury in preterm neonates: a review of potential therapies. *Neonatology* 2016;110:155–162.
8. Chen T, Chen C, Zhang Z, Zou Y, Peng M, Wang Y. Toll-like receptor 4 knockout ameliorates neuroinflammation due to lung-brain interaction in mechanically ventilated mice. *Brain Behav Immun* 2016;56:42–55.
9. Bassi T, Rohrs E, Reynolds S. Systematic review of cognitive impairment and brain insult after mechanical ventilation. *Crit Care* 2021;25:99.
10. Giordano G, Pugliese F, Bilotta F. Neuroinflammation, neuronal damage or cognitive impairment associated with mechanical ventilation: a systematic review of evidence from animal studies. *J Crit Care* 2021; 62:246–255.
11. Chen C, Zhang Z, Chen T, Peng M, Xu X, Wang Y. Prolonged mechanical ventilation-induced neuroinflammation affects postoperative memory dysfunction in surgical mice. *Crit Care* 2015;19:159.
12. Kamuf J, Garcia-Bardon A, Ziebart A, Thomas R, Folkert K, Frauenknecht K, *et al.* Lung injury does not aggravate mechanical ventilation-induced early cerebral inflammation or apoptosis in an animal model. *PLoS One* 2018;13:e0202131.
13. Ataya A, Silverman E, Aranya B, Aarti S, Gerard J, David L. Temporary transvenous diaphragmatic neurostimulation in prolonged mechanically ventilated patients: a feasibility trial (RESCUE 1). Critical Care Explor 2020;2:e0106.
14. Dres M, Gama De Abreu M, Similowski T. Temporary transvenous diaphragm neurostimulation in difficult-to-wean mechanically ventilated patients - results of the RESCUE 2 randomized controlled trial [abstract]. *Eur Respir J* 2020;56:4352.
15. Rohrs E, Ornowska M, Bassi T, Fernandez K, Reynolds S. Temporary transvenous phrenic-nerve-stimulated diaphragm neurostimulation during mechanical ventilation preserves homogeneity in non-injured lungs [abstract]. *Am J Respir Crit Care Med* 2020;201:A4120.
16. Rohrs E, Bassi T, Fernandez K, Ornowska M, Nicholas M, Wittmann J, *et al.* Diaphragm neurostimulation during mechanical ventilation reduces atelectasis and transpulmonary plateau pressure, preserving lung homogeneity and $Pa_{O_2}/F_{I_{O_2}}$. *J Appl Physiol* 2021;131:290–301.
17. Grasso S, Stripoli T, De Michele M, Bruno F, Moschetta M, Angelelli G, *et al.* ARDSnet ventilatory protocol and alveolar hyperinflation: role of positive end-expiratory pressure. *Am J Respir Crit Care Med* 2007;176:761–767.
18. Reynolds S, Ebner A, Meffen T, Thakkar V, Gani M, Taylor K, *et al.* Diaphragm activation in ventilated patients using a novel transvenous phrenic nerve pacing catheter. *Crit Care Med* 2017;45:e691–e694.
19. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, *et al.*; Acute Lung Injury in Animals Study Group. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. *Am J Respir Cell Mol Biol* 2011;44:725–738.
20. Voldby AW, Brandstrup B. Fluid therapy in the perioperative setting-a clinical review. *J Intensive Care* 2016;4:27.
21. Yasuda Y, Shimoda T, Uno K, Tateishi N, Furuya S, Tsuchihashi Y, *et al.* Temporal and sequential changes of glial cells and cytokine expression during neuronal degeneration after transient global ischemia in rats. *J Neuroinflammation* 2011;8:70.
22. Janz DR, Abel TW, Jackson JC, Gunther ML, Heckers S, Ely EW. Brain autopsy findings in intensive care unit patients previously suffering from delirium: a pilot study. *J Crit Care* 2010;25:538.e7–538.e12.
23. Papa L, Brophy GM, Welch RD, Lewis LM, Braga CF, Tan CN, *et al.* Time course and diagnostic accuracy of glial and neuronal blood biomarkers GFAP and UCH-L1 in a large cohort of trauma patients with and without mild traumatic brain injury. *JAMA Neurol* 2016;73:551–560.
24. Papa L, Zonfrillo MR, Welch RD, Lewis LM, Braga CF, Tan CN, *et al.* Evaluating glial and neuronal blood biomarkers GFAP and UCH-L1 as gradients of brain injury in concussive, subconcussive and non-concussive trauma: a prospective cohort study. *BMJ Paediatr Open* 2019;3:e000473.
25. Marchi N, Rasmussen P, Kapural M, Fazio V, Kight K, Mayberg MR, *et al.* Peripheral markers of brain damage and blood-brain barrier dysfunction. *Restor Neurol Neurosci* 2003;21:109–121.
26. Scaccianoce S, Del Bianco P, Pannitteri G, Passarelli F. Relationship between stress and circulating levels of S100B protein. *Brain Res* 2004;1004:208–211.
27. Fan S, Wang H, Yin J. Increase of plasma S100B level in patients with moderate and severe traumatic brain injury. *Int J Clin Exp Pathol* 2016; 9:12130–12135.
28. Ercole A, Thelin EP, Holst A, Bellander BM, Nelson DW. Kinetic modelling of serum S100b after traumatic brain injury. *BMC Neurol* 2016;16:93.
29. Tsuruta R, Oda Y, Shintani A, Nunomiya S, Hashimoto S, Nakagawa T, *et al.*; Japanese Epidemiology of Delirium in ICUs (JEDI) Study Investigators. Delirium and coma evaluated in mechanically ventilated patients in the intensive care unit in Japan: a multi-institutional prospective observational study. *J Crit Care* 2014;29:472.e1–472.e5.
30. Turon M, Fernández-Gonzalo S, de Haro C, Magrans R, López-Aguilar J, Blanch L. Mechanisms involved in brain dysfunction in mechanically ventilated critically ill patients: implications and therapeutics. *Ann Transl Med* 2018;6:30.
31. Pellegrini M, Hedenstierna G, Roneus A, Segelsjö M, Larsson A, Perchiazzi G. the diaphragm acts as a brake during expiration to prevent lung collapse. *Am J Respir Crit Care Med* 2017;195:1608–1616.
32. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest* 2007;117:289–296.
33. Frasch MG, Szynkaruk M, Prout AP, Nygard K, Cao M, Veldhuizen R, *et al.* Decreased neuroinflammation correlates to higher vagus nerve activity fluctuations in near-term ovine fetuses: a case for the afferent cholinergic anti-inflammatory pathway? *J Neuroinflammation* 2016;13: 103.
34. Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology. Heart rate variability: standards of measurement, physiological interpretation, and clinical use. *Eur Heart J* 1996;93:1043–1065.

35. Jo A, Blasi A, Valladares E, Juarez R, Baydur A, Khoo M. Determinants of heart rate variability in obstructive sleep apnea syndrome during wakefulness and sleep. Am J Physiol Heart Circ Physiol 2005;288: H1103–H1112.
36. Lotufo PA, Valiengo L, Benseñor IM, Brunoni AR. A systematic review and meta-analysis of heart rate variability in epilepsy and antiepileptic drugs. *Epilepsia* 2012;53:272–282.
37. Kamuf J, Garcia Bardon A, Ziebart A, Frauenknecht K, Folkert K, Schwab J, *et al.* Experimental lung injury induces cerebral cytokine mRNA production in pigs. *PeerJ* 2020;8:e10471.
38. López-Aguilar J, Fernández-Gonzalo MS, Turon M, Quílez ME, Gómez-Simón V, Jódar MM, *et al.*; GT-IRA de la SEMICYUC. Lung-brain interaction in the mechanically ventilated patient [in Spanish]. *Med Intensiva* 2013;37:485–492.
39. Castle M, Comoli E, Loewy AD. Autonomic brainstem nuclei are linked to the hippocampus. *Neuroscience* 2005;134:657–669.
40. Masmoudi H, Persichini R, Cecchini J, Delemazure J, Dres M, Mayaux J, *et al.* Corrective effect of diaphragm pacing on the decrease in cardiac output induced by positive pressure mechanical ventilation in anesthetized sheep. *Respir Physiol Neurobiol* 2017;236:23–28.