Blockage of the Upregulation of Voltage-Gated Sodium Channel $\text{Na}_v1.3$ Improves Outcomes after Experimental Traumatic Brain Injury

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Abstract

Excessive active voltage-gated sodium channels are responsible for the cellular abnormalities associated with secondary brain injury following traumatic brain injury (TBI). We previously presented evidence that significant upregulation of $\text{Na}_v1.3$ expression occurs in the rat cortex at 2 h and 12 h post-TBI and is correlated with TBI severity. In our current study, we tested the hypothesis that blocking upregulation of $\text{Na}_v1.3$ expression *in vivo* in the acute stage post-TBI attenuates the secondary brain injury associated with TBI. We administered either antisense oligodeoxynucleotides (ODN) targeting $\text{Na}_v1.3$ or artificial cerebrospinal fluid (aCSF) at 2 h, 4 h, 6 h, and 8 h following TBI. Control sham animals received aCSF administration at the same time points. At 12 h post-TBI, $\text{Na}_v1.3$ messenger ribonucleic acid (mRNA) levels in bilateral hippocampi of the aCSF group were significantly elevated, compared with the sham and ODN groups ($p < 0.01$). However, the $\text{Na}_v1.3$ mRNA levels in the uninjured contralateral hippocampus of the ODN group were significantly lowered, compared with the sham group ($p < 0.01$). Treatment with antisense ODN significantly decreased the number of degenerating neurons in the ipsilateral hippocampal CA3 and hilar region ($p < 0.01$). A set of left-to-right ratio value analyzed by magnetic resonance imaging T2 image on one day, three days, and seven days post-TBI showed marked edema in the ipsilateral hemisphere of the aCSF group, compared with that of the ODN group ($p < 0.05$). The Morris water maze memory retention test showed that both the aCSF and ODN groups took longer to find a hidden platform, compared with the sham group ($p < 0.01$). However, latency in the aCSF group was significantly higher than in the ODN group ($p < 0.05$). Our *in vivo* $\text{Na}_v1.3$ inhibition studies suggest that therapeutic strategies to block upregulation of $\text{Na}_v1.3$ expression in the brain may improve outcomes following TBI.

Key words: antisense oligodeoxynucleotides; intracerebroventricular administration; $\text{Na}_v1.3$; traumatic brain injury; voltage-gated sodium channels

Introduction

TRAUMATIC BRAIN INJURY (TBI) is an important public health problem, and leads to death and disability. Although great advances have been made to improve outcomes of TBI patients in the 21st century, there are still great limitations to restoring high levels of function for patients.¹ TBI can be classified as primary mechanical injury or secondary injury. The secondary injury is considered to consist of complex biomolecular and pathophysiological changes that still are not completely understood.² Excessive activation of voltage-gated sodium channels (VGSCs) is known to occur during secondary brain injury and results in vasogenic and cytotoxic edema, both being harmful to cellular stability in both *in vivo* and *in vitro* studies.^{3,4}

VGSCs consist of α and β subunits. Nine Na_v1 α subunit genes ($\text{Na}_v1.1$ – 9), which contain the ion-selective pore and act as voltage

sensors within the cell membrane, have been identified in mammals.⁵ Among the nine Na_v1 α subunits, $\text{Na}_v1.1$, $\text{Na}_v1.2$, and $\text{Na}_v1.6$ are abundant in the central nervous system (CNS), whereas $\text{Na}_v1.3$ is mostly present during embryonic development.⁶ $\text{Na}_v1.3$, a tetrodotoxin-sensitive channel, has been reported to play an important role in different neurogenic diseases, such as traumatic spinal cord injury, epilepsy, and neuropathic pain.^{7–12} Our previous study demonstrated that expression of $\text{Na}_v1.3$ messenger ribonucleic acid (mRNA) and protein were significantly upregulated in the rat ipsilateral-injured cortex at the very early stage post-TBI and correlated with TBI severity.¹³

Antisense oligodeoxynucleotides (ODN) are single-stranded deoxyribonucleic acid (DNA) or RNA molecules, which are complementary to the target gene's mRNA. There are two effects of antisense ODN: inducing the degradation of target mRNA and inhibiting the progression of relative protein translation. Antisense

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ODN knock-down strategies, used for silencing gene expression, have been used widely in the laboratory for decades. Sodium channel blockers have been shown to reduce cerebral edema and improve cognitive function in several experimental TBI studies^{14–16} but these drugs are not translatable to TBI patients due to their unwanted adverse effects.

In this study, we tested the hypothesis that blocking upregulation of $\text{Na}_v1.3$ expression in the brain may improve outcome by intracerebroventricular (ICV) administration of antisense ODN targeting $\text{Na}_v1.3$. We assessed both the molecular expression of $\text{Na}_v1.3$ in the hippocampi and cognitive-behavioral outcome.

Methods

Subjects

Male Sprague-Dawley rats ($n=98$; 316–342 g) were used in this study. All experimental protocols were conducted according to the Principles of Laboratory Animal Care formulated by National Committee of Medical Research and the Guide for the Use of Laboratory Animals issued by Institute of Laboratory Animal Resources of China, and approved by the Shanghai Jiao Tong University School of Medicine Research Animal Care and Use Committee. Animals were housed in a conventional rodent facility at 22–25°C on a 12 h light/dark cycle with free access to food and water.

Experimental design

Rats were randomized into three groups: ODN, aCSF, and sham. All animals were subjected to ICV cannulation surgery two days prior to lateral fluid percussion injury. On the injury day, rats were subjected to TBI (2.9–3.0 ATM) or sham-TBI. Each TBI rat received a total of four ICV administrations of a vehicle (aCSF) or an antisense ODN targeting $\text{Na}_v1.3$, while sham rats received only aCSF. The time points for both drug or vehicle administration in all animals were at 2 h, 4 h, 6 h, and 8 h post-TBI. In a separate experiment, a subgroup of ODN rats received ICV administration of cyanine dye 3 (Cy3)-tagged antisense ODN only at 8 h post-TBI ($n=3$).

We conducted five separate studies with all animals undergoing the exact procedures as described above. In study one, we examined expression of mRNA for $\text{Na}_v1.3$ in the bilateral hippocampi of all rats at 12 h post-TBI ($n=6$ per group) by real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). In study two, brains were harvested at 12 h post-TBI and sections were stained with Fluoro-Jade B (FJ-B) for degenerating CA3 and hilar neurons ($n=7$ in the ODN group, $n=6$ in the aCSF group, and $n=3$ in the sham group). Double labeling with $\text{Na}_v1.3$ antibody and neuronal nuclei-specific antibody (NeuN) was performed to verify that immunoreactivity was observed in neurons. In study three, ODN rats ($n=3$) that received one single administration of Cy3-tagged antisense ODN 8 h post-TBI were sacrificed at 12 h post-TBI, and brain sections were stained with NeuN to examine ODN penetration and uptake. In study four, magnetic resonance imaging was performed three days prior to TBI and on Day 1, Day 3, and Day 7 post-TBI to quantify hemispheric midline shift ($n=10$ in the ODN group, $n=9$ in the aCSF group, and $n=8$ in the sham group). In study five, Morris water maze (MWM) was performed on 21–25 days post-TBI to evaluate for chronic cognitive behavior deficits ($n=10$ in the ODN group, $n=9$ in the aCSF group, and $n=8$ in the sham group).

Surgical preparation

Two days prior to TBI, rats were anesthetized by 0.4% sodium pentobarbital (40 mg/kg, intraperitoneal injection) and mounted in a stereotaxic frame. A scalp incision was made along the midline and a craniectomy (4.8 mm) was performed on the left parietal bone

(midway between the bregma and lambda). Two stainless steel screws (1.5 mm diameter, 5.5 mm length) were placed 1 mm rostral to bregma and 1 mm caudal to lambda.

Animals were stereotactically implanted with a stainless steel cannula into the right lateral ventricle of the brain (ventral-dorsal-3.5 mm; anterior-posterior-0.8 mm; medial-lateral-1.5 mm).¹⁷ The cannula was made from a Novofine 30G needle (Novo Nordisk, Denmark) and the PE-10 catheter (0.28 mm ID, 0.61 mm OD). The needle was cut to a length of 11 mm and its end was connected to a PE-10 catheter (35 mm length). The cannula was connected to a micro-syringe and filled with aCSF before implantation. Correct cannula placement into the lateral ventricle was verified by the outflow of CSF after disconnecting the syringe from the cannula. The cannula was heat sealed to prevent leakage of CSF.

A rigid plastic injury tube (modified 22 gauge needle hub, 2.6 mm inside diameter) was placed on the exposed, intact dura. Then, the injury tube and the intracerebroventricular cannulation were secured to the skull with dental cement. Bone wax was used to cover the open needle hub connector after the dental cement hardened. Then, the scalp was sutured closed and the cannula was left subcutaneous, and animals were returned to their cages for recovery.

Lateral fluid percussion

Rats were subjected to TBI 48 h after the surgical preparation in order to minimize possible confounding effects of surgical trauma. The fluid percussion injury device (VCU Biomedical Engineering, Richmond, VA) has been described previously by Dixon and colleagues and McIntosh and colleagues.^{18,19} In brief, the device consisted of a plexiglas cylindrical reservoir filled with 37°C isotonic saline, bounded at one end by a rubber-covered plexiglas piston mounted on O-rings, and the opposite end had a pressure transducer housing with a 2.6 mm inside diameter male needle hub opening. Injury was induced by a metal pendulum, which struck the piston of the device from a predetermined height. This technique produces a small volume of saline epidurally into the closed cranial cavity. The amplitude of pressure pulse (in ATM), was measured by the pressure transducer (Statham PA 85-100; Gould, Oxnard, CA) and recorded on a storage oscilloscope (Tektronix 5111; Tektronix, Beaverton, OR).

On TBI day, rats were anesthetized with 2% isoflurane in a 2:1 nitrous oxide/oxygen mixture, intubated, and mechanically ventilated with a rodent ventilator (ALC-V8S, Shanghai Alcott Biotech Ltd., Shanghai, China). Rectal temperature ($37 \pm 0.4^\circ\text{C}$) was continuously monitored and maintained by a feedback temperature-controlled pad. Brain temperature ($36 \pm 0.4^\circ\text{C}$) was measured by insertion of a 29-gauge needle probe between the temporalis muscle and skull. The suture was opened and bone wax was removed. Rats were disconnected from the ventilator and the injury tube was connected to the fluid percussion cylinder. A fluid percussion pulse (2.9–3.0 ATM) mimicking a TBI was delivered directly onto the exposed dura within 10 msec. Immediately after TBI, rats were ventilated with a 2:1 nitrous oxide/oxygen mixture without isoflurane, and the rectal and temporalis muscle temperature were recorded. Then, the scalp was sutured closed and the cannula was left outside of the suture for further administration. As soon as spontaneous breathing was observed, rats were extubated and placed in a supine position, and their righting time was recorded. The righting reflex measures the ability to spontaneously recover to prone position, is used as an indicator of level of unconsciousness and injury severity. Sham-TBI group rats were subjected to all anesthetic and surgical procedures but without injury.

Antisense ODN synthesis and delivery

At 2 h, 4 h, 6 h, and 8 h post-TBI, under brief (< 1 min) isoflurane sedation (4% by facial mask), ICV administration of an antisense

ODN sequence corresponding to the translation initiation site of Na_v1.3 (5'-CAGTGCCTGGGCCATCTTTTC-3' (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., Shanghai, China) was initiated in the ODN group. A total of four injections of antisense ODN (35 µg/5 µL) in aCSF (containing in mM: 1.3 CaCl₂·2H₂O, 2.6 KCl, 0.9 MgCl₂, 21.0 NaHCO₃, 2.5 Na₂HPO₄·7H₂O, and 125.0 NaCl, prepared in sterile H₂O) was injected followed by 10 µL of aCSF flush. Fifteen µL of aCSF only was injected by the same manner at the same time points post-TBI in both the aCSF and sham groups. An additional three rats in the ODN group had a different ICV administration of Cy3-tagged antisense ODN at 8 h post-TBI. The cannula was sealed by heat after each injection. Immediately after the last injection, the needle hub, screws, intracerebroventricular cannula, and dental cement were removed from the skull. The burr hole used for drug administration was filled with bone wax and covered with dental cement; then, the scalp was sutured closed.

By basic local alignment search tool search, there was no similarity between the 21 nucleotides of the antisense sequence and any other sodium channel of other genes. Further, there was no homology to Na_v1.1, Na_v1.2, Na_v1.6, or other sodium channels that are known to be expressed in the brain.

Tissue collection and sectioning

Rats were anesthetized deeply by sodium pentobarbital (100 mg/Kg, i.p.) at 12 h post-TBI. For Na_v1.3 mRNA analysis, rats were perfused transcardially with 200 mL of 4°C 0.9% saline, and the bilateral hippocampi were collected. The tissue samples were frozen in liquid nitrogen, and kept at -80°C for real-time quantitative polymerase chain reaction (RT-qPCR).

For brain sections, rats were perfused transcardially with 200 mL of 4°C 0.9% saline, followed by 250 mL of ice-cold 4% PFA (PH=7.4). Brains were removed and post-fixed for 4 h in 4% PFA at 4°C, then cryoprotected overnight in 20% sucrose solution followed by 1 day in a 30% sucrose solution. Serial coronal sections (25 µm) were collected between -2.30 mm bregma and -4.30 mm bregma. Three sets of 16 sections collected from every fifth section were selected for appropriate staining.

RT-qPCR

Total RNA was extracted with TRIZOL Reagent (Invitrogen Ltd., Life Technologies, Paisley, Scotland) according to the manufacturer's instructions. The complementary DNA (cDNA) synthesis from the isolated RNA was performed using a reverse transcriptional system. Briefly, 2 µg of total RNA was reversely transcribed using 1 µL of oligo (dT)₁₅ primer (Shanghai Sangon Biological Engineering Technology and Service Co., Ltd.). The tube was heated to 70°C for 5 min, and then cooled on ice for 2 min. A volume of 5 µL of 5×M-MLV Reaction Buffer, 1.25 µL of 10 mmol/L each of dATP, dCTP, dGTP, and dTTP, 25 units of RNasin RNase Inhibitor, and 200 units of M-MLV RT RNase H⁻ (Promega Biotech CO., Ltd., Beijing, China) were added to the reagent to give a total reaction volume of 25 µL. And then the tube was incubated at 42°C for 60 min and kept at -20°C. The cDNA was amplified by PCR using specific oligonucleotide primers. Follow as Na_v1.3: forward and reverse primers were 5'-CCCACTACGGCTACACAAGC-3', 5'-CCTGAGTCATGAGTCGAAACAGG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward and reverse primers were 5'-ATCACTGCCACTCAGAA GAC-3' and 5'-AAGTCACAGGAGACAACCTG-3'. A total volume of 20 µL PCR reaction included a 2 µL cDNA sample, 10 µL of 2×SYBR Premix Ex Taq (TaKaRa, Ltd., Tokyo, Japan) and 2 µL of each gene-specific forward and reverse primer as described above. All PCR reactions were performed in a Lightcycler PCR detection system (Roche Diagnostics Ltd.). The Na_v1.3/ GAPDH product ratios were calculated and considered as an index of Na_v1.3 mRNA expression. The samples were performed in triplicate.

Acute neuronal degeneration

To evaluate for acute neuronal damage following TBI, one set of selected sections were stained for degenerating neurons using FJ-B at 12 h post-TBI. FJ-B histofluorescence staining procedures were conducted according to the previous study.²⁰ Tissue sections were rinsed in 0.1 M phosphate buffered saline (PBS) and dehydrated in 50°C for 30 min. The sections were first immersed in a solution containing 1% sodium hydroxide in 80% alcohol (5 min), and followed by 70% alcohol (2 min). After being rinsed in dH₂O (2 min), sections were transferred to a solution of 0.06% potassium permanganate (10 min). After a second wash in dH₂O (2 min), sections were incubated in 0.0004% FJ-B staining solution for 20 min (Histochem, Jefferson, AZ). After staining, the sections were mounted on slides and placed in a slide warmer (50°C for 10 min). After drying, the sections were immersed in xylene and coverslipped for further examination by fluorescence microscopy.

Cell counting

Anatomical regions of interesting for cell counting were conducted according to the previous study.²¹ From our previous experience, FJ-B positive neurons were nonexistent in the contralateral hippocampus; therefore, we focused our cell counts in the ipsilateral CA3 and hilus. Degenerating neurons were identified as FJ-B green fluorescing, morphologically distinct neuronal cell bodies with at least one clearly identifiable dendrite. For each animal, every fifth coronal section for a total 16 sections (400 µm) were stained with FJ-B. Digital microscopic images from these 16 sections were collected at 20× magnification using a fluorescence microscope with a camera. We conducted a modified stereological approach to count the degenerating neurons in hippocampi from different groups. In the x-y plane, we defined the subgranular zone of the CA3 and hilus as the area within two cell bodies (~20 µm) of the inner edge of the molecular layer. In the z-plane, a modified optical disector method was employed to ensure that the layer with the largest amount of clear degenerating neurons was chosen. The FJ-B positive neurons per sections were counted using the Cell Counter tool of the software IMAGEJ 1.43 (National Institute of Health, United States). The total number of degenerating neurons was estimated by multiplying the number counted by five as previous described.²² An investigator blinded to the drug groups collected all stereological data for the study.

Double immunofluorescent staining

To verify for immunoreactivity in neurons, double labeling with Na_v1.3 antibody and NeuN antibody was performed on the second set of selected cryosections described above. Sections were first blocked with 0.1% Triton X-100 for 2 h in order to avoid non-specific background staining. First, the sections were incubated with the primary rabbit polyclonal antibody for Na_v1.3 (1:50 dilution; Alomone Labs, Jerusalem, Israel) at 37°C for 1 h. After three washes with 0.1 M PBS, sections were then incubated with the second antibody of Alexa Fluor 555 goat anti-rabbit immunoglobulin G (IgG; Invitrogen Ltd., Life Technologies, Paisley, Scotland) at 37°C for 30 min. After the second immunofluorescent staining, tissues were rinsed with three washes of 0.1 M PBS using the same procedure described above with the primary mouse anti-human NeuN protein monoclonal antibody (maixin.bio, Co., Ltd., Fuzhou, China) and the second antibody of Alexa Fluor 488 goat anti-mouse IgG (Invitrogen Ltd., Life Technologies). The stained sections were examined with a Leica fluorescence microscope (Leica, DM 5000B; Germany).

Fluorescent tracer of Antisense ODN

Sections from three rats with administration of Cy3-tagged antisense ODN were counterstained with NeuN to detect penetration

of antisense ODN around the cerebral ventricles. These sections were rinsed in 0.1M PBS and dehydrated in 50°C for 30 min. Sections were then incubated with the primary mouse anti-human NeuN protein monoclonal antibody (maixin.bio, Co., Ltd., Fuzhou, China) at 37°C for 30 min. After three washes with 0.1 M PBS, sections were then incubated with the second antibody of Alexa Fluor 488 goat anti- mouse IgG (Invitrogen Ltd., Life Technologies). After being washed in 0.1 M PBS and sealed with a coverslip, the slides were analyzed using a fluorescence microscopy.

Magnetic resonance T2-weighted imaging

Images were performed on a 3.0-Tesla magnetic resonance imaging (MRI) system (Signa HDx, GE Medical System, Milwaukee, WI). Rats were anesthetized by 0.4% sodium pentobarbital (40 mg/kg, i.p.) at three days prior to injury as well as one day, three days, and seven days post-TBI, and placed in a prone position on a 50 mm diameter transmit-receiving birdcage coil, parallel to the B0 magnetic field, in the magnet bore.

T2-weighted anatomical images were acquired with the following parameters: repetition time (TR)=1200 msec; echo time (TE)=60 msec; matrix=384×256; number of excitation=6; echo train length=16; bandwidth=31.2 kHz; field of view=11×11 cm²; slice thickness=2.4 mm; and intersection gap=0.0 mm.

The midline shift was evaluated as an indicator of the severity of brain edema post-TBI.²³ MRI scans were analysed with National Institutes of Health ImageJ 1.43 (National Institutes of Health, Bethesda, MD), and the degree of midline shift were measured directly by constructing a straight line from the midpoint of the longitudinal cerebral fissure to the middle of the mammillary body. The length from the lateral surface of the cortex to the midline was measured and a left-to-right ratio was calculated (Fig. 6A). A value higher than 1.0 indicated a midline shift that resulted from edema in the left hemisphere.

Morris water maze

Spatial memory performance was evaluated on Day 21–25 post-TBI using a Morris water maze.²⁴ A circular tank with dimensions of 200 cm in diameter and 60 cm in height was filled with water (24–28 °C) at a depth of 30 cm. The hidden escape platform was submerged 1.5 cm below water. Four consistent visual cues were located on the test room wall outside the maze. On each test day, rats were placed randomly in one of four directions (north, south, east, west) and allowed to swim (maximum 90 sec) until they located the hidden escape platform placed in the middle of the southeast quadrant of the pool. If the rat did not find the platform within 90 sec, the experimenter placed the rat on the platform for 20 sec before being removed from the maze. Rats received a total of four trials per day, one from each starting point and with an inter-trial interval of 4 min. The data were analyzed for mean latency to find the platform.

Statistical analysis

Data analysis was performed by using statistical software (SPSS Version 13.0, SPSS Inc, Chicago, USA). A *p* value of less than 0.05 was considered statistically significant for all comparisons. Data for body weights, fluid percussion injury magnitudes, temperature measurements, righting times, apnea duration of the survivors, mRNA levels, and degenerating neuronal counts from FJ-B staining were presented as mean±standard deviation (STD) and analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test. Data for MWM latency and left-to-right ratio in magnetic resonance T2 images were presented as mean±standard error of the mean, and were analyzed using repeated measures ANOVA with assessment days as the repeated variable within subjects followed by Bonferroni's *post hoc* analysis. Chi-square (χ^2) test was employed for the analysis of mortality in all groups.

Results

ICV cannulation and injection

CSF outflow could be seen in the cannula after implantation in the cerebral ventricles. At 2 h, 4 h, 6 h, and 8 h post-TBI, after cutting the sealed end of the catheter, CSF outflow could be seen due to the high intracranial pressure in the brain. There were no obstructions to flow during any ICV injections. The correct placement of the injection needle into the right ventricle was verified with gross histology.

Measurement of physiological parameters

There were no significant differences between groups in body weight (pre-surgery and pre-TBI), temporalis muscle and rectal temperature values pre-TBI or post-TBI (Table 1). There were no significant differences between the ODN and aCSF groups in injury magnitude and righting time.

The mortality rate in the first seven days post-TBI in the ODN and aCSF groups were much higher than that in the sham group ($p<0.01$) but there was no significant difference between the ODN and aCSF groups. Gross pathology revealed the main cause of mortality was extensive subarachnoid blood underneath the injury site. Cerebral hemorrhage was extensive in the posterior cranial fossa and surrounding the brainstem.

Although there were no significant differences in the righting time between the ODN and aCSF groups, they were both significantly higher than that in the sham group ($p<0.01$). An occurrence of pulmonary edema was defined as the presence of pink fulminating exudate from the mouth and nostrils. No survivors among the groups showed occurrence of pulmonary edema but the occurrence of pulmonary edema in non-survivors of the ODN group and the aCSF group were 64.3% and 60%, respectively.

Apneic episodes were observed only in TBI animals. The incidence of apneic episodes in non-survivors of the ODN group and the aCSF group were 71.4% and 70%, respectively. With regard to non-survivors, it should be noted that irreversible apnea occurred in one non-survivor from the ODN group and two non-survivors in the aCSF group, and the apneic duration was recorded as 300 sec. With respect to the incidence of apneic episodes and the mean apneic duration in the survivors, there was no significant difference between the ODN and aCSF groups.

mRNA expression of *Nav1.3*

The mRNA levels of *Nav1.3* at 12 h post-TBI were detected by RT-qPCR and illustrated in Figure 1. In the ipsilateral hippocampus, the mRNA levels of *Nav1.3* in the aCSF group was much higher than that in the ODN group and the sham group (1.14 ± 0.04 in the aCSF group vs. 0.91 ± 0.06 in the ODN group, $p<0.01$; and 1.14 ± 0.04 in the aCSF group vs. 0.89 ± 0.04 in the sham group, $p<0.01$). There was no significant difference between the ODN and sham groups.

In the contralateral hippocampus, *Nav1.3* mRNA level in the aCSF group was much higher than in the ODN and sham groups (1.60 ± 0.19 in the aCSF group vs. 0.76 ± 0.05 in the ODN group, $p<0.01$, and 1.60 ± 0.19 in the aCSF group vs. 1.21 ± 0.06 in the sham group, $p<0.01$). Interestingly, *Nav1.3* mRNA level in ODN group was significantly decreased, compared with the sham group (0.76 ± 0.05 in the ODN group vs. 1.21 ± 0.06 in the sham group, $p<0.01$).

When comparing *Nav1.3* level in the two hemispheric hippocampi within the same group, we found significant differences in

TABLE 1. PHYSIOLOGICAL PARAMETERS

Group	Sample (n=98)	Transient apnea															
		Body weight (g)		Body weight (g) (Pre-TBI)	Injury (ATM)	Rectal Temp (°C)		Temporalis Temp (°C)		Righting Time (Min)		Pulmonary edema (%)		Survivors			
														Non-survivors		Survivors	
		Pre-surgery				T	Pre- TBI	Post- TBI	Pre- TBI	Post- TBI	Survivors	Non-survivors	Survivors	Incidence (%)	Median duration (sec)	Incidence (%)	Mean duration (sec)
ODN	40	327.2±6.1	293.3±5.9	2.96±0.02	37.2±0.3	37.0±0.3	36.0±0.2	35.9±0.3	28.1±3.1*	64.3	0	71.4	67.5 ^{\$}	34.6	49.1±5.8	35.0*	
aCSF	41	329.6±6.3	292.1±6.8	2.95±0.03	37.2±0.4	37.1±0.3	35.9±0.3	35.8±0.4	29.4±3.8*	60.0	0	70.0	63.0 ^{\$}	33.3	51.6±6.8	48.8*	
Sham	17	328.9±5.6	291.8±6.8	-	37.3±0.3	37.1±0.3	35.9±0.3	35.8±0.3	4.0±0.3	-	0	0	-	0	-	0	0

- = not applicable; * = significantly different ($p < 0.01$ vs. sham-TBI group); \$ = standard deviation not shown because of abnormal distribution. TBI, traumatic brain injury; ODN, oligodeoxynucleotides; aCSF, artificial cerebrospinal fluid.

FIG. 1. Expression of $\text{Na}_v1.3$ messenger ribonucleic acid in the ipsilateral and contralateral hippocampi of the oligodeoxynucleotides (ODN) group (grey bar), the artificial cerebrospinal fluid (aCSF) group (black bar), and the sham group (white bar) at 12 h post-traumatic brain injury ($n=6$ per group). Values marked with an asterisk were significantly different between the selected group and the sham group at $p < 0.01$. Values marked with # were significantly different between the ODN and aCSF groups at $p < 0.01$. Values marked with % and \$ were significantly different between the bilateral hippocampi in the ODN and sham groups, respectively, at $p < 0.01$.

the ODN group (0.76 ± 0.05 in contralateral hippocampus vs. 0.91 ± 0.06 in ipsilateral hippocampus, $p < 0.01$) and the sham group (1.21 ± 0.06 in contralateral hippocampus vs. 0.89 ± 0.04 in ipsilateral hippocampus, $p < 0.01$). The data for $\text{Na}_v1.3$ mRNA represent values normalized to glyceraldehyde-3-phosphate dehydrogenase.

Degenerating neurons cell counts

In the sham group, no FJ-B positive neurons were observed in either hippocampus region. Some uncommon FJ-B positive neurons could be visualized in the contralateral hippocampus of the ODN group and the aCSF group (Fig. 2B). Visual inspection of the ipsilateral hippocampus of the ODN group and the aCSF group revealed a greater number of degenerating neurons in CA3 and hilar regions, and sparse positive cells in CA1 fields (Fig. 2A).

In the CA3 and hilar regions of the ipsilateral hippocampus, FJ-B positive neurons were more pronounced in the aCSF group than in the ODN group (Fig. 3). The number of degenerating neurons were significantly different in the ipsilateral CA3 field (2371 ± 486 in the ODN group vs. 4325 ± 624 in the aCSF group, $p < 0.01$) and hilar region (1557 ± 278 in the ODN group vs. 2201 ± 327 in the aCSF group, $p < 0.01$).

Localization of $\text{Na}_v1.3$ in hippocampus

Sections from the three groups were double-immunofluorescence labeled for $\text{Na}_v1.3$ and NeuN. As shown in Figure 4, strong signal of $\text{Na}_v1.3$ was expressed as red on the membrane of neurons in the ipsilateral hippocampus of the aCSF group (Fig. 4E), whereas very weak immunofluorescence of $\text{Na}_v1.3$ were observed in the ODN and sham groups (Fig. 4D, 4F). This was an indication that $\text{Na}_v1.3$ expression level was much higher in the aCSF group than that of both the ODN and sham groups. The orange circles in the merged photomicrographs revealed expression of $\text{Na}_v1.3$ occurring on cell membranes of neurons in the hippocampus (Fig. 4H).

FIG. 2. Fluoro-Jade B (FJ-B) histofluorescence of degenerating neurons 12 h post-traumatic brain injury in the ipsilateral hippocampus (**A**) and contralateral hippocampus (**B**) of the oligodeoxynucleotides group (merged pictures). The numbered rectangular frames in the two panels represent the regions of CA1, hillus/dentate gyrus (DG), and CA3, respectively. In panel A, degenerating neurons, labeled with bright fluorescence, were found primarily in hillus/DG and CA3, but a few scattered in CA1. However, rare FJ-B α -positive neurons were found in panel B (scale bar = 200 μ m).

Antisense ODN delivery

A one-time intracerebroventricular injection of antisense ODN labeled with Cy3 resulted in red fluorescence being detected in bilateral cerebral ventricles, the third cerebral ventricle, and the subarachnoid space 4 h post-injection (Fig. 5A). Cy3 penetration was observed to be very strong within the bilateral hippocampi (Fig. 5B, 5C). NeuN staining (Fig. 5D, 5E) revealed that neurons from both hippocampi exhibited strong uptake of Cy3-labeled antisense ODN (Fig. 5F, 5G). These important observations suggested successful delivery of antisense ODN into the cerebral ventricles, and that penetration and uptake occurred in both hippocampi after ODN administration.

Midline shift in MRI

Prior to TBI, equal intensities were displayed in the brain tissue in T2 images of all the groups (Fig. 6A). The left-to-right ratios were about equal to 1 in the naïve rats. Dramatic changes were observed in these MRI scans from both the ODN and aCSF group post-TBI (Fig. 6C). Diffuse hyperintensities could be observed in the ipsilateral hemisphere brain (including the cortex and the hippocampus) in these two groups after TBI, notably at one day post-

TBI. Decreased signal intensities could be found in the ipsilateral external capsule and the ipsilateral region between hippocampus and thalamus in injured animals. This is indicative of hemorrhage occurring in these regions post-TBI. Midline shifts and deformations of the ipsilateral hemisphere were obvious at one day post-TBI and approached to normal baseline at seven days post-TBI. As shown in Figure 6B, both the ODN and aCSF groups showed a significant increase in left-to-right ratio, reflecting edema in the ipsilateral hemisphere, when compared to the sham group ($p < 0.01$). There is a significant difference between the ODN group and the aCSF group with respect to left-to-right ratio post-TBI ($p < 0.05$). This was an indication that brain edema was more severe in the aCSF group than that of the ODN group.

Morris water maze

As shown in Figure 7, there are differences in latency between the different groups from 21 days to 25 days post-TBI. A repeated measure ANOVA revealed a significant main effect of group on latency to find the platform ($p < 0.01$). Bonferroni's *post hoc* tests indicated that the ODN group and the aCSF group took significantly more time to locate the hidden platform compared with the sham group ($p < 0.01$). The ODN group had significantly shorter

FIG. 3. Fluoro-Jade B–positive neurons in the ipsilateral hippocampus in the oligodeoxynucleotides (ODN) and artificial cerebrospinal fluid (aCSF) groups at 12 h post-traumatic brain injury. A significant increase in the number of degenerating neurons was observed in both CA3 and hilus in the aCSF group (C and D), compared with ODN group (A and B). Quantification analysis of degenerating cells is shown in (E) and (F) (ODN group in white bar, and aCSF group in black bar). Values marked with asterisk were significantly different between ODN and aCSF group at $p < 0.01$. Scale bar = 100 μm .

latency to find the hidden platform, compared with the aCSF group ($p < 0.05$).

Discussion

We demonstrated that by inhibiting upregulation of $\text{Na}_v1.3$ expression in the hippocampus post-TBI, we could improve outcome measures, such as reducing severity of brain edema, acute neuronal degeneration, and subsequent long-term functional deficits.

Activated ion channels, including calcium channel and sodium channel, are considered to play critical roles in secondary brain injury post-TBI, and these two ion channel complement each other. Wolf and colleagues demonstrated that traumatic deformation of axons induced abnormal sodium influx, which subsequently triggered an increase in intra-axonal calcium and reversal of the Na^+ - Ca^{2+} exchanger.⁴ Iwata and associates reported that mechanical trauma of axons resulted in Na^+ influx and triggered rapid increases of intracellular Ca^{2+} . This initial increase of Ca^{2+} resulted in proteolysis of the sodium channel α subunit but blockade of sodium channel inhibited influx of Ca^{2+} and prevented proteolysis of sodium channel α subunit.²⁵ These results were further confirmed by von Reyn and colleagues, that calpain-mediated proteolysis of the sodium channel α subunit within the I - II and III-IV

loops of $\text{Na}_v1.2$; however, the authors indicated that sites of proteolysis might differ between α subunit.²⁶ Their further study reported that calpain activation and subsequent proteolysis of sodium channel α subunit were mediated by NMDA receptors and sodium channels following stretch injury.²⁷ A recent *in vivo* study employed a novel calpastatin overexpressing transgenic mouse model and demonstrated that augmenting calpastatin levels attenuated calpain-mediated proteolysis of sodium channels.²⁸

According to the previous studies mentioned above and our study on $\text{Na}_v1.3$ expression, we consider that proteolysis and up-regulated expression may occur in different sodium channel α subunits due to their different structures and varied abundance following brain injury. These changes contribute to abnormal sodium influx and subsequent calcium influx. Therefore, the neuroprotective effect of pharmacological inhibition of VGSCs is believed to include but is not limited to 1) reducing Na^+ influx followed by increased intracellular Na^+ concentration which leads to cellular swelling, 2) reducing Ca^{2+} influx via voltage-dependent calcium channel activation and reversal of Na^+ / Ca^{2+} exchange, 3) reducing excitability and cellular ATP consumption, and 4) reducing release of excitotoxic glutamate.

Many studies demonstrated that blocking the sodium channels has a neuroprotective effect in TBI. A number of drugs with sodium

FIG. 4. Double immunofluorescence staining for $\text{Na}_v1.3$ and neuronal nuclei-specific antibody (NeuN) markers in the ipsilateral hippocampus in the oligodeoxynucleotides (ODN) group (**A**, **D**, and **G**), the artificial cerebrospinal fluid (aCSF) group (**B**, **E**, and **H**), and the sham group (**C**, **F**, and **I**) at 12 h post-traumatic brain injury. Representative NeuN labeled cells, and $\text{Na}_v1.3$ labeled cells were seen in the photomicrographs. When double-stained for NeuN and $\text{Na}_v1.3$, it showed $\text{Na}_v1.3$ expressions on neural cell membranes in the aCSF group (**H**). However, the $\text{Na}_v1.3$ expressions were not substantial in the ODN and sham groups. Scale bar = 50 μm .

channel-blocking properties were reported to have neuroprotective effects in rat models of brain injury. For example, BW1003C87 can attenuate regional cerebral edema²⁹; riluzole can attenuate cortical lesion size and improve cognitive and neuromotor outcome.^{14,15} Another study indicated that amiloride, which inhibited the reverse mode of sodium-calcium exchanger, type 1 sodium-proton antiporter and acid-sensing ion channel type 1a, afforded robust reduction of astrocyte pathology and neuronal degeneration.¹⁶

The method of ICV administration of antisense ODN has been reported in experimental TBI rat models for more than ten years.^{30,31} In those studies, expression level of the target gene in the brain was reduced effectively by application of the antisense ODN. To our knowledge, we are the first to perform an ICV administration of antisense ODN targeting sodium channel α subunit in the brain. VGSCs are located in different tissues and organs of mammals. Hence, non-specific sodium channel blockers are not suitable for therapy because they are not specific to the brain. Hains and colleagues showed that downregulation of expression of $\text{Na}_v1.3$ mRNA and protein can be achieved by intrathecal administration of antisense ODN targeting $\text{Na}_v1.3$ in spinal cord injury models.⁸ Our method has the advantage of targeting brain-specific local certain sodium channel α subunit and avoiding the adverse effects in peripheral organs. With selective blockade of $\text{Na}_v1.3$, we were able to attenuate increased of $\text{Na}_v1.3$ expression level specific to hippocampus in an experimental TBI model. There are many obstacles toward progress being made with application of antisense ODN drugs in patients despite its many trials in the past two decades. Recent encouraging reports demonstrated that one antisense ODN drug, mipomersen, an apolipoprotein B synthesis inhibitor, was escalated to advanced clinical trial (phase III) with some mild toxic effects.^{32,33} It is estimated that 20 antisense ODN drugs are in clinical trials.³⁴ Indeed, there is great promise for using antisense ODN as a therapeutic strategy for brain injury.

ICV injection was commonly use as the prefer method for direct CNS drug delivery in most experimental TBI models.^{16,35–38} Based on our previous study, the $\text{Na}_v1.3$ expression was shown to increase at 2 h post-TBI and peaked at 12 h post-TBI. Hence, we've chosen 2 h as the critical timepoint during the very early stage post-TBI to administer antisense ODN.¹³ In this study, we used the modified cannula made of the Novofine 30G needle for the ICV injection. The outside diameter of the needle was only 0.3 mm which is much smaller than the regular ICV cannula. The small size injection needle is beneficial because no hemorrhage was found in MRI scans of any animals. Although midline shift had been documented at the early stage post-TBI, the labeled antisense ODN still could be identified in the ventricular system. These results validated the proper placement and administration of labeled antisense ODN into the ventricle. Further, this ICV injection method would effectively model a practical therapeutic strategy to clinical TBI patients because they would have had ventricular cannulation to alleviate rises in intracranial pressure.

In this study, Cy3-labeled antisense ODN was proven to permeate through and be absorbed by the hippocampal neurons, and the double immunofluorescence staining for $\text{Na}_v1.3$ and NeuN markers in the hippocampus represented the expression of $\text{Na}_v1.3$ occurring on neural cell membranes. Thus, we conclude that the antisense ODN therapy focused specifically on neurons in the hippocampus and our positive experimental outcomes can be regarded as the direct result from this novel therapy.

In regards to the physiological parameters being recorded, the ODN and aCSF groups showed significant differences in righting time, mobility of pulmonary edema and transient apnea, and increased mortality, compared with the sham group. There were no significant differences in these measured parameters between the ODN and aCSF groups. However, further analysis of mortality revealed that rats in the ODN group died only in the first two days

FIG. 5. Cryosection with one single intracerebroventricular injection of Cy3-labeled antisense oligodeoxynucleotides (ODN) in the ODN group at 12 h post-traumatic brain injury. In the upper merged picture (A), Cy3 fluorescence labeled with fluorescence is detectable in bilateral cerebral ventricles, the third cerebral ventricle, and the subarachnoid space, and can be seen to penetrate in the hippocampus. In the higher magnification image of ipsilateral hippocampus (B) and contralateral hippocampus (C), the density of the fluorescence indicated the antisense ODN concentrations decreased along with the distance from cerebral ventricles. When stained with neuronal nuclei-specific antibody (fluorescence in D and E), the antisense ODN was demonstrated to be absorbed by the hippocampal cells (in the merged images F and G). Scale bar in A = 2 μ m; in B, C, D, E, F, and G = 200 μ m.

post-TBI but rats in the aCSF group died during the first week post-TBI. This suggested that ICV injection of antisense ODN targeting $\text{Na}_v1.3$ may play a critical role in attenuating the associated secondary brain injury at the early stage post-TBI.

The level of $\text{Na}_v1.3$ mRNA found in the bilateral hippocampi varied in different groups. It can be suggested that antisense ODN is effective in prohibiting the upregulation of $\text{Na}_v1.3$. As shown in Figure 1, the $\text{Na}_v1.3$ mRNA level expression in bilateral hippocampi in the aCSF group was the highest among the three groups.

However, $\text{Na}_v1.3$ mRNA level in the ODN group did not show a similar spike post-TBI. This suggested that the antisense ODN may block the upregulation of $\text{Na}_v1.3$ expression.

Interestingly, a significant downregulation of $\text{Na}_v1.3$ level was observed in the contralateral hippocampus in the ODN group, compared with the contralateral hippocampus in the sham group and the ipsilateral hippocampus in the ODN group. The long and narrow ipsilateral cerebral ventricle indicated that brain edema was more severe in the ipsilateral brain than that in the contralateral brain. We speculated that the narrow ipsilateral cerebral ventricle might contribute to the limited permeability of antisense ODN, and the ICV administration was far more effective in the contralateral ventricle than in the ipsilateral ventricle. This may be the reason for the difference of $\text{Na}_v1.3$ level between bilateral hippocampi.

In the sham group, a significant upregulation of $\text{Na}_v1.3$ expression was detected in the contralateral hippocampus compared to the ipsilateral hippocampus. We speculated that the ICV cannulation and administration procedure alone might contribute to the upregulation of $\text{Na}_v1.3$ expression in contralateral hippocampus.

The present findings demonstrated that antisense ODN targeting $\text{Na}_v1.3$ resulted in a significant reduction in the numbers of degenerating neurons in the CA3 and hilus at 12 h post-TBI. Previous TBI studies showed that F-J positive neurons were observed in ipsilateral cortex as early as 3–4 h and peaked at 24 h post-injury.^{21,39} FJ-B, used in our present study, is a useful fluorescein derivative for histological staining of neurons undergoing degeneration, and results in noticeably higher definition and contrast labeling of neuronal processes than its predecessor F-J.²⁰ We chose 12 h as the evaluation time in order to correlate the time we analyzed the mRNA expression level and avoid the intense FJ-B positive neurons in the hippocampus. Our data suggest that ICV administration of antisense ODN targeting $\text{Na}_v1.3$ is sufficient to lessen acute hippocampal neuronal degeneration.

Midline shift, which indicates cerebral herniation, is a common occurrence in many brain diseases, including injury, stroke, hemorrhage, and tumor. Computed tomography and MRI are widely-used means to assess midline shift in clinic and sensitive predictors of patient's outcome. Midline shift quantification had been correlated with brain edema in the stroke and TBI models in rats.^{23,40} In this study, rats in the ODN and aCSF groups revealed significant midline shifts in the MR images compared to the sham group. However, rats in the aCSF group showed a larger shift of the midline, compared with rats in the ODN group. At seven days after injury, midline shifts in the ODN and aCSF groups resemble that of normal level of the sham group, which indicated a recovery in acute pathology at that particular timepoint. This might be the reason why there are differences in time of death between the ODN and aCSF groups. It is noteworthy that antisense ODN may have alleviated the secondary brain injury by reducing brain edema.

Many TBI studies demonstrated that cognitive impairments occurred in the presence of hippocampal damage.^{41,42} In this study, a significant improvement in MWM performance in the ODN group was observed suggesting that antisense ODN ICV administration consequently improved chronic cognitive outcome of rats following TBI. We presumed that differences in $\text{Na}_v1.3$ expression level resulted from the variable secondary brain injury, which eventually led to differences in outcome, including degeneration during the acute stage and long-term cognitive behavior dysfunction.

FIG. 6. Magnetic resonance images and data analysis on Day -3, Day 1, Day 3, and Day 7 post-traumatic brain injury in all groups. The schematic diagram showed the measurements used to calculate midline shift ratio (A). The magnetic resonance images displayed different consecutive changes on Day -3, Day 1, Day 3, and Day 7 in these three groups (C). The left-to-right ratios analysis was shown in (B). Data were expressed as mean $\pm$ standard error of the mean. Values marked with an asterisk were significantly different between the selected group and the sham group at $p < 0.01$. Values marked with # were significantly different between the oligodeoxynucleotides and artificial cerebrospinal fluid groups at $p < 0.05$.

FIG. 7. Morris water maze test for cognitive performance on Days 21–25 post-traumatic brain injury. Data were expressed as mean $\pm$ standard error of the mean, and each data point represented the mean of four daily trials. Values marked with an asterisk were significantly different between the selected group and the sham group at $p < 0.01$. Values marked with # was significantly different between the oligodeoxynucleotides and artificial cerebrospinal fluid groups at $p < 0.05$.

In conclusion, our results suggest that ICV administration of antisense ODN targeting $Na_v1.3$ may inhibit upregulation of $Na_v1.3$ expression in hippocampus post-TBI in rats. Further, brain edema may be alleviated and results in reduction of degenerating neurons in hippocampus and improved functional cognitive outcome. Further studies are needed to find out the most effective therapeutic dose of the antisense ODN targeting $Na_v1.3$ and the most effective therapeutic timepoints to block this sodium channel α subunit post-TBI.

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Author Disclosure Statement

No competing financial interests exist.

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