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Isolated Primary Blast Alters Neuronal Function with Minimal Cell Death in Organotypic Hippocampal Slice Cultures

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Abstract

An increasing number of U.S. soldiers are diagnosed with traumatic brain injury (TBI) subsequent to exposure to blast. In the field, blast injury biomechanics are highly complex and multi-phasic. The pathobiology caused by exposure to some of these phases in isolation, such as penetrating or inertially driven injuries, has been investigated extensively. However, it is unclear whether the primary component of blast, a shock wave, is capable of causing pathology on its own. Previous *in vivo* studies in the rodent and pig have demonstrated that it is difficult to deliver a primary blast (i.e., shock wave only) without rapid head accelerations and potentially confounding effects of inertially driven TBI. We have previously developed a well-characterized shock tube and custom *in vitro* receiver for exposing organotypic hippocampal slice cultures to pure primary blast. In this study, isolated primary blast induced minimal hippocampal cell death (on average, below 14% in any region of interest), even for the most severe blasts tested (424 kPa peak pressure, 2.3 ms overpressure duration, and 248 kPa*ms impulse). In contrast, measures of neuronal function were significantly altered at much lower exposures (336 kPa, 0.84 ms, and 86.5 kPa*ms), indicating that functional changes occur at exposures below the threshold for cell death. This is the first study to investigate a tolerance for primary blast-induced brain cell death in response to a range of blast parameters and demonstrate functional deficits at subthreshold exposures for cell death.

Key words: blast; hippocampus; *in vitro*; neuron; shock tube

Introduction

As much as 80% of all wounds suffered by U.S. soldiers in Operation Enduring Freedom and Operation Iraqi Freedom are the result of improvised explosive devices.[1,2] Blast exposure may be exceedingly complex, and injury can result from a combination of blast mechanisms. Primary blast loading results from the shock wave, which is a fast-rising pressure transient and is the only injury mechanism unique to blast. Secondary blast injury includes penetration from ejected materials, such as shrapnel. Tertiary blast injury encompasses inertially driven injuries, the result of the body being propelled by the blast into a surface. Inertially driven brain injury is well studied in the literature.[3,4] Quaternary blast injury captures other mechanisms, such as thermal, chemical, or radiation exposure, after detonation.[5] The complexity of the exposure confounds attribution of the pathology to a specific biomechanical mechanism. Improved understanding of injurious biomechanics could inform the design of future head protection to reduce the likelihood for blast-induced neurotrauma.

A current controversy is whether shock wave loading, referred to as primary blast, can cause damage to the brain in the absence of other injury mechanisms, specifically penetrating or inertially driven brain injury. An increasing number of experimental studies have investigated the effect of blast injury in a number of animal species using shock or blast-tube injury models.[6–9] However, these studies present seemingly contradictory evidence for tolerance of the brain to primary blast, some of which may be attributed to concomitant, but uncontrolled, head accelerations, making it difficult to differentiate the contribution of primary versus tertiary injury mechanisms to the ensuing pathobiology.[7,10] Interpretation of some studies is further complicated because the thorax was not protected; exposure of the unprotected thorax to blast readily damages the lungs and bowels, resulting in secondary central nervous system (CNS) deficits.[11]

The current study was undertaken to answer two important questions: 1) Can a shock wave cause brain cell death?, and 2) What are the safe exposure limits to pure shock wave loading? These data are critical for understanding the pathophysiology of blast and may

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lead to improvements in protective equipment, including combat helmets. Current military helmets have been shown to be effective at preventing penetrating impact and are somewhat less effective at reducing neurotrauma from blunt impact and inertially driven brain injuries.[3,9] However, if a shock wave is capable of causing neurodegeneration or other pathobiology, being able to assess the trade-offs for blast protection is essential for military personnel.

To determine the safe exposure limits of isolated primary blast without the confounds of inertially driven injury, we have previously developed a simplified blast injury model comprised of a shock tube and sample receiver for exposing organotypic hippocampal slice cultures (OHSC) to a shock wave.[12,13] This unique design translates the primary shock wave in air to a pressure transient in fluid, which interacts with tissue and has a similar profile to intracranial pressure during blast.[12,13] As previously reported, finite element modeling predicted tissue strain to be less than 5%, which is below the threshold for stretch-induced cell death in OHSC.[13,14] This design mitigates the confounding effects of inertial loading (i.e., stretch), so that the effects of pure shock wave loading can be studied.[12,13]

In the present study, we investigated acute and delayed hippocampal cell death in response to a wide range of relevant blast exposures. Neuronal function was quantified to determine whether blast-induced functional deficits were produced in the absence of cell death. Our results suggest not only that a shock wave can kill brain cells, but also that functional deficits occur in the absence of cell death. However, as compared to other mechanisms of injury, isolated primary blast exposure resulted in low levels of cell death.

Methods

Organotypic hippocampal slice culture

All animal procedures were approved by the Columbia University Institutional Animal Care and Use Committee (New York, NY). OHSC were generated as previously described from approximately 25 dams and 100 pups. In brief, P8-11 Sprague-Dawley rat pups were decapitated and the brains removed.[14–16] Hippocampi were excised, sectioned into 400 μm thick slices and separated aseptically in ice-cold Gey's salt solution supplemented with 25 mM of D-glucose (Sigma-Aldrich, St. Louis, MO). Two to three slices were plated onto each porous Millipore Millicell cell-culture inserts (Millipore, Billerica, MA) in Neurobasal medium supplemented with 1 mM of L-GlutaMAX, 1×B27 supplement, 10 mM of HEPES, and 25 mM of D-glucose (Life Technologies, Grand Island, NY). Every 2–3 days, half of the medium was replaced with full-serum medium containing 50% minimum essential medium (MEM), 25% Hank's balanced salt solution (HBSS), 25% heat-inactivated horse serum, 2 μM of L-GlutaMAX, 25 mM of D-glucose, and 10 mM of HEPES (Sigma-Aldrich). Cultures were grown for 10–14 days before testing.

Blast injury

Blast injury methods have been described in detail previously.[12,13] In brief, a shock wave was generated with a 76-mm-diameter aluminum shock tube with an adjustable-length driver section (25, 50, and 190 mm used for the current studies) pressurized with helium or nitrogen and a 1240 mm long driven section.[12,13] Piezoresistive pressure transducers (Endevco, San Juan Capistrano, CA) were flush-mounted at the exit of the shock tube and within the fluid-filled blast receiver, at the location of the culture, and were oriented perpendicular to the direction of propagation to record side-on (incident) pressure. Transducer outputs were amplified (50× gain) and low-pass filtered (corner frequency of 40 kHz; Alligator Technologies, Costa Mesa, CA) before being digitized at 125 kHz. Peak overpressure, overpressure duration, and impulse were calculated with custom MATLAB scripts (MathWorks, Natick, MA).[12] For in-air and in-fluid pressure histories, peak overpressure was defined as the maximum pressure of the positive overpressure phase, duration was defined as the length of time of the positive overpressure phase, and impulse was defined as the integral over time under the pressure history trace for the duration of the positive overpressure exposure (i.e., area).

Primary blast levels were chosen for their relevance to real-world blast-loading conditions.[7,17] Among those parameters investigated here, the range of peak overpressures tested was 92.7–534 kPa, the range of durations tested was 0.25–2.3 ms, and the range of impulses was 9.2–248 kPa*ms determined from in-air measurements (Table 1), which are nominally referred to as levels 1–9.

At the time of experimentation, individual culture wells were sealed inside sterile polyethylene bags (Whirl Pak, Fort Atkins, WI) filled with prewarmed, serum-free culture medium containing 75% MEM, 25% HBSS, 2 μM of L-glutamine, 25 mM of D-glucose, 10 mM of HEPES that had been equilibrated with 5% CO_2/95% O_2. These polyethylene bags were specifically chosen because their acoustic impedance matched that of water so as not to attenuate the pressure transient, which was validated previously.[12] Samples were immediately placed in the fluid-filled receiver, which was also maintained at 37°C. For injured cultures, the shock tube was then fired; sham cultures were treated identically, except that the shock tube was not fired. After blast or sham exposure, the sample was immediately removed from the receiver and returned to the incubator in fresh, full-serum medium. In preliminary studies with naïve OHSC, we found that maintaining cultures in serum-free medium caused cell death in the dentate gyrus (DG). Therefore, OHSC were maintained in full-serum medium after blast or sham exposure to eliminate this confounding cell death resulting from serum withdraw.

Cell death quantification

Propidium iodide (PI) fluorescence was used to quantify cell death before and after injury at indicated time points. OHSC were incubated in 2.5 μM of PI (Life Technologies) in serum-free medium for 1 h before imaging. Images were acquired using an Olympus IX81 microscope (Olympus, Tokyo, Japan) with 568/24 nm (peak/width) excitation and 610/40 nm emission filters and a CoolSNAP ES camera (Photometrics, Tucson, AZ). After the initial imaging to determine baseline health, cultures were immediately injured. At the indicated time points, cell death was determined for all regions of interest (ROIs; DG, CA3, and CA1) of OHSC, as previously described, using MetaMorph (Molecular Devices, Downingtown, PA).[14] OHSC were evaluated for cell death at one time point only after exposure. In brief, the same threshold for fluorescence was used to analyze all images at each time point. The threshold was chosen just high enough to exclude background signal in preinjury images of both sham- and blast-exposed OHSC. Tissue damage was quantified as the percentage area of a specific region exhibiting fluorescence above the threshold. Any OHSC with 5% or greater cell death in any ROI at the preinjury time point was excluded from the study.

Electrophysiology

Neuronal function was quantified for cultures receiving a 336 kPa, 0.84 ms, 86.5 kPa*ms (level 4; subthreshold for cell death) blast; a 424 kPa, 2.3 ms, and 248 kPa*ms (level 9; suprathreshold for cell death) blast; or the sham injury. Electrophysiological recordings were performed 4–6 days after blast or sham exposure with 60-channel microelectrode arrays (MEAs; 8×8 electrode grid, 10 μm electrode diameter, 100 μm electrode spacing; MultiChannel Systems, Reutlingen, Germany). Recordings were taken

from multiple slices. (For mossy fiber [MF] stimulation, the number of slices used for sham, level 4, and level 9 exposure was as follows: 9, 5, and 7 slices, respectively; for Schaffer collateral [SC] stimulation, the number of slices used for sham, level 4, and level 9 exposure was as follows: 10, 5, and 9 slices, respectively.) Before use, each MEA was plasma cleaned (Harrick Plasma, Ithaca, NY) and coated with 5 μL of 0.01% nitrocellulose in methanol (GE Healthcare Life Sciences, Piscataway, NJ) to ensure tissue adhesion. Each sample was excised from its Millipore insert, cutting around each tissue slice, and inverted onto an MEA. OHSC were held stationary with a nylon mesh harp-slice grid (ALA Scientific Instruments, Farmingdale, NY). Samples were perfused with artificial cerebral spinal fluid containing 125 mM of NaCl, 3.5 mM of KCl, 26 mM of $NaHCO_3$, 1.2 mM of KH_2PO_4, 1.3 mM of $MgCl_2$, 2.4 mM of $CaCl_2$, and 10 mM of glucose (pH 7.40), which was bubbled with 5% CO_2/95% O_2 and prewarmed to 37°C. Recordings were taken with an MEA1060-BC amplifier and data acquisition system (Multi-Channel Systems). The system recorded neural signals at 20 kHz with a 6-kHz analog, antialiasing filter.

Stimulus-response (S/R) curves were generated with a programmable stimulator (STG1004; Multi-Channel Systems), applying a constant current, bipolar, biphasic stimulus (100 μs positive phase followed by a 100 μs negative phase) of varying magnitude (0×200 μA in 10 μA increments) to electrodes located in either the MF or SC pathways. The evoked response was recorded on all electrodes simultaneously. As in previous studies, each electrode's recording was fit to a sigmoidal curve based upon the following equation[19]:

$$R(S) = \frac{R_{max}}{1 + e^{m(I_{50} - S)}}$$

where R_{max} represents the maximum amplitude of the evoked response and I_{50} represents the current necessary to generate a half-maximal response. The term m, which is proportional to the slope of the sigmoidal fit, represents the spread in the firing threshold for the population of neurons. These three parameters were calculated for each electrode in each slice, and results were grouped by electrode location in an ROI (DG, CA3, and CA1), stimulus location (MF and SC), and exposure level.

Excitotoxic injury

A subset of OHSC exposed to the highest blast level (level 9) were subjected to an excitotoxic injury (10 mM of glutamate for 3 h) 4 days after blast exposure (i.e., immediately after measuring blast-induced cell death on day 4). Glutamate-containing medium was subsequently changed to fresh serum-free medium, and cultures were imaged for resultant cell death 24 h after glutamate exposure.

Statistical analysis

All data reported are from at least two experimental trials from cultures generated from at least two different litters. Experimental outcomes were measured at only one time point for each culture, so analysis by repeated measures was not appropriate. Statistical significance of the time course of blast-induced cell death at 24-h intervals over the first 4 days after exposure was determined with an independent t-test for each time point of time-matched blast- and sham-injured groups (SPSS v. 19; IBM, Armonk, NY; significance, $p < 0.05$; for time points 0, 24, 48, 72, and 96 h: sham, $n = 75$, 8, 11, 6, and 51 slices, respectively; blast, $n = 31$, 5, 6, 11, and 36 slices, respectively). Statistical significance of blast-induced cell death at 4 days for a range of primary blast levels was determined with a univariate general linear model for each ROI separately, with cell death as the dependent variable and experimental group (sham and all blast injury levels) as the fixed factor with Tukey's post-hoc analysis (significance, $p < 0.05$; for sham, levels 1 through 9, and level 9 plus glutamate exposure: $n = 89$, 17, 9, 39, 14, 9, 20, 14, 25, 35, and 17 slices, respectively). For statistical analysis of S/R parameters calculated for each electrode, a univariate general linear model was used, with each measure (m, I_{50}, and R_{max}) as the unique dependent variable and experimental group (sham and both blast injury levels) as the fixed factor (significance, $p < 0.05$; for MF stimulation DG recording after sham, level 4, and level 9 exposure: $n = 60$, 30, and 82 electrodes, respectively; for MF stimulation CA3 recording after sham, level 4, and level 9 exposure: $n = 94$, 63, and 135 electrodes, respectively; for MF stimulation CA1 recording after sham, level 4, and level 9 exposure: $n = 71$, 61, and 110 electrodes, respectively; for SC stimulation DG recording after sham, level 4, and level 9 exposure: $n = 83$, 45, and 134 electrodes, respectively; for SC stimulation CA3 recording after sham, level 4, and level 9 exposure: $n = 126$, 56, and 175 electrodes, respectively; for SC stimulation CA1 recording after sham, level 4, and level 9 exposure: $n = 82$, 56, and 123 electrodes, respectively), with Bonferroni's *post hoc* analysis. Data for each ROI and stimulation site (MF or SC) were treated independently.

Results

Blast-induced cell death is protracted over several days after exposure

Cell death was evaluated every 24 h for 4 days after sham injury or exposure to level 9 (424 kPa peak pressure, 2.3 ms duration, and 248 kPa*ms impulse; Table 1) primary blast. This level was the most severe used in the current study, delivering the greatest impulse. Cell death reached significance in the DG and CA1 on day 4, as compared to time-matched sham-injured controls. Cell death did not increase significantly before day 4 for any ROI (Fig. 1).

Primary blast-induced cell death in the hippocampus is minimal

OHSC were exposed to a range of relevant primary blasts levels (Table 1). Cell death increased marginally (< 5%) in all ROIs at the 4-day time point after sham injury. Cell death increased significantly in the DG, as compared to sham-injured, controls at day 4 after a level 8 blast. Additionally, cell death increased significantly in the DG and CA1 for cultures exposed to a level 9 blast. OHSC appeared darker in bright-field images 4 days after exposure to the highest levels of primary blast tested, indicating ultrastructural changes and injury (Fig. 3).[20] In response to other blast levels tested, cell death in all ROIs at day 4 after exposure was not significantly different from sham-injured controls (Fig. 2). Cell death did not increase significantly in the CA3 for any of the levels tested (levels 1–9). The greatest amount of cell death was observed in CA1 after a level 9 blast, yet was below 14% (Figs. 2 and 3). In contrast, glutamate exposure 4 days after level 9 blast significantly increased cell death in all ROIs, as compared to OHSC exposed to sham or a level 9 primary blast only.

Mild primary blast decreased excitability

In response to MF stimulation, I_{50} increased significantly in the DG after a level 4 blast (336 kPa, 0.84 ms, and 86.5 kPa*ms), as compared to sham (Fig. 4A1). After exposure to a level 9 blast, I_{50} returned to baseline in the DG. I_{50} increased significantly in the CA1 after a level 9 blast. In response to SC stimulation, I_{50} was significantly increased in the DG after a level 4 blast, as compared to sham (Fig. 4A2). After a level 9 blast exposure, I_{50} returned to baseline in the DG, similar to MF stimulation. I_{50} for SC stimulation increased significantly in the CA3 after a level 9 blast only.

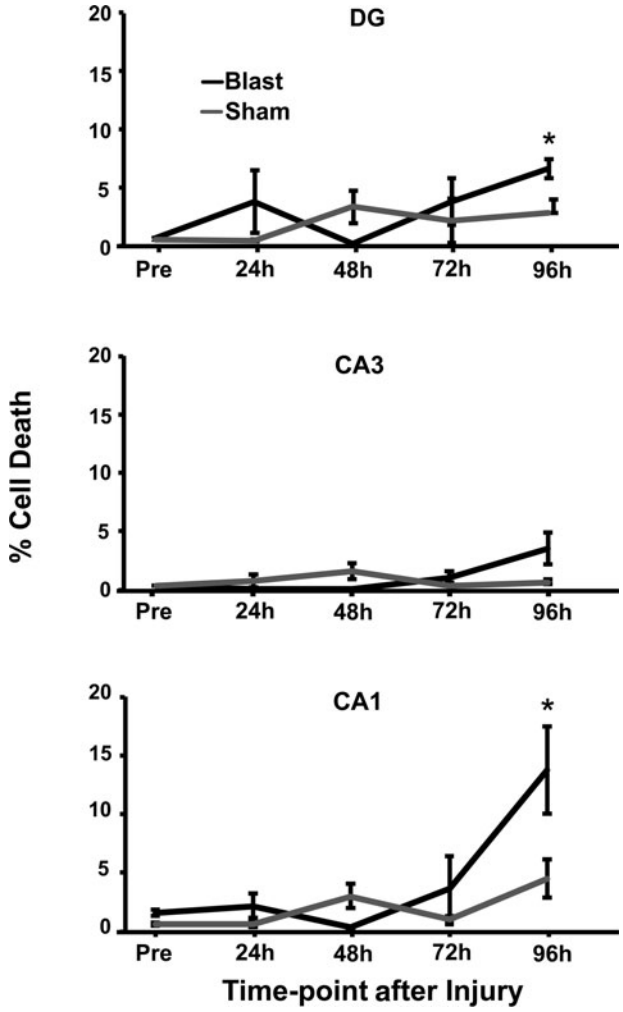

FIG. 1. Time course for cell death development over 4 days (preinjury and every 24 h) after sham or a level 9 blast. Cell death did not change from sham, except at day 4, as compared to time-matched controls in the DG and CA1 (± standard error of the mean; $n \geq 5$; $^*p < 0.05$, as compared to sham for the same ROI and time point).

Mild primary blast decreased neuronal firing

After MF stimulation, R_{max} decreased significantly in the CA3 and CA1 after exposure to a level 4 blast (Fig. 4B1). After a level 9 blast exposure, there was a return to baseline in R_{max} in both of these regions. In response to SC stimulation, R_{max} (Fig. 4B2) decreased in the DG after both blast exposures, as compared to sham. After a level 9 blast, R_{max} increased significantly in the CA3, as compared to a level 4 blast.

Mild and moderate primary blast decreased neuronal firing synchrony

In both the DG and CA3, m decreased significantly in response to MF stimulation for both blast injury levels (Fig. 4C1). In the CA1, m decreased significantly after a level 9 blast, as compared to sham. After SC stimulation, there were no (Fig. 4C2) significant decreases at either injury level in any region.

TABLE 1. BLAST EXPOSURE LEVELS

	Level	*Peak overpressure (kPa)*	*Duration (ms)*	*Impulse (kPa*ms)*
Open-tube	1	106 ± 2.2	0.25 ± 0.01	9.2 ± 1.6
Receiver	1	134 ± 1.9	1.5 ± 0.01	88.8 ± 0.02
Open-tube	2	92.7 ± 2.6	1.4 ± 0.01	38.5 ± 0.7
Receiver	2	270.1 ± 15	2.6 ± 0.2	295.1 ± 56
Open-tube	3	190 ± 2.1	1.2 ± 0.01	73.1 ± 1.3
Receiver	3	516 ± 31	1.3 ± 0.03	254 ± 35
Open-tube	4	336 ± 8.3	0.84 ± 0.01	86.5 ± 1.4
Receiver	4	598 ± 15	1.85 ± 0.3	440 ± 13
Open-tube	5	377 ± 8.3	0.89 ± 0.01	95.5 ± 1.5
Receiver	5	817 ± 22	1.53 ± 0.04	472 ± 16
Open-tube	6	469 ± 21	0.99 ± 0.005	143 ± 1.5
Receiver	6	1258 ± 26	1.46 ± 0.02	658 ± 22
Open-tube	7	364 ± 2.8	1.6 ± 0.01	151 ± 1.3
Receiver	7	956 ± 43	3.6 ± 0.1	1258 ± 43
Open-tube	8	534 ± 3.6	1.0 ± 0.007	184 ± 2.1
Receiver	8	991 ± 53	2.1 ± 0.2	686 ± 23
Open-tube	9	424 ± 6.4	2.3 ± 0.3	248 ± 3.4
Receiver	9	1510 ± 91	2.8 ± 0.1	1420 ± 87

Experimental loading conditions were characterized by the primary blast peak pressure, duration, and impulse calculated from the pressure histories recorded by pressure transducers at different locations. In-air values (open-tube) are characteristic of the shock tube alone without the receiver in place and were determined from pressure histories recorded at the exit of the shock tube in the incident configuration. In-fluid values (receiver) were determined from pressure transducers located in the fluid-filled receiver at the location of the sample (± standard error of the mean; $n \geq 3$).

Discussion

In the current study, we have demonstrated that 1) a shock wave in isolation can cause delayed brain cell death, 2) the safe exposure limit to pure shock wave loading for significant cell death is below 534 kPa, 1.0 ms, and 184 kPa*ms (level 8), and 3) blast-induced, electrophysiological (functional) deficits can occur in the absence of significant cell death (level 4). Whereas cell death increased significantly after exposure to both level 8 and 9 primary blast exposures, average resultant cell death in any ROI was less than 14% (Fig. 2). However, primary blast-induced functional deficits were observed after a level 4 primary blast exposure (Fig. 4), which did not increase cell death.

The delayed cell death observed is suggestive of cell death resulting from second-messenger cascades initiated by the mechanical stimulus.[14,16,21] These data, scaled to humans by lifespan and physiology, are consistent with clinical findings in U.S. soldiers.[5] Similarly, stretch injury of OHSC, which simulates inertially driven injury, has been reported previously to produce protracted cell death over several days.[14,21,22] Inertially driven injuries are characterized by relatively high strains at lower strain rates, in contrast to the primary blast injuries of the current study, which are characterized by low strains at high strain rates.[13] The different biomechanics of these injuries resulted in a similar delayed progression of cell death. Future studies will be necessary to determine the cell death pathways and secondary messenger systems influential in these injury models and whether they differ.

Primary blast levels were chosen for their relevance to real-world blast-loading conditions.[7,17] Among those parameters investigated here, the range of peak overpressures tested was 92.7–534 kPa, the range of durations tested was 0.25–2.3 ms, and the range of impulses was 9.2–248 kPa*ms (Table 1). One study has

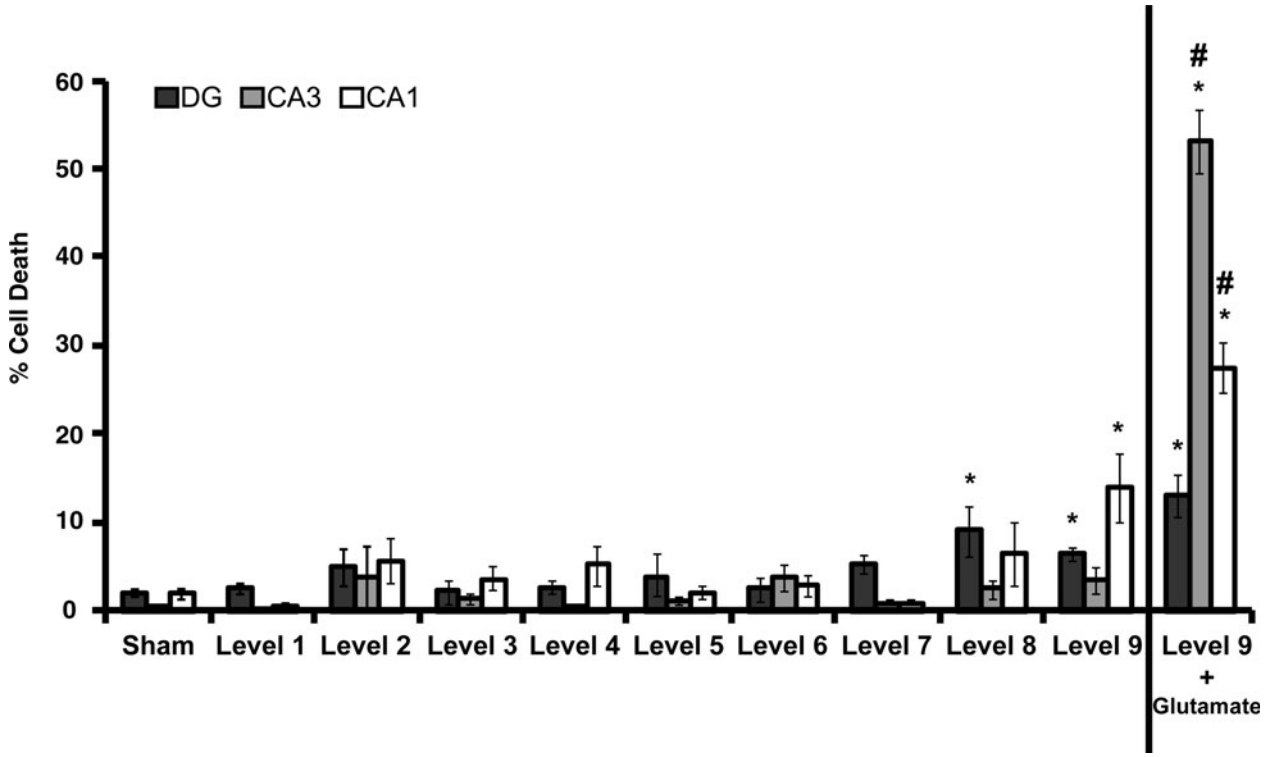

FIG. 2. Cell death response for each ROI of the hippocampus (DG, CA3, and CA1) on day 4 after injury. Groups are in order of increasing impulse from left to right. Additional OHSC were exposed to glutamate after a level 9 blast to evaluate tissue health and integrity after blast. Cell death increased significantly after a level 8 and 9 blast, but did not exceed 14% in any ROI ($\pm$ standard error of the mean; $n \geq 9$; $^{*}p < 0.05$, as compared to sham for the same ROI; $^{\#}p < 0.05$, as compared to level 9 for the same ROI). After a level 9 blast, exposure to glutamate significantly increased cell death, confirming the presence of living cells in OHSC after a level 9 blast.

previously investigated effects of scaling of the blast biomechanics in different species and demonstrated that blast injury scales with duration only.[17] The unscaled in-air values for levels tested were within this relevant range for exposures with relatively short durations. Alternatively, given that the tissue samples used in these studies were derived from rat brain, Bowen scaling to the mass of the rat brain can be used to interpret these blast levels.[18] Again, this scaling demonstrates that parameters fall within a relevant range for real-world blast exposure, but have relatively long durations. Both unscaled and scaled parameters equate to a range of real-world blast exposure conditions from a small mortar round (M49A4 60 mm mortar; standoff distance, 0.25–2.0 m) to a large bomb (M118 bomb; standoff distance, 10–32 m; conventional weapons effects [CONWEP]). The unique design of the fluid-filled receiver translates the shock wave into a pressure wave that travels 8 cm before it interacts with tissue. Therefore, it is likely that scaling for this injury is more complicated, yet falls between the extremes of the unscaled and rat-scaled interpretations and within a range of real-world blast exposure levels.

Only those levels that were on the high range with respect to the typical exposure for a U.S. soldier were capable of inducing cell death. Similar amounts of cell death in OHSC on day 4 after injury have been reported after exposure to a 10–20% strain stretch injury, which has been characterized as a mild stretch injury level.[14,16] Low levels of PI staining could be the result of cells not dying after primary blast or a lack of cells in OHSC to stain. To rule out the latter possibility, an excitotoxic injury was delivered after the highest level of blast tested (level 9), which confirmed the presence of living cells in OHSC after primary blast exposure (Figs. 2 and 3). Therefore, we conclude that whereas isolated primary blast exposure can result in cell death, primary blast exposure in the absence of higher-order blast injury mechanisms is capable of a mild degree of cell death.

Although OHSC were exposed to varying peak pressures, durations, and impulses, cell death increased significantly only after exposure to the two blasts with the highest levels of impulse tested. Cell death was not significantly increased after other exposures with either greater peak pressures or longer durations, suggesting that impulse, which is dictated by the shape of the pressure history and influenced by both peak pressure and duration, may form an appropriate basis for a tolerance criterion to primary blast for brain injury. Blast impulse has previously been shown to correlate with increased poration of human promyelocytic leukemia cells (HL-60).[23] Investigation of apnea after blast suggests that, across species, blast scales with duration.[17] These finding shed light on the importance of reporting complete characterization of blast injury parameters (peak pressure, duration, and impulse) for better understanding of the observed pathology.

Our findings may have implications for physical protection of the U.S. soldier, including the future design of improved protective headgear. The protective capability of current military helmets for shock-wave loading and blast is mostly unknown.[39] The injury criterion described here may help to inform design criteria for blast-protective helmets over a range of blast impulse. Future studies are necessary to determine the correlation of these biomechanical parameters to resultant cell death.

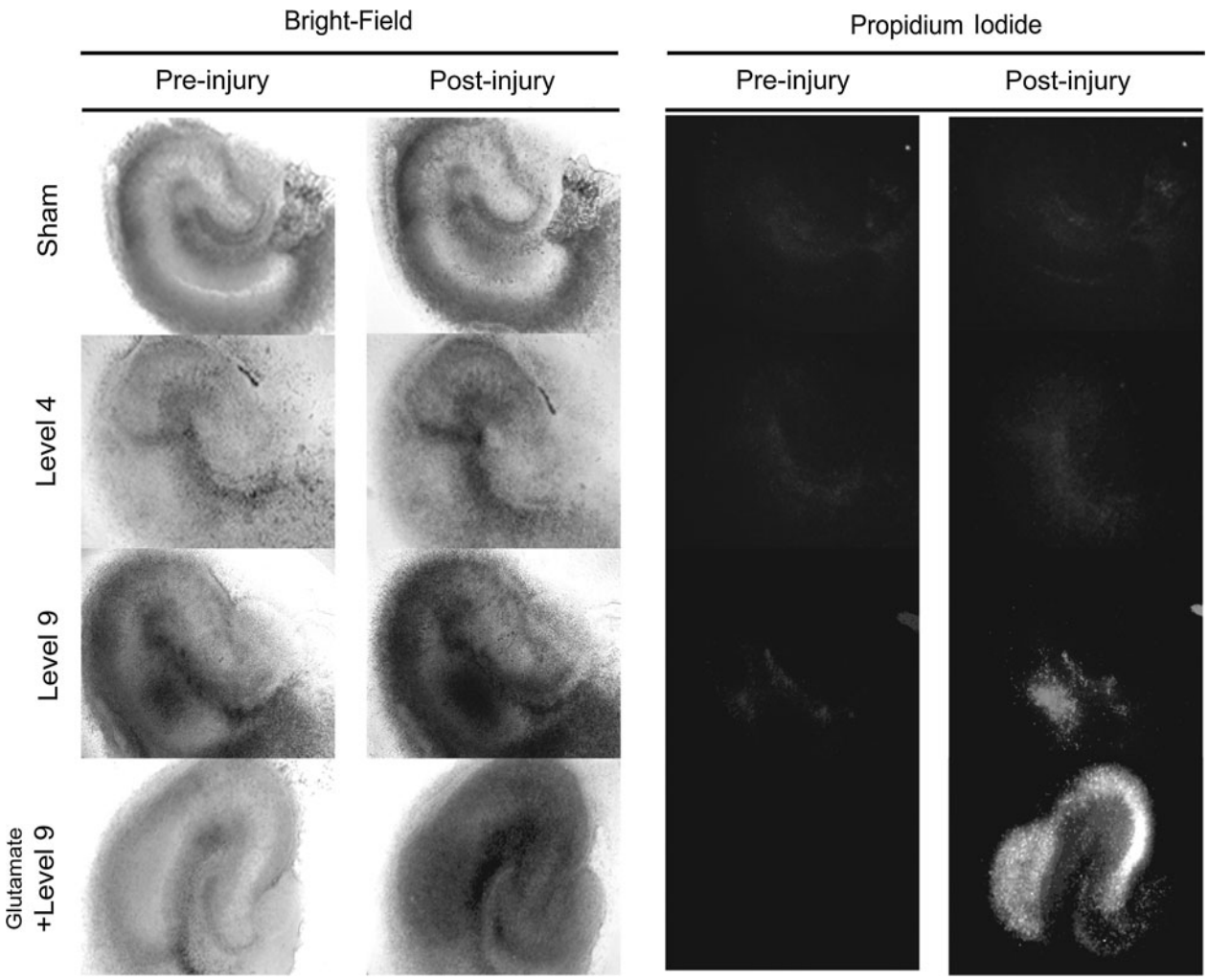

FIG. 3. Bright-field images and fluorescent images of PI staining of OHSC. Images were taken immediately before injury and 4 days after blast exposure. (**A**) Tissue slices appeared darker in bright-field images after low and high levels of blast. (**B**) PI fluorescence confirmed tissue health before experimentation. Fluorescence increased minimally after low and high levels of blast. Glutamate exposure after a level 9 blast confirmed OHSC viability after blast. All cultures shown met the *a priori* criteria for inclusion with cell death of less than 5% before experimentation.

Previous studies have demonstrated neurodegeneration *in vivo* after blast injury pressure histories with lower peak overpressures, but longer durations. In the rat, damage has been observed for peak pressures of 48.9–240 kPa with durations between 2 and 18.2 ms.[24,25] In the nonhuman primate, free-field blast exposure with peak pressures of 80 and 200 kPa and 10-ms duration were injurious.[26] These results, taken together, emphasize that peak pressure and duration are not sufficient to predict blast-induced injury and further suggest the relevance of impulse as a predicative measure of primary blast-induced brain injury. However, experimental limitations of some *in vivo* blast injury models may complicate interpretation of the observed pathologies in these studies. In one of these studies, lung injury was found upon necropsy in rats postblast.[25] In this case, free-field explosive exposure without thoracic protection exposes the whole body to blast, which can result in damage to the gut and/or lungs with downstream influence on CNS damage.[5,11,27] An *in vivo* study in the rat has similarly reported minimal neurodegeneration in the absence of tertiary blast loading after more-severe blast injury exposure.[10] When the head was not restrained, exposure to a 77 kPa, 4.8 ms (impulse not reported) shock wave resulted in head accelerations in excess of 950 krad/s^2, myelinated axonopathy, neuroinflammation, as well as hippocampal-dependent learning and memory deficits.[10] Critically, this same study mitigated tertiary blast loading by restraining the head, producing minimal neurodegeneration and reduced learning and memory deficits after isolated primary blast.[10] These findings support that primary blast in the absence of inertial loading results in minimal neurodegeneration.

In the current study, significant functional changes were observed in the absence of blast-induced cell death (Fig. 4). Additionally, we observed changes in neuron function in conjunction with cell death after the highest level of blast studied. The parameters I_{50} and R_{max} were altered after mild primary blast exposure, but then returned to sham levels when exposed to a more-severe blast intensity. Alterations in the parameter m suggested that primary blast preferentially altered the MF pathway. In recent studies, electrophysiological changes after blast have been investigated infrequently. After exposing isolated slices of guinea pig spinal cord white matter to increasing blast severities (30–70 kPa overpressure, $\sim$200 μs durations), compound action potential (CAP)

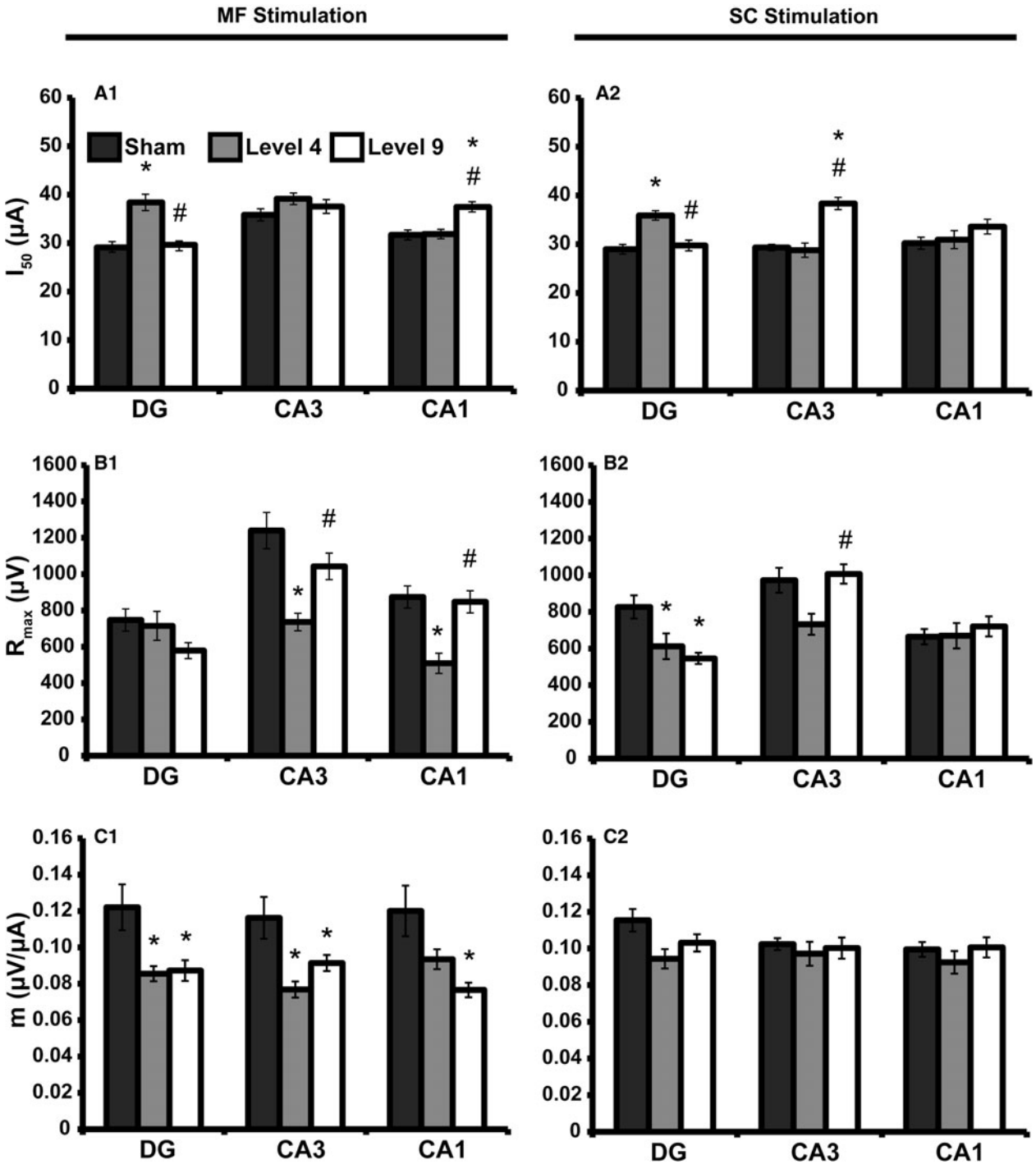

FIG. 4. OHSC functional response after mild and moderate blast exposures. After a level 4 (subthreshold for cell death) and level 9 (suprathreshold for cell death) blast injury, electrophysiological parameters (I_{50} [**A**], R_{max} [**B**], and m [**C**]) were measured 4–6 days after blast exposure. Responses were measured in all three ROI after stimulation of either the MF (1) or SC (2) pathways (± standard error of the mean; $n \geq 30$; $^{*}p < 0.05$, as compared to sham for the same parameter, stimulation location, and ROI; $^{\#}p < 0.05$, as compared to level 4 exposure for the same parameter, stimulation location, and ROI).

amplitude (analogous to R_{max}) was reduced up to a maximum of 56%.[28] All injury exposures in that study caused significant deficits in membrane permeability, demonstrating that morphological disruptions are linked to functional changes. Other electrophysiological studies after blast exposure have presented mixed results. After low-level primary blast exposure in rats, in which increased cell death was measured in the corpus callosum at 3 days postinjury, a significant reduction in CAP amplitude was measured for unmyelinated fibers. However, no significant change in CAP duration was measured for either myelinated or unmyelinated fibers in acute brain slices.[29] It is important to note that the blast parameters in that study were not operationally relevant, being of low magnitude and

very short duration (66 kPa, $\sim 200\,\mu s$), and no evidence of damage was reported in the hippocampus or cortex.

Previous studies using inertially driven TBI models have similarly reported functional deficits in the absence of cell death. For example, reductions in miniature inhibitory postsynaptic currents, inhibitory synaptic transmission, and hippocampal excitability in DG and CA1 were measured in acute hippocampal slices generated from mice subjected to a moderate lateral fluid percussion (LFP) injury sufficient to cause cell death.[30] In the current study, neuron function was altered after a level 9 blast accompanied by cell death. Stretch injuries (10%, $5\,s^{-1}$ equibiaxial strain) suprathreshold for inducing cell death in OHSC reduced R_{max} and increased I_{50} (reduced excitability) in all hippocampal regions.[19]

Injury models replicating inertially driven injuries (i.e., tertiary blast injury), such as LFP injury, can also induce behavioral and memory deficits with no gross morphological damage to the cerebral cortex or hippocampus.[31] Stretch injuries (5%, $5\,s^{-1}$ equibiaxial strain) subthreshold for inducing cell death in OHSC reduced R_{max} and increased I_{50} across all hippocampal regions.[19] Our data confirmed that neuron function changes after a non-cell-death–inducing injury (level 4). Our data substantiate the hypothesis that cell machinery controlling neuron function can be damaged and yet still transmit electrical signals, such that, after primary blast exposure, cellular death is not a requirement for alterations in hippocampal function.

Our results suggested that excitatory neurons may be more susceptible to changes from primary blast exposure than inhibitory neurons. This hypothesis is based upon the measured changes in I_{50} and R_{max} across both stimulation pathways. After mild blast (level 4), excitability decreased (I_{50} increased) and the number of firing neurons decreased (R_{max} decreased), but both parameters returned to sham levels after more-severe blast exposure (level 9). Though no single study has reported direct evidence of a bimodal electrophysiological response after injury, separate experiments have reported damage to excitatory processes after mild, non-cell-death–inducing injuries and to inhibitory processes after more-severe, cell-death–inducing injuries. After a 74 kPa blast exposure to rats, which caused no morphological damage, axonal initial segment length was shortened, which resulted in decreased neuronal excitability, when utilized in computational simulations of network functionality.[32] After exposing rats to cell-death–inducing LFP injury, excitability increased when measured in either acute hippocampal slices *in vitro* or with hippocampal electrodes implanted *in vivo*.[33–36]

Additional studies suggest that severe injuries decrease excitability. After moderate LFP injury to mice, which induced significant morphological changes, field excitatory postsynaptic potential recordings measured decreased excitability in the CA1 region by nearly 100%, out to 2 days postinjury in acute *in vitro* hippocampal slices. In another study, after moderate LFP injury in the mouse, inhibition was increased in the CA1, although excitability was increased in the DG.[30,37] Whereas our hypothesis of a bimodal electrophysiological response after injury is indirectly supported by previous studies, it requires direct testing in future experiments.

Recovery of S/R parameters after more-severe primary blast exposure may be a result of the role each region plays within the hippocampal circuit. The DG has been hypothesized to be the ''filter'' for the trisynaptic circuit.[38] When stimulated, neurons in the DG may be able to dynamically adapt to prevent loss of this filtering function. After MF stimulation, these changes may be evidenced in the parameter m, which decreased after blast, suggesting greater heterogeneity in excitability.

Although we can conclude that primary blast results in altered functional changes and mild cell death, there are several limitations to this study to be considered. Further studies are necessary to evaluate the appropriate scaling between our fluid-filled receiver and human exposure. Additionally, cell death was evaluated at day 4 after exposure, given results from the time-course evaluation and an in light of previous literature using OHSC; it is possible that cell death continues to increase after this time point.[14,16] This is similarly true for the electrophysiological data, which were recorded 4–6 days postinjury. In future studies, the time course of functional changes will be evaluated. In the current study, blast levels were chosen given their relevance to real-world blast exposure; however, these blast injury parameters were not decorrelated from one another. Though the data suggest that impulse is an important criterion for primary blast-induced injury, further experiments over an even larger range of parameters are necessary to elucidate this correlation in greater detail. Last, functional data show close comparisons between subinjurious stretch (5%, $5s^{-1}$) and subthreshold blast results (level 4). Finite element simulations have demonstrated that maximum strains after blast are approximately 5%.[13] Tissue-level biomechanics after blast are limited to computational results, and direct comparisons between injury models are prevented until strains can be directly measured during a blast exposure, which may be possible in future studies.

Here, we have utilized an *in vitro* model of the brain parenchyma to identify the effect of a range of operationally relevant, pure blast-loading conditions on structure and function. This is the first study to expose the brain to a range of relevant pure primary blast-loading conditions and elucidate a threshold for blast-induced cell death. Primary blast injury was capable of killing brain cells; however, an isolated severe primary blast exposure resulted in minimal delayed cell death. Function may be more sensitive than cell death as an outcome measure for the brain after primary blast. However, identification of a blast-induced functional threshold may be more complex than that of blast-induced cell death, given the bimodal effect of blast severity on S/R parameters. Future studies will be necessary to determine these thresholds and elucidate the correlation of individual blast injury parameters to structural and functional outcome measures.

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Author Disclosure Statement

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