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Primary Blast Injury Depressed Hippocampal Long-Term Potentiation through Disruption of Synaptic Proteins

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Abstract

Blast-induced traumatic brain injury (bTBI) is a major threat to United States service members in military conflicts worldwide. The effects of primary blast, caused by the supersonic shockwave interacting with the skull and brain, remain unclear. Our group has previously reported that *in vitro* primary blast exposure can reduce long-term potentiation (LTP), the electrophysiological correlate of learning and memory, in rat organotypic hippocampal slice cultures (OHSCs) without significant changes to cell viability or basal, evoked neuronal function. We investigated the time course of primary blast-induced deficits in LTP and the molecular mechanisms that could underlie these deficits. We found that pure primary blast exposure induced LTP deficits in a delayed manner, requiring longer than 1 hour to develop, and that these deficits spontaneously recovered by 10 days following exposure depending on blast intensity. Additionally, we observed that primary blast exposure reduced total α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor 1 (GluR1) subunit expression and phosphorylation of the GluR1 subunit at the serine-831 site. Blast also reduced the expression of postsynaptic density protein-95 (PSD-95) and phosphorylation of stargazin protein at the serine-239/240 site. Finally, we found that modulation of the cyclic adenosine monophosphate (cAMP) pathway ameliorated electrophysiological and protein-expression changes caused by blast. These findings could inform the development of novel therapies to treat blast-induced loss of neuronal function.

Keywords: electrophysiology; *in vitro* studies; learning and memory; military injury; TBI

Introduction

Blast-induced traumatic brain injury (bTBI) poses a substantial problem for both training and active duty members of the military. Nearly 83% of military TBIs are considered mild TBI.[1] Although TBI can occur from blunt impact and penetrating injury, blast forces are considered the cause of the majority of mTBIs.[2] Although the physiological consequences of brain deformation; that is, tertiary injury, are well studied,[3,4] the pathobiology of TBI caused by primary blast, or shock-wave exposure, remains debated.[5]

Animal studies that investigated the effects of primary blast injury have produced conflicting results for motor[6–11] and cognitive deficits.[8,9,12,13] One potential reason for these discrepancies is the lack of standardization in several critical factors in the bTBI injury models, such as verification of head immobilization, thoracic protection, blast exposure magnitude and duration, and subject orientation. In comparison, appropriately developed *in vitro* models of blast injury allow one to overcome these complications of *in vivo* models and focus on the precise effect of blast loading to brain tissue.[14] A major feature of our *in vitro* injury model is that it isolates the shock-wave component of blast from the other, confounding phases of injury including secondary (transection caused by shrapnel), tertiary (large deformations caused by inertial loading), and quaternary blast loading.[15] By using a system with controlled biomechanics, we can determine whether primary blast in isolation affects neuronal function of the hippocampus.

Common symptoms of bTBI include memory deficits and loss of spatial navigation,[16] which implicate damage to the hippocampus. Many studies showed that bTBI impairs memory processing in animal models through cognitive tests;[8–13] however, fewer reveal their underlying electrophysiological basis.[8,17–19] Long-term potentiation (LTP) is thought to functionally represent experience-driven neural circuitry changes,[20,21] and numerous TBI studies have reported deficits in LTP *in vitro* following non-blast injury;[22–26] however, there is a dearth of studies showing LTP deficits following bTBI.[8,19] Our group was the first to observe that LTP was disrupted after pure primary blast exposure,[19] and this report examines the molecular mechanisms behind primary blast-induced LTP deficits.

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We observed that primary blast reduced LTP in a delayed manner, requiring >1 h to develop. LTP spontaneously recovered 10 days after exposure to an 87 kPa·ms impulse blast, but not after a 248 kPa·ms blast. Blast significantly reduced phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-glutamate receptor 1 (AMPA-GluR1) subunits at the serine-831 (Ser831), site and reduced phosphorylation of stargazin at the serine-239/240 (Ser239/240) site. Expression of GluR1 subunits and postsynaptic density protein-95 (PSD-95) were significantly decreased by primary blast exposure. Post-exposure treatment with the United States Food and Drug Administration (FDA)-approved phosphodiesterase-4 (PDE4) inhibitor roflumilast prevented blast-induced LTP deficits. The observed improvement in LTP with roflumilast treatment warrants its further investigation as a potential therapy for blast-induced LTP-loss.

Methods

Organotypic hippocampal slice culture

All animal procedures were approved by the Columbia University Institutional Animal Care and Use Committee (IACUC). Organotypic hippocampal slice cultures (OHSCs) were generated from P8-P10 Sprague Dawley rat pups as previously described.[15,18,19,27] In brief, the hippocampus was isolated, cut into thin sections (400 μm) with a McIlwain tissue chopper (Ted Pella, CA), and plated onto Millicell inserts (EMD Millipore, Billerica, MA) in Neurobasal medium supplemented with 2 mM GlutaMAX™, 1X B27 supplement, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 25 mM D-glucose (Life Technologies, Grand Island, NY). Thereafter, cultures were fed every 2–3 days with full serum medium, containing 50% Minimum Essential Medium, 25% Hank's Balanced Salt Solution, 25% heat inactivated horse serum, 2 mM GlutaMAX, 25 mM D-glucose, and 10 mM HEPES (Sigma). Prior to blast injury, cultures were maintained for 10–14 days.

Primary blast exposure

Blast injury methods have been previously described in detail [15,18,19,28–30] Piezoresistive pressure transducers (Endevco 8530B-500, San Juan Capistrano, CA) recorded side-on (incident) pressure at the shock tube exit and inside the fluid-filled receiver. Peak overpressure, overpressure duration, and impulse were recorded, processed, and quantified as previously described.[15,18,19,29]

For injured cultures, the shock tube was fired; sham cultures were treated identically except that the shock tube was not fired. Two blast exposure levels were utilized (Table 1, levels referenced to Vogel and coworkers[19]), characterized by the peak pressure (kPa), duration (ms), and impulse (kPa•ms) of the in-air shockwave and the in-fluid pressure transient. Blast levels (specific parameters given in Table 1) simulated real-world exposures and were chosen both below and above the threshold for causing cell death, based on previous studies.[18,19] Following blast or sham exposure, the culture was immediately removed from the receiver and returned to the incubator in fresh, full serum medium. Cultures were maintained in full serum medium for up to 10 days post-exposure.

Cell death measurement

Propidium iodide (PI) fluorescence was used to measure cell death immediately prior to injury, and at 1 h and 10 days following injury using 2.5 μM PI (Life Technologies) in serum-free medium; previous studies with this injury model have reported that cell death remains minimal between 1 and 4 days after blast.[18,19] Cell death was determined for specific regions (dentate gyrus [DG], cornu ammonis 1 [CA1], cornu ammonis 3 [CA3]), as previously described, using MetaMorph (Molecular Devices, Downingtown, PA), and reported as percentage area.[15,18,19,31] To confirm OHSC viability after blast, a subset of cultures was exposed to the highest blast level tested (Level 9) and subsequently subjected to an excitotoxic injury (10 mM glutamate for 3 h) 10 days following blast exposure. OHSC were returned to fresh serum-free medium following excitotoxic exposure, and cultures were imaged for cell death 24 h later. Cell death was analyzed by analysis of variance (ANOVA), followed by Dunnett *post-hoc* tests with statistical significance set at $p < 0.05$ (SPSS v22, IBM, Armonk, NY).

Electrophysiology

In a separate set of cultures, electrophysiological activity within the OHSC was recorded using 60 channel MEAs (8×8 electrode grid without the corners, 30 μm electrode diameter, 200 μm electrode spacing) at either 1 h, 1 day, 2 days, 4 days, 6 days, or 10 days following blast injury (60MEA200/30iR-Ti-gr, Multi-Channel Systems, Reutlingen, Germany). In our previous studies, electrophysiological deficits were measured at only 4–6 days after blast.[18,19] The MEAs were prepared and slices were placed onto the arrays as previously described.[18,19] OHSCs were perfused with artificial cerebral spinal fluid (norm-aCSF) containing 125 mM NaCl, 3.5 mM KCl, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10mM HEPES, and 10 mM glucose (pH = 7.40), which was bubbled with 5% CO_2/95% O_2 and warmed to 37°C, as previously described.[32] Recordings were acquired with an MEA1060-BC amplifier and data acquisition system (Multi-Channel Systems).

Stimulus-response (SR) curves

SR curves were generated by applying a constant current, biphasic, bipolar stimulus (100 μs positive phase followed by 100 μs negative phase) of increasing magnitude (0–200 μA in 10 μA increments) to electrodes located in the Schaffer collateral (SC) pathway. Evoked responses were recorded from each electrode throughout the hippocampal trisynaptic circuit. As in previous studies, each electrode's response was fit to a sigmoidal curve, and three parameters were quantified: R_{max}, represented the maximum amplitude of the evoked response, I_{50} represented the current necessary to generate a half-maximal response, and the term *m*, represented the slope of the sigmoidal fit.[32] Each parameter (I_{50}, m, R_{max}) for an electrode was averaged within a region to determine

Table 1. Blast Exposure Levels Tested in this Study

	In-air			*In-fluid*		
Exposure level	*Pressure (kPa)*	*Duration (ms)*	*Impulse (kPa•ms)*	*Pressure (kPa)*	*Duration (ms)*	*Impulse (kPa•ms)*
Level 4	336 ± 8	0.84 ± 0.01	87 ± 2	598 ± 15	1.85 ± 0.30	440 ± 13
Level 9	424 ± 6	2.31 ± 0.03	248 ± 3	1510 ± 91	2.80 ± 0.10	1420 ± 87

As reported earlier by Effgen and coworkers.[18]

that regional response for any given slice. Data reported for each region are the average across slices within a given experimental group. Individual parameters were analyzed by ANOVA followed by Dunnett *post-hoc* tests with statistical significance set as $p < 0.05$ (SPSS v22, IBM).

LTP

Following SR evaluation, the ability to induce LTP was measured. Baseline response was evoked by stimulating at I_{50} once every minute for 30 min. LTP was then induced by stimulating across the SC pathway with a high frequency stimulus, which consisted of three trains of 100 Hz pulses applied for 1 sec at I_{50}, with each train separated by 10 sec.[33,34] Immediately following LTP induction, post-LTP responses were evoked by stimulating at I_{50} once every minute for 60 min. LTP induction was calculated as percent potentiation above baseline based on the last 10 min of recording in each recording window. To ensure that only stable responses were included for analysis, electrodes were discounted if the coefficient of variance (pre- or post-induction) was >20%.[35] LTP induction was averaged among electrodes within the CA1 and analyzed by ANOVA followed by Dunnett *post-hoc* tests with statistical significance set as $p < 0.05$, as compared with time-matched shams (SPSS v22, IBM).

Chemically induced LTP (chemLTP)

In separate sets of cultures, LTP was chemically induced with two distinct protocols. Baseline electrical activity was recorded for 30 min as described. The first chemLTP protocol replaced electrical LTP induction with a 3 min perfusion with a modified aCSF solution (gly-aCSF)[36]: norm-aCSF containing 200 μM glycine and 0 mM MgCl (reduced from 1.3mM). The perfusate was then switched back to norm-aCSF and washed out for 20 min prior to post-induction electrical stimulation. Percent potentiation was calculated by comparing the average of the final 10 min of post-induction responses to the average of the final 10 min of pre-induction responses, as described. The gly-aCSF solution activates synaptic *N*-methyl-D-aspartate (NMDA) receptors, allowing Ca^{2+} ions to enter the dendritic spine, similar to electrically induced LTP.[36]

The second chemLTP protocol replaced electrical LTP induction with a 20 min perfusion with a modified aCSF solution (cyclic adenosine monophosphate [cAMP]-aCSF)[37]: norm-aCSF containing 1 mM MgCl (reduced from 1.3 mM), 50 μM forskolin, 50 μM picrotoxin, and 100 nM rolipram. Forskolin, rolipram, and picrotoxin stocks were dissolved in dimethyl sulfoxide (DMSO) with a final DMSO concentration in cAMP-aCSF of 0.07%. The perfusate was then switched back to norm-aCSF and washed out for 20 min prior to post-induction stimulation for 60 min. Percent potentiation was calculated as described. The cAMP-aCSF solution acts to upregulate the cAMP-protein kinase A (PKA) pathway through inhibition of phosphodiesterase-4 (rolipram) and activation of adenylate cyclase (forskolin), which results in elevated cAMP levels and consequently PKA activation.[37] Picrotoxin, a γ-aminobutyric acid $(GABA)_A$ inhibitor, reduced inhibition and enhanced stability of the potentiated signal, while not inducing LTP on its own.[37]

Chemically induced LTP was averaged among electrodes within the CA1 and analyzed by ANOVA followed by Dunnett *post-hoc* tests with statistical significance set as $p < 0.05$, as compared with time-matched shams (SPSS v22, IBM).

Western blotting

For each condition tested by Western blotting, eight slices from two different animals were collected for protein extraction. Groups receiving chemLTP induction (gly-aCSF or cAMP-aCSF) were treated, switched back to normal aCSF after respective treatment times, incubated for appropriate induction times, and then lysed. Slices were rinsed twice with ice-cold phosphate-buffered saline (PBS) and immediately placed in chilled lysis buffer A (40 mM HEPES, 120 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, and 10 mM β-glycerophosphate, Sigma). Samples were sonicated (Sonicator 3000, Misonix), incubated on ice, and then centrifuged to remove cellular debris. Protein concentrations were determined by the bicinchoninic acid assay (BCA) according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA), and 50 μg of protein per sample were loaded in a 4–12% Bis-Tris gel (Life Technologies). Proteins were separated by electrophoresis (150 V, 1.5 h) and transferred (100 mA, 40 min) to a nitrocellulose membrane (Life Technologies) using a semi-dry apparatus (Fisher Scientific, NY). The membrane was blocked in Tris-buffered saline (TBS, pH 7.4) with 1% bovine serum albumin (BSA) for 1 h. Membranes were incubated overnight at 4°C with primary antibodies (total GluR1 [Millipore, #04-855], phosphorylated GluR1-Ser^{831} [Millipore, #04-823], phosphorylated GluR1-Ser^{845} [Millipore, #04-1073], total CaMKII [Sigma, #SAB4503250], phosphorylated CaMKII-Thr286 [Abcam, #ab5683], total NMDA NR2B [Millipore, MAB5778], total PSD-95 [Thermo Fisher, MA1-046], phosphorylated stargazin-Ser239/240 [Millipore, AB3713], and β-actin [Sigma, #A1978]) in TBS with Tween (TBS-T, 0.1% Tween-20, pH 7.4) and 0.25% BSA. Following the primary antibody incubation, membranes were washed 3 times for 10 min in TBS-T. To detect protein bands, the membranes were labeled with a corresponding secondary antibody (Donkey anti-Rabbit Alexa Fluor 488 or Goat anti-Mouse Alexa Fluor 647, Life Technologies). Fluorescence was detected using a CRi Maestro 2 Imaging System (Perkin Elmer). The bands were quantified using ImageJ software. Average fluorescence was quantified from, at minimum, four lysates per exposure group (8 slices per lane, 32 slices in total) and analyzed by ANOVA with statistical significance set as $p < 0.05$, as compared with treatment-matched shams (SPSS v22, IBM). A *post-hoc* Bonferroni analysis revealed statistically significant differences between injury-matched treatments for each antibody, with significance set as $p < 0.05$.

Roflumilast treatment

To test the ability of a PDE4 inhibitor to rescue LTP after blast exposure, 1 μM roflumilast (SML1099, Sigma-Aldrich; dissolved in 0.07% DMSO) in full serum media was delivered to sham and Level 4-exposed cultures immediately after blast exposure. For comparison, DMSO vehicle was also delivered to a second set of cultures that had received either sham or Level 4 blast exposure. The cultures' ability to generate LTP through high frequency electrical stimulation was evaluated at 24 h post-exposure. Potentiation was averaged in the CA1 region of the hippocampus and analyzed by ANOVA, with statistical significance set as $p < 0.05$, as compared with similarly treated shams.

Results

Primary blast exposure inhibited LTP in a delayed manner

Potentiation (Fig. 1) was not reduced when measured 1 h (Day 0) after Level 4 or Level 9 blast exposures, as compared with sham. When measured at 24 h (Day 1) post-blast exposure, potentiation was significantly reduced in cultures exposed to either a Level 4 or Level 9 exposure. This deficit was maintained at Day 2, Day 4, and Day 6 post-Level 4 and Level 9 blast exposure. At 10 days after Level 4 blast, potentiation had partially recovered and was no longer significantly different from time-matched sham-exposed cultures; however, potentiation remained significantly depressed in the Level 9 exposed cultures at the same time point.

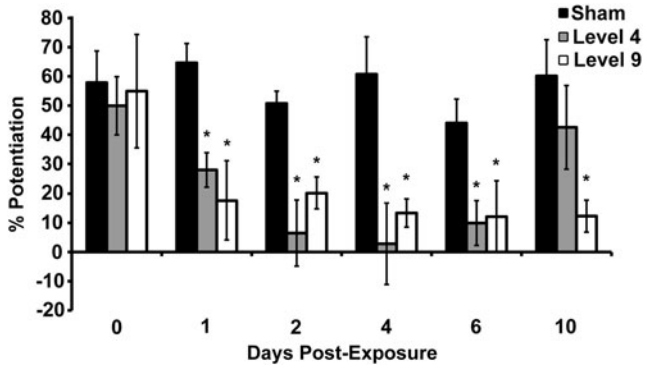

FIG. 1. Long-term potentiation (LTP) measured in the cornu ammonis 1 (CA1) at multiple time points after primary blast injury. Groups are organized by increasing time after exposure (Day 0, Day 1, Day 2, Day 4, Day 6, Day 10) and then by increasing impulse from left to right (sham, Level 4, Level 9). LTP was significantly reduced 1 day following Level 4 or Level 9 blast exposures. This deficit persisted out to 6 days following either injury exposure. LTP spontaneously recovered 10 days after Level 4 blast exposure, but not after Level 9 blast exposure (mean ± SEM.; $n \geq 5$; $^*p < 0.05$, as compared with time-matched sham).

Primary blast exposure did not induce neuronal death

One possible explanation for changes in potentiation is neuronal degeneration and loss after blast exposure. Previous work has reported that this injury model does not induce substantial cell death at the time points previously examined; that is, between Days 1 and 4 following blast exposure.[18,19] We evaluated cell viability (Fig. 2) at a longer time point (Day 10) to confirm that cells remained alive at extended periods following Level 9 blast exposure, the most severe blast condition used in the study. We found that cell death was not significantly increased for any region of interest (ROI) at 10 days following Level 9 exposure compared with sham cultures. Although cell death was slightly higher in the DG (~10%) than in other regions following blast, this pattern was also present in sham cultures. To confirm the presence of living neurons, exposure to toxic levels of glutamate 10 days following Level 9 primary blast caused significant cell death (>80%) across all ROIs, confirming the presence of cells not killed by primary blast exposure alone.

Primary blast exposure did not induce deficits in basal evoked function

Alterations in basal evoked function, the ability to stimulate local circuitry or transmit stimulation to downstream networks, could potentially explain the observed change in LTP following blast injury. We have previously reported that our injury model induced minimal changes in basal evoked function, when stimulating across the SC pathway, at 4–6 days post-primary blast exposure.[19] In the current study, we observed that neither blast level significantly altered the maximum voltage response (Fig. 3A, R_{max}), the current necessary to generate a half-maximal response (Fig. 3B, I_{50}), or the spread in the firing threshold for the population of neurons (Fig. 3C, m) for any ROI at any time point following injury. These results confirmed the previous findings from this system, and revealed that basal evoked function was not disrupted at acute or longer time points following blast exposure.

Primary blast exposure reduced potentiation when induced with gly-aCSF

To further explore blast-induced deficits in LTP, we investigated the effect of blast injury on chemical induction of LTP using gly-aCSF. We observed that potentiation (Fig. 4A) was significantly reduced in Level 4 blast-exposed cultures measured at 1 day after injury when induced with gly-aCSF. When measured at 10 days following Level 4 blast exposure, gly-aCSF-induced potentiation (Fig. 4A) recovered and was not significantly different from time-matched, sham-exposed cultures. These findings matched the time course of blast-induced LTP deficits when induced electrically. To verify that the shorter treatment duration with gly-aCSF did not confound results, we extended gly-aCSF treatment from 3 to 20 min, and observed that Level 4 blast exposure significantly decreased potentiation (27 ± 13%) as compared with sham (78 ± 15%) exposure (data not shown).

Primary blast exposure did not reduce potentiation when induced with cAMP-aCSF

In contrast, potentiation (Fig. 4B) was not significantly reduced at 1 day following a Level 4 blast exposure using a chemLTP

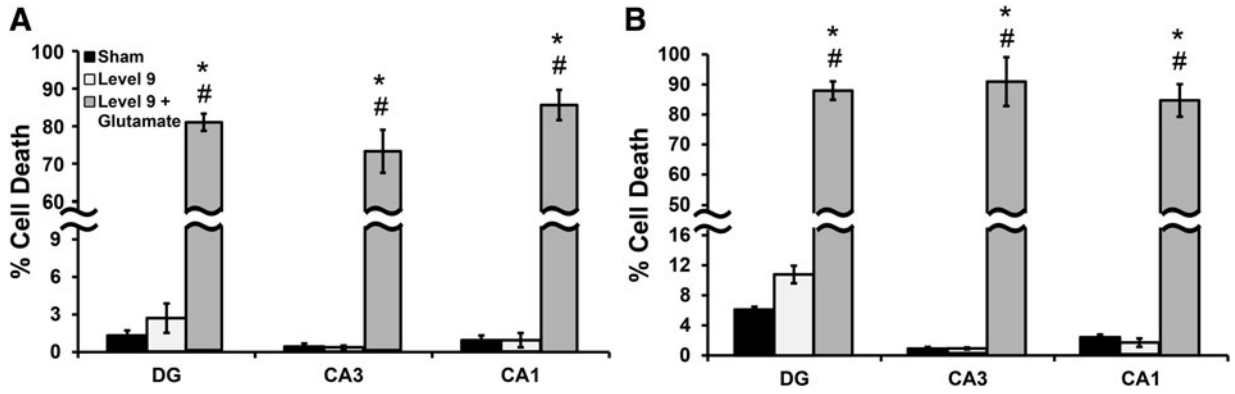

FIG. 2. Cell death measured for each region of interest (ROI) of the hippocampus at (**A**) 1 h and (**B**) 10 days after Level 9 blast. Cell death was not significantly induced after Level 9 blast exposure in any ROI compared with sham-injured cultures. Glutamate exposure at (A) 1 h and (B) 10 days following Level 9 blast induced significant cell death in all ROIs (mean ± SEM.; $n = 12$; $^*p < 0.05$ as compared with sham, $\#p < 0.05$ as compared with Level 9).

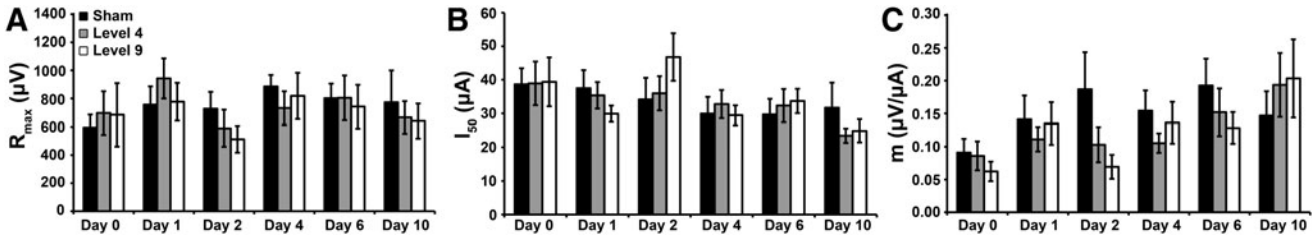

FIG. 3. Blast injury minimally affected stimulus-response parameters in the cornu ammonis 1 (CA1) when stimulated across the Schaffer collateral (SC) pathway. There was no significant effect on R_{max} (**A,** $p > 0.35$), I_{50} (**B,** $p > 0.42$), m (**C,** $p > 0.10$) at any time point following blast exposure, as compared with sham. (Data not shown for the cornu ammonis 3 [CA3] and dentate gyrus [DG], mean ± SEM; $n \geq 5$.)

induction protocol that upregulated the cAMP/PKA pathway (cAMP-aCSF). To verify that the extended treatment duration with cAMP-aCSF did not confound results, we shortened cAMP-aCSF treatment from 20 to 3 min, and observed that blast exposure did not significantly reduce potentiation (48 ± 11%) as compared with sham (42 ± 17%) exposure (data not shown). These results suggested that modulation of the cAMP/PKA pathway may act therapeutically against primary blast-induced LTP loss.

Phosphorylated GluR1-Ser831 and total GluR1 expression was significantly reduced by primary blast injury

Blast prevented a significant increase in phosphorylation of AMPA receptor (AMPAR)-GluR1 subunits at the Ser831 site at 24 h post-injury as compared with shams, when LTP was induced with gly-aCSF (Fig. 5A). There was no observed effect of blast with vehicle-treated cultures or with the cAMP-aCSF treatment. Treatment with either gly-aCSF or cAMP-aCSF significantly increased GluR1-Ser831 phosphorylation over vehicle-treated sham cultures; that is, no induction of LTP; however, only cAMP-aCSF significantly increaseed GluR1-Ser831 phosphorylation over vehicle-treated blast cultures.

Blast also significantly decreased the expression of total GluR1 subunits (Fig. 5B) as compared with shams, when LTP was induced with gly-ACSF. Although this deficit was not observed with vehicle-treated cultures or with the cAMP-aCSF treatment, it is important to note that there was an increase in total GluR1 expression for both sham and blast-exposed cultures receiving cAMP-aCSF treatment, as compared with the vehicle-treated cultures.

We observed no significant change in the phosphorylation state of AMPAR-GluR1 subunits at the Ser845 site (Fig. 5C) between blast and sham cultures for any chemical treatment; however, both chemical treatments significantly increased GluR1-Ser845 phosphorylation over vehicle-treated cultures.

There also was no significant change to NMDA receptor (NMDAR)-NR2B subunits (Fig. 5D) as a result of either injury exposure or chemical treatment.

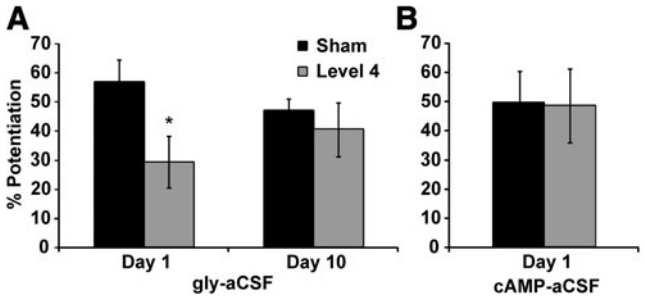

FIG. 4. Blast injury significantly reduced glycine-induced long-term potentiation (LTP), but not rolipram/forskolin-induced LTP. (**A**) LTP was significantly reduced when induced by glycine-artificial cerebral spinal fluid (gly-aCSF) at 1 day following Level 4 blast exposure. There was no significant change in glycine-induced LTP at 10 days following Level 4 blast exposure. (**B**) LTP was not reduced when induced by cyclic adenosine monophosphate (cAMP)-aCSF at 1 day following Level 4 blast exposure (mean ± SEM; $n \geq 8$, $^*p < 0.05$, as compared with sham).

Primary blast injury did not affect total CaMKII or phosphorylated CaMKII-Thr286

Another important target in the LTP pathway is CaMKII, as CaMKII phosphorylation is necessary for the induction of some forms of LTP.[38–40] We observed that phosphorylation of CaMKII at the Threonine-286 (Thr-286) site was not affected by injury (Fig. 6A). There was an expected increase in phosphorylation with both chemLTP treatments. We also observed that total expression of CaMKII (Fig. 6B) was not altered as a result of injury ($p > 0.33$) or chemical treatment ($p = 1.0$).

Primary blast injury significantly reduced PSD-95 and pStargazin-Ser239/240

We also examined two key proteins responsible for receptor integration at the synapse: PSD-95 and stargazin. We found that blast decreased expression of total PSD-95 for both vehicle- and gly-aCSF-treated cultures as compared with shams, but not for cAMP-aCSF-treated cultures (Fig. 7A). We observed that blast exposure reduced the phosphorylation of stargazin (Ser239/240) after gly-aCSF treatment, but not for vehicle or cAMP-aCSF-treated cultures (Fig. 7B).

Roflumilast treatment immediately after exposure prevented blast-induced LTP deficits

Roflumilast treatment immediately following blast exposure prevented a deficit in electrically induced LTP at 24 h post-injury (Fig. 8). Level 4 blasted cultures that were treated with vehicle (0.07% DMSO in aCSF) immediately following injury were unable to potentiate at 24 h post-exposure, as compared with sham-

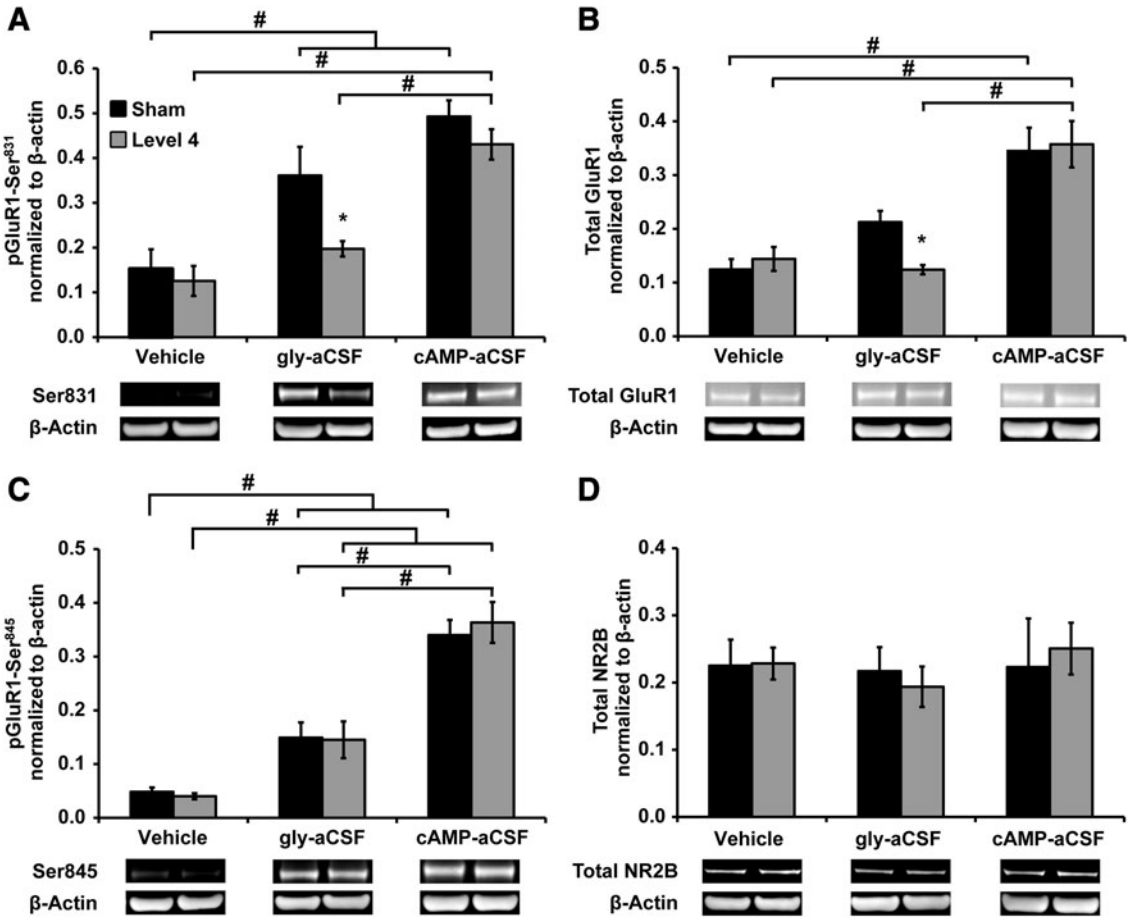

FIG. 5. Phosphorylation of glutamate receptor 1 (GluR1)- serine-831 (Ser831) and total GluR1 expression was reduced at 24 h following Level 4 blast injury. Protein expression (normalized to loading control β-actin) was evaluated at 24 h following Level 4 blast injury for four different synaptic membrane protein targets: pGluR1-Ser831 (**A**), total GluR1 (**B**), pGluR1-Ser845 (**C**), and total *N*-methyl-D-aspartate (NMDA)-NR2B (**D**). Cultures were either untreated (vehicle) or long-term potentiation (LTP) was induced by either glycine-artificial cerebral spinal fluid (gly-aCSF) or cyclic adenosine monophosphate (cAMP)-aCSF prior to cell lysis. Phosphorylation of GluR1-Ser831 and total GluR1 expression were significantly reduced when LTP was induced by gly-aCSF, but not by cAMP-aCSF, at 1 day following Level 4 blast exposure. Representative bands from each group are shown below the graphs (mean ± SEM; $n \geq 4$, $^*p < 0.05$, as compared to treatment-matched sham, $\#p < 0.05$ as compared with injury-matched treatment).

vehicle-treated cultures. This indicated that PDE4 inhibitors have the potential to prevent primary blast-induced LTP loss.

Discussion

This study demonstrated important details regarding the mechanisms behind LTP deficits following primary blast exposure. The onset of blast-induced LTP deficits was delayed (1–24 h post-exposure) and the deficit recovered over time (6–10 days post-exposure), depending on blast intensity. Primary blast exposure reduced expression and disrupted phosphorylation of proteins critical to LTP induction. Blast reduced phosphorylation of GluR1-Ser831 and expression of total GluR1. Blast also decreased PSD-95 expression and phosphorylation of stargazin-Ser239/240. Treatment with FDA-approved roflumilast immediately post-blast prevented LTP deficits measured at 24 h. We hypothesize that a mechanism for blast-induced LTP deficits is the disruption of PSD-95, which prevents the increase, and subsequent phosphorylation, of synaptic AMPAR-GluR1 at the PSD, thereby preventing the induction of LTP.

Our study is the first to report that primary blast produced deficits in LTP at acute time points following injury. *In vivo* blast injury induced LTP deficits at 2 and 4 weeks following blast in mice.[8,41] Non-blast, fluid percussion injury (FPI) models induce LTP deficits in rodents at 2–4 h,[42] 1–2 days,[22,23] and 1 week[25] post-injury. One study did not observe FPI-induced LTP deficits at 1 week post-injury, but did observe deficits at 8 weeks post-injury.[24] The difference in injury biomechanics between our injury model and FPI is important to note when interpreting results. Our blast model applied a pressure transient *in vitro* that led to minimal tissue deformation,[28] whereas FPI is a mix of pressure and deformation throughout the brain.[43,44]

Glycine-LTP is similar to high-frequency stimulus-induced LTP because they both directly activate NMDARs enabling an influx of Ca^{2+} ions.[36] Conversely, rolipram/forskolin-LTP activates secondary messengers (namely cAMP) to induce LTP.[37] In sham cultures, both

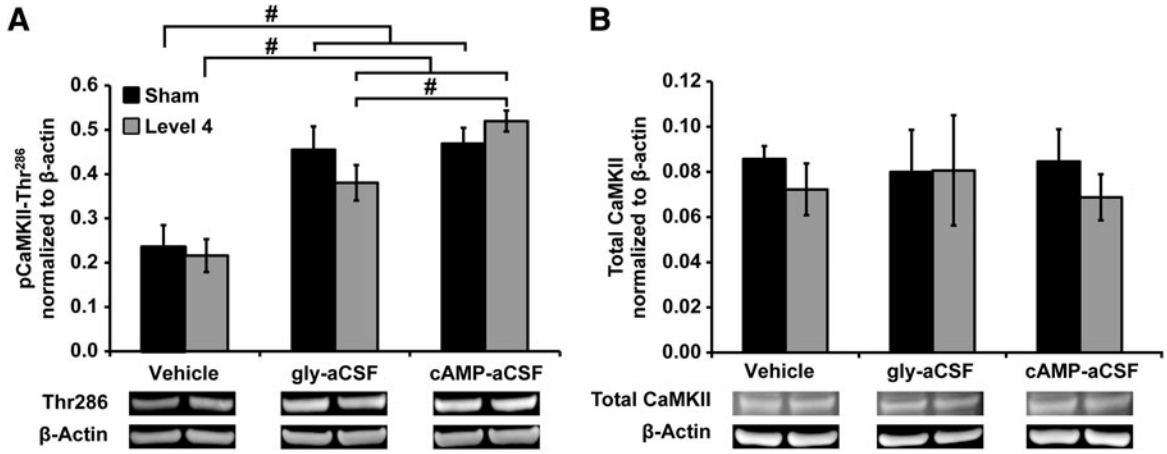

FIG. 6. Primary blast exposure did not affect (**A**) phosphorylation of CaMKII-Thr286 or expression of (**B**) total CaMKII at 24 h post-injury with or without induction of long-term potentiation (LTP). Representative bands from each group are shown below the graphs (mean ± SEM; $n \geq 4$, $\#p < 0.05$ as compared with injury-matched treatment).

protocols induced hallmark signs of LTP, including GluR1-containing AMPAR insertion into the PSD, phosphorylation of CaMKII, phosphorylation of GluR1-Ser831, and increased electrophysiological response.[37,45,46] No previous study has investigated the effect of TBI on glycine-LTP; however, one study observed that blast exposure significantly reduced rolipram/forskolin-LTP at 2 and 4 weeks following injury.[8] Although the results of our study contradict those findings, it is important to note the substantial differences between the studies including the post-injury time point, injury biomechanics, and concentration of rolipram.

We observed that primary blast affected postsynaptic receptors, specifically total GluR1 expression and phosphorylation of GluR1-Ser831 (Fig. 5). Conversely, one non-blast TBI study observed an increase in phosphorylation of GluR1-Ser831 between 1 and 4 h following stretch injury in cortical neurons; however, their findings corroborate our observations that total GluR1 expression or phosphorylation of GluR1-Ser845 did not change with injury.[47] This difference may be linked to different injury models, brain region, and use of chemical LTP induction. We observed that cAMP-aCSF increased the phosphorylation of GluR1-Ser845 over that of gly-aCSF for both blast and sham cultures. This difference suggests a mechanism for LTP recovery with cAMP-aCSF treatment. We also chose to investigate the NMDAR-NR2B subunit, as previous studies reported that this subunit governed NMDAR mechanosensitivity.[48] We observed no change to this subunit after blast exposure; however, previous non-blast TBI studies reported mixed results for this target.[49–51] The effect of primary blast on postsynaptic receptors, namely the AMPAR-GluR1 subunit, is likely linked to the LTP deficits measured electrophysiologically.

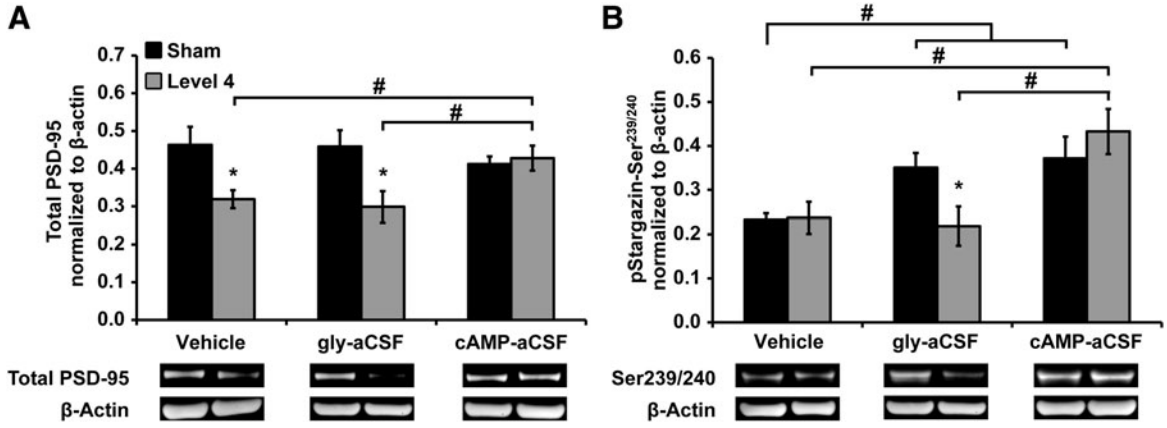

FIG. 7. Total postsynaptic density protein-95 (PSD-95) expression (**A**) was significantly reduced at 24 h following Level 4 blast exposure in vehicle treated and glycine-artificial cerebral spinal fluid (gly-aCSF)-treated cultures, but not in cyclic adenosine monophosphate (cAMP)-aCSF-treated cultures. Phosphorylation of stargazin-serine-239/240 (Ser239/240) (**B**) was significantly reduced at 24 h following Level 4 blast exposure in gly-aCSF-treated cultures, but not in cAMP-aCSF-treated cultures. Representative bands from each group are shown below the graphs (mean ± SEM; $n \geq 4$, $*p < 0.05$, as compared to treatment-matched sham, $\#p < 0.05$ as compared to injury-matched treatment).

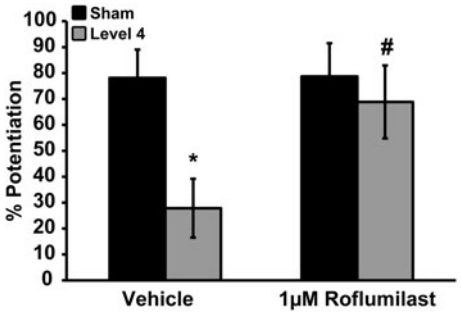

FIG. 8. Roflumilast treatment prevented blast-induced long-term potentiation (LTP) deficits at 24 h post-injury. Cultures were exposed to Level 4 blast and immediately treated with either 1 μM roflumilast or vehicle (0.07% dimethyl sulfoxide [DMSO] in medium). LTP was electrically induced and evaluated 24 h post-injury (mean ± SEM; $n \geq 5$, $^*p < 0.05$, as compared with treatment-matched sham, $\#p < 0.05$ as compared with injury-matched vehicle).

CaMKII is a protein critical for induction of hippocampal LTP.[52] Our data suggested that blast did not affect either total CaMKII expression or phosphorylation at the Thr-286 site (Fig. 6). These results suggest that the mechanism to phosphorylate CaMKII was not impacted by blast. Studies with non-blast TBI have reported a decrease in total CaMKII expression at varying time points after FPI.[25,53] Conversely, CaMKII phosphorylation was reported to increase acutely following *in vivo* and *in vitro* non-blast TBI in rodents.[47,50,53,54] Most of these studies reported resolution of altered phosphorylation of CaMKII by 24 h post-injury, which corroborates our finding that CaMKII phosphorylation was not affected at 24 h following primary blast exposure; however, reduction of total CaMKII by non-blast-TBI suggested a different injury cascade from blast. Interestingly, we observed that blasted cultures that were subsequently treated with cAMP-aCSF exhibited significantly increased phosphorylation of CaMKII-Thr286 over blasted cultures that subsequently were treated with gly-aCSF, suggesting that CaMKII phosphorylation may modulate LTP-rescue by rolipram/forskolin.

The immobilization of GluR1-containing AMPARs at the PSD through PSD-95 is a required step for LTP induction.[55] We observed that primary blast reduced PSD-95 expression, in both vehicle and glycine-LTP groups (Fig. 7). PSD-95 was the only target for which blast, without subsequent LTP-induction, reduced expression. These findings corroborate our observation that blast did not affect all neuronal function, but rather that deficits after blast were LTP specific. Combined primary and tertiary blast exposure did not affect cortical PSD-95 expression 2 weeks post-injury in rats[56]; however, the rise time of the pressure profile in that model was extended ($\sim$2 ms) compared with typical shock exposures ($\sim$20 μs).[28] Several non-blast TBIs reduced hippocampal PSD-95 expression between 18 h and 7 days post-injury.[57–59] Stargazin is an auxiliary AMPA protein that mediates the binding of AMPARs to PSD-95 upon phosphorylation. We observed that blast exposure reduced pStargazin-Ser239/240 when LTP was induced with glycine, which could explain the reduction in total GluR1 after blast, as stargazin binds to the GluR1 subunit. Genetically altered stargazin expression was shown to decrease the duration that AMPARs resided in the PSD.[60] Our study is the first to report that TBI affected stargazin

Our working hypothesis is that blast disrupts PSD-95 which, in turn, reduces induction of LTP caused by decreased GluR1 immobilization at the PSD and phosphorylation at the Ser831 site

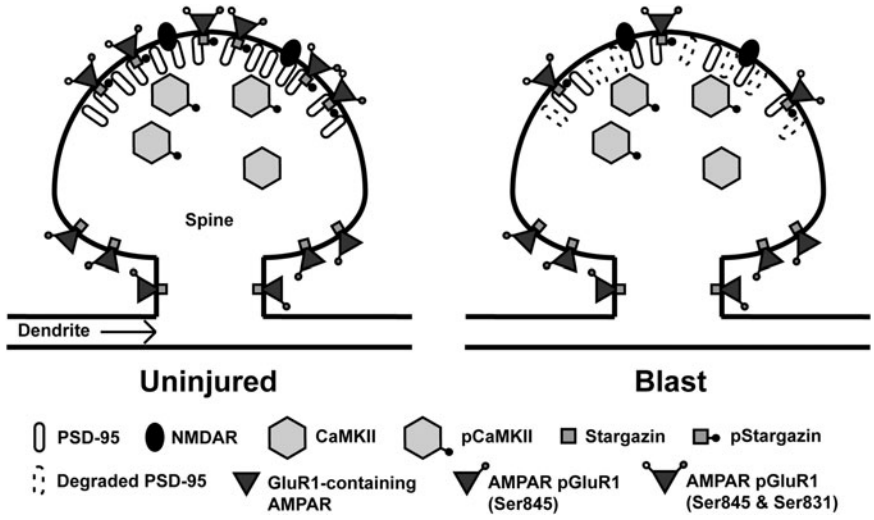

FIG. 9. Hypothesized injury mechanism for primary blast-induced long-term potentiation (LTP) deficits. This diagram compares an uninjured dendritic spine to a spine exposed to primary blast. Our findings suggest that primary blast exposure degrades or reduces the expression of postsynaptic density protein-95 (PSD-95), the major postsynaptic membrane scaffolding protein. We hypothesize that when attempting to induce LTP after blast, glutamate receptor 1 (GluR1)-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are less able to immobilize at the PSD, a required step for the increase in AMPAR signaling that enables potentiation. Therefore, pCaMKII is unable to phosphorylate GluR1 subunits at the serine-831(Ser831) site and potentiation is reduced. Our data suggest that NR2B-containing *N*-methyl-D-aspartate receptors (NMDARs), CaMKII, and extrasynaptic AMPAR priming (via phosphorylation of Glur1-Ser845) were unaffected by primary blast.

(Fig. 9). Phosphorylation of GluR1-Ser831 requires immobilization of GluR1-containing AMPARs to PSD-95. We also investigated the effect of blast on protein expression when LTP was chemically induced through secondary messengers, specifically cAMP. We observed that modulation of the cAMP/PKA pathway restored phosphorylation of GluR1-Ser831 and PSD-95 expression. One study previously observed that activation of the cAMP pathway with forskolin prevented NMDA-induced PSD-95 loss.[61] Our findings, in conjunction with previously reported results, warrant further investigation into the modulation of cAMP/PKA pathway as a therapeutic target for primary blast injury.

In this study, we report that the PDE4 inhibitor, roflumilast, reduced LTP-deficits after blast (Fig. 8). Although it has not been investigated in TBI, treatment with roflumilast improved cognition in hypertensive rats.[62] Another PDE4 inhibitor, rolipram, rescued FPI-induced LTP deficits in rats at 2 weeks post-injury.[63] Together, these studies suggest that the modulation of the cAMP pathway may hold therapeutic potential for TBI.

There are limitations to consider when interpreting these results. A goal of this study was to focus on the effects of primary blast injury in isolation. This approach required an *in vitro* injury model to ensure precise control over the injury biomechanics. Subsequently, it is difficult to translate the observed functional deficits from this study to macroscopic behavioral and cognitive changes. In studying the time course of primary blast-induced LTP deficits, we chose to measure LTP out to 10 days post-injury. Although we did not observe spontaneous recovery for Level 9 exposed cultures in this time frame, it is possible that LTP could recover at later time points. As previously mentioned, PSD-95 was the only target for which blast reduced expression when LTP was not induced. This finding supports our observation that blast did not affect basal function, but rather affected an LTP-specific pathway. We concluded that modulation of the cAMP/PKA pathway may act therapeutically against primary blast-induced LTP loss; however, it is possible that the multiple stimulatory components within the cAMP-aCSF solution comprise a more powerful potentiating stimulus than electrical or glycine-induced LTP. In this case, our results suggest that a supraphysiological induction (i.e., non-synaptic) of LTP is required following primary blast exposure.

Conclusion

In summary, we report that primary blast disrupted hippocampal LTP in a delayed manner (>1 h) and, depending on the severity of blast exposure, LTP spontaneously recovered by 10 days post-injury. We observed blast-induced deficits in phosphorylation or expression of proteins critical for LTP induction; namely, AMPAR-GluR1 (total and pGluR1-Ser831), PSD-95, and pStargazin-Ser239/240. Finally, we observed that modulation of the cAMP/PKA pathway using PDE4 inhibitors ameliorated blast-induced deficits in LTP and protein expression. Future studies will investigate the potential of PDE4 inhibitors to prevent the effects of primary blast injury.

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Author Disclosure Statement

No competing financial interests exist.

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