Effect of mild blast-induced TBI on dendritic architecture of the cortex and hippocampus in the mouse

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Traumatic brain injury (TBI) has been designated as a signature injury of modern military conflicts. Blast trauma, in particular, has come to make up a significant portion of the TBIs which are sustained in warzones. Though most TBIs are mild, even mild TBI can induce long term effects, including cognitive and memory deficits. In our study, we utilized a mouse model of mild blast-related TBI (bTBI) to investigate TBI-induced changes within the cortex and hippocampus. We performed rapid Golgi staining on the layer IV and V pyramidal neurons of the parietal cortex and the CA1 basilar tree of the hippocampus and quantified dendritic branching and distribution. We found decreased dendritic branching within both the cortex and hippocampus in injured mice. Within parietal cortex, this decreased branching was most evident within the middle region, while outer and inner regions resembled that of control mice. This study provides important knowledge in the study of how the shockwave associated with a blast explosion impacts different brain regions.
Data currently suggests that there are major differences which exist in the pathophysiology between blast-related and non-blast related TBI. One of the difficulties of assessing blast injuries is the different spectra of clinical manifestations that can appear due to mTBI after a direct blow to the head, blast waves, or a mix of both. One animal study even suggests that mTBI can occur as a result of blast pressure waves directed at the chest of the subject.

Several studies have investigated the effects of mechanical brain trauma on neuronal morphology. Results consistently suggest decreases in neuronal density within the cortex and hippocampus on the ipsilateral side to the injury. Interestingly, this dendritic damage extends well beyond the area of injury; relatively minimal cell death still results in significant dendritic changes. This may account for the cognitive deficits observed in both humans and animals exposed to a mild TBI. While there is significant evidence to suggest that even mild mechanical TBI induces relatively widespread dendritic changes, there have been few studies investigating these changes as a result of blast TBI.

The purpose of this study was to evaluate and characterize the effects of blast-induced mTBI on the dendritic morphology of neurons in the parietal cortex and hippocampal of an animal model (the mouse brain) at the cellular level. 95% of the volume of the neuron is in the dendritic arbor and changes in dendritic parameters are a very sensitive index of neuronal damage. Neurons were visualized using the Rapid Golgi staining method. This technique randomly stains the soma and the complete dendritic arbor of about 5% of all neurons and was the method of choice to permit visualization and quantification of the dendritic branching. The Golgi Staining technique is a powerful visualization tool in the study of neuroanatomy. Modifications of the original stain continue to be some of the best for small-scale analysis and have become a popular choice of neuroscientists examining the effects of TBI on dendritic morphology over the past six years. Golgi staining, because of its high contrast, completeness in staining and selectivity of less than ten percent of cells can be translated exceptionally with camera lucid drawings as well as light microscopy; these qualities allow images to be compiled from multiple levels of depth without muddiness from neighboring neurons. From these images, numerical characterizations can be made to determine the complexity of dendritic branching including Sholl analysis which counts the amount and length of branches and Dendritic Branching analysis which tracks the amount of branch splitting.

Previous experiments involving our model of blast injury, at the same peak over-pressure, have yielded cognitive and behavioral changes in blast exposed mice similar to that TBI in humans at both acute and chronic timepoints, such as deficits in spatial and recognition memory and increased anxiety-like behavior. One previous study also noted significant neurodegeneration within the neurons of the cortex and hippocampus at 3 days post-injury via FluoroJade B staining. The goal of our study was to identify morphological changes within the neurons of the cortex and hippocampus occurring as a result of blast exposure at this timepoint to better understand how small morphological changes occurring within the brain following injury may induce cognitive deficits and how we can identify treatments to prevent these changes from progressing.

Materials and Methods

Animal husbandry. Male ICR mice were obtained from Harlan Sprague Dawley Inc. (now Envigo, Jerusalem, Israel) weighing approximately 25–30 g were kept under a constant 12-h light/dark cycle at room temperature, with up to 5 animals per cage. Ad libitum food and water was provided. The ethics committee of the Sackler Faculty of Medicine approved the experimental protocol, in compliance with the guidelines for animal experimentation of the National Institutes of Health. For this experiment, 6 brains from bTBI mice and 4 brains from sham mice were analyzed. Animals from different experimental groups were housed in the same room, but not in the same cages. No instances of fighting prior to or following bTBI exposure was noted.

Blast trauma model. The blast-induced TBI protocol took place in the experimental site of “Tamar Explosives” and of “Sadwin Consultancy” in central Israel. Mice (both bTBI and sham controls) were anesthetized via I.P. injection with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), a combination that induces deep anesthesia and still enables spontaneous respiration. Mice were placed, with noses facing the source of the explosion device, in a restricted plastic net in a holder which is clamped to the floor with screws. This device maintained their position but allowed exposure to the blast shockwave (Fig. 1a). A gauge measuring blast levels (in PSI) and temperature was secured to each holder next to the mice. The explosion device used was a constructed cast of 500 g TNT. The animals, gauges, and the explosive charge was were elevated 1.0 m above ground level. In order to reduce confounding variables, all animals used in this experiment were exposed to one explosion, during the same trial, such that all experimental animals receive the same blast under the same conditions. Animals were positioned such that the angle and distance relative to the blast were identical. Placed at a distance of 4 m, mice were exposed to 5.5 PSI peak over-pressure during the blast, measured ‘side-on’ with Free-Field ICP® Blast Pressure Sensor “pencil gauges” (PCB Piezoelectronics, Depew, NY, USA; Model 137). Photographs of the blast apparatus and site have been published previously. Following explosion, mice receive an impact of the “direct” shockwave, as well as the reflected wave from the ground. A reflected wave from the embankment surrounding the arena can also be expected, but is likely insignificant relative to the initial direct and reflected waves. Each of these shockwaves combine to form a complex blast injury representative of what would be experienced by a human exposed to an explosive blast. The sampling rate was 61.25 kHz or 16 microseconds between readings on all channels. The pressure-time curves were recorded for each experiment.

Dendritic analysis. Dendritic analysis was performed as described in a previous study investigating dendritic changes within the amygdala of this mouse cohort following blast. Mice were perfused 72 hours post injury with phosphate buffered saline followed by 4% neutral buffered formalin. Brains were then removed and placed in 10% formalin overnight at 4 °C. The following day, brains were cryoprotected in 15% sucrose and incubated at room temperature for 24 hours. The cortical samples were then incorporated into formalin-fixed tissue.
blocks (2–3 mm thick in the coronal plane) and stained using the Rapid Golgi method. Fixed tissue blocks were first placed in potassium dichromate and osmium tetroxide for approximately 6 days, then transferred to 0.75% silver nitrate for approximately 40 hours. Increasing concentrations of alcohol solutions and ethyl ether were used to dehydrate the blocks, followed by infiltration with increasing concentrations of nitrocellulose solutions (5%, 10%, 20%, 30%; 1–2 days each). Blocks were then placed in plastic molds and hardened using chloroform vapors. Tissue sections containing the parietal cortex and underlying hippocampus within the region approximately \(-1.7\) mm to \(-2.7\) mm from Bregma (Fig. 1b) were cut to a thickness of 120 microns in the coronal plane using an AO sliding microtome, cleared in alpha-terpineol, rinsed with xylene, and mounted on slides using Permount. Strict selection criteria were used to select neurons for analysis. Neurons must have been well impregnated; branches had to be unobscured by other neurons, glia, blood vessels, or undefined precipitate (a staining by-product), and the soma must have been located within the middle third of the section (Fig. 1c). Representative images of individual Golgi stained neurons for within both the parietal cortex and hippocampus are shown in Fig. 1d. A Zeiss brightfield microscope with long-working distance oil-immersion objective leesee and drawing tubes was used to prepare camera lucida drawings for analysis.

Dendritic arbors were analyzed from both brain hemispheres using two methods: the Sholl Analysis and Branch Point Analysis (BPA), as previously described\(^{31-33}\). Sholl analysis defines the mean amount and

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**Figure 1.** Mouse model of mild blast TBI. (a) Prior to blast, anesthetized mice were placed in a net within a holder with adjacent pressure gauges at a distance of 4 m from an explosive charge containing 500g TNT. The explosion generates approximately 5.5 psi peak over-pressure at this distance. The measured peak over-pressure for this experiment was 37.9 kPa (~5.5 psi). The animals, pressure gauges, and the explosive charge was elevated 1.0 m above ground level. The rows are situated 4 meters from the TNT cast. Each row had space for 12 mice and two pressure gauges were mounted at the ends of each platform. Brain regions were analyzed via Rapid Golgi stain. (b) Coronal blocks of tissue incorporating the parietal cortex (and underlying hippocampus) were removed and neurons were Golgi impregnated using the Rapid Golgi method, shown in (c) (Mulherkar (2017) (https://www.nature.com/articles/s41598-017-11113-3) under the conditions set forth by Creative Common BY 4.0 license (http://creativecommons.org/licenses/by/4.0/)). Individual Golgi stained neurons of the parietal cortex and hippocampus are shown in (d). The parietal cortex was broken down into inner, middle, and outer regions for further analysis (e) (Mulherkar (2017) (https://www.nature.com/articles/s41598-017-11113-3) under the conditions set forth by Creative Common BY 4.0 license (http://creativecommons.org/licenses/by/4.0/)).
distribution of the dendritic arbor. Concentric circles or “shells” are superimposed over the camera lucida drawings. The radius of the first shell was 10 μm from the cell soma and subsequent shells increased by 10 μm increments out to 200 μm away from the soma. Interactions are measured by the number of times a dendrite intersects with a shell. BPA measures the complexity of the dendritic arbor by quantifying the number of dendrite branch bifurcations. A first-order branch is a branch emanating directly from the soma. The point where this branch splits into two is the first-order branch point and the resultant branches are second-order branches. This pattern continues until the branches reach an endpoint. More branch points indicate increased dendritic branching complexity.

Statistical analysis. GraphPad Prism software was used for statistical analysis. Mean values are depicted ± standard deviation and were compared using the two tailed t test. For Sholl and Branch Point Analyses, the Wilcoxon signed-rank test was used with animal n being used for analysis. In both tests p < 0.05 indicates significance.

Results

Sholl analysis. Sholl Analysis was performed to assess the dendritic distribution of the dendritic arbor at increasing distances from the soma. Within the infragranular layer IV and V pyramidal neurons of the parietal cortex, we saw a highly significant reduction in dendritic intersections per shell as a result of injury (Fig. 2a). The same pattern was observed in the hippocampus, with a significant reduction in the number of dendritic intersections per shell in bTBI mice (n = 6, 54 total neurons analyzed) compared to sham controls (n = 4, 36 total neurons analyzed) (p < 0.0001).

Dendritic length. The data from the Sholl analysis were used to approximate the total dendritic length (in microns). Within the layer IV and V pyramidal neurons, we did not see any significant changes in dendritic length with injury, 1449 ± 127.7 μm (100 ± 8.81%) in the sham mice and 1281 ± 63.83 μm (88.41 ± 4.41%) in the injured mice (Fig. 3a). However, we do see a significant reduction in dendritic length with injury in the hippocampus, 2086 ± 82.04 μm (100 ± 4.12%) in the sham mice and 1813 ± 69.65 μm (86.87 ± 4.55%) in the injured mice (Fig. 3b).

Branch point analysis. Branch Point Analysis shows the complexity of the dendritic arbor based on the number of dendritic bifurcations at each branch order. More branch points indicate more dendritic branching complexity. Branch Point Analysis of neurons within the parietal cortex indicates that there is a reduction in dendritic

Figure 2. Sholl analysis of parietal layer V and VI pyramidal neurons and hippocampal CA1 neurons. Sholl analysis was performed to evaluate the amount and distribution of the dendritic arbor following bTBI. (a) In parietal cortex, there was a significant reduction in the number of dendritic interactions per shell in bTBI mice (n = 6, 54 total neurons analyzed) when compared to sham controls (n = 4, 36 total neurons analyzed) (p = 0.0002). (b) The same pattern was observed in the hippocampus, with a significant reduction in the number of dendritic interactions per shell in bTBI mice (n = 6, 54 total neurons analyzed) compared to sham controls (n = 4, 36 total neurons analyzed) (p < 0.0001).

Figure 3. Dendritic length per neuron in cortex and hippocampus. Sholl analysis was also used to approximate the average dendritic length per neuron. (a) In cortex, bTBI mice had a slightly reduced average dendritic length per neuron, however, this was not significant (p = 0.352). (b) In hippocampus, bTBI mice had significantly reduced dendritic length compared to controls (p = 0.013).
complexity for the first 4 branch orders in bTBI mice, though this reduction is only statistically significant for the first branch order (Fig. 4a). In hippocampus, we see a significant reduction in branch points for all branch orders (Fig. 4b). Overall, we see that our injury model does induce a reduction in dendritic complexity in both the parietal cortex and hippocampus.

Analysis of parietal cortex regions. To further understand how our model of bTBI is influencing specific areas of the parietal cortex, we divided the parietal cortex into three regions, each representing a third of the parietal cortex: inner, middle, and outer (Fig. 1e). One third of the previously analyzed parietal neurons are represented in each of these regions. When we examine the layer IV and V pyramids from these individual regions using Sholl analysis, we see that there is no loss of the dendritic arbor in the inner (Fig. 5a) and outer (Fig. 5c) regions. There is, however, a highly significant reduction in the distribution of the dendritic arbor in the middle region (Fig. 5b), accounting for the reduction shown in the Sholl analysis of the entire region explained previously. Further, we also see a significant reduction in dendritic length per neuron in this middle region, 1599 ± 175.7 μm (100 ± 10.99%) in the sham mice and 1125 ± 95.19 μm (70.36 ± 5.95%) in the injured mice (Fig. 6b). This was not seen in the inner (Fig. 6a) and outer (Fig. 6c) regions. As such, we see that the middle region of the parietal cortex experiences the most damage as a result of the blast injury, despite the entire brain being exposed to the blast.

Results summary. Overall, we saw that mild bTBI resulted in reduced distribution of the dendritic arbor in both the parietal cortex and in the hippocampus. Further analysis shows that the middle region of the parietal cortex accounts for the majority of this reduction. We also saw that dendritic length was decreased in hippocampus, but not in parietal cortex. However, we do see a decrease in dendritic length when we isolate the middle region of the parietal cortex. Further, we also see a decrease in dendritic complexity in both cortex and hippocampus following mild bTBI.

Discussion
Our results indicate that mild blast-related TBI induced morphological changes within both the parietal cortex and the hippocampus of the model mice. Sholl and branch point analyses indicated that there was a significant decrease in dendritic distribution and density. This is consistent with previous rodent studies of mechanical TBI20–23,30. Our branch point analysis also indicated that there was decreased dendritic branching in parietal cortex and hippocampus. Additionally, we saw region-specific changes within the cortex. The middle section of the parietal cortex appears to make up the majority of the changes in dendritic distribution and density, while the inner and outer sections are relatively unchanged. Unlike controlled mechanical injury, our explosive blast exposes the whole brain to the shockwave. It is interesting that, despite this, damage still appears to be relatively localized to the middle region.

Interactions between the amygdala and hippocampus have been shown to be integral to the association between emotion and memory35. In our bTBI mouse model, we see a similar pattern where we have observed increases in dendritic spine density in the basolateral amygdala coupled with the decreased dendritic branching within the hippocampus observed in this study. Our results within the parietal cortex are also consistent with fMRI studies in humans who have experienced mTBI from both mechanical and blast injury, which indicate decreased activity within that region36,37. We saw decreases in dendritic length and complexity which may suggest a decrease in connectivity within the hippocampus. One previous study showed that changes in branch point morphology within the hippocampus had a direct effect on the ability of neurons to generate, propagate, and time action potentials38. These observations could also account for the decreases in spatial and recognition memory that we have observed in our mouse model previously29,29.

There do seem to be similarities and differences between blast-induced TBI compared to fluid percussion or controlled cortical impacts with respect to the effects on the dendritic arbor. At three days after blast exposure, we observed reduced dendritic arbors, specifically in the pyramidal neurons of layers IV and V of the parietal cortex.
and in the hippocampal CA1 region. This is in contrast to our previous study in the amygdala, which did not show decreased dendritic arbors or dendritic length as a result of bTBI. Winston et al. reported reductions in spine densities in several brain regions in mice at 24 hours after controlled cortical impact and the impact resulted in similar reductions even in the contralateral hemisphere. Rats that received CCI at a young age displayed, at 1 month after injury, the largest reductions (measuring dendritic length and also branch points) in dendritic arbors in the hippocampal CA1 region. Semple et al. showed (by dendritic lengths, branching, and Sholl intersections) that male mice were more sensitive to CCI induced reductions in prefrontal cortex layer III pyramidal neurons and dentate gyrus granule cells and these morphological changes were reflected in spatial memory deficits and hyperactivity in similarly treated mice in their study. In another recent blast TBI mouse model, Konan et al. observed decreased synapse density within the cortex, which mirror our results indicating a reduction in the dendritic arbor within the cortex. In a previous study using the same blast TBI mouse model, myelinated axonal damage and mitochondrial abnormalities were also observed. These morphological changes were also accompanied by deficits in neurobehavioral outcomes.

Limitations and strengths. This study provides useful information regarding the morphological changes observed within the cortex and hippocampus following blast TBI during the acute phase. However, it would be useful to characterize temporal changes in response to the injury at longer intervals. Future studies should cover an extended time course and provide increased sample sizes to further strengthen statistical significance. Additionally, the inclusion of detailed morphological analyses, including quantification and categorization of dendritic spines within the regions of interest, would further add to our understanding of post-injury phenomena. Despite these limitations, the findings of this study provide a useful basis for future study of morphological changes following blast TBI.

Conclusion
These results from our blast TBI model provide an important connection between morphological changes within the brain and associated behavioral and cognitive changes which have been previously observed. Losses in neuronal connectivity can be a major contributor to the symptoms observed after injury. In previous studies using our model, we have observed increases in motor dysfunction and anxiety-like behavior, as well as deficits in...
spatial and recognition memory in blast-injured mice \textsuperscript{27,28,46}. We have seen decreased complexity of the dendritic arbor in parietal cortex and hippocampus which suggests a morphological change which could account for the cognitive and memory deficits observed previously, as well as in blast-injured humans \textsuperscript{29-30}.

**Data availability**

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
B.S. and V.R. designed and performed the experiments. B.A.C., S.S., and C.G.P. supervised and designed the study. R.F.M. performed the Golgi staining and analysis. W.A.R. analyzed the data and wrote the manuscript.

Competing interests
The authors declare no competing interests.

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