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Engineering advanced neural tissue constructs to mitigate acute cerebral inflammation after brain transplantation in rats

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Abstract

Stroke, traumatic brain injuries, and other similar conditions often lead to significant loss of functional brain tissue and associated disruption of neuronal signaling. A common strategy for replacing lost neurons is the injection of dissociated neural stem cells or differentiated neurons. However, this method is unlikely to be suitable for replacing large brain cavities, and the resulting distribution of neurons may lack the necessary architecture to support appropriate brain function. Engineered neural tissues may be a viable alternative. Cell death is a prominent concern in neuronal grafting studies, a problem that could be magnified with the transplantation of engineered neural tissues. Here, we examined the effect of one contributor to cell death, acute cerebral inflammation, on neuronal survival after the transplantation of bioengineered constructs based on silk scaffolds. We found evidence of a high degree of inflammation and poor neuronal survival after introducing engineered constructs into the motor cortex of rats. Integrating a corticosteroid (methylprednisolone) into the constructs resulted in significantly improved neuron survival during the acute phase of inflammation. The improved construct survival was associated with decreased markers of inflammation and an anti-inflammatory state of the immune system due to the steroid treatment.

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Author contributions

The original idea for the study was proposed by H.I.C., V.L., D.J., H.I.C., D.L.K. and M.W. then designed the study, and planned experimental approach. V.L. conducted in vitro studies with the help of A.N.B., D.J.B., and H.H.M. M.W. assisted with injury data interpretation. D.J. conducted in vivo studies in assistance with B.J.G. and V.L. V.L. and D.J. performed data analysis. H.I.C. and D.L.K. supervised the work. V.L., D.J., H.I.C. and D.L.K. prepared the manuscript.

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Conflicts of interests

The authors declare no competing financial interests.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of ongoing study.

Keywords

Tissue Engineering; brain tissue; brain damage; transplantation; neuroinflammation; cortical tissue

1. Introduction

Brain damage from traumatic brain injury, stroke, and other neurological disorders is a devastating condition that can lead to massive neuron extinction and encephalomalacia, resulting in functional impairment, long-term disabilities, and a significant decrease in quality of life [1, 2]. A primary reason for these sequelae is the limited ability of the brain to repair itself after injuries [3]. Currently, there are no therapies that effectively restore brain architecture and neurological function after injury. The majority of available treatments focus on mitigating symptoms, but they do not address the underlying cause of these symptoms [4]. Cellular injections of dissociated stem cells or differentiated neurons have been pursued [5] as a potential treatment for various neurological conditions. These studies showed neuronal survival [6] and integration of injected cells into native brain circuits [7]. However, there remain significant limitations to neuron transplantation therapy, such as identifying suitable cell sources for clinical translation, generating construct architecture that is capable of adopting sophisticated cerebral network function, and developing strategies for replacing large brain defects. Recent advances in tissue engineering allow the fabrication of brain-like tissue constructs *in vitro* to recapitulate the complexity of native brain architecture and offer new options for transplantation of engineered neural tissue to fill large brain defects [8–11]. Several studies showed the successful transplantation of neural stem cells using microparticles [12], synthetic or [13, 14] natural biomaterials [15]. Regardless of the approach utilized, neuroinflammation is a major limitation after cell, biomaterial, or tissue graft transplantation [6, 16]. Hyperacute, acute, and chronic neuroinflammation is initiated and guided by the immune cells of the brain, including microglia and macrophages [17–21]. Neuroinflammation is a critical defense mechanism in the mammalian brain, but it also plays an irreplaceable role in wound healing and tissue regeneration [22]. Inflammatory response is initiated and guided by microglia and peripheral macrophages (in the case of blood barrier disruption). The original concept of immune cell activity included polarization into two phenotypes based on inflammatory signature and function: M1 pro-inflammatory prevalently expressed nitric oxide species (iNOS), tumor necrosis factor alpha (TNF α), interleukin 1beta (IL-1 β), cluster of differentiation 86 (CD86), and M2 anti-inflammatory state with highly expressed arginase 1 (Arg1), cluster of differentiation 163 (CD163), interleukin-4 and 10 (IL-4, IL-10). Later M2 polarized microglia were subdivided into three more groups: M2a stimulates Th2 immune response in the presence of parasites; M2b resembles the M1 polarized state on the molecular and cellular levels and regulates the immune response; and M2c regulates tissue repair and matrix remodeling [23]. Recent studies showed multiple subtypes of immune cells polarization in various disease states, based on the inflammatory profile they secrete [24–26]. Thus, a better understanding of neuroinflammation and the refined design of grafts is required to modulate the inflammatory responses to optimize graft survival and integration.

Therefore, brain grafts should mimic native brain architecture and be composed of inert or low immunogenic materials, neural progenitors or mature neurons, and factors that can mitigate acute inflammation to improve graft survival. To address this need, the corticosteroid methylprednisolone (MP) was integrated in a 3D engineered brain tissue construct [11] to modulate the acute inflammatory cascades after transplantation *in vivo*. These MP-enriched constructs showed improved survival during the acute phase of inflammation, decreased pro-inflammatory responses through downregulation of surface marker cluster of differentiation 86 (CD86), cytokines C-C Motif Chemokine Ligand 2 (CCL2), interleukin 1beta (IL-1 β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF α), and their upstream regulator signal transducers and activators of transcription 1 (STAT1), and (3) upregulated expression of anti-inflammatory markers, such as surface marker cluster of differentiation 163 (CD163), cytokines interleukin 4, 10 (IL-4 and IL-10), and transcription factors STAT3 and Neurogenic Locus Notch Homolog Protein 1 (Notch1) [27–29].

2. Results

2.1. 3D brain-like tissue constructs do not survive the acute inflammatory response of the host tissue

In brief, 3D engineered brain tissue constructs were prepared 3 days prior to transplantation by seeding embryonic rat cortical neurons into silk fibroin scaffolds, followed by embedding into a hydrogel (detailed in Methods) [11]. In order to determine if the brain-like tissue constructs were suitable for transplantation into rats, a preliminary motor cortex transplantation study was performed. Three experimental groups were evaluated to understand the brain's response to the transplants: no injury (i.e., constructs placed on top of the uninjured brain after a craniotomy), acute transplantation (i.e., constructs transplanted immediately after an aspiration injury was made in the cortex), and delayed transplantation (i.e., constructs transplanted 7 days after cortical aspiration injury was performed) (Fig. 1a–d). All constructs contained green fluorescent protein (GFP)-labeled neurons to distinguish the implanted cells from host cells. Three days after transplantation, the brains were harvested and analyzed for neuronal survival and inflammatory responses. Only the no injury group showed significant neuronal survival (Fig. 1e). Further immunohistochemical analysis of immune cell morphology evaluated with CD11b suggested the activation of immune cells in the injured groups compared to the ramified morphology of microglia in the uninjured group (Fig. 1f). The presence of induced nitric oxide synthase (iNOS) and cluster of differentiation 68 (CD68) positive immune cells in acute and delayed transplants provided further evidence of inflammatory response in the aspiration lesion groups (Fig. 1g). Based on these preliminary studies, the goal of subsequent studies was to modulate the acute inflammatory response to support improved survival of the constructs. Thus, the anti-inflammatory drug, methylprednisolone, commonly used in clinic to treat inflammatory challenges, was incorporated into subsequent iterations of the engineered brain tissue construct.

2.2. Methylprednisolone has no negative effects on neuronal viability and delays their maturation in the engineered cortical tissue constructs *in vitro*

Two different hydrogels, fibrin and collagen, were studied for methylprednisolone release when loaded at various concentrations with the goal of finding a biomaterial with a burst release of the drug in the first 2–3 days of acute inflammation [6], and prolonged smaller doses released over two weeks to support engineered brain tissue construct integration post-transplantation *in vivo*, but not suppress immune cells from expressing anti-inflammatory cytokines and clearing debris [31]. MP was incorporated by mixing a range of MP concentrations, from 0 to 8.7 mM with 250 U/ml thrombin or water prior to the addition of 25 mg/ml fibrinogen or 10mg/ml collagen accordingly (Table S1), followed by embedding the brain tissue constructs with premixed hydrogels. Media with the drug was collected every other day for quantification. A reverse-phase high performance liquid chromatography (HPLC) method was employed to separate methylprednisolone from media components for quantitation (Fig. 2a–d, Fig. S1) (detailed in Methods). Fibrin groups with the highest methylprednisolone concentration (6 mM and 8.7 mM) demonstrated a 1.4- and 1.8-fold higher release of MP, respectively, in the first 48 hours compared to the collagen group with the same amounts of loaded drug (Fig. 2a, b). This consisted of 50–60% (215 µg) of the total amount of drug loaded, followed by a slower release (20% more) out to day 8. Groups with lower drug concentrations (1.5 and 3 mM) showed a similar release from both hydrogels during the first week. From day 10 on, the release for all conditions was in the nanogram range, with two-fold higher release of MP in collagen groups compared to the fibrin groups (data not shown). For the purposes of this study, fibrin was selected as optimal hydrogel, due to its high MP release in the first 48 hours, and controlled release of smaller doses over extended period of time.

To determine if higher doses of methylprednisolone negatively affected neuronal viability, Live/Dead assay was used to count dead cells, and analyze the neurites outgrowth (Fig. 2e, f, Fig. S3); DNA quantification to quantify total cell number (Fig. 2g), Lactate dehydrogenase (LDH) assay to evaluate death markers release (Fig. S2a), and Reactive oxygen and nitrogen species (ROS/RNS) assay to assess hypoxia (Fig. S2b). Calcein stain (Fig. 2e) showed extensive neuronal network formation in the control (0 MP) and all drug-loaded groups at later time points (7 and 14 days), however, at early time points in the 6 and 8.7 MP groups, shorter neurite outgrowth was observed. This finding suggested delayed neuronal maturation compared to the control group, which consisted of mature neurons, based on the neuronal network formation (Calcein staining), spontaneous activity (LFP), and expression of progenitor markers (Pax6), and mature neurons markers (PSD95 and Gephyrin positive) (Fig S4). MP treated neurons showed higher levels of neuronal progenitor marker Paired box 6 (PAX6) expression in 2D and 3D cultures and, higher membrane depolarization and responsiveness after GABA agonist treatment, typical for premature neurons (Fig. S4) [32].

There was no statistically significant difference in dead cell counts at the first three time points. At later time points, a general increase in the number of dead cells was observed in all but the 8.7 MP groups (Fig. 2f, Fig S3). These results were confirmed with LDH release, where the overall increase of LDH was detected at day 14 in all groups except for the 8.7 MP (Fig. S2). The 8.7MP group showed an increase of LDH release only at day 3. The total

amount of DNA showed a general decline in cell numbers at later time points (Fig. 2g), as observed in our original studies [11]. Lastly, the ROS/RNS assay suggested a general increase in ROS/RNS species in the engineered brain tissue constructs, however, no significant differences were observed between different experimental groups (Fig. S2). The data demonstrated that the high doses of MP prevented neuronal death at later time points and delayed neuronal maturation. These findings supported the benefit of MP treatment for transplantation studies due to the higher survival, and plasticity of premature vs mature neurons [33].

2.3. Methylprednisolone decreases the inflammatory response of immune cells and triggers expression of anti-inflammatory markers

In order to understand the effect of MP on immune cells (primary microglia and macrophages) activity and behavior in various polarization states (Fig. S5), experiments were pursued in *in vitro* 2D conditions to establish a controlled baseline for comparison with further *in vivo* experiments. Immune cells were polarized towards the M1 pro-inflammatory state with interferon-gamma (INF-gamma) and lipopolysaccharide (LPS) treatment, and the M2 anti-inflammatory activation was achieved with interleukin-4 treatment (IL-4) (Fig. S5). As control M0 or non-activated immune cells were analyzed in all experiments.

Methylprednisolone did not affect immune cell proliferation or cell death, based on DNA quantification (Fig. 3a, Fig. S6a). A significant upregulation of extracellular adenosine triphosphate (ATP) was observed in the M1 polarized microglia treated with 8.7 mM MP (Fig. S7). Upregulated ATP level in the extracellular environment is a hallmark of immune cell polarization towards pro-inflammatory state, followed by increased secretion of pro-inflammatory cytokines such as IL-1 β , TNF α and CCL2 [34]. We observed an increased release of ATP from only M1 polarized microglia treated with MP, however, the level of pro-inflammatory cytokines was down-regulated in M1-MP treated groups (Fig. 4a, b), suggesting that MP acts downstream of ATP release in the extracellular environment, potentially through the purinergic receptors [27]. Furthermore, we observed an elevated level of intracellular ATP in M2 microglia and macrophages (Fig. S7b) that was shown in previous work to be a trigger for activation of inhibitor of nuclear factor kappa B kinase subunit beta (IKK β) [34], followed by canonical NF- κ B activation and translocation to the nuclei, where it triggers the expression of pro-inflammatory cytokines. However, in all M2-MP groups with upregulated NF- κ B expression the level of pro-inflammatory cytokines was downregulated (Fig. 4a, b), suggesting MP blocks either the translocation of NF- κ B complex to the nucleus, or the transcription of pro-inflammatory cytokines [35], which was supported by the mRNA level for the CCL2 cytokine (Fig 4b).

Immunocytochemistry results showed significant down-regulation of the pro-inflammatory marker iNOS [28, 29] in M1 polarized microglia after MP treatment (Fig 3, also see Fig. S6). The expression of mannose receptor (MR), an anti-inflammatory marker, was similar in the M2 groups and M0 in microglia and macrophages, however, this marker was significantly upregulated in the M2-MP treated microglia and macrophage groups, whereas the M0 group showed a significant downregulation of MR after MP treatment (Fig.3 b and c, also see Fig. S6 b and c). MR expression remained unchanged in M1 polarized cells after

treatment with MP (Fig. 3d and Fig. S6d). Gene expression analysis of mRNA supported the above data and showed significant upregulation of CD163 (M2 surface marker) in the M2 treated groups (Fig. 3d and Fig. S6d). In contrast, no differences were observed in the M1 polarized cells (Fig. 3d and Fig. S6d). A general decrease in CD86, a pro-inflammatory marker, was observed in the M1 polarized cells, however upregulation of CD86 in the MP-treated M2 microglia and M0 cells was also observed (Fig. 3d and Fig. S6d). Further analysis showed MP converted the M0 macrophages towards the M2b polarization state, with upregulated CD86, interleukin (IL-4) interleukin-6 (IL-6) and interleukin-10 (IL-10) (Fig. 4b) [32, 33]. M2 microglia treated with MP showed downregulation of IL-4, IL-6 and IL-10 expression compared to M2-noMP, however the level of these markers was still significantly higher than in the M0-MP group, what also points to the M2b polarization state of these cells [33]. M0-MP microglia showed that most of the pro and anti-inflammatory markers were downregulated, with only CD86 (mRNA) (Fig.3d) upregulated. This implies initiation of the transition to one of the activation states. Further analysis of cytokines panel for both microglia and macrophages showed significant downregulation of many pro-inflammatory markers in most but not all MP treated groups (both microglia and macrophages), in particular C-C Motif Chemokine Ligand 2 (CCL2), tissue inhibitor of matrix metalloproteinases (TIMP1) vascular endothelial growth factor-A (VEGF-A), granulocyte- macrophage colony-stimulating factor (GM-CSF), interleukin-1 alpha (IL-1 α), interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF α) (Fig. 4a and b). The MP treatment triggered significant upregulation of anti-inflammatory markers (IL-4, IL-10, IL-6) in M0 and M2 macrophages treated with MP. The M1 group showed a slight down-regulation. Microglia expression of anti-inflammatory markers was stable, or slightly downregulated, after treatment with MP, and the level was significantly higher than in the control M0 group (Fig. 4a and b). Expression of several markers showed no similarities between the different groups with or without MP, such as cytokine-induced neutrophil chemoattractant 2 and 3 (CINC2–3), and C-X-C motif chemokine 5 (LIX). Additional studies will be needed to fully understand some of these differences (Fig. 4a and b).

To better understand the potential molecular pathways activated/deactivated after MP treatment, the expression of several upstream markers of CCL2 and TIMP1 chemokines were quantified through RT-qPCR. Activation of signal transducers and activators of transcription 1 and 3 (STAT1 and STAT3), known upstream regulators for CCL2 and TIMP1 expression, were analyzed [36]. STAT1 is a transcription factor activated in response to pro-inflammatory molecules like interferon-gamma (IFN γ) and LPS and leads to upregulation of CCL2 and TIMP1 expression. We observed the downregulation of STAT1 expression in all groups, except for upregulation in the M2 macrophages treated with MP (Fig. 4a and b). The data corresponded to the results from the cytokine panel and mRNA expression levels, where CCL2 and TIMP1 were significantly downregulated in all groups. STAT3 is another transcription factor activated upon contact with inflammatory molecules, and it triggers activation of anti-inflammatory cytokines and chemokines [36]. Significant upregulation of STAT3 expression was observed in most groups treated with MP, except for the M1-MP microglia where expression did not change. TIMP1 expression downregulates matrix metalloproteinases (MMPs) production. In order to validate this, we tested the expression of two MMPs (2 and 9) and observed increased expression of MMP2 in most groups (microglia

and macrophages) compared to the control M0 group. The M2-noMP group showed no significant differences from the control, however, MMP2 expression was significantly upregulated after MP treatment (Fig. 4a, b, also see Fig. S6d). Interestingly, MMP9 expression in all microglia groups was higher than in the control M0 group, whereas in macrophages the expression in all groups was lower than in the control M0. This MMPs expression emphasizes the difference in function of these cells, and the critical contribution of microglia in the brain in terms of remodeling (Fig. 4a, b, also see Fig. S6d). NF- κ B is a key regulator of most processes in the cell, including survival, proliferation, differentiation and inflammation. NF- κ B triggers acute inflammation through upregulation of various pro-inflammatory cytokines, such as IL-1 β , TNF α and others. Downregulation of NF- κ B p50 expression was observed after treatment with MP in most experimental groups together with downregulated pro-inflammatory cytokine production and gene expression. However, in the M2 treated microglia and macrophages, NF- κ B was significantly upregulated. A potential mechanism is that the alternative activation of the canonical NF- κ B can occur through the upregulation of the upstream activator beta-NGF (Fig. 4a, b), which leads to cell survival and differentiation [36]. Co-activation of NF- κ B and STAT3 was shown in literature to be crucial for the proliferation and prevention of differentiation in hematopoietic progenitors (microglia/macrophages precursors), which can also explain the observed upregulation of NF- κ B [19].

2.4. Methylprednisolone transplants survive the acute inflammation state post-transplantation

Having characterized the effects of MP on both neurons in the silk sponges and inflammatory cells, we evaluated our next-generation constructs (loaded with MP) after transplantation. To test if the MP loaded constructs can survive during the acute inflammation phase, a set of implantations in the rat M1 motor cortex area was performed using the MP-loaded silk constructs. A significant increase in the survival of MP constructs was observed in all animals compared to the non-MP constructs (Fig. 5a-e). Further analysis showed a general increase of cluster of differentiation 11b (CD11b) positive cells in the non-MP transplants near the injury sites, but not in the transplant itself (Fig. 5f-j). The immunostaining data were supported with mRNA gene expression analysis of CD11b, CD86 and CD163 markers. Expression of pan marker CD11b was significantly higher in both non-MP, MP transplants, and in the contralateral hemisphere of MP transplants (Fig. 5k). The pro-inflammatory surface marker CD86 was significantly upregulated in the contralateral hemisphere of the non-MP transplants. In contrast, both hemispheres of the animals that received the MP-transplants had upregulated levels of anti-inflammatory CD163 expression and downregulated CD86 (Fig. 5j, k).

2.5. MP transplants survive due to a decrease in inflammation and a shift to anti-inflammatory marker expression

In this study inflammatory response of native brain tissue against transplants was evaluated. The immune response was studied in both hemispheres, the one that received a transplant and the contralateral tissue, to evaluate the progression of inflammation. The transplants itself and 2 mm of the surrounding brain area (for contralateral hemisphere only brain tissue of equal volume) was collected for molecular analysis through cytokines antibody array and

mRNA gene expression. The effect of methylprednisolone on immune response after transplantation is summarized in the schematic (Fig. 6c). A significant change in cytokine production was observed in both MP and non-MP transplants compared to the uninjured brain (Fig. 6a, b), based on the results of the cytokine antibody array. Non-MP transplants had significantly upregulated levels of CCL2, IL-1 α , IL-1 β , IFN γ , TNF α , GM-CSF and TIMP-1 among the pro-inflammatory markers, and beta-NGF, IL-4 and IL-10 among anti-inflammatory markers. Moreover, fractalkine (CX3CL1) expression was significantly downregulated in both non-MP and MP groups relative to uninjured brain. Even with downregulated fractalkine expression in MP transplants, its level was still higher than in the non-MP group, especially in the contralateral hemisphere. Lastly, leptin production was significantly downregulated in MP transplants compared to non-MP transplants as well as uninjured brain.

Further, to support proteomics analysis and molecular pathways involvement, targeted gene expression was studied. mRNA levels of CCL2 and TIMP1 were significantly downregulated in MP transplants, together with their upstream regulator STAT1 [36]. Upregulation of STAT3 transcription factor was observed in both non-MP and MP transplants, responsible for the activation of anti-inflammatory markers, which corresponds to upregulated levels of IL-4, IL-10, beta-NGF in MP and non-MP transplants compared to the uninjured brain (Fig. 6a, b). However, despite having TIMP1 downregulated in MP transplants, there was no detected upregulation of metalloproteinases MMP2 and 9, suggesting the involvement of other proteases in ECM remodeling. Finally, significantly upregulated expression of p50 NF- κ B subunit was observed in MP transplants. Upregulation of NF- κ B and downregulation of pro-inflammatory cytokines points towards NF- κ B activation with non-inflammatory function, such as angiogenesis which corresponds with the observed upregulation of VEGF-A and IL-6 markers. Another potential function of activated NF- κ B together with Notch1 and STAT3 upregulation is control of astrogliosis and radial glia recruitment to the site of injury [27, 28, 37–40]. The presence of Nestin positive cells at the site of injury supports the molecular data (Fig. 6) and points towards either neurogenesis or astrogliosis (Fig. S4c) [39, 40].

Discussion

The present study demonstrates successful transplantation of 3D bioengineered tissue constructs into rat brains as potential treatment for large brain defects. Here, the volume of constructs used was roughly 6.3mm³, representing approximately one percent of the total cortical brain volume in the rat. Moreover, modulation of the acute inflammatory response after graft transplantation using the engineered brain tissue constructs was shown to augment neuronal survival.

We pursued bioengineered 3D cortical tissue transplants that could mitigate acute inflammation after transplantation through the introduction of methylprednisolone (MP). MP is a corticosteroid that is FDA-approved and used in the clinic to treat various inflammatory challenges, including multiple sclerosis, lupus and other autoimmune diseases [41–44]. Clinical application of MP used at high concentrations, from 5.4 mg/kg to 1 g/kg, has shown significant improvement in terms of patient outcomes with various inflammatory

challenges [41–44]. Moreover, previous studies have shown that even low concentrations of steroids from 1nM to 1mM significantly decreased tissue inflammation [45, 46]. We used the highest possible concentration of MP (8.7mM) that can be loaded into the hydrogel to achieve a burst release (300µM) during the acute stage of inflammation, and with a prolonged release of the drug during chronic inflammation. Next, we showed that high doses of MP do not adversely affect neuronal survival *in vitro*; on the contrary, we observed delayed neuronal maturation in the MP group compared to non-MP group, which could be beneficial for transplantation purposes, since premature neurons are more plastic and are expected to better withstand inflammatory stresses.

Thorough analysis of immune cells activation has been shown in multiple publications, including the molecular pathways that regulate the expression of pro- or anti-inflammatory markers [17, 19, 20] and including basic analysis of the effect of steroids on immune cell polarization and activity. However, there is still a lack of understanding of the molecular pathways that lead to decreased inflammation in the brain after treatment with steroids, methylprednisolone in particular. Thus, after demonstrating that high doses of MP do not have a negative impact on neuronal viability and functionality, we analyzed the effect of MP on the expression of multiple pro- and anti-inflammatory markers and on related molecular mechanisms that guide the decrease in inflammation. We showed that MP acts downstream of NF-κB p50 subunit activation, either preventing the NF-κB complex translocation to the nucleus or within the nucleus blocking the transcription of inflammatory cytokines. These data provide a better understanding of the effect of steroids on neuroinflammation and may help inform future drug design and broaden the application of steroids for treatment of other inflammatory challenges.

Another novel effect of MP was observed on the expression of TIMP1-MMP complex, critical matrix remodeling proteins. Downregulated TIMP1 allows MMPs to perform ECM degradation and matrix remodeling: this may be an important aspect in future studies of brain neuroinflammation and potentially a target during transplantation to improve survival and prevent scar formation.

We achieved survival of 3D bioengineered tissue constructs in rat M1 motor cortex during the acute phase of inflammation, which was shown to be a critical step for transplant survival and further integration in neural stem cell transplantation studies [6, 47]. High survival rate correlates with decreased levels of pro-inflammatory cytokines and a shift of immune cells from an M1 pro-inflammatory state towards an M2 anti-inflammatory state. Moreover, we showed the importance of the NF-κB pathway and ECM remodeling in transplant survival. Downregulation of TIMP1 in MP transplants supporting the *in vitro* results and the importance of matrix remodeling for successful constructs survival and integration. Finally, we observed the co-activation of Notch1, STAT3, and NF-κB pathways in MP transplants, with downregulated cytokines, which points towards the presence of reactive astrocytes or potentially radial glia or neural stem cells recruitment and proliferation at the site of injury. Significant downregulation of leptin protein indicates the importance of communication between the brain and the gut even for transplant survival and integration; this is a potential target for future research [48].

In summary, MP loaded transplants survived the acute stage of inflammation through downregulation of upstream targets for most inflammatory cytokine and chemokine regulators. We also achieved a better understanding of the effect of steroids on inflammation, which can be used in future work to develop more targeted therapies for brain inflammatory challenges.

3. Materials and Methods

Poly-L-lysine (PLL), 6 α -Methylprednisolone 21-hemisuccinate sodium salt (MP), Lactate Dehydrogenase kit (MAK066), Propidium iodide, Bovine Serum Albumin, TritonX were purchased from Sigma-Aldrich (St Louis, MO, USA). B-27 Supplement (50X), penicillin/streptomycin, Glutamax, Trypsin-EDTA (0.25%), Defined Trypsin Inhibitor, Dulbecco's Modified Eagle Medium (DMEM), NeuroBasal medium, CalceinAM, Paraformaldehyde, Goat serum; 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI); Dulbesco's Phosphate-Buffered Saline (DPBS), Hank's Balanced Salt solution (HBSS) were obtained from ThermoFisher Scientific (Grand Island, NY, USA). DNase was provided by Roche Applied Sciences (Indianapolis, IN, USA). Salmon fibrinogen and Salmon thrombin were purchased from Sea Run Holdings (Freeport, MN, USA).

3.1. Silk scaffold preparation

Liquid silk and salt-leached scaffolds were prepared from *Bombyx mori* cocoons following our published protocols [49]. Scaffolds were biopsy punched (McMaster-Carr, Princeton, NJ, USA) for *in vitro* experiments with 6 mm diameter, for in *in vivo* with 2mm diameter, and 1–2 mm height. Prior to cell seeding scaffolds were autoclaved and coated with poly-L-lysine (10 μ g/mL; Sigma) overnight in a CO₂ incubator. The next day they were extensively washed with PBS (ThermoFisher) and equilibrated for one hour in NeuroBasal medium (ThermoFisher) supplemented with 2% B27 (ThermoFisher), penicillin/streptomycin (100U/mL and 100 μ g/mL) (ThermoFisher) and 2mM Glutamax (ThermoFisher). Concentrated cell suspensions (10 million cells/mL) were added to slightly dehydrated scaffolds and incubated for 24 hours, washed with PBS and prepared for hydrogel embedding the next day. Scaffolds were embedded in fibrin hydrogels loaded with/without drug concentrations for 30 min, before immersion in media.

3.2. Cell cultures

3.2.1. Macrophages—Rat bone marrow macrophages were purchased from ScienCell Research Laboratories (Calrsbad, CA, USA). Cells were handled according to the manufacturers' protocol. In brief, 20,000 cells/cm² macrophages were seeded on 0.1 mg/ml Poly-L-lysine (PLL) coated plates with rat macrophage media (ScienCell Research Laboratories). The cells were used the next day for the specific experiments.

3.2.2. Microglia isolation from neonatal brain tissue—A neonatal brain tissue isolation protocol was approved by the Tufts University Institutional Animal Care and Use Committee and complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee M2015–151). Microglia isolation was performed following established protocols [50]. In brief, P2

(postnatal) Sprague-Dawley rats (Charles River) were utilized, all brains were removed, cleared from meninges and collected in cold HBSS (ThermoFisher). Collected brains were dissociated with trypsin (0.25%; Thermo Fisher) mixed with fresh DNase (0.3 mg/ml, Roche Applied Sciences), followed by incubation with trypsin inhibitors. Before centrifugation, cell suspension was gently vortexed, and clumps were removed with 40µm filters. After centrifugation cells were resuspended in glial media (DMEM basal medium (ThermoFisher) supplemented with 10% fetal bovine serum, and penicillin/streptomycin (100U/mL and 100 ug/mL) and plated in T75 flasks for 2–3 weeks, until microglia reached a high number. Microglia were isolated by shaking for 1 hour at 100 rounds/minute on a shaker (TYZDIII Orbital shaker, Jiangsu Tenlin Instrument CO, LTD). Media with microglia in suspension was collected and spun down, counted and plated according to the experimental design on 0.1 mg/ml poly-L-lysine pre-coat plates.

3.2.3 Cortical neuron isolation—A fetal brain tissue isolation protocol was approved by the Tufts University Institutional Animal Care and Use Committee and complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee M2015–28), and by the University of Pennsylvania Institutional Animal Care and Use Committee and complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institutional Animal Care and Use Committee 805600).

Cortices were isolated from embryonic day 18 (E18) Sprague-Dawley rats (Taconic). Cortices were dissociated using a mix of trypsin (0.25%; ThermoFisher) and DNase solution (0.2%; Roche Applied Sciences) and followed with incubation with trypsin inhibitors (ThermoFisher). Cells were collected in pellets by centrifugation, resuspended in fresh neural medium and counted.

3.3. 3D engineered brain tissue constructs preparation with methylprednisolone loaded into the hydrogel

Following our published protocol [11], 100µl of cortical neurons were seeded in silk sponges (10mln cells/mL), and incubated at 37°C overnight. The following day, the sponges were embedded in a fibrin or collagen hydrogel mixed with methylprednisolone. In the original work [11] collagen gel was used to support the formation of axon connections, in this study besides collagen, salmon fibrinogen and thrombin was tested as well, as it was shown to have a better support for neuronal growth and functional network formation [51]. Methylprednisolone (Sigma) was mixed with thrombin or PBS to achieve a final concentration of MP in a range from 0 to 8.7mM (Table S1) and afterwards combined with fibrinogen or collagen accordingly. The upper limit for loading methylprednisolone into the fibrin or collagen hydrogel was determined to be 8.7mM, based on the solubility limit of MP in water (10mM), and the drug loading capacity of thrombin and collagen. After all components were combined, 100µl of this solution was added to the seeded sponges and incubated at 37°C for 30 minutes to promote gelation. After samples were transferred to a 24 well plates containing 1ml NB media per well for MP release measurements. Sampling for release of the drug included complete media replacement for more precise quantification and was carried out over the first 24 hours and up to 2 weeks. To study the viability with lactate

dehydrogenase (LDH, Sigma) assay the media was collected at Days 1, 3, 7 and 14, and samples were stained for Live and Dead imaging.

3.4. Preparation of fibrin gel loaded with methylprednisolone

Fibrin hydrogels were prepared to a final concentration of 3 mg/ml fibrinogen (Sea Run Holdings) and 1.5 U/ml thrombin (Sea Run Holdings), using water and/or 10 mM methylprednisolone (Sigma) in sterile water (ThermoFisher) as diluent. All steps were carried out on ice. At first, 6 µl of thrombin stock (250U/mL) was combined with variety of water volumes (0, 0.27, 0.57, 0.72, 0.87 mL) and methylprednisolone (0.87, 0.6, 0.3, 0.15, 0 mL) to achieve hydrogels with MP final concentrations of 8.7, 6, 3, 1.5, and 0 mM, respectively. Finally, fibrinogen was added to pre-mixed solutions of thrombin with the drug and immediately pipetted on top of the cell seeded scaffolds. Samples were incubated for 30 minutes at 37°C to allow for fibrin gelation, and afterwards were immersed in media.

3.5. Preparation of collagen gel loaded with methylprednisolone

Collagen solution was prepared according to Chwalek et al. [52], with the addition of 10 mM MP to achieve collagen hydrogels containing methylprednisolone in the range of 0–8.7 mM. Briefly, 10x PBS, sterile water, MP solution and NaOH were combined in a 15 ml falcon tube on ice, followed by collagen type 1, and the components mixed carefully several times with a pipette, avoiding air bubble formation. A brain tissue construct was transferred with sterile forceps to a fresh, dry well of a 96-well plate, and 100 µl of collagen solution was then added above the center of the sponge, avoiding air bubble formation. Sponges were then incubated at 37 C for 30 minutes to induce gelation. To increase the available volume for drug addition it is recommended that a concentrated collagen stock be used (i.e. 10 mg/ml).

3.6. Drug release measurements with high performance liquid chromatography (HPLC)

A reverse-phase HPLC method was employed to separate methylprednisolone from media components for quantitation. Methylprednisolone was quantified via an Agilent 1260 HPLC using an Agilent Zorbax Eclipse Plus C18 reverse phase column (4.6 × 75 mm, 3.5 µm; Agilent Technologies, Santa Clara, CA, USA); flow rate of 1 ml/minute, running a gradient of 95% (water + 0.1% TFA)/ 5% acetonitrile toward 5% (water + 0.1% TFA) / 95% ACN from 2 to 6 minutes, followed by a 2 minute hold and subsequent re-equilibration. A standard curve of methylprednisolone in neurobasal medium was produced over a concentration range of 500–0.39 µg/ml, with an R squared value of 0.999, by integrating the area under the curve for the peak corresponding to MP (retention time = 5.9–6.2 minutes).

3.7. Cell viability assays

Cell viability was evaluated with calcein/propidium iodide staining (Live/Dead; ThermoFisher, Sigma) and LDH assay. In brief, samples were washed in PBS and after that incubated for 30 min at 37°C in the solution of calcein and propidium iodide (PI) (2 µm calcein and 25 µm PI in PBS). After incubation samples were extensively washed with PBS, and then imaged with Leica SP8 CARS (Leica Microsystems) immediately. LDH release was measured and quantified following the manufacturers protocol (Sigma, MAK066). In

brief, media was collected from drug-loaded samples according to the experimental design and analyzed for LDH activity. Experimental values were compared to standard curves.

3.8. Cell proliferation

Cell number was determined with PicoGreen assay following the manufacturers' protocol. In brief, samples were treated with 0.05% TritonX to permeabilize cell membranes, after which samples were diluted twice with 1xTE buffer provided with a DNA quantification kit, and incubated with 1xPicoGreen solution. The fluorescent intensity of DNA-PicoGreen complexes was detected in 96-well black plates using a plate reader (485 nm excitation, 538 emission). To correlate fluorescent intensity with DNA concentration, a calibration curve was generated using DNA standards.

3.9. ROS/RNS evaluation

Release of reactive oxygen and nitrogen species was measured with OxiSelect™ in vitro ROS/RNS assay kit (STA-347, Cell Biolabs, INC.) following the provided instructions. Cell supernatant was collected according to the experimental design and treated with ROS/RNS catalyst, and fluorescent signal was quantified after incubation with dichlorodihydrofluorescein (DCFH) using a plate reader at 480 excitation/530 emission. Collected data were normalized against DCF standards.

3.10. Immunocytochemistry/histochemistry

Samples were fixed with 4% paraformaldehyde (PFA) (Thermo Fisher) for 30 minutes, washed and permeabilized with 0.1% TritonX supplemented with 10% donkey serum (Sigma), and 4% BSA (Sigma) for 1 hour. Primary antibody incubation for 2D cultures was for 1 hour at room temperature; for 3D cultures overnight at 4°C. Samples were washed three times with PBS with gentle shaking, and afterwards incubated with secondary antibodies for 1 hour at room temperature, followed by 5 minutes DAPI incubation and triple washing with PBS. Fluorescent images were taken on a Leica SP8 CARS (Leica Microsystems) and Nikon C1 Confocal Microscope (Nikon).

For immunohistochemistry, animals were perfused transcardially with saline and heparin followed by 10% Formalin (Sigma), the brains were extracted and post-fixed for 24 hours. The brains were transferred into Sucrose 30% solution allowed to sink and then frozen. Coronal sections of 20 µm were collected. Sections were pre-incubated in permeabilization solution (5% normal goat serum and 0.25% Triton X-100 in 0.1 M PBS) and then incubated at 4°C overnight with primary antibodies diluted in blocking solution (5% normal goat serum and in 0.1 M PBS). Fluorophore-conjugated secondary antibodies were diluted in blocking solution and applied for 1 hour at room temperature, followed by three washes with PBS. Nuclei were visualized with DAPI stain for 15 min followed by three rinses and sections embedded in fluoromountG.

Primary antibodies included: anti-beta III tubulin (mouse; 1:200); anti-MAP2 (rabbit; 1:200); anti-iNOS (rabbit; 1:200); anti-il1beta (rabbit; 1:200); anti-MR (rabbit; 1:200); anti-CD11b (mouse; 1:200); anti-CD68 (1:500); Anti-Mannose (1:500); Anti-iNOS purchased

from Abcam; anti-iba1 (rabbit; 1:200) purchased from Wako. Secondary antibodies: goat anti-mouse or rabbit Alexa 488 and 568 (1:500; Thermo Fisher).

3.11. Polarization of microglia and macrophages to M1 and M2 stages

Both immune cells, microglia and macrophages were seeded in 24 or 96-well plates with 50 or 20×10^3 cells per well accordingly, and allowed to equilibrate overnight in 100 μ l macrophages medium. The next day all media was replaced with either control media (macrophages); or control media + 20 ng/ml interferon gamma (M1 activator) and 48 hours later + 100ng/ml of LPS; or control media + 20 ng/ml interleukin 4 (M2 activator). Cells were incubated for 72 hours, and after treatment with 300 μ M MP or control media to evaluate the effect of the drug on immune cells. MP concentration was established based on the release data obtained with HPLC analysis. Immune cell activation was assessed with immunocytochemistry analysis against M1 and M2 markers, iNOS and MR, accordingly, before MP treatment. Cells were treated with 300 μ M MP for 72 hours. Media was collected for subsequent analysis of the released cytokines, and ATP. Cells were analyzed for expression of specific surface markers, secretion of cytokines and chemokines, ATP production and release, and cell proliferation was based on DNA quantification assay.

3.12. ATP analysis

The extracellular and intracellular ATP level of immune cells was detected with the Luminescent ATP detection assay kit (ab113849, Abcam) following the provided instructions. In brief, adherent cells or media were treated with detergent and substrate solution, and the luminescence was measured with a plate reader. The blank was subtracted from all samples. At least 6 samples were used for quantification.

3.13. Quantitative reverse transcription polymerase chain reaction in real time (RT-qPCR)

Gene expression was quantified with RT-qPCR. Briefly, RNA from cells was isolated with Qiagen RNA extraction kit following the manufacturers' protocol. Total RNA was resuspended in RNase-free water and quantified using NanoDrop. Reverse transcription was performed in a final volume of 20 μ l with 1 μ g of RNA using BioRad gDNA clear cDNA synthesis kit (1725035). Transcribed cDNA was diluted to use 5ng per each PCR reaction. RPS18, CD11b, CD86, CD163, STAT1, STAT3, CCL2, and TIMP1, NF-kappaB, Notch1 primers were purchased from BioRad, and MMP2, MMP9 were purchased from Qiagen (Table S2). Reactions were conducted in 20 μ l volumes: 1 μ l cDNA, 1 μ l primers, 10 μ l SYBR green and 8 μ l of water. Amplification and detection were carried out using a CFX96 Touch™ Real-time PCR machine (Biorad, UK).

3.14. Cytokine panel

Inflammatory cytokines and chemokines were quantified with RayBio® C-series Rat Cytokine antibody array C1 kit (AAR-CYT-1-8, RayBioTech, Norcross, GA, USA) following the provided protocol. In brief, membranes provided with the kit were incubated with supernatant collected from non- or polarized immune cells at 4°C overnight. After washing, the membranes were incubated with secondary antibodies and then treated with chemiluminescence detection solution. Images of the membranes were acquired on the

G:Box Chemi XR5 (Syngene, Cambridge, United Kingdom). Results were normalized against controls on each membranes.

3.15. Animal surgeries and histology

Acute Transplants—All procedures were conducted in accordance with Institutional Animal Care and Use Committee 805600 protocol and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In brief, adult male Sprague Dawley rats (200–250 g, Charles River) were anesthetized and 5×5 mm craniotomy was performed around M1 motor cortex area. Dura was removed and the brain was injured using 2mm diameter biopsy punch. The brain tissue was aspirated by the vacuum suction. After stopping the bleeding, 50µl of 0mM MP or 8.7mM MP loaded fibrinogen was injected to cover the bottom of the injury, and immediately after a 2 mm² sized silk sponge seeded with cortical GFP+ neurons was transplanted into the injury site. Finally the injury with the transplant was covered with fibrinogen hydrogel (0 mM or 8.7mM MP). And with craniotomy cap was sewn on top of the injury. The skin was sutured. All animals survived for 3 days.

3.16. Statistical analysis

Statistical analysis was performed with one way or two way analysis of variance using GraphPad Prism 7 software. Tukey's *post-hoc* test was used to evaluate the significant difference among the experimental and control groups. A p value less than 0.05 was considered statistically significant. Gene expression analysis was performed using the *ddC_t* approach, and normalized against RPS18 gene expression. Expression of all targeted genes was compared to non-activated and non-treated (without drugs) immune cells (Microglia or macrophages) in *in vitro* samples, and to uninjured brain in *in vivo* samples. All experiments were repeated at least three times, and technical replicas (3–6) were used for every assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

3D brain-like tissue transplants do not survive due to acute inflammation. (a) Schematic representation of transplant preparation and implantation in rat M1 motor cortex area (see Methods). 2mm biopsy punch was used to make a 2mm² injury and tissue was removed by aspiration. (b) Silk sponge seeded with GFP-positive cortical neurons embedded in fibrin with/without methylprednisolone (MP). (c) 3D bioengineered cortical tissue +/- methylprednisolone (MP) transplanted in the created cavity. (d) Cranial cap sutured to cover the cavity and transplant. Scale bar 5 mm. (e) Evaluation of tissue constructs survival 3 days

after being placed on top of uninjured brain, implanted as a delayed transplant, or implanted as an acute transplant. GFP positive cells were distinguished as bright green, well-defined areas (approximately 20 μm in diameter) against the weaker, diffuse green autofluorescence of silk, which has no defined geometries. Scale bar: 100 μm . **(f)** Immunohistochemistry detects the shift in morphology of the immune cells with CD11b staining, and **(g)** the presence of inflammation with MR, CD68, and iNOS markers in the no transplant group, and the delayed and acute transplant groups, respectively. The markers label both microglia and macrophages.

Fig. 2. Methylprednisolone (MP) at concentrations from 1.5 to 8.7mM had no negative effect on neuronal viability within 2 weeks of treatment.

Daily concentration and cumulative release of MP from (a–b) fibrin and (c–d) collagen within 10 days. (e) Neuron viability by Live/Dead assay (representative images of calcein positive cells, and PI positive cells (Fig S3). Red arrows points to premature neurons, white arrows point to mature healthy neurons. (f) Statistical analysis of PI positive cells in samples with different MP concentrations at day 1, 3, 5, 7, and 14 from top to the bottom, accordingly. The average number of dead cells counted (in a frame) per culture condition.

Error bars represent mean $\pm$ SD (n=6). (g) DNA quantification analysis with PicoGreen in samples with various MP concentration 0, 1.5, 3, 6, and 8.7 from left to the right accordingly at different time points. Statistically significant groups are marked with * to correspond to $p^* < 0.05$ –0.001. Scale bar: 100 μ m. Data represent the mean $\pm$ SD (n=3).

Fig. 3. Methylprednisolone significantly downregulated pro-inflammatory markers expression and triggered transition towards the anti-inflammatory state without affecting the proliferation potential of immune cells. **(a)** Proliferation of M0, M1 and M2 polarized microglia (MG) treated/non-treated with methylprednisolone (MP) measured by DNA quantification. **(b)** Quantification of iNOS and MR positive cells against the total CD11b count. **(c)** Representative immunocytochemistry images against the iba1, CD11b (pan markers); iNOS (pro-inflammatory marker), and mannose receptor (MR) (anti-inflammatory marker) of

microglia 3 days post-treatment with 8.7 mM methylprednisolone. (**d**) Total mRNA level quantification of CD11b (pan), CD86 (pro-inflammatory) and CD163 (anti-inflammatory) markers with RT-qPCR. Statistically significant groups were marked with * and \$. $P^{*,*} < 0.05$, $P^{***} < 0.001$. * - was used to show difference within same polarization group, \$ - shows the difference between experimental (polarized or MP treated) and control (untreated) groups. Scale bar: 100 μ m. Gene expression in **d**, was normalized to RPS18 house-keeping gene expression, and all groups were compared to M0 non-treated microglia expression.

Fig. 4.

Quantification of inflammatory protein production and gene expression pointed towards NF- κ B pathway and TIMP-MMP complex involvement post-MP treatment. (a-b) Analysis of inflammatory cytokine production and gene expression in (a) microglia and (b) macrophages polarized towards pro- and anti-inflammatory states and treated with MP. Gene expression analysis was done against the inflammatory cytokine CCL2, and the ECM remodeling regulator TIMP1, and their up-stream regulators STAT1, STAT3, and NF- κ B p50 subunit. Gene expression was normalized to the RPS18 gene, and the expression of the

genes of interest was compared to the M0 non-activated control group. Error bars represent mean $\pm$ SD (n=6). Statistically significant groups were marked with * that corresponds to $p^* < 0.05$; $p^{**} < 0.01$; $p^{***} < 0.001$; $p^{****} < 0.0001$.

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Fig. 5. Methylprednisolone loaded transplants had significantly higher survival rates due to increased levels of M2 polarized immune cells. **(a-d)** Representative images of transplant survival with **(a, b)** MP and **(c, d)** without MP. **(a, c)** Immunostaining against inflammatory markers CD68 and CD11b and **(b, d)** GFP positive neurons survived the acute inflammation. Scale bar 100μm. **(e)** Quantification of surviving GFP cells in animals received non-MP and MP transplants. At least 4 animals per condition received a transplant. **(f-i)** Immune response analyzed with IHC against **(f, h)** anti-inflammatory MR (left) and **(g, i)** pro-inflammatory iNOS markers expression in **(f, g)** the transplants with/w-out MP and **(h, i)** near the transplant, injury site. Scale bar 100μm. **(j)** Quantification analysis of iNOS and MR positive cells in -/+ MP transplants. **(k)** RT-qPCR quantification of immune pan: CD11b, pro-inflammatory: CD86 and anti-inflammatory: CD163 markers expression. Gene

expression was normalized to the RPS18 gene, and the expression of the genes of interest was compared to the mRNA levels of uninjured brain. Error bars represent mean $\pm$ SD (n=4). Statistically significant groups were considered with $p < 0.05$ – 0.001 , * - corresponds with statistically significant differences between the study groups and uninjured brain; \$ - corresponds with statistically significant difference between study groups and the transplant without MP.

regulation of survival, proliferation and neural stem cells recruitment: NF- κ B and Notch1. Gene expression normalized to RPS18 housekeeping gene expression and compared to the mRNA level of uninjured brain. (c) Schematic representation of the molecular pathways activated/deactivated in MP/nonMP transplanted constructs. Error bars represent mean $\pm$ SD (n=4). Statistically significant groups were considered with $p < 0.05$ – 0.001 , * - corresponds with statistically significant difference between the studying groups and uninjured brain; \$ - corresponds with statistically significant difference between studying groups and the transplant without MP.