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Comparison of the Effect of Minocycline and Simvastatin on Functional Recovery and Gene Expression in a Rat Traumatic Brain Injury Model

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Abstract

The goal of this study was to compare the effects of minocycline and simvastatin on functional recovery and brain gene expression after a cortical contusion impact (CCI) injury. Dosage regimens were designed to provide serum concentrations in a rat model in the range obtained with clinically approved doses; minocycline 60 mg/kg q12h and simvastatin 10 mg/kg q12h for 72 h. Functional recovery was assessed using motor and spatial learning tasks and neuropathological measurements. Microarray-based transcriptional profiling was used to determine the effect on gene expression at 24 h, 72 h, and 7 days post-CCI. Gene Ontology analysis (GOA) was used to evaluate the effect on relevant biological pathways. Both minocycline and simvastatin improved fine motor function, but not gross motor or cognitive function. Minocycline modestly decreased lesion size with no effect of simvastatin. At 24 h post-CCI, GOA identified a significant effect of minocycline on chemotaxis, blood circulation, immune response, and cell to cell signaling pathways. Inflammatory pathways were affected by minocycline only at the 72 h time point. There was a minimal effect of simvastatin on gene expression 24 h after injury, with increasing effects at 72 h and 7 days. GOA identified a significant effect of simvastatin on inflammatory response at 72 h and 7 days. In conclusion, treatment with minocycline and simvastatin resulted in significant effects on gene expression in the brain reflecting adequate brain penetration without producing significant neurorestorative effects.

Key words: cortical contusion injury model; gene expression; minocycline; simvastatin; traumatic brain injury

Introduction

Traumatic brain injury (TBI) is a serious problem facing the medical community. In the United States, 1.7 million TBIs occur annually.[1] Despite many years of research, there are currently no approved pharmacotherapies for the treatment of patients with TBI. This may in part be because of the complicated secondary cascade of damage that occurs after the initial impact. The etiology of the secondary cascade resulting from TBI is likely from interrelated processes including mitochondrial energy failure, excessive generation of reactive oxygen species, activation of destructive enzymes, membrane disruption, neuronal death, thrombosis from intravascular coagulation in small vessels, increased synaptic concentrations of excitatory amino acids, and activation of innate inflammatory responses.[2–4]

The inflammatory response to TBI has been noted as being considerably up-regulated, even months after injury.[5] In addition, many researchers have noted that the inflammatory response likely plays a dual-role in TBI, assisting in mitigating immediate consequences of injury, but that extended action likely contributes to detrimental consequences.[6] Preventing the inflammatory response is considered a high potential target for neuroprotection after TBI.[3,7] Two such agents, minocycline and simvastatin, have come to the attention of the field and represent particularly interesting targets because they are already clinically approved for other uses.

Minocycline is a lipid-soluble antibiotic. There have been more than 300 publications demonstrating neuroprotection properties in experimental models of ischemic injury, Parkinson disease, Huntington disease, Alzheimer disease, multiple sclerosis, amyotrophic lateral sclerosis (for review, see Plane and associates[8]), and TBI.[9–17] Minocycline has been shown to have anti-inflammatory, anti-apoptotic, and anti-oxidant properties.[8] Simvastatin is a member of the HMG-reductase inhibitors, a group of anti-cholesterol drugs, many of which have been found to have anti-inflammatory properties.[18] Simvastatin has been found to be as beneficial or, in some cases, more

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beneficial than atorvastatin, for the management of experimental TBI.[19,20] In a cortical contusion injury (CCI) rodent model, simvastatin at a dose of 1 mg/kg/day selectively down-regulated the pro-inflammatory cytokine, interleukin-1β (Il-1β) 72 h post-CCI, while not reducing the secretion of anti-inflammatory cytokine, interleukin-6,[21] which some data suggest is neuroprotective.[22] Simvastatin also may reduce apoptosis by means of Akt-mediated pathways.[23]

The objective of the current study was to characterize the effects of minocycline and simvastatin on changes in gene expression and behavioral function after a unilateral TBI. This study is part of an on-going project that assesses and compares the effectiveness of several drugs on TBI. The goal is to identify the most beneficial treatment and also identify the mechanism of action using micro-array analysis. The ultimate goal is to use these data to design a multi-drug treatment therapy that will also be tested in our TBI model. Before the CCI studies, pharmacokinetic studies were performed in uninjured animals to determine the dose regimens needed to provide average serum concentrations in the range of the concentrations obtained clinically with Food and Drug Administration (FDA) approved doses.

Methods

Animals and housing

Male Sprague-Dawley rats, obtained from Harlan Laboratories (Indianapolis, IN), approximately 3 months old with mean body weight of 350 g, were used in this study. For the pharmacokinetic studies, the rats were obtained with surgically implanted jugular vein catheters. All animal and surgical procedures were adhered to as described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Southern Illinois University Institutional Animal Care and Use Committee (IACUC) and the University of Washington's IACUC reviewed and approved all experimental procedures. Animals were housed in a university-maintained, Association for Assessment and Accreditation of Laboratory Animal Care accredited vivarium, with a 12-h light/dark schedule and a controlled environmental temperature of 22°C in standard housing cages with food and water available *ad libitum.*

Pharmacokinetic studies

Based on initial single dose studies, minocycline 60 mg/kg ($n=4$) and simvastatin 10 mg/kg ($n=4$), obtained from Sigma-Aldrich, St. Louis, were administered by oral gavage every 12 h for 72 h. Minocycline is a highly sclerosing agent[24] and has been shown to cause tissue damage, scarring, and inflammation if administered by intraperitoneal (i.p.) injection.[25] Simvastatin is a lactone pro-drug (inactive itself), which is hydrolyzed to its active metabolite simvastatin acid and other metabolites in the intestinal wall and liver. Blood specimens were collected from the jugular catheter, at 0, 1, 2, 4, 6, 11.5, 23.5, 26, 47.5, 50, 71.5, and 74 h for the minocycline study and 0, 1, 2, 4, 6, 14, 26, 38, 50, 62, and 74 h for the simvastatin study. Samples were collected in microtubes, separated using a centrifuge, and stored at -80^{o} C until assayed. The terminal exponential rate constant () was used to determine elimination half-life ($T_{1/2}$) as 0.693/.

Drug assays

Minocycline was analyzed in serum using high pressure liquid chromatograph with UV detection at 352 nm on a Varian Pro Star 210 HPLC system. Hydrochloride salts of minocycline and the internal standard demeclocycline were obtained from Sigma-Aldrich (St. Louis). Serum samples (40 μL) containing the internal standard and an equal volume of 0.1M sodium phosphate were adjusted to a pH of 2.05, deproteinized with 1 mL methanol, and centrifuged. The supernate was dried under nitrogen at 40–50°C and reconstituted with 100 uL 0.1M sodium phosphate buffer, pH 2.05. Samples were injected onto a Sunfire C-18, 3.5 μm, 150 × 4.6 mm column kept at 40°C (Waters, Milford, MA) and separated using a mobile phase gradient of 10–22% acetonitrile in 0.1M sodium phosphate at pH 2.05 over 4 min, held at 22% for 3 min, then returned to 10% until end of the run at 16.5 min. Minocycline concentrations were targeted at 5–20 μg/mL.[26]

Because simvastatin is a pro-drug with active metabolites, HMG-CoA activity in plasma was estimated using a HMG-CoA Reductase Assay Kit (Sigma-Aldrich, St. Louis) adapted for quantitation of enzyme inhibition by pravastatin. Calibration standards ranged from 10–200 ng/mL of pravastatin in a 0.03125M K_2HPO_4:0.75 % KCl: 0.1% bovine serum albumin buffer adjusted to pH 6.22 with o-phosphoric acid. Aliquots of reconstituted kit components and calibration standards were stored at -80°C and used only once after thawed and kept on ice. Plasma samples to be analyzed were deproteinized with acetonitrile and centrifuged at 4°C. The supernate was evaporated under N_2 and reconstituted in the buffer used for the calibration standards.

Sixty μL of blank buffer, calibrators, or plasma samples, 29.5 μL kit assay buffer, 2 μL reconstituted NADPH, 6 μL HMG-CoA, and 2.5 μL of HMG-CoA reductase solution diluted in 82 μL of kit assay buffer were added to 96 well microplates equipped with half area wells (Corning Inc., Corning). Plates were immediately placed in a Benchmark microplate reader (BioRad Laboratories) set at 37°C and incubated for 5 min. After mixing for 1 min, using the kinetic mode, readings were taken every 20 sec (with 10 sec of mixing before every read) at 340 nm to monitor the reduction in absorbance from the oxidation of NADPH.

Average velocity over the measuring interval from 2.5–23.3 min was computed for each well. A linear regression equation was computed using log pravastatin concentration versus average velocity for the calibration standards. The average velocity measured for the rat plasma samples was used to calculate as pravastatin equivalents by regression analysis. Simvastatin concentrations were targeted based on pravastatin equivalents. A 19.2 mg oral dose of pravastatin, a dose within the therapeutic dosing range, resulted in average peak concentrations of 27 ± 11 ng/mL in a group of healthy subjects.[27]

Surgery

Aseptic surgery was performed according to previous protocols.[28,29] Animals were anesthetized with a combination of isofluorane (2–4%) and oxygen (0.8L/min), and body temperature was maintained at 37°C. A midline incision was made through the skin and underlying fascia. A circular craniotomy (5.0 mm) was centered 2.4 mm posterior to and 2.4 mm lateral (left) to bregma. A 4.0 mm stainless steel impactor tip, attached to an electromagnetic impactor (myneurolab.com), was used to induce the CCI injury. The cortex was impacted at 3.0 m/sec to a depth of 2.5 mm for 0.5 sec. After the injury was induced, the incision was sutured closed, and the animal was allowed to recover in a heated chamber until locomotor function returned.

Functional behavioral studies

Rats were randomly assigned to four groups. Doses of minocycline (60 mg/kg), simvastatin (10 mg/kg), or vehicle (0.9% saline, 4.8 mL/kg), were administered via oral gavage beginning at 2 h post-surgery and continued every 12 h until 72 h post-surgery. Group one received CCI and was administered minocycline ($n=8$). Group two received CCI and was administered simvastatin ($n=7$). Group three received CCI and was administered vehicle ($n=8$). Group four received sham procedures and was administered vehicle ($n=8$). Sham animals received an ''intact sham'' procedure according to previous protocols [30] in which they were anesthetized, a midline incision was made, sutured closed, and then they were placed in the recovery chamber.

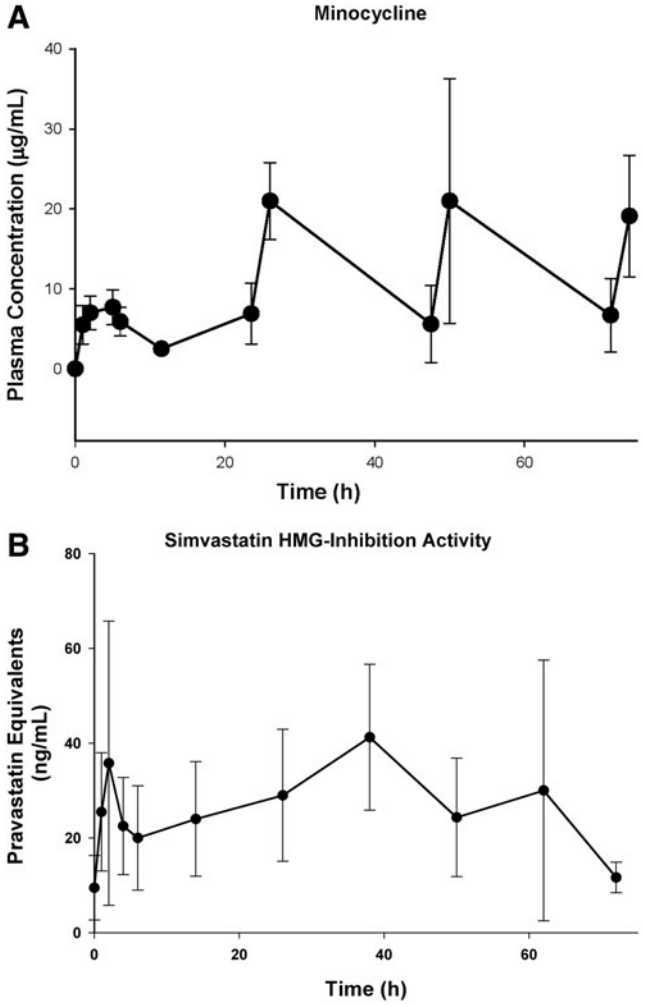

FIG. 1. Concentration-time curves. (**A**) Minocycline 60 mg/kg oral every 12 h for 72 h. (**B**) Simvastatin 10 mg/kg orally every 12 h for 72 h. After 12 h, only peak concentrations were obtained. Simvastatin activity was determined as pravastatin equivalents.

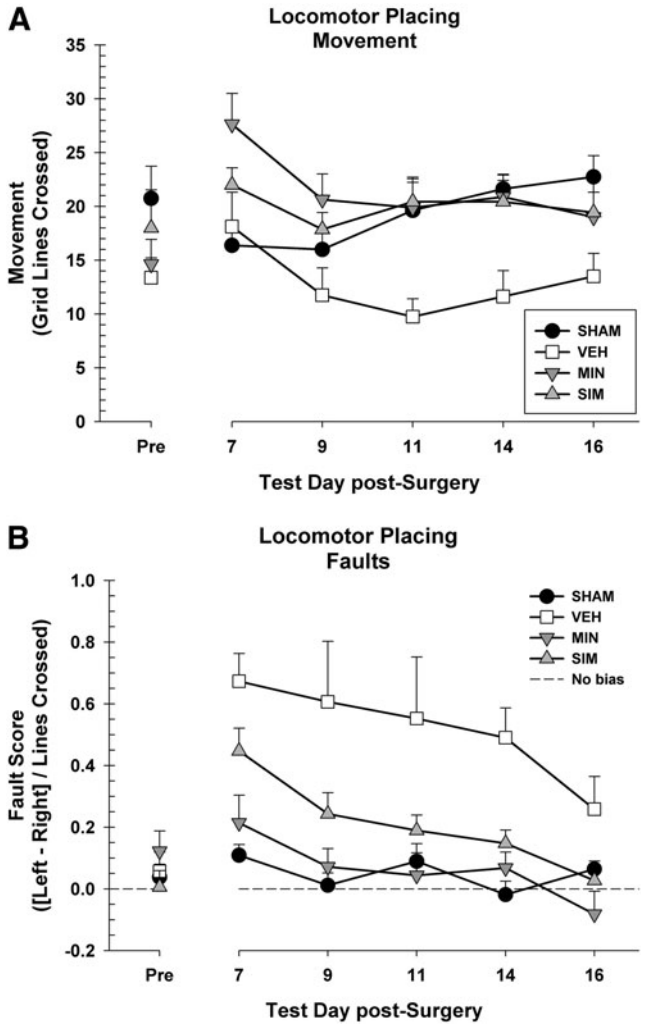

FIG. 2. (**A**) The locomotor placing task showing movement (+standard error of the mean [SEM]) for days 2, 4, 6, 8, and 10 post-cortical contusion impact (CCI). Overall, both the minocycline (MIN $p = 0.004$) and simvastatin (SIM $p = 0.019$) animals moved more than the vehicle (VEH) animals during the task. (**B**) The locomotor placing task showing the average fault scores (+SEM) for days 2, 4, 6, 8, and 10 post- CCI. Overall, both the minocycline ($p < 0.001$) and simvastatin ($p = 0.003$) animals performed better than the vehicle animals.

Locomotor placing task

To assess fine motor function, rats were tested on the locomotor placing task according to previous protocols.[29,31] Rats were pre-tested on the day before surgery to assess baseline performance. Rats were placed on an elevated grid surface (56 × 54 cm) with openings measuring 3.2 × 3.2 cm and allowed to freely explore. The number of grid lines crossed was recorded (movement) as were the number of ''foot faults'' when an animal placed a limb through one of the openings. The following equation was used to calculate foot faults on the impaired limb as a function of lines crossed: ([Right Faults–Left Faults]/Lines Crossed). One trial of 180 sec was given on days 7, 9, 11, 14, and 16 post-surgery.

Rotarod task

To evaluate gross locomotor dysfunction after brain injury, rats were tested on the rotarod according to a previous protocol.[32] A 7 cm diameter cylinder was positioned 1.2 m above a foam pad while speed and acceleration were controlled by computer interface (San Diego Instruments, San Diego, CA). Pre-training occurred on the 2 days preceding injury. Initially, an adaptation trial was performed in which rats were familiarized with the consequences of falling by repeatedly replacing them on the cylinder while it was stationary. Once they were able to stay on for 1 min, training began. A constant acceleration paradigm was used (0.055 cm/sec^2), and the latency to fall was recorded. Four trials were given per day with an intertrial of approximately 10 min. After injury, rats were re-tested on the rotarod task on days 7–11 post-surgery.

Morris water maze (MWM)

To determine the extent of cognitive deficits, rats were tested on the MWM according to a previous protocol.[29] Two phases of testing were conducted: reference memory and working memory. The reference memory paradigm testing occurred on days 15–18 post-surgery. A clear Plexiglas platform (10 × 10 cm) was submerged in 32 cm of room temperature water in the center of the northeast quadrant of a 1.5 m diameter pool. Rats were lowered from a random start position into the water facing the wall of the tank. A trial ended when 90 sec had elapsed or the rat had reached the platform. Rats unable to complete the task in 90 sec were guided by hand to the

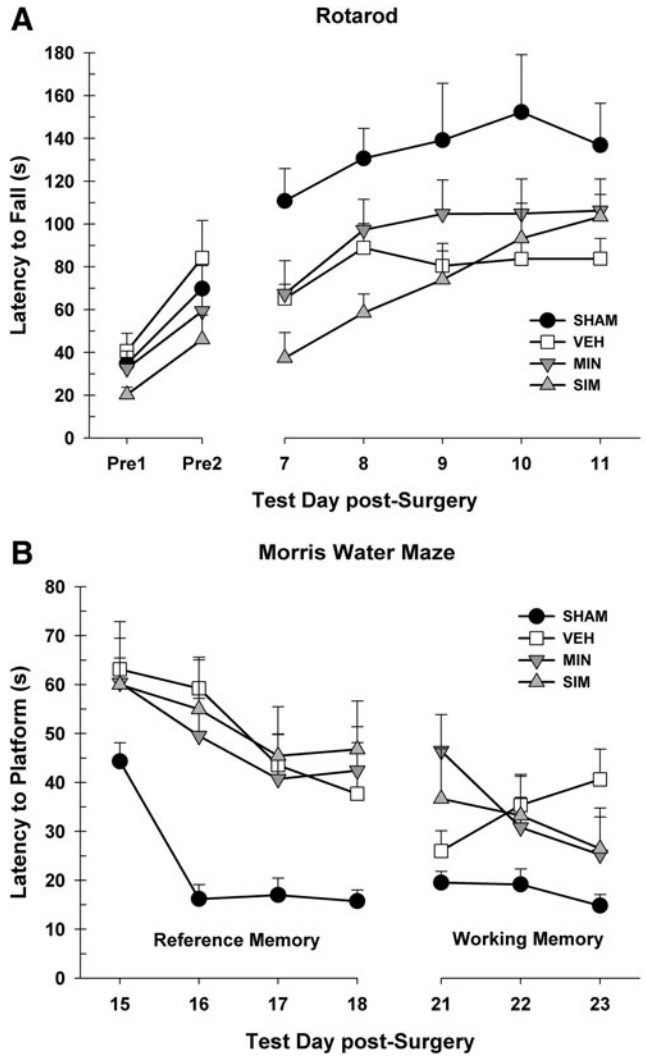

FIG. 3. (**A**) The Rotarod test showing the average latency to fall (+standard error of the mean [SEM]) off of the rotating cylinder for days 8–12 post-cortical contusion impact (CCI). No significant differences were found between the treated animals and vehicle (VEH) animals. (**B**) The MWM reference memory task showing the average latency (+SEM) to reach the platform on days 14–17 post-CCI. No significant differences were found between the treated animals and VEH animals. The MWM working memory task showing the average latency (+SEM) to reach the platform on days 21–23 post-CCI. No significant differences were found between the treated animals and VEH animals. SIM, simvastatin; MIN, minocycline.

platform. Rats were allowed to remain on the platform for 10 sec and then removed and placed in a heated cage. There were four trials per day with a 15 min intertrial interval. The latency and distance to reach the platform was recorded for each trial.

The working memory paradigm was assessed on days 21–23 post-surgery. The procedure was the same as above, except the position of the platform was changed on each test day (northwest, southwest and southeast quadrant). There were four trials per day with a 15 min inter-trial interval. The first trial was considered to be an acquisition trial and not analyzed.

Lesion analysis

At day 25 post-surgery, rats were given a lethal dose of a sodium pentobarbital solution (Euthasol, Virbac Animal Health; 0.3 mL, i.p.). Once eye-blink and pedal responses disappeared, rats were transcardially perfused with ice-cold 0.9% phosphate-buffered saline, followed by 10% phosphate-buffered formalin. Brains were then coronally sliced, frozen, on a sliding microtome at 40 μm and mounted to gelatin-subbed slides. Five coronal sections transversing the lesion were selected (-0.4, -1.4, -2.4, -3.4, -4.4) and stained with cresyl-violet. An Olympus microscope (BX-51) with an Olympus 13.5 megapixel camera (DP-70) was used to image the sections. The area of each hemisphere was measured using ImageJ software (NIH, Bethesda, MD). A percent reduction score was then calculated by dividing the left (injured) hemisphere by the right (intact) hemisphere.[28,29,32,33] These scores were then averaged together to calculate the extent of the injury.

Statistical analyses

All data were analyzed without knowledge of group assignment. The mean and standard error of the mean were calculated for all the data. The effects of treatment on behavioral outcome for each task were analyzed in repeated measures analysis of variance (ANOVA). The Huynh-Feldt correction was used to correct for repeated measures. A one-way ANOVA was used to evaluate the effect of treatment on lesion size. The Fischer least significant difference (LSD) test was used for planned *post-hoc* comparisons between the vehicle and minocycline groups and the vehicle and simvastatin groups. A p value of less than 0.05 was considered to be statistically significant.

Gene expression studies

Rats were randomly assigned to four groups. Doses of minocycline (60 mg/kg), simvastatin (10 mg/kg), or vehicle (0.9% saline, 4.8 mL/kg), were administered via oral gavage beginning at 2 h post-surgery and continued every 12 h until 72 h post-surgery or sacrifice. Group one received CCI and was administered minocycline ($n=15$). Group two received CCI and was administered simvastatin ($n=15$). Group three received CCI and was administered vehicle ($n=15$). Group four did not receive surgery and was administered vehicle (sham, $n=5$). Animals were sacrificed and tissues collected at 24 h, 72 h, and 7 days after injury according to previously published protocols.[33]

At each time point, five animals from each treatment group were sacrificed. Control animals were sacrificed at 72 h so that they

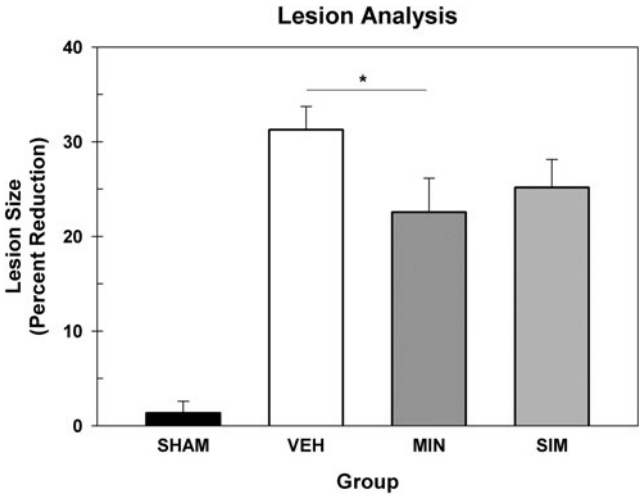

FIG. 4. Lesion analysis. The average (+standard error of the mean) percent reduction score. The minocycline (MIN)-treated group had reduced lesion volumes compared with the vehicle (VEH)-treated group, but the simvastatin (SIM)-treated group showed no improvement. $*=p<0.05$.

Table 1. Total Number of Genes that Were Differentially Expressed (>1.5-Fold up or Down, $p<0.05$) at 24 h, 72 h, and 7 Days

	24 h			*72 h*			*7 days*		
	Down	*Up*	*Total*	*Down*	*Up*	*Total*	*Down*	*Up*	*Total*
Vehicle Sham	1012	1382	2394	1710	2149	3859	1380	1899	3279
Minocycline Vehicle	103	20	123	71	116	187	55	126	181
Simvastatin Vehicle	5	5	10	67	47	114	30	99	129

received an equivalent amount of experience with gavage vehicle administration to account for any effects related to the stress response. Animals were put under deep anesthesia with a mixture of CO_2 (80%) and O_2 (20%), a cardiac blood sample taken, and then decapitated. Brains were then rapidly extracted, sliced into a 4 mm coronal slab, placed on a cold plate, and a 5 mm tissue punch taken, which included the injured cortex and a small amount of peri-injury cortex. Tissue samples were then placed in centrifuge tubes, snap frozen, and stored at −80° C. Cardiac blood samples were collected at the time of sacrifice and from the tail vein at 72 h in the 7 day group to verify serum drug levels. Blood serum was separated by microcentrifuge, snap frozen and stored at −80° C.

All samples were shipped by overnight carrier to the University of Washington on dry ice. Minocycline concentrations were assayed as described above. Simvastatin activity was not determined in the CCI studies because of problems with stability of HMG-reductase activity during transport.

The processing and analysis of the RNA samples, the microarray analysis using the AffymetrixGeneChip® 3000 scanner, and Bioconductor package p.adjust[34] were performed as described previously.[30,31] We performed Gene Ontology category analysis via the cumulative hypergeometric distribution method to determine enhanced Gene Ontology categories[35] using the Bioconductor package GOstats.[36] We used differentially expressed genes (more than 1.5 fold up or down regulated, $p<0.05$) for this analysis to identify Gene Ontology categories by evidence of overrepresentation of significant genes. The validation of the data obtained with the microarrays was performed using fluorogenic 5' -nuclease-based assay and quantitative reverse transcription-polymerase chain reaction (RT-PCR) as previously described.[30,31] The RT-PCR data were normalized to the housekeeping genes, -actin and glyceraldehyde 3-phosphate dehydrogenase (GADPH).

Results

Pharmacokinetics

In the non-injured animals, minocycline peak concentrations ranged from 5.5–10.5 μg/mL, with an average $T_{1/2}$ of 4.6 h after the first oral dose (Fig. 1A). Subsequent doses resulted in average peak concentrations of 21 ± 5 μg/mL, 21 ± 15 μg/mL, and 19 ± 8 μg/mL when sampled 2 h after the 24 h, 48 h, and 72 h doses, respectively, demonstrating a prolonged variable absorption and accumulation. In the gene expression studies, minocycline concentrations were 8 ± 6 μg/mL, and 18 ± 10 μg/mL at 24 h and 72 h time points, consistent with the findings in the non-injured animals. In the functional behavioral study, minocycline concentrations were 17 ± 11 μg/mL after the last minocycline dose at 72 h. Average peak simvastatin concentrations activity measured as pravastatin equivalents were 31 ± 7 ng/mL in the non-injured animals (Fig. 1B) and were within the range of the concentrations attained with a therapeutic dose of pravastatin.[27]

Functional behavioral study

Locomotor placing. Before injury, there were no differences between the groups in terms of movement, $F(3, 27)=1.59$, $p=0.215$, or fault score, $F(3, 27)=0.65$, $p=0.588$. Movement on the task post-surgery was analyzed in a 4×5 repeated measures ANOVA (group [sham, vehicle, minocycline, simvastatin]×day [7, 9, 11, 14, 16]) (Fig. 2A). There was a significant main effect of day because of changes in movement across the testing time, $F(3.58, 96.74)=5.92$, $p<0.001$. There was a significant difference between the groups, $F(3, 27)=3.88$, $p=0.021$. A *post-hoc* comparison showed that the minocycline group had increased locomotion compared with the vehicle group, $LSD\ (14)=8.65$, $p=0.004$. The simvastatin group also moved more compared with the vehicle group, $LSD\ (13)=7.08$, $p=0.019$. There was also a day×group interaction on movement, $F(10.75, 96.74)=3.96$, $p<0.001$, because of the vehicle group decreasing in total movement considerably as testing went on (Fig. 2A).

The post-surgery fault scores were analyzed in a 4×5 repeated measures ANOVA (group [sham, vehicle, minocycline, simvastatin]×day [7, 9, 11, 14, 16]). There was a significant main effect of day, suggesting that rats improved over time, $F(3.36, 90.81)=8.89$, $p<0.001$. There was a significant difference between the groups, $F(3, 27)=11.82$, $p<0.001$. *Post-hoc* tests revealed that minocycline administration improved performance compared with the vehicle group, $LSD(14)=0.45$, $p<0.001$. The simvastatin group also showed improved performance relative to the vehicle group, $LSD(13)=0.31$, $p=0.003$ (Fig. 2B). There was no day×group interaction on fault score, $F(10.09, 90.81)=1.18$, $p=0.317$.

Rotarod. The 2 pre-injury days were averaged together and analyzed in a one-way ANOVA. There were no differences between the groups, $F(3, 27)=2.33$, $p=0.097$. Latencies to fall were recorded on the task post-surgery and analyzed in a 4×5 repeated measures ANOVA (group [sham, vehicle, minocycline, simvastatin]×day [7, 8, 9, 10, 11]). There was a significant main effect of day, suggesting that rats improved over time, $F(3.59, 96.92)=10.35$, $p<0.001$. There was a significant difference between the groups, $F(3, 27)=4.50$, $p=0.011$. There were no significant improvements in the minocycline group, however, compared with the vehicle group, $LSD\ (14)=15.71$, $p=0.385$, or the simvastatin group compared with the vehicle group, $LSD(13)=7.06$, $p=0.704$. There was no day×group interaction, $F(10.77, 96.92)=1.05$, $p=0.407$ (Fig. 3A).

MWM. Latencies to reach the platform were recorded on the reference memory paradigm of the MWM and analyzed in a 4×4

Table 2. Gene Ontology Analysis: Effect of Minocycline Compared with Vehicle

GO ID	*GO Term*	*Annotated*	*Significant genes*	p *value*
24 h				
GO:0006935	chemotaxis	284	Bmp4, C3ar1, Ccr2, Egr2, Trp2,Ntf3,Tgfb2	0.00105
GO:0008015	blood circulation	297	C3ar1, Htr2c, Kng1l1, Nts, Scn4b, Sult1a1, Tgfb2	0.00136
GO:0050801	ion homeostatis	582	C3ar1, Egr2, Htr2c, Ntf3, Pkp2, Scn4b, Slc9a2, Slc9a4, Tgfb2	0.00140
GO:0006584	catecholamine metabolic process	40	Htr2c, Sult1a1, Tgfb2	0.00146
GO:0007420	brain development	487	Aldh1a2, Bmp4, Egr2, Gabra5, Neurod1, Neurod6, Ntf3, Tgfb2	0.00154
GO:0045055	regulated secretory pathway	42	Btk, Ccr2, Steap2	0.00168
GO:0042129	regulation of T cell proliferation	94	Bmp4, Ccr2, Cd3e, RT1-Ba	0.00180
GO:0008285	negative regulation of cell proliferation	407	Aldh1a2, Bmp4, Ifi30, Ogn, RT1-Ba, Rxfp2, Tgfb2, Xdh	0.00195
GO:0015812	GABA transport	12	Htr2c, Trpc4	0.00190
GO:0021542	dentate gyrus development	12	Neurod1, Neurod6	0.00190
GO:0042063	gliogenesis	165	Bmp4, Ccr2, Egr2, Ntf3, Tgfb2	0.00230
GO:0007210	serotonin receptor signaling pathway	13	Htr1f, Htr2c	0.00235
GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class II	13	Ifi30, RT1-Ba	0.00235
GO:0045601	regulation of endothelial cell differentiation	13	Bmp4, Xdh	0.00235
GO:0007184	SMAD protein import into nucleus	14	Bmp4, Tgfb2	0.00273
GO:0046641	positive regulation of alpha-beta T cell proliferation	14	Ccr2, Cd3e	0.00273
GO:0008347	glial cell migration	15	Ccr2, Tgfb2	0.00313
GO:0007267	cell-cell signaling	661	Bmp4, Btk, C1ql3, Egr2, Gabra5, Htr2c, Neurod1, Ntf3, Rgs14, Trpc4	0.00365
GO:0050678	regulation of epithelial cell proliferation	183	Aldh1a2, Bmp4, Mmp12, Tgfb2, Xdh	0.00365
72 h				
GO:0006953	acute-phase response	41	Ass1, Il1rn, Kng1, Kng1l1, Ptgs2, Reg3a, Reg3b, Reg3g	5.26e-09
GO:0007218	neuropeptide signaling pathway	64	Calca, Crhr1, Glra1, Grp, Nmb, Npy	2.72e-06
GO:0002526	acute inflammatory response	100	Ass1, Il1rn, Kng1, Kng1l1, Ptgs2, Reg3a, Reg3b, Reg3g	2.80e-06
GO:0007204	elevation of cytosolic calcium ion concentration	164	Agtr1a, Calca, Crhr1, Cxcl2, Kng1, Nmb, Npy2r, Tgfb2	7.87e-06
GO:0051051	negative regulation of transport	270	Acvr1c, Calca, Crhr1, Il1rn, Nmb, Npy2r, Ostn, Ptgs2, Tgfb2	4.38e-05
GO:0008015	blood circulation	297	Agtr1a, Calca, Hcn4, Kng1, Kng1l1, Nppc, Npy, Ptgs2, Sult1a1, Tgfb2	9.14e-05
GO:0006874	cellular calcium ion homeostasis	235	Agtr1a, Calca, Crhr1, Cxcl2, Kng1, Nmb, Npy2r, Tgfb2	1.04e-04
GO:0030808	regulation of nucleotide biosynthetic process	84	Calca, Crhr1, Nppc, Npy2r, Ostn	1.75e-04
GO:0045638	negative regulation of myeloid cell differentiation	46	Calca, Lrrc17, Tnfrsf11b, Zbtb16	1.88e-04
GO:0007200	phospholipase C-activating G-protein coupled receptor signaling pathway	48	Agtr1a, Calca, Crhr1, Gpr139	2.22e-04
GO:0032845	negative regulation of homeostatic process	21	Calca,Tgfb2, Tnfrsf11b	2.88e-04
GO:0030155	regulation of cell adhesion	209	Calca, Il1rn, Kng1, Npy2r, Plau, Serpini1, Tgfb2	3.18e-04
GO:0033555	multicellular organismal response to stress	64	Calca, Crhr1, Gabra5, Npy2r	6.73e-04
GO:0070555	response to interleukin-1	73	Cxcl2, Hnmt, Il1rn, Mmp9	1.11e-03
GO:0006805	xenobiotic metabolic process	34	Fmo2, Gsta3, Sult1a1	1.22e-03
GO:0006584	catecholamine metabolic process	40	Sult1a1, Sult1d1, Tgfb2	1.96e-03
7 days				
GO:0042573	retinoic acid metabolic process	17	Aldh1a1, Aldha2, Cyp26b1	6.60e-05
GO:0042445	hormone metabolic process	148	Aldh1a1, Aldha2, Bmp5, Cyp26b1, Igf2	6.66e-04
GO:0046890	regulation of lipid biosynthetic process	97	Bmp5, Igf2, Prox1, Thrsp	1.14e-03
GO:0021542	dentate gyrus development	12	Neurod6, Prox1	1.42e-03
GO:0007210	serotonin receptor signaling pathway	13	Htr1a, Htr5b	1.68e-03

(*continued*)

Table 2. (Continued)

GO ID	*GO Term*	*Annotated*	*Significant genes*	p *value*
GO:0050810	regulation of steroid biosynthetic process	50	Bmp5, Igf2, Prox1	1.70e-03
GO:0019216	regulation of lipid metabolic process	185	Chrm5, Igf2, Prox1, Thrsp	1.81e-03
GO:0021766	hippocampus development	58	Neurod6, Prox1	2.61e-03
GO:0007155	cell adhesion	573	Cdh1, Cdh7, Col1a1, Cpxm2, Pkp2, Sell,Tbx18, Thbs1, Wisp2	5.36e-03
GO:0050920	regulation of chemotaxis	86	Il16, Sell, Sema3a, Thbs1	7.89e-03
GO:0050678	regulation of epithelial cell proliferation	183	Aldh1a2, Bmp5, Prox1, Tbx18, Thbs1	1.10e-02
GO:0008285	negative regulation of cell proliferation	407	Aldh1a2, Bmp5, Ogn, Prox1, RT1-Bb, Thbs1, Wisp2	1.24e-02
GO:0050679	positive regulation of epithelial cell proliferation	105	Bmp5, Prox1, Tbx18	1.36e-02
GO:0006821	chloride transport	55	Gabra5, Slc26a7	2.81e-02
GO:0016051	carbohydrate biosynthetic process	139	Chst9, Igf2	2.83e-02

Acvr1c (activin A receptor, type IC), Agtr1a (angiotensin II receptor, type 1a), Aldh1a1 (aldehyde dehydrogenase 1 family, member A1), Aldh1a2 (aldehyde dehydrogenase 1 family, member A2), Ass1 (argininosuccinate synthase 1), Bmp4 (bone morphogenetic protein 4), Bmp5 (bone morphogenetic protein 5), Btk (Bruton agammaglobulinemia tyrosine kinase), Calca (calcitonin-related polypeptide alpha), C1ql3 (complement component 1, q subcomponent-like 3), C3ar1 (complement component 3a receptor 1), Ccr2 (chemokine (C-C motif) receptor 2), Cd3e (CD3 molecule, epsiolon), Cdh1 (cadherin 1), Cdh7 (cadherin 7, type 2), Chrm5 (cholinergic receptor, muscuarinic 5), Chst9 (carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9),Cish (cytokine inducible SH2-containing protein), Col1a1 (collagen, type I, alpha 1), Cpxm2 (carboxypeptidase X (M14 family), member 2), Crhr1 (corticotropin releasing hormone receptor 1), Cxcl2 (chemokine (C-X-C motif) ligand 2), Cyp1b1 (cytochrome P450, family 1, subfamily b,polypepetide 1), Cyp27b1 (cytochrome P450, family 27, subfamily b,polypepetide 1) Cyp26b1 (cytochrome P450, family 26, subfamily b,polypepetide 1)Egr2 (early growth response 2), F13a1 (coagulation factor X111, A1 polypeptide), Fmo2 (flavin containing monooxygenase 2), Gabra5 (gamma-aminobutyric acid (GABA) A receptor, alpha 5), GPr139 (G protein-coupled receptor 139), Grp (gastrin releasing peptide), Gsta3 (glutathione S-transferase A3), Hcn4 (hyperpolarization activated cyclic nucleotide-gated potassium channel 4), Hnmt (histamine N-methyltransferase), Hspb1 (heat shock protein 1), Htr1a (5-hydroxytryptamine (serotonin), receptor 1A, G-protein coupled), Htr1f (5-hydroxytryptamine (serotonin), receptor 1F, G-protein coupled), Htr2c (5-hydroxytryptamine (serotonin) receptor 2C, G-protein coupled), Htr5b (5-hydroxytryptamine (serotonin), receptor 5B), Il1rn (interleukin 1 receptor antagonist), Il16 (interleukin 16), Igf2 (insulin-like growth factor 2), Ili30 (interferon gamma inducible protein 30), Kng1 (kiniogen 1),Kng1l1 (kiniogen 1-like 1), Lrrc17 (leucine rich repeat containing 17), Mmp9 (matrix metallopeptidase 9), Mmp12 (matrix metallopeptidase 12), Mustn1 (musculoskeletal, embryonic nuclear protein 1), Neurod1 (neurogenic differentiation 1), Neurod6 (neurogenic differentiation 6), Nmb (neuromedin B), Nppc (natriuretic peptide C), Npy (neuropeptide Y), Npy2r (neuropeptide Y receptor Y2), Nrp2 (neuropilin 2), Ntf3 (neurotrophin 3), Nts (Neurotensin), Ogn (osteoglycin), Ostn (osteocrin), Pkp2 (plakophilin 2), Plau (plasminogen activator, urokinase), Prox1 (prospero homeobox 1), Ptgs2 (prostaglandin-endoperoxide synthase 2), Reg3a (regenerating islet-derived 3 alpha), Reg3b (regenerating islet-derived 3 beta), Reg3g (regenerating islet-derived 3 gamma), Rgs14 (regulator of G-protein signaling 14), RT1-Ba (RT1 class II, locus Ba), RT1-Bb (RT1 class II, locus Bb), Rxfp2 (relaxin/insulin-like family peptide receptor 2), Scn4b (sodium channel, voltage-gated), Sell (selectin L), Sema3a (sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A), Serpini1 (serpin peptidase inhibitor, clade I, member 1),Slc26a7 (solute carrier family 26, member 7), Steap2 (Steap family member 4), Sulf1 (sulfatase1), Sult1a1 (sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1), Sult1d1 (sulfotransferase family, cytosolic, 1D, member 1),Tbx18 (T-box18), Tgfb2 (transforming growth factor, beta 2), Tgfbi (transforming growth factor, beta induced), Thbs1 (thrombospondin 1), Thrsp (thyroid hormone responsive), Tnfrsf11b (tumor necrosis factor receptor superfamily, member 11b), Trpc4 (transient receptor potential cation channel, subfamily C, member 4), Wisp2 (WNT1 inducible signaling pathway protein 2), Xdh (xanthine dehydrogenase), Zbtb16 (zinc finger and BTB domain containing 16).

GO, gene ontology.

repeated measures ANOVA (group [sham, vehicle, minocycline, simvastatin] × day [15, 16, 17, 18]). There was a significant main effect of day, suggesting that rats improved over time, $F(2.99, 80.81) = 11.83$, $p < 0.001$. There was a significant difference between the groups, $F(3, 27) = 5.62$, $p = 0.004$. *Post-hoc* tests revealed no differences between the minocycline and vehicle groups, $LSD(14) = 2.64$, $p = 0.745$, or the simvastatin and vehicle groups, $LSD(13) = 0.89$, $p = 0.915$. There was no day × group interaction, $F(8.98, 80.81) = 0.87$, $p = 0.552$ (Fig. 3B).

Latencies to reach the platform were recorded on the working memory paradigm of the MWM and analyzed in a 4 × 3 repeated measures ANOVA (group [sham, vehicle, minocycline, simvastatin] × day [21, 22, 23]). There was no significant main effect of day, $F(2, 54) = 1.01$, $p = 0.370$. There was no significant difference between the groups, $F(3, 27) = 2.90$, $p = 0.053$ (Fig. 3B). There was no significant day × group interaction, $F(6, 54) = 2.18$, $p = 0.059$. There were also no differences in motor ability (average velocity) in the MWM during the reference memory paradigm, $F(3, 27) = 0.68$, $p = 0.570$, or working memory paradigm, $F(3, 27) = 1.02$, $p = 0.400$.

Lesion analysis

Lesion size (percent reduction of hemisphere) was evaluated in a one-way ANOVA. There was a significant difference between the groups, $F(3, 27) = 24.06$, $p < 0.001$. *Post-hoc* tests revealed that the minocycline group had smaller lesions compared with the vehicle, $LSD(14) = 8.69$, $p = 0.028$, but the simvastatin group showed no difference, $LSD(13) = 6.08$, $p = 0.128$ (Fig. 4).

Gene expression study

The microarray data passed all the standard and advanced quality control metrics. The number of differentially expressed genes (> 1.5-fold change, $p < 0.05$) at 24 h, 72 h, and 7 days are presented in Table 1. The vehicle to sham comparison reflects the effect of the TBI without treatment relative to sham controls. The minocycline or simvastatin (CCI animals that received treatment) to vehicle (CCI animals that received vehicle) comparison evaluates the effect of treatment on gene expression in the context of TBI.

Both minocycline and simvastatin treatments resulted in significant changes in brain gene expression in the CCI model demonstrating acceptable brain penetration. At 24 h post-CCI, Gene Ontology analysis (GOA), which shifts the emphasis from evaluation of single genes to evaluation of pathways, networks, and functions, identified a significant effect of minocycline on chemotaxis, blood circulation, immune response, and a variety of cell to cell signaling pathways (Table 2). Inflammatory pathways were only affected by minocycline at the 72 h time point.

Table 3. Effect of Minocycline and Traumatic Brain Injury (Vehicle/Sham) on Genes of Interest (1.5 Fold Change, $P<0.05$)

Affymetrix ID	Gene symbol	Genes	Minocycline / Vehicle	Vehicle / Sham
24 h				
10911380	Aldh1a2	aldehyde dehydrogenase 1 family, member A2	0.61	0.48
10782891	Bmp4	bone morphogenetic protein 4	0.63	n.s.
10939293	Btk	Bruton agammaglobulinemia tyrosine kinase	0.66	2.33
10799733	C1ql3	complement component 1, q subcomponent-like 3	0.65	n.s.
10914614	Ccr2	chemokine (C-C motif) receptor 2	0.64	6.19
10916955	Cd3e	CD3 molecule, epsilon	0.51	n.s.
10848165	Chrm5	cholinergic receptor, muscarinic 5	0.62	n.s.
10722347	Gabra5	gamma-aminobutyric acid (GABA) A receptor, alpha 5	0.56	n.s.
10787491	Ifi30	interferon gamma inducible protein 30	0.66	4.11
10755135	Kng1l1	kininogen 1-like 1	0.51	3.26
10907869	Mmp12	matrix metallopeptidase 12	0.44	7.84
10846685	Neurod1	neurogenic differentiation 1	0.41	n.s.
10862698	Neurod6	neurogenic differentiation 6	0.50	0.60
10923835	Nrp2	neuropilin 2	0.67	2.13
10902047	Nts	Neurotensin	0.18	n.s.
10865724	Ntf3	neurotrophin 3	0.63	n.s.
10797648	Ogn	osteoglycin	0.46	0.36
10752576	Pkp2	plakophilin 2	0.64	n.s.
10797274	Rgs14	regulator of G-protein signaling 14	0.65	n.s.
10768357	Rgs18	regulator of G-protein signaling 18	0.54	n.s.
10828351	RT1-Ba	RT1 class II, locus Ba	0.63	2.81
10922909	Slc9a2	solute carrier family 9 (sodium/hydrogen exchanger), member 2	0.44	n.s.
10922895	Slc9a4	solute carrier family 9 (sodium/hydrogen exchanger), member 4	0.26	n.s.
10725782	Sult1a1	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	0.61	0.58
10815352	Trpc4	transient receptor potential cation channel, subfamily C, member 4	0.66	n.s.
10882933	Xdh	xanthine dehydrogenase	0.63	2.75
72 h				
10845416	Acvr1c	activin A receptor, type IC	0.59	n.s.
10798194	Agtr1a	angiotensin II receptor, type 1a	0.29	4.17
10835355	Ass1	argininosuccinate synthase 1	0.64	1.94
10911711	Bmp5	bone morphogenetic protein 5	0.66	n.s.
10725051	Calca	calcitonin-related polypeptide alpha	0.50	9.91
10912908	Cish	cytokine inducible SH2-containing protei	0.57	1.97
10816405	Crabp2	cellular retinoic acid binding protein 2	0.49	3.81
10775896	Cxcl2	chemokine (C-X-C motif) ligand 2	2.43	n.s.
10771649	Cxcl11	chemokine (C-X-C motif) ligand 11	0.59	3.87
10887947	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	0.49	6.77
10894200	Cyp4f4	cytochrome P450, family 4, subfamily f, polypeptide 4	1.57	0.41
10794734	F13a1	coagulation factor XIII, A1 polypeptide	2.00	1.99
10718934	Fcar	IgA Fc receptor	1.68	n.s.
10769370	Fm02	flavin containing monooxygenase	1.97	n.s.
10722347	Gabra5	gamma-aminobutyric acid (GABA) A receptor, alpha 5	1.57	0.33
10935811	Gabre	gamma-aminobutyric acid (GABA) A receptor, epsilon	0.50	2.22
10891679	Gpr68	G protein-coupled receptor 68	0.66	1.62
10725286	Gpr139	G protein-coupled receptor 139	1.60	n.s.
10926958	Gsta3	glutathione S-transferase A3	1.54	0.52
10843184	Hnmt	histamine N-methyltransferase	1.58	0.36
10761128	Hspb1	heat shock protein 1	0.65	14.24
10834109	Il1rn	interleukin 1 receptor antagonist	0.50	13.14
10888131	Kcng3	potassium voltage-gated channel, subfamily G, member 3	0.59	0.58
10751988	Kng1	kininogen 1	0.38	4.47
10842239	Mmp9	matrix metallopeptidase 9	0.60	4.54
10786532	Mustn1	musculoskeletal, embryonic nuclear protein 1	0.57	2.32
10723220	Nmb	neuromedin B	1.60	n.s.
10929606	Nppc	natriuretic peptide C	1.55	0.53
10855506	Npy	neuropeptide Y	0.61	1.52
10815655	Npy2r	neuropeptide Y receptor Y2	0.39	3.42
10850783	Nrsn2	neurensin 2	1.52	0.60

(*continued*)

Table 3. (Continued)

Affymetrix ID	Gene symbol	Genes	Minocycline / Vehicle	Vehicle / Sham
10755008	Ostn	osteocrin	0.43	1.58
10782271	Plau	plasminogen activator, urokinase	0.58	12.43
10764551	Ptgs2	prostaglandin-endoperoxide synthase 2	0.43	2.60
10856481	Reg3a	regenerating islet-derived 3 alpha	0.45	2.55
10856474	Reg3b	regenerating islet-derived 3 beta	0.47	2.07
10863410	Reg3g	regenerating islet-derived 3 gamma	0.10	34.41
10827686	RT1-M6-1	RT1 class I, locus M6, gene 1	0.53	n.s.
10827691	RT1-M6-2	RT1 class I, locus M6, gene 2	0.59	1.79
10815962	Serpini1	serpin peptidase inhibitor, clade I, member	1.51	0.38
10874981	Sulf1	sulfatase 1	0.65	1.84
10725782	Sult1a1	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	2.10	0.64
10771919	Sult1d1	sulfotransferase family 1D, member 1	1.69	0.30
10770577	Tgfb2	transforming growth factor, beta 2	0.58	1.98
10797138	Tgfbi	transforming growth factor, beta induced	0.64	6.93
10903725	Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b	0.59	n.s.
10917116	Zbtb16	zinc finger and BTB domain containing 16	1.94	0.56
7 days				
10714323	Aldh1a1	aldehyde dehydrogenase 1 family, member A1	1.71	0.61
10911380	Aldh1a2	aldehyde dehydrogenase 1 family, member A2	0.61	2.82
10807542	Cdh1	cadherin 1	0.60	n.s.
10763401	Cdh7	cadherin 7, type 2	0.64	n.s.
10848165	Chrm5	cholinergic receptor, muscarinic 5	1.74	0.62
10803315	Chst9	carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 9	1.77	n.s.
10737532	Col1a1	collagen, type I, alpha	0.64	3.57
10863608	Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1	0.67	n.s.
10722347	Gabra5	gamma-aminobutyric acid (GABA) A receptor, alpha 5	1.60	0.45
10812879	Htr1a	5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled	1.63	0.45
10767186	Htr5b	5-hydroxytryptamine (serotonin) receptor 5B	1.89	0.51
10726999	Igf2	insulin-like growth factor 2	0.59	2.18
10723351	Il16	interleukin 16	1.54	n.s.
10907913	Mmp8	matrix metallopeptidase 8	0.63	14.59
10862698	Neurod6	neurogenic differentiation 6	2.10	n.s.
10752576	Pkp2	plakophilin 2	1.80	0.57
10770680	Prox1	prospero homeobox	1.53	n.s.
10831567	RT1-Bb	RT1 class II, locus Bb	0.48	4.06
10817053	S100a3	S100 calcium binding protein A3	1.72	2.76
10765186	Sell	selectin L	0.64	2.02
10860481	Sema3a	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	0.66	0.66
10875581	Slc26a7	solute carrier family 26, member 7	0.63	n.s.
10919175	Tbx18	T-box18	0.66	2.18
10723720	Thrsp	thyroid hormone responsive	1.51	0.58
10842043	Wisp2	WNT1 inducible signaling pathway protein 2	0.60	1.88

The effect of minocycline treatment (minocycline/vehicle) and TBI (vehicle/sham) on differentially expressed genes of interest and their specific fold changes in expression are presented in Table 3. Of note, the expression of matrix metallopeptidase 8 (Mmp8), Mmp9, Mmp12, chemokine (C-C motif) receptor 2 (Ccr2), and heat shock protein 1 (Hspb1) were significantly increased by TBI and decreased by minocycline. Minocycline also decreased the expression of interleukin 1 receptor antagonist (Ilrn) and increased the expression of interleukin 16, a pro-inflammatory cytokine, at 72 h and 7 d, post-CCI.

There was minimal effect of simvastatin on gene expression 24 h after injury, with increasing effects at 72 h and 7 days post- CCI (Table 1). GOA identified a significant effect of simvastatin on inflammatory response and cell chemotaxis at both the 72 h and 7 day time points (Table 4). The effect of simvastatin treatment (simvastatin/vehicle) and TBI (vehicle/sham) on differentially expressed genes of interest and their specific fold changes in expression are presented in Table 5. Of note, the primary effect of simvastatin on the chemokines, selectin, interleukin 18 (Il-18), Il1rn, S100 binding proteins, and other genes was to increase the expression of genes that were also increased by TBI itself. As has been reported by others,[21] Il-1 was increased by TBI 3.7 fold, 2.2 fold, and 1.7 fold at 24 h, 72 h, and 7 days, respectively. There was no effect, however, of simvastatin on Il-1 expression at any time point measured.

To validate gene expression changes, Figure 5 shows the gene expression of 14 genes selected from specific pathways of interest that are affected by TBI. The data generated via microarray and quantitative-PCR (qPCR) were normalized to GADPH and β-actin. The qPCR findings were highly correlated with the microarray data, the Pearson correlation, were 0.82 and 0.81 when normalized for GADPH and β-actin, respectively.

Table 4. Gene Ontology Analysis: Effect of Simvastatin Compared with Vehicle

GO ID	*GO term*	*Annotated*	*Significant genes*	p *value*
72 h				
GO:0006954	inflammatory response	341	Ace, Adcyap1, Agtr1a, Ccl3, Ccl7, Ccl12, Ccl24, Ccr41, Ccr2, Cxcl2, Fabp4, Olr1, Ptgs2, Reg3b, S100a9, Tac1	1.05e-11
GO:0030595	leukocyte chemotaxis	93	Ccl3, Ccl7, Ccl12, Ccl24, Ccr1, Ccr2, Cxcl2, S100a9, Sell, Tgfb2	3.76e-11
GO:0050729	positive regulation of inflammatory response	56	Ace, Ccl3, Ccl24, Ccr2, Fabp4, Ptgs2, Tac1	1.35e-08
GO:0030335	positive regulation of cell migration	202	Ccl3, Ccl7, Ccl24, Ccr1, Ccr2, Fgf1, Ptgs2, Sell, Tac1, Tgfb2	7.51e-08
GO:0050727	regulation of inflammatory response	156	Ace, Adcyap1, Ccl3, Ccl24, Ccr1, Ccr2, Fabp4, Ptgs2, Tac1	9.71e-08
GO:0055074	calcium ion homeostasis	243	Adcyap1, Agtr1a, Ccl3, Ccr1, Ccr2, Cxcl2, Cyp27b1, Gnas, Npy2r, Tac1, Tgfb2,	4.21e-07
GO:0002690	positive regulation of leukocyte chemotaxis	43	Ccl3, Cc17, Ccr1, Ccr2, Sell	2.64e-06
GO:0002548	monocyte chemotaxis	20	Ccl3, Cc12, Ccr1, Ccr2	3.00e-06
GO:0007270	neuron-neuron synaptic transmission	102	Adcyap1, Glra1, Htr1b, Npy2r, Ptgs2, Tac1	1.39e-05
GO:0008015	blood circulation	297	Ace, Adcyap1, Agr1a, Alb, Gnas, Hcn4, Ptgs2, Tac1, Tgfb2	2.03e-05
GO:2000403	positive regulation of lymphocyte migration	11	Ccl3, Ccl7, Ccr2	2.14e-05
GO:0050921	positive regulation of chemotaxis	66	Ccl3, Ccl7, Ccr2, Sell	2.24e-05
7 d				
GO:0002690	positive regulation of leukocyte chemotaxis	43	Ccl2, Ccl3, Ccl4, Ccl7, Ccr2, Cxcl10, Lbp, Sell, Serpine1	6.90e-12
GO:0050920	regulation of chemotaxis	86	Ccl2, Ccl3, Ccl4, Ccl7, Ccr2, Cxcl9, Cxcl10,Lbp, Sell, Sema3a, Serpine1 Thbs1	8.08e-12
GO:0050921	positive regulation of chemotaxis	66	Ccl2, Ccl3, Ccl4, Ccl7, Ccr2, Cxcl9, Cxcl10,Lbp, Sell, Serpine1 Thbs1	1.34e-11
GO:0006955	immune response	533	Ccl2, Ccl3, Ccl4, Ccl7, Ccl12, Ccr2, Cd14, Cd36, Col3a1, Crip1, Cxcl9, Cxcl10, Cxcl11, Fcnb, Il18, Il1rn, Lbp, Lcn2, Serpina3n, Thbs1	1.37e-10
GO:0006954	inflammatory response	341	Anxa1, Calca, Ccl1, Ccl3, Ccl4, Ccl7, Ccl12, Ccr2, Cd14, Cxcl10, Il18, Il1rn, Lbp, Serpina3n, Serpine1, Thbs1	4.80e-09
GO:0071706	tumor necrosis factor superfamily cytokine production	63	Ccl3, Ccl4, Ccr2, Cd14, Cd36, Il18, Lbp, Thbs1	1.70e-07
GO:0016525	negative regulation of angiogenesis	46	Ccl2, Ccr2, Cd36, Cxcl10, Serpine1, Sulf1, Thbs1	5.16e-07
GO:0001944	vasculature development	422	Calc1, Ccl2, Ccl12, Ccr2, Cd36, Col3a1, Cxcl10, Il18, Loxl2, Serpine1, Sox18, Srpx2, Sulf1, Tgfbi, Thbs1	5.48e-07

Ace (angiotensin I converting enzyme (peptidyl-dipeptidase A) 1), Adcyap1 (adenylate cyclase activating polypeptide 1), Agtr1a (angiotensin II receptor, type 1a), Alb (albumin), Anxa1(annexin A1), Calca (Calcitonin-related polypeptide alpha), Ccl2 (chemokine (C-C motif) ligand 2), Ccl3 (chemokine (C-C motif) ligand 3), Ccl4 (chemokine (C-C motif) ligand 4). Ccl7 (chemokine (C-C motif) ligand 7), Ccl12 (chemokine (C-C motif) ligand 12), Ccl24 (chemokine (C-C motif) ligand 24), Ccl7, Ccr41, Ccr2 (chemokine (C-C motif) receptor 2), Cd14 (CD 14 molecule), Cd36 (CD36 molecule (thrombospondin receptor), Col3a1 (collagen, type III, alpha 1), Crip1 (cysteine-rich protein 1), Cxcl2 (chemokine (C-X-C motif) ligand 2), Cxcl9 (chemokine (C-X-C motif) ligand 9), Cxcl10 (chemokine (C-X-C motif) ligand 10), Cxcl11 (chemokine (C-X-C motif) ligand 11), Cyp27b1 (cytochrome P450, family 27, subfamily b, polypeptide 1), Fabp4 (fatty acid binding protein 4, adipocyte), Fcnb (ficolin B), Fgf1 (fibroblast growth factor 1), Glra1 (glycine receptor, alpha 1), Gnas (GNAS complex locus), Htr1b (5-hydroxytryptamine (serotonin) receptor 1B, G protein-coupled), Il18 (nterleukin 18), Il1rn (interleukin 1 receptor antagonist), Lbp (lipopolysaccharide binding protein), Lcn2 (lipocalin 2), Loxl2 (lysyl oxidase-like 2), Npy2r (neuropeptide Y receptor Y2), Olr1 (oxidized low density lipoprotein (lectin-like) receptor 1), Ptgs2 (prostaglandin-endoperoxide synthase 2), Reg3b (regenerating islet-derived 3 beta), S100a9 (S100 calcium binding protein A9), Sell (Selectin L), Sema3a (sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A), Serpine1 (serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1), Sox18 (SRY (sex determining region Y)-box 18), Srpx2 (sushi-repeat-containing protein, X-linked 2), Sulf1 (sulfatase 1), Tac1 (tachykinin, precursor 1), Tgfb2 (transforming growth factor, beta 2), Thbs1 (thrombospondin 1).

GO, gene ontology.

Discussion

The overall goal of this study was to evaluate the change in transcriptome profiles of brain injured rats as a result of two therapeutic agents and to investigate the correlated behavioral outcomes. The initial dose of minocycline and simvastin resulted in prolonged absorption in the rodents. Therefore, doses were administered 2 h post-injury to attain targeted concentrations in the range by 4 h post-injury. Oral gavage was used to prevent the complication of an additional treatment-induced inflammatory response from the sclerosing properties of minocycline when administered i.p. or subcutaneously.[24] Clinically, minocycline is available as a parenteral formulation and can be administered by the intravenous route in patients with TBI resulting in immediate

Table 5. Effect of Simvastatin and Traumatic Brain Injury (Vehicle/Sham) on Genes of Interest (1.5 Fold Change, $P < 0.05$)

Affymetrix ID	Gene symbol	Genes	Simvastatin / Vehicle	Vehicle / Sham
72 h				
10739035	Ace	angiotensin I converting enzyme (peptidyl-dipeptidase A)	0.62	1.85
10930539	Adcyap1	adenylate cyclase activating polypeptide 1	0.60	n.s.
10798194	Agtr1a	angiotensin II receptor, type 1	0.45	4.17
10775968	Alb	Albumin	1.85	0.49
10744425	Alox15	arachidonate 15-lipoxygenase	2.83	n.s.
10736712	Ccl12	chemokine (C-C motif) ligand 12	1.55	1.60
10745677	Ccl3	chemokine (C-C motif) ligand 3	1.67	2.97
10736702	Ccl7	chemokine (C-C motif) ligand 7	1.76	10.83
10757632	Ccl24	chemokine (C-C motif) ligand 24	2.07	1.77
10921163	Ccr1	chemokine (C-C motif) receptor 1	1.90	4.29
10914614	Ccr2	chemokine (C-C motif) receptor 2	1.66	13.15
10775896	Cxcl2	chemokine (C-X-C motif) ligand 2	2.06	n.s.
10887947	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	0.62	6.77
10781404	Dok2	docking protein 2	1.51	1.89
10794734	F13a1	coagulation factor XIII, A1 polypeptide	1.86	1.99
10811126	Fa2h	fatty acid 2-hydroxylase	0.66	n.s.
10814286	Fabp4	fatty acid binding protein 4, adipocyte	1.67	4.40
10804127	Fgf1	fibroblast growth factor 1	0.63	1.73
10935811	Gabre	gamma-aminobutyric acid (GABA) A receptor, epsilon	0.64	2.22
10742820	Glra1	glycine receptor, alpha 1	0.49	5.47
10842657	Gnas	GNAS complex locus	0.65	1.83
10823412	Gpr149	G protein-coupled receptor 149	0.64	n.s.
10724315	Hbb	hemoglobin, beta	1.65	n.s.
10724319	Hbb-b1	hemoglobin, beta adult major chain	1.77	n.s.
10910473	Hcn4	hyperpolarization activated cyclic nucleotide-gated potassium channel 4	0.52	2.01
10918979	Htr1b	5-hydroxytryptamine (serotonin) receptor 1B, G protein-coupled	0.62	n.s.
10729791	Ifit1	interferon-induced protein with tetratricopeptide repeats 1	1.77	n.s.
10888131	Kcng3	potassium voltage-gated channel, subfamily G, member 3	0.56	n.s.
10760290	Nptx2	neuronal pentraxin II	0.59	n.s.
10815655	Npy2r	neuropeptide Y receptor Y2	0.57	3.42
10866030	Olr1	oxidized low density lipoprotein (lectin-like) receptor	1.55	11.06
10764551	Ptgs2	prostaglandin-endoperoxide synthase 2	0.63	2.60
10856474	Reg3b	regenerating islet-derived 3 beta	0.66	2.07
10824695	S100a9	S100 calcium binding protein A9	1.74	2.23
10765186	Sell	selectin L	1.66	4.04
10874981	Sulf1	sulfatase 1	0.66	1.84
10853683	Tac1	tachykinin, precursor 1	0.53	n.s.
10770577	Tgfb2	transforming growth factor, beta 2	0.60	1.98
7 days				
10729269	Anxa1	annexin A1	1.64	5.84
10843229	Cacna1b	calcium channel, voltage-dependent, N type, alpha 1B subunit	0.64	0.66
10746327	Cacna1g	calcium channel, voltage-dependent, T type, alpha 1G subunit	0.65	n.s.
10725051	Calca	calcitonin-related polypeptide alpha	1.72	n.s.
10907834	Casp12	caspase 12	1.58	2.31
10736697	Ccl2	chemokine (C-C motif) ligand 2	2.09	13.29
10745677	Ccl3	chemokine (C-C motif) ligand 3	1.55	2.77
10736863	Ccl4	chemokine (C-C motif) ligand 4	1.54	1.54
10736702	Ccl7	chemokine (C-C motif) ligand 7	2.47	1.59
10736712	Ccl12	chemokine (C-C motif) ligand 12	1.83	1.69
10914614	Ccr2	chemokine (C-C motif) receptor 2	1.88	4.33
10803991	Cd14	CD14 molecule	1.90	4.41
10853240	Cd36	CD36 molecule (thrombospondin receptor)	1.74	2.83
10923052	Col3a1	collagen, type III, alpha 1	1.59	2.90
10887622	Crip1	cysteine-rich protein 1 (intestinal)	1.84	1.58
10771660	Cxcl9	chemokine (C-X-C motif) ligand 9	1.52	1.60
10771655	Cxcl10	chemokine (C-X-C motif) ligand 10	1.96	6.47
10771649	Cxcl11	chemokine (C-X-C motif) ligand 11	1.87	4.43
10845681	Fap	fibroblast activation protein, alpha	1.62	1.56

(*continued*)

Table 5. (Continued)

Affymetrix ID	Gene symbol	Genes	Simvastatin / Vehicle	Vehicle / Sham
10844005	Fcnb	ficolin B	1.58	2.72
10909874	Il18	interleukin 18	1.59	3.21
10834109	Il1rn	interleukin 1 receptor antagonist	1.68	2.39
10734952	Kcnab3	potassium voltage-gated channel, shaker-related subfamily, beta member 3	0.66	n.s.
10890609	Kcnh5	potassium voltage-gated channel, subfamily H (eag-related), member 5	0.54	n.s.
10904329	Kcnk9	potassium channel, subfamily K, member 9	0.66	0.66
10903585	Kcnv1	potassium channel, subfamily V, member 1	0.66	0.50
10841693	Lbp	lipopolysaccharide binding protein	1.93	2.84
10844331	Lcn2	lipocalin 2	1.83	5.81
10781304	Loxl2	lysyl oxidase-like 2	1.64	3.16
10912439	Rbp1	retinol binding protein 1, cellular	1.52	2.92
10817053	S100a3	S100 calcium binding protein A3	1.99	2.76
10817057	S100a4	S100 calcium-binding protein A4	1.69	4.37
10817065	S100a6	S100 calcium binding protein A6	1.54	2.27
10765186	Sell	selectin L	1.73	2.02
10860481	Sema3a	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	0.64	0.66
10886621	Serpina3n	serine (or cysteine) peptidase inhibitor, clade A, member 3N	2.16	4.01
10761047	Serpine1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	3.05	2.29
10852595	Sox18	SRY (sex determining region Y)-box 18	1.50	n.s.
10934865	Srpx2	sushi-repeat-containing protein, X-linked 2	1.55	n.s.
10874981	Sulf1	sulfatase 1	1.53	n.s.
10797138	Tgfbi	transforming growth factor, beta induced	1.63	2.25

targeted concentrations. Simvastatin is not available in a parenteral formulation and would need to be administered by nasogastric tube clinically, similar to the oral gavage used in this study.

Assuming that the primary effect of minocycline and simvastatin is on attenuating the TBI-induced inflammatory effect, a 72 h treatment duration was chosen. Experimental studies have demonstrated both a neurotoxic as well as neuroprotective function of the inflammatory response.[6] The response is pro-inflammatory during the acute phase and anti-inflammatory during the chronic phase, which theoretically assists in the repair and recovery processes.[37] The time-dependent dual neurotoxic and neuroprotective effects of the inflammatory response after TBI suggest that the time of initiation and the duration of anti-inflammatory treatment will be essential factors in optimizing therapy. Therefore, a short duration was chosen to target the acute inflammation without reducing the beneficial effects on recovery.[6]

Transcriptome profiling can be used to evaluate the potential effect of treatment, in addition to increasing the understanding of the mechanism of the treatment effect. Injured rats were compared with sham rats who had undergone anesthesia but no craniotomy to provide an adequate control for the effects of isoflurane anesthesia on gene expression, because it has previously been shown to increase some pro-inflammatory cytokines in the blood.[38] Only a time point of 72 h after surgery, however, was used for the sham rats, which may slightly limit the interpretation at the 24 h and 7 day intervals. Minocycline significantly decreased lesion size, although the effect was significantly less than we found with nicotinamide and progesterone in the same CCI model.[28] The behavioral outcomes from minocycline and simvastatin showed even less promise. Both drugs improved performance on the locomotor placing task, a measure of fine motor control. Neither drug improved performance on the rotarod (gross locomotor) or the Morris water maze (cognition). This suggests that while there is some benefit to these drugs, there may also be considerable limits in terms of behavioral improvement.

Inflammatory pathways were affected by minocycline only at the 72 h time point. Minocycline decreased the expression of several inflammatory genes, including chemokines and several Mmps. Mmps are increased in TBI and may play a key role in TBIs by degrading components of the basal lamina leading to disruption of the blood–brain barrier.[39,40] Specifically, in our CCI model, minocycline decreased the expression of Mmp9 at 72 h post-CCI. In an exploratory clinical trial of minocycline in stroke, minocycline treatment also resulted in decreased serum Mmp9 when measured 72 h after the stroke.[41]

The majority of the previous work evaluating minocycline in TBI used a close head injury mouse model with 45 mg/kg or 90 mg/kg doses administered i.p. 30 min after injury. Minocycline showed improvement in reducing locomotor hyperactivity, modest gains in rotarod performance, and transient improvement on beam-walking in mice after TBI.[9–11,13–16] In the mouse closed head injury model, minocycline 45 mg/kg injected i.p. 30 min post-injury and every 12 h thereafter resulted in differentially expressed Gene Ontology groups involving chemokines, signal transduction, neuronal growth factors ,and pro-inflammatory cytokines when evaluated 2, 6, and 24 h post-injury.[42] The authors noted a concern about the suitability of minocycline for neuroprotection because minocycline also upregulated several genes involved in the cell death pathway.

In a CCI rat model, minocycline 50 mg/kg i.p. administered 1 h after injury and then daily for 2 days resulted in a modest improvement of active avoidance and had no effect on myelin loss or preventing cognitive defects.[12] Therefore, despite considerable enthusiasm for minocycline as a neuroprotectant in TBI, the majority of the positive pre-clinical studies have used doses initiated immediately after injury. Initiating therapy within 30 minutes of a TBI is not clinically feasible; therefore, minocycline would not provide a practical treatment. A recent study in a mild-blast induced TBI rat model did find that minocycline 50 mg/kg i.p. administered

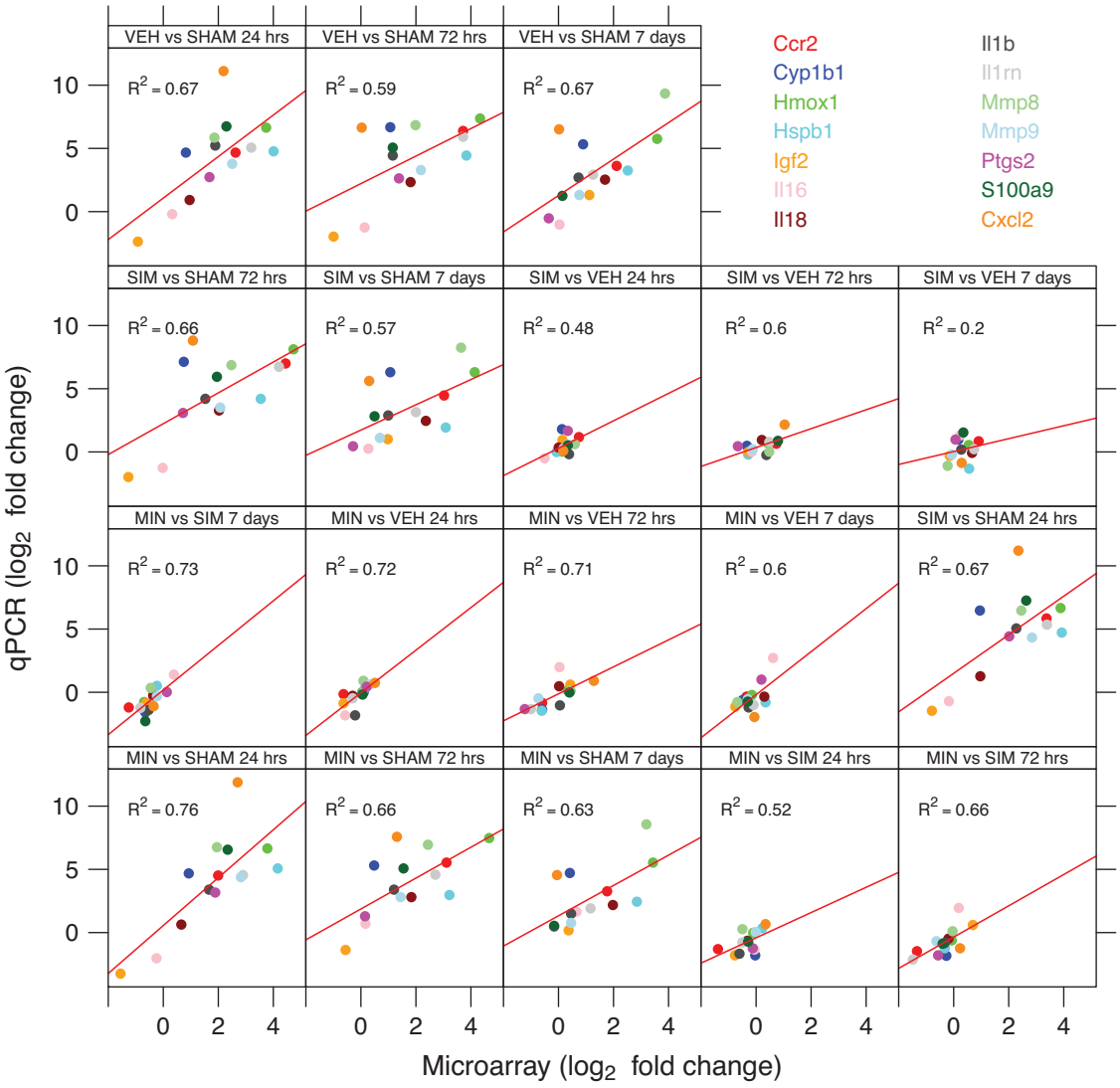

FIG. 5. TaqMan based RT-PCR validation of the microarray data for the selected genes: Ccr2 (chemokine (C-C) receptor 2), CxCl2 (chemokine [C-X-C motif], ligand 2), Cyp1b1 (cytochrome P450 1b1), Hmox1 (hemeoxygenase 1), Hspb1 (heat shock protein b1), IGF2 (insulin like growth factor 2), Il1b (interleukin 1 beta), Il16 (interleukin 16), Il18 (interleukin 18), IL1rn (interleukin 1 receptor antagonist), Mmp8 (matrix metallopeptidase 8), Mmp9 (matrix metallopeptidase 9), Ptgs2 (prostaglandin-endoperoxide synthase 2), S100a9 (S100 calcium binding protein A9. The RT-PCR data shown in the figure were normalized to the housekeeping gene -actin.

4 h after injury and then daily for 4 days prevented the development of neurobehavioral abnormalities and normalized serum levels of inflammatory biomarkers.[43]

One factor that is important to note in comparing this current study to previous studies is that the CCI model is more severe than the weight-drop model used in many of the mouse studies and the mild-blast TBI rat model. In addition, surgery in the present study was also performed with 100% O_2 delivered by nose cone, which is common in the field, and may have increased oxidative damage.

Simvastatin has been used in a range of doses from 0.5 mg/kg to 100 mg/kg, with beneficial effects found across the spectrum. Chen and colleagues[44] evaluated simvastatin doses ranging from 25 mg/kg to 100 mg/kg administered orally at 1 and 6 h post-TBI and determined that the 37.5 mg/kg dose was the most effective at reducing post-traumatic neurological and motor coordination dysfunction and edema. The majority of the experimental TBI studies have evaluated doses of 0.5 to 1.0 mg/kg/day initiated from 1 to 24 h post-injury. Mahmood and associates[45] demonstrated that both the 0.5 and 1.0 mg/kg/day doses administered orally for 14 days starting 1 day post-TBI improved functional outcome. Only the 1 mg/kg dose increased the density of neurons in the hippocampus. In a series of studies evaluating the 1 mg/kg daily for 14 days, the same investigators found beneficial effects on reduction of IL-expression,[46] angiogensis,[47,48] improved spatial learning,[47] reduction of neuronal loss,[47] and stimulated neurite outgrowth.[49]

Our pharmacokinetic studies found that 10 mg/kg every 12 h provided HMG-reductase inhibition activity in the therapeutic range based on pravastatin equivalence. Using HMG-reductase inhibition activity as a measure of simvastatin activity assumes that the neuroprotective effects of simvastatin are correlated with the effects on HMG-reductase, which is a limitation. Our data suggest that the doses needed for HMG-reductase inhibition may be significantly higher than those optimized for neuroprotection. We only evaluated one dosage regiment of simvastatin. Administering a lower dose,

initiated at a later time, and/or increasing our duration of treatment may have improved our functional and histological outcomes.

There is evidence for a U-shaped dose-effect relationship (i.e., decreasing effect at higher doses) in TBI for other neuroprotective agents, including rosuvastatin,[50] progesterone,[51–53] and vitamin D.[54] In a TBI murine model, rosuvastatin 1 mg/kg daily for 5 days was effective at reducing neuronal degeneration at 24 h, reducing microgliosis at 7 days post-TBI, and preserving neuronal density at 35 days post-injury.[50] A higher dose (5 mg/kg) was ineffective. Therefore, the lack of a significant neuroprotective effect may be because of the high dose of simvastatin or the short duration of treatment.

Both nicotinamide and progesterone demonstrated significant effects on functional recovery of cognition and motor behavior when administered for 72 h in the same CCI model.[33,51] Nicotinamide treatment counteracted the changes in genes differentially expressed from TBI,[33] while progesterone treatment primarily altered expression of genes not affected directly by TBI itself.[51] In contrast, simvastatin significantly increased the expression of inflammatory genes that TBI also increased when administered for 72 h. The gene expression data suggest that simvastatin may have increased the inflammatory response by increasing chemokines, S100a9, and arachidonate 15-lipoxygenase (Alox15) at 24 h. Specifically, Alox15 is considered to have a pro-inflammatory effect and may play a key role in the acute inflammatory response as it reportedly leads to the generation of unstable lipid products from arachidonate.[55] The S100 binding proteins are involved in a variety of intracellular calcium dependent functions including cell motility and apoptosis. S100a9 regulates vascular inflammation and contributes to vascular injury by increasing leukocyte recruitment.[58] In patients with moderate to severe TBI, increased S-100 concentrations are associated with unfavorable outcomes.[58]

Conclusion

A major limitation of concentration target dosing based on non-TBI approved indications is the assumption that the neuroprotection effect of the drug occurs at the same concentrations attained in the non-TBI indication. The targeted concentrations will provide information regarding the maximum concentration or dose for drugs with narrow therapeutic ranges but may not be an effective study design for drugs with U-shape pharmacology. In this study, minocycline and simvastatin initiated at dosage regimens designed to produce concentrations in the range reported in patients receiving FDA approved doses did result in significant effects on gene expression in the brain reflecting adequate penetration; however, the dose regimens were only sufficient to produce mild functional neurorestorative effects.

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Author Disclosure Statement

No competing financial interests exist.

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