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Transcriptional re-programming in rat central nervous system two weeks after burn trauma: the impact of nephrilin treatment on the expression of oxidative stress-related genes

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Abstract

Introduction: Survivors of severe burns suffer lifetime neuroinflammatory consequences manifested by higher incidence of major depression and neurodegenerative disease. In a scald model, nephrilin peptide has previously been shown to protect rats from loss of lean body mass, kidney function and glycaemic control, complications that have also been shown to endure in burn patient populations. Nephrilin's mechanism of action has been suggested to involve protection from excessive oxidative stress.

Methods: Using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) amplification of transcripts in total RNA extracted from dorsal root ganglia of male rats 14 days after exposure to thermal insult, we query the relative levels of expression of 34 genes believed to be associated with oxidative stress biology in the central nervous system (CNS). We use these data to explore the central role of oxidative stress in astrogliosis, immunosuppression and mitochondrial homeostasis.

Results and Discussion: Rats that received nephrilin treatment (4 mg/kg by subcutaneous bolus injection once daily for seven days after scald injury) showed significantly reduced elevations in gene expression of some key genes such as NOX2, GFAP, AQP4 and RAC1, but not of others such as NOX4, STEAP4, ARG1 and CCL2.

Conclusion: The implications of these data with reference to nephrilin's potential clinical utility for mitigating the enduring effects of burn trauma on the CNS are discussed. Nephrilin reduces the expression of some genes implicated in neurodegeneration after burn insult.

Lay Summary

Nephrilin peptide is a novel treatment for short- and long-term systemic effects of burn trauma. This study measures the capability of nephrilin to address post-traumatic neurodegenerative disease by looking at the expression of genes in the central nervous system, in a rat scald model. Nephrilin appears to have beneficial effects by reducing the expression of some key genes known to be relevant in neurodegenerative processes, but not others.

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Keywords
Nephrilin, systemic burn injury, neurodegeneration, quantitative reverse transcription polymerase chain reaction, oxidative stress, astrocyte, immunosuppression, mitochondrial homeostasis

Introduction

Nephrilin peptide is an inhibitor of Rictor complex that is actively transported into cells *in vivo*.[1] Nephrilin peptide has been used to reverse the systemic effects of traumatic, metabolic and xenobiotic stress in a number of rodent models.[2] In a well-characterised rat scald model, we previously demonstrated the pleotropic effects of nephrilin peptide in combating post-burn systemic neuroinflammation, loss of glycaemic control, lean body mass and kidney function, and impaired wound healing. These short-term effects mirror more enduring consequences of thermal injury in survivors of severe burns.[3,4] Burn injury has been implicated in the development of long-term neurodegenerative conditions such as severe depression.[5,6] In this study, we set out to look at the role of oxidative stress in nephrilin peptide's protective mechanisms post-burn in the central nervous system (CNS) of rats. Nephrilin's mechanism of action has previously been shown to involve protection from excessive oxidative stress in kidney.[7]

In this study, using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) amplification of transcripts in total RNA extracted from dorsal root ganglia (DRG) of male rats 14 days after exposure to thermal insult, we query the relative levels of expression of 34 genes believed to be associated with oxidative stress biology in the CNS, with an emphasis on astrogliosis, immunosuppression and mitochondrial homeostasis. These phenomena appear to be inextricably linked to one another, and to CNS oxidative stress post-traumatic insult in a variety of contexts.[8,9] Astrocytes are the most abundant cell type in the CNS. Activation of astrocytes and microglia is a key early step in the propagation of the defensive immune response from the CNS after insult.[8] Upon traumatic injury, activated astrocytes participate in the protection of neural cells from excessive oxidative stress, but astrogliosis can proceed to pathology.[9] Furthermore, immunosuppression mediated by myeloid-derived suppressor cells (MDSC)—a major complication of traumatic insult—is enhanced by oxidative stress.[10] The context in which all these related dysfunctions occur is, in turn, believed to be inextricably linked to mitochondrial homeostasis.[11] In this study, I analysed the expression of 34 genes selected based on their implication in the above processes, as described in the scientific literature.[8,9] The analysis was performed with qRT-PCR on RNA templates extracted from DRG of male rats 14 days after exposure to thermal insult.

Materials and methods

Nephrilin peptide

Nephrilin peptide, a 40-mer peptide carrying a sequence derived from PRR5/Protor (the sequence is conserved in human, rat and mouse species) was synthesised by Lifetein LLC (Hillsborough, NJ, USA) and purified to > 80% purity by HPLC. The design and synthesis of nephrilin have been previously described.[1]

Dissection of dorsal root ganglia and extraction of RNA

In a subset analysis of a previously reported burn study,[4] DRG were dissected from male adult Sprague Dawley rats (250–300 gm, Charles River Laboratories, Wilmington, MA, USA) using a procedure previously reported.[3] As tissue was not available from all animals, DRG from three randomly selected animals in each treatment group (sham, burn + vehicle, burn + nephrilin 4 mg/kg/day) were pooled for further analysis by qRT-PCR. Experimental outcomes from the original study[4] were re-calculated for this subset and are shown in Table 1. Total RNA was extracted from each pool using the RNeasy Midi Kit (Qiagen, Germantown, MD, USA). Yield was ~30 ug RNA per pool and A260/A280 ratio was in the range of 1.87–2.04 in all cases. The high quality of each RNA was further confirmed by electrophoresis (using Eukaryote Total RNA Nano).

Quantitative reverse transcriptase polymerase chain reaction

RNAs were diluted in RNase/DNase free water and aliquoted into wells in triplicate. Approximately 100 ng of RNA was used per well. A one-step qPCR method was performed using Luna Universal One-Step RT-qPCR kit (New England Biolabs, Ipswich, MA, USA) containing reverse

Table 1. Clinically relevant variables in the three treatment groups of the study.

	Sham	Burn + vehicle	Burn + nephrilin
Lean body mass (DEXA)	343.6 ± 15.7*	304.9 ± 9.2	328.5 ± 5.1*
Glycaemic control (GTT AUC mg.dL.h)	44.7 ± 19.0†	117.0 ± 19.1	69.0 ± 18.1*
Kidney function (eGFR, calculated)	1.21 ± 0.23*	0.58 ± 0.18	1.29 ± 0.32*
Urinary 8-isoprostate (ng/pg cystatin)	5.02 ± 2.91†	26.44 ± 1.60	4.53 ± 1.21†

*$p<0.05$, † $p<0.01$ versus Burn+Vehicle group
DEXA, dual-energy X-ray absorptiometry; eGFR, estimated glomerular filtration rate; GTT AUC, glucose tolerance test, area under the curve.

transcriptase enzyme mix. Primer pairs for each gene were synthesised for the SYBR assay. Primer sequences are listed in Table 2. The following standard qPCR cycling conditions were used: 55 °C for 10' (for RT), 95 °C for 1' followed by 40 cycles at 95 °C for 10 s, 58 °C for 30 s. Background was set at 3–10 cycles and the threshold was set at 0.02 for all runs. Ct values were collected and analysed using the 'delta-delta Ct' method. All samples amplified well within acceptable Ct range. Expression of each gene was normalised to GAPDH, a house-keeping gene. Comparisons between treatment groups in each case were done by setting the sham group value to 1.

Statistical analysis

Data are presented as mean ± SD unless otherwise indicated. Probability values (*P* values) were computed using Student's *t*-test and expressed as relative values to sham or saline-treated group.

Results

Astrocyte activation

After CNS injury, formation of an astrocytic scar adjacent to the 'lesion' is a characteristic histopathologic feature that can be demonstrated by immunohistochemistry with primary antibodies against glial fibrillary acidic protein (GFAP) or connective tissue growth factor (CTGF).[9,12–14] Aquaporin-4 (AQP4), a membrane-bound protein that regulates water permeability is expressed in the endfeet of astrocytes in the CNS. Recently, AQP4 has been extensively examined for its role as a neuroimmunological inducer.[15] After insult, circulating monocytes and lymphocytes are known to enter the CNS. Peptidase Pi16 is a master regulator of T-cell subsets, a key function in the adaptive immune response in all tissues.[16]

Figure 1a shows the results obtained by RT-PCR of GFAP, CTGF, AQP4 and Pi16 genes using RNA extracted from CNS of scalded rats (with or without nephrilin treatment), with sham-treated rats as a control. The results show that burn injury causes significant increase in the expression of all four genes; nephrilin treatment significantly reduces those elevations.

MDSC-mediated immunosuppression

The co-induction of chronic low-grade inflammation and MDSC-mediated immunosuppression is a hallmark of post-traumatic responses, aging and neurodegenerative disease.[17–19] MDSC-mediated immunosuppression is dependent upon oxidative stress[10] and is brought about by the interplay of a number of genes. NADPH oxidase 2 (NOX2) and neuropeptide Y (NPY) are both key players in this interplay and in the effects of oxidative stress in the CNS.[20–22] S100A9 production is dependent upon reactive oxygen species (ROS) and is specifically exported by exosomes produced by MDSC in the inflammatory environment,[23,24] while galanin (GAL) protects rat astrocytes from oxidative stress.[25]

Figure 1b shows the results obtained by RT-PCR of GAL, NPY and S100A9 expression in the CNS of scalded rats (with or without nephrilin treatment) and sham-treated rats. The results indicate that burn insult causes significant increase in the expression of all three genes, and nephrilin treatment significantly reduces those elevations.

Surprisingly, Figure 1c shows no change in the expression of NRF2, a master regulator of the response to oxidative stress in other environments,[26] but significant elevation of transcript levels for SLC7A11, ARG1, CD63 and CCL2. However, nephrilin treatment did not affect the elevation of these transcripts. SLC7A11, which codes for the

Table 2. Genes and primers used in the study.

RefSeqID	Description	Gene Symbol	Amplicon bp		Primer sequence
NM_134366	Ras-related C3 botulinum toxin substrate 1	RAC1	111	Fwd	TGCCTGCTCATCAGTTACACG
				Rev	GCCCAGATTCACTGGTTTTCCA
NM_023965	NADPH oxidase 2	NOX2	121	Fwd	TCTTTGTCATTCTGGTGTGGTTGG
				Rev	AGAGCCAGTGCTGACCCAA
NM_017316	Solute carrier family 23 (nucleobase transporters), member 2	SLC23A2	136	Fwd	TCCCGGTGGTGATCAATGGT
				Rev	CAGTGCTGTCCAGGGTCTCT
NM_057194	Phospholipid scramblase 1	PLSCR1	199	Fwd	CTTCTGGAAGTCTTAACAGGCTTTGA
				Rev	TGCATCTCAGGGGTCTCTCCA
NM_031530	Chemokine (C-C motif) ligand 2	CCL2	152	Fwd	GCCAACTCTCACTGAAGCCAG
				Rev	TGAGTAGCAGCAGGTGAGTGG
NM_134372	Aminocarboxymuconate semialdehyde decarboxylase	ACMSD	118	Fwd	AGCAAGGCAAGGGAGAAGCA
				Rev	ACTGTCACTCCTTTCTGGTTCATTTC
NM_017073	Glutamate-ammonia ligase (glutamine synthetase)	GLUL	153	Fwd	CATGTATATCTGGGTTGATGGTACCG
				Rev	GGAGGTACATGTCGCTGTTGG
NM_031347	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	PPARGC1A	151	Fwd	GTCTCTACTTAAGAAGCTCTTACTGGC
				Rev	ATTGCTCCATGAATTCTCGGTCTT
NM_001105919	Fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae)	FIS1	94	Fwd	GAGGAGCTGTTGCCCAAAGG
				Rev	CCTTTTCATATTCCTTGAGCCGGT
NM_130894	Mitofusin 2	MFN2	193	Fwd	TGGGGGCCTACATCCAAGAG
				Rev	CTCTTCCCATTGCTCGTCCG
NM_001106694	PTEN induced putative kinase 1	PINK1	149	Fwd	CCTGTCAGGAGATCCAGGCAA
				Rev	GGCTTCATACACAGCGGCA
NM_053517	SHC (Src homology 2 domain containing) transforming protein 1	SHC1	135	Fwd	GACTCAGGTCACCAGGGAGG
				Rev	CCAGCAAACTTCAGGTTACTCCT
NM_031326	Transcription factor A, mitochondrial	TFAM	211	Fwd	AGAAACCTATGAGTTCATACCTTCGATT
				Rev	AGCTGCTCTTTATACTTGCTCACAG
NM_001044265	STEAP family member 4	STEAP4	119	Fwd	CTGGGCTCTCCAGTCAGGAA
				Rev	CCAGTGGAGTGAGCCCAAGA
NM_019354	Uncoupling protein 2 (mitochondrial, proton carrier)	UCP2	165	Fwd	CAGAGCACTGTCGAAGCCTAC
				Rev	TGTCATGAGGTTGGCTTTCAGG

(Continued)

Table 2. (Continued)

RefSeqID	Description	Gene Symbol	Amplicon bp		Primer sequence
NM_017312	BCL2-related ovarian killer	BOK	151	Fwd	CGCTTGGGAGATGAGCTGGA
				Rev	TGCCCCATGTGATACCTGCT
NM_001142366	Aquaporin 4	AQP4	178	Fwd	CACCACGGTTCATGGAAACCTC
				Rev	ATTGATTGCAAACAAATGTCCAATTGC
NM_019204	Beta-site APP cleaving enzyme 1	BACE1	222	Fwd	GGAGATGGTGGACAACCTGAGG
				Rev	CCCTGGGTGTAGGGCACATAC
NM_017125	Cd63 molecule	CD63	97	Fwd	GTCTCATGATTACATTTGCCATCTTCC
				Rev	GACTTCACCTGGTCTCTAAACACATAG
NM_013064	Hypocretin (orexin) receptor 1	HCRTR1	231	Fwd	TYCTCATAGCCTTGGTGGGCAA
				Rev	CTGCCACTGACACCGACAC
NM_134363	Solute carrier family 12 member 5	SLC12A5	204	Fwd	CCTGTTTGAGGAGGAGATGGACA
				Rev	ACACCAAAGATGTTCTGCAGGC
NM_031798	Solute carrier family 12 member 2	SLC12A2	132	Fwd	CGATGAGCTGGAAAAGGAACCT
				Rev	ACACCCTTGATCCAGCCAAAC
NM_031789	Nuclear factor, erythroid 2-like 2	NRF2	116	Fwd	CTACTCCCAGGTTGCCCACA
				Rev	TATCCAGGGCAAGCGACTCA
NM_031588	Neuregulin 1	NRG1	154	Fwd	ACTGGGACCAGCCATCTCAT
				Rev	CGTAGTTTTGGCAACGATCACC
NM_001197332	Oxidation resistance 1	OXR1	170	Fwd	ACCCCAGTGAACTCTTACTGCC
				Rev	CACCATCAGCACCGGAGTGT
NM_001170481	Peptidase inhibitor 16	Pi16	111	Fwd	ACTACACTCAGGTAGTGTGGAGCA
				Rev	AGTTGCACACCAGCAAATGGATG
NM_001107673	Solute carrier family 7 (cationic amino acid transporter), member 11	SLC7A11	137	Fwd	CTGGAGTTATACAGCTAATTAAAGGGCA
				Rev	GTTGAGGTAAAACCAGCCAGCA
NM_053524	NADPH oxidase 4	NOX4	110	Fwd	GGATCACAGAAGGTCCCTAGCA
				Rev	GCTACATGCACACCTGAGAAAATAC
NM_022266	Connective tissue growth factor	CTGF	138	Fwd	CAAGCAGCTGGGAGAACTGT
				Rev	CCACCGAAGACACAGGGTG
NM_053587	S100 calcium binding protein A9	S100A9	136	Fwd	TGGACATCCTGACACCCTGA
				Rev	GTCCTGGTTTGTGTCCAGGTC
NM_017009	Glial fibrillary acidic protein	GFAP	150	Fwd	CCTGAGGCAGAAGCTCCAAG
				Rev	AAGAACTGGATCTCCTCCTCCAG

(Continued)

Table 2. (Continued)

RefSeqID	Description	Gene Symbol	Amplicon bp		Primer sequence
NM_033237	Galanin prepropeptide	GALANIN	154	Fwd	CCACATGCCATTGACAACCAC
				Rev	TGAGAAACTCCATTATAGTGCGGAC
NM_012614	Neuropeptide Y	NPY	111	Fwd	CATCACCAGACAGAGATATGGCAAG
				Rev	GAAGGGTCTTCAAGCCTTGTTCT
NM_017134	Arginase	ARG1	135	Fwd	TGCTGGGTGGAGACCACAG
				Rev	GCAGATTCCCAGAGCTGGTTG
NM_017008	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	138	Fwd	CTGAGTATGTCGTGGAGTCTACTGG
				Rev	CGTGGTTCACACCCATCACA

cystine/glutamate antiporter, modulates T-cell activation by depleting local cysteine.[27,28] ARG1, an archetypal marker of immunosuppression, helps MDSCs deplete local arginine,[29] while CD63 is a marker for exosome production (see S100A9, above). C-C motif chemokine ligand 2 (CCL2) is a powerful recruiter of MDSCs.[30]

Mitochondrial homeostasis

Dysfunction in genes key to mitochondrial homeostasis, such as TFAM,[31] UCP2,[32] NOX2 and NOX4,[33] HCRTR1,[34] OXR1,[35] BOK[36] and RAC1[37] result in profound disruption of mitochondrial dynamics, oxidative stress and, in the context of the CNS, severe gliosis. Figure 1d shows the results obtained by RT-PCR of TFAM, UCP2, BOK, RAC1, NOX2, NOX4, OXR1 and HCRTR1 genes. The results demonstrate that burn injury causes significant increases in the expression of all genes except OXR1, which remained unchanged, and nephrilin treatment significantly reduces transcript elevations for all remaining genes except NOX4.

Several additional genes induced by burn injury and known to play key roles in mitochondrial biogenesis, function, mitophagy, fission and fusion were not affected by nephrilin treatment (Figure 1e): STEAP4,[38] SHC1,[39] GLUL,[40] SLC23A2,[41] PINK1[42] and MFN2.[43] Expression of ACMSD,[44] PPARGC1A[45] and FIS1[46] genes was not changed by burn injury or nephrilin treatment.

Downstream effects of oxidative stress

Indirect effects of oxidative stress can provide clues to possible regulatory pathways for nephrilin's protective effects in the CNS. Two cases (two transcripts each) were examined:

PLSCR1 and BACE1 are physically and functionally associated products in the CNS. BACE-1 is associated with elevated oxidative-stress and CNS pathologies such as Alzheimer's.[47,48] Figure 1G shows that PLSCR1 and BACE1 are similarly elevated by burn injury. Nephrilin treatment does not seem to affect these levels.

Another case of possible downstream regulons was examined by measuring transcripts for potassium/chloride channel carriers SLC12A5 and SLC12A2. Both gene products are coordinately upregulated by OSR1 kinase, which responds to oxidative stress.[49] The results in Figure 1g show that burn injury causes significant increase in the expression of these two genes, but nephrilin treatment does not have a significant effect on the levels of these elevated transcripts.

Discussion

In rodent models of stress, nephrilin peptide has been shown to reverse elevations in neuroimmune and oxidative stress consequent to thermal, metabolic and xenobiotic insult.[1–4,7] In particular, nephrilin's efficacy in reversing the systemic effects of sepsis and burn trauma, including inflammation, catabolism and loss of kidney function[3,4] suggests a mechanistic link to the body's immunological response to stress challenge.

In this study, the focus was on the effects of burn injury on gene expression in the CNS, with an emphasis on the inter-related phenomena of astrogliosis, oxidative stress, immunosuppression and mitochondrial homeostasis. In each category, a number of gene transcripts were carefully quantified using qRT-PCR. In general, in each category, nephrilin peptide reverses burn-induced

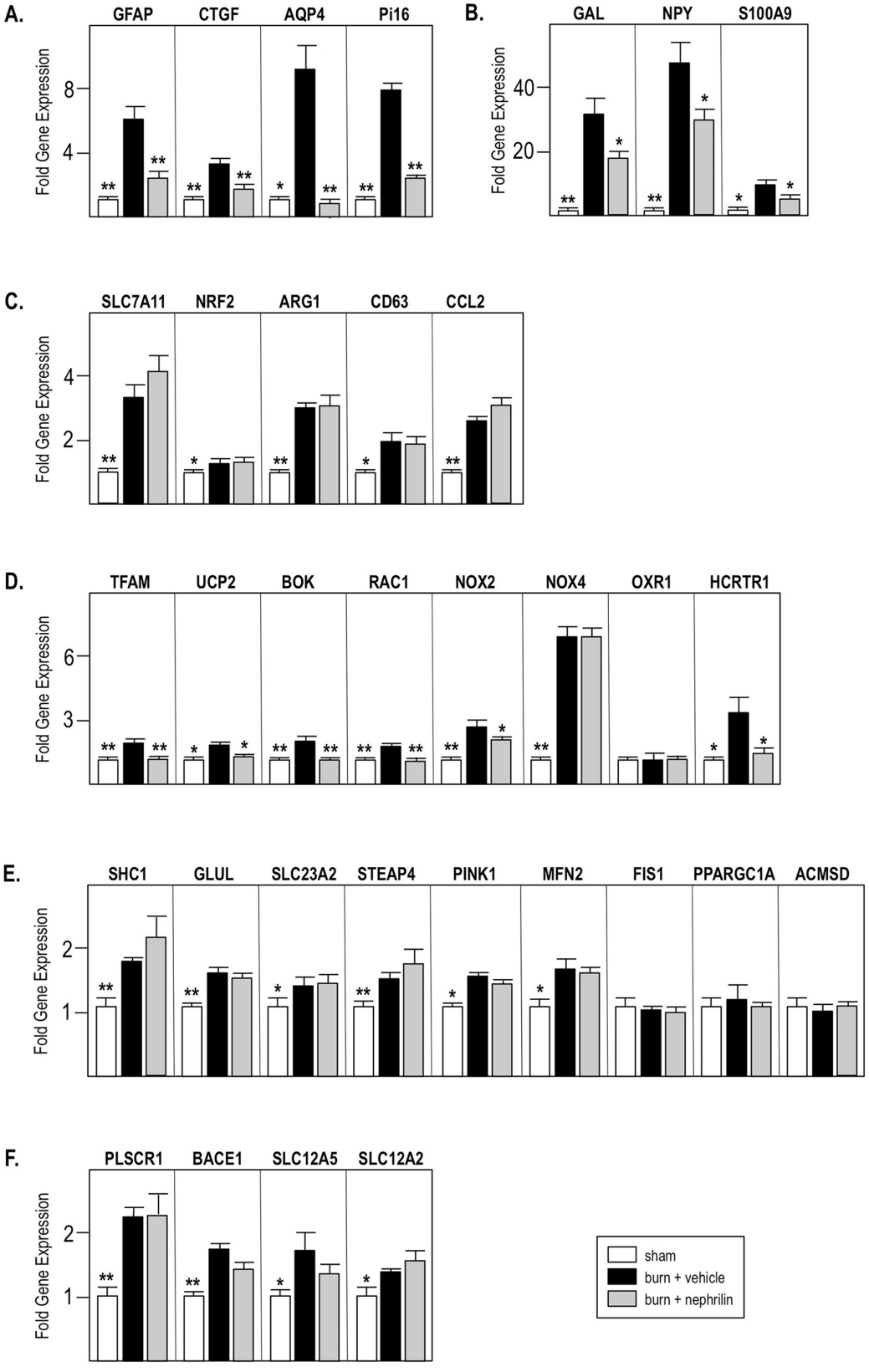

Figure 1. Relative gene expression in rat CNS 14 days after scald injury was determined by qRT-PCR as described in the 'Material and methods' section. Sham values (white bars) are set to = 1. Black bars are scald + vehicle, grey bars are scald + nephrilin. *$P < 0.05$, **$P < 0.01$ versus scald + vehicle group. CNS, central nervous system; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

transcript elevation for some key genes but not others. Additional studies will be needed to uncover the underlying mechanisms that may explain this observed dichotomy.

Based on these data, it does seem clear that nephrilin treatment might be expected to ameliorate some of the long-term consequences of burn trauma on the CNS. Whether the pathways affected by nephrilin are instrumental in changing quality-of-life outcomes, especially those relating to depression and dementia, remains to be established.

Some genes whose transcripts were elevated by burn injury in this study have been shown to cluster into well-characterised regulons. The TLR4/MyD88 regulon is known to regulate production of ROS, immunosuppression and chronic inflammation in response to lipopolysaccharide challenge via NOX4, ARG1 and CCL2,[50–52] three genes whose burn trauma-induced elevations were not reversed by nephrilin in the current study. One possible line of investigation suggested by this result is co-treatment of thermal insult with both nephrilin and a modifier of TLR4 signalling such as fenofibric acid.[53] Co-treatment concepts may also include the use of valproic acid, which not only downregulates TLR4-mediated inflammation[55] but also downregulates gliosis[56] and the immunosupressive function of MDSCs.[54] The use of anti-epileptic agents such as valproic acid as co-treatments with nephrilin makes additional sense, given the known link between trauma and seizure.[57]

In conclusion, these results show the efficacy of nephrilin treatment in mitigating the transcriptional effects of burn trauma in the CNS, but also raise intriguing questions for future study. Is it possible to modify the treatment regimen to include additional agents that address the areas that nephrilin cannot? Can this peptide be coupled to other moieties to further improve effective treatment of burn trauma? We intend to address these questions in future experiments.

Declaration of conflicting interests

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