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TNF α causes thrombin-dependent vagal neuron apoptosis in inflammatory bowel disease

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Abstract

Background—The role of peripheral tumor necrosis factor- α (TNF α) in inflammatory bowel disease (IBD) is well-established, but its central nervous system (CNS) effects are not understood. Thrombin, another mediator of inflammation in IBD, has been implicated in CNS vagal neuron apoptosis in the Dorsal Motor Nucleus of the Vagus (DMV). This study evaluates DMV TNF α exposure, characterizes effects of TNF α on DMV neurons, and identifies a relationship between DMV TNF α and thrombin in IBD.

Methods—2,4,6-trinitrobenzene sulfonic acid was administered via enema to induce colonic inflammation in rats. [TNF α] in serum, cerebrospinal fluid (CSF), and DMV tissues were determined by ELISA, and DMV TNF α expression by quantitative RT-PCR. TNF α was administered into the 4th intracerebral ventricle (4V) adjacent to the DMV, with and without blockade of TNF receptor I (TNFR1) and the thrombin receptor PAR1. Immunofluorescence was used to evaluate microglial activation (Cd11b) and prothrombin presence in DMV sections. Apoptosis was examined using TUNEL and activated caspase-3 immunofluorescence.

Results—IBD is associated with increased TNF α protein in serum, CSF, and DMV tissue; DMV TNF α transcription is also increased. TNF α (4V) caused a 54% increase in microglial activation, a 27% increase in DMV prothrombin protein, and a 31% increase in vagal neuron apoptosis by TUNEL. There was a 52% increase in activated caspase-3 immunofluorescence in TNF α -treated animals ($p < 0.05$). All effects of 4V TNF α were prevented by TNFR1 blockade. TNF α -induced apoptosis was prevented by PAR1 blockade.

Conclusions—IBD is associated with DMV exposure to TNF α , causing excess DMV prothrombin and vagal apoptosis.

Keywords

TNBS Colitis; TNF α ; Vagus

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Inflammatory bowel disease (IBD) has the potential to impact nearly every organ system, including the autonomic nervous system. Patients with IBD and autonomic dysfunction have increased health care resource utilization and diminished quality of life. The pathophysiology leading to autonomic dysfunction has not been fully elucidated.

The vagus nerve constitutes one of the main autonomic circuits regulating the gastrointestinal (GI) tract. Afferent vagal fibers transmit sensory information from the GI tract to the dorsal motor nucleus of the vagus (DMV) in the brainstem. The DMV houses efferent vagal neurons which project back to the GI tract. In IBD, thrombin mediates DMV neuronal apoptosis and impaired response to neurotransmitters, suggesting a mechanism for the development of autonomic dysfunction. The means by which IBD leads to increased DMV thrombin exposure is not known.

TNF α is an inflammatory cytokine with an established role in IBD and the capacity to impact the central nervous system (CNS). Patients with IBD have elevated concentrations of circulating TNF α as well as increased TNF α expression in inflamed intestinal mucosa(1). TNF α traverses the blood brain barrier and is produced locally within the brain(2, 3). Central nervous system (CNS) neurons and glia express TNF receptor I (TNFRI), and are known to be affected by TNF α in other states of neuroinflammation(3-6). The following studies link TNF α to DMV thrombin exposure, and establish a role for TNF α in the vagal neuronal apoptosis associated with IBD.

MATERIALS and METHODS

Chemicals and solutions

Neurobasal A medium, B27 supplement, penicillin/streptomycin, L-glutamine, trypsin type I, fibroblast growth factor, fura-2-AM, trizol reagent, goat serum, and ProLong anti-fade with DAPI were purchased from Life Technologies, formerly Invitrogen (Carlsbad, CA). Recombinant DNase I and the Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) in situ Cell Death Detection Kit, TMR red, were obtained from Roche Diagnostics (Indianapolis, IN). Triton X-100 and recombinant rat TNF α were obtained from ThermoFischer (Waltham, MA). The small molecule PAR-1 inhibitor FR171113 was purchased from Tocris Bioscience (Ellisville, MO). TNF α ELISA was accomplished using a rat TNF α DuoSet ELISA kit and reagents from R&D Systems (Minneapolis, MN). 5-thio-D-glucose was purchased from MP Biomedicals, LLC (Solon, OH). 2,4,6-trinitrobenzenesulfonic acid (TNBS) and the small molecule TNF α inhibitor, SPD304, were obtained from Sigma-Aldrich (St. Louis, MO). Primary antibodies used in these studies include mouse anti-Hu from Invitrogen (Carlsbad, CA), mouse anti-Cd11b, clone OX-42 from AbD Serotec (Raleigh, NC), rabbit anti-prothrombin from Abbiotec, LLC (San Diego, CA). FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG obtained from Jackson ImmunoResearch (West Grove, PA) were used as secondary antibodies. Intracerebroventricular cannulas were purchased from and cut to 7.4mm by Plastics One (Roanoke, VA).

Animal Care

All animal studies were approved by the University of Michigan Committee on the Use and Care of Animals. Animals were housed in a temperature controlled environment with 12-hr light and dark cycles, and access to food and water ad libitum.

IBD Model

Male Sprague-Dawley rats weighing 220-250g were briefly anesthetized with isoflurane. To induce intestinal inflammation, a 0.5mL enema containing 30mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in 50% ethanol was administered. Control animals received a 0.5mL enema of 0.9% saline. Weight and food intake were monitored daily; animals were sacrificed humanely on post-treatment day 4. Rats were first deeply anesthetized. Where indicated, whole blood was collected from the orbital sinus and allowed to clot. It was then centrifuged at 4° for 20minutes at approximately 1500G and the serum preserved at -80° until use. Cerebrospinal fluid (CSF) was collected from the cisterna magna as previously described(7), then stored at -80°. For all other cases, rats were deeply anesthetized prior to intra-cardiac perfusion with PBS. For collection of fresh DMV tissue, anesthetized rats were decapitated, their brains removed and quickly frozen, then stored at -80° until use. Those animals whose brains were intended for immunofluorescence were also perfused with 4% paraformaldehyde. Brains were subsequently removed, post-fixed in 4% paraformaldehyde overnight, transferred to 20% sucrose in PBS for 5 days, and finally embedded in 20% sucrose and OCT. Tissue blocks were sectioned in 10 micron slices through the DMV using a cryostat. Sections were mounted such that each slide contained samples from each treatment group.

Quantitating TNF α

ELISA for rat TNF α was performed according to the manufacturer's instructions, with all samples run in duplicate. Samples included rat serum and CSF collected as described above, as well as protein extract from DMV tissue. Rat brains stored at 80° were transferred to dry ice and placed into a metal form (Braintree Scientific, Braintree, MA) where 1 millimeter sections of medulla were created. The DMV was identified directly ventral to the nucleus of the solitary tract, and dorsal to the hypoglossal nucleus. This area was excised with a tissue punch, and total protein extracted using triton X-100.

TNF α production in rat DMV tissue was also evaluated with quantitative RT-PCR. DMV tissue samples obtained as for protein extraction were instead homogenized in Trizol reagent, and total RNA was isolated by phase separation and precipitation. RNA was then converted to cDNA with reverse transcriptase. PCR primers and probe were developed using published sequences for the rat TNF α gene. Forward: 5' – CTT CTC ATT CCT GCT CGT GG – 3', reverse: 5' – TGA TCT GAG TGT GAG GGT CTG – 3', and probe: 5' – /56-FAM/AAC TTC TCC/ZEN/TCC TTG TTG GGG ACC G/31ABkFQ/- 3'. Real time quantitative PCR was performed in triplicate for each sample, and mRNA quantified using the comparative cross threshold (CT) method. Values were normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Reaction products were also visualized by agarose gel electrophoresis.

4th Ventricle Cannula

Male Sprague-Dawley rats weighing 270-310g were anesthetized with intra-peritoneal ketamine and xylazine. A 26G single lumen cannula was introduced into the 4th intracerebral ventricular stereotactically according to coordinates published by Paxinos and Watson: midline, 13mm caudal to bregma, and 7.4mm deep with a head tilt of 0:–3.3mm(8). The cannula was secured with craniofacial cement. A sterile internal cannula was placed to maintain patency and prevent infection. On post-procedure day 3, cannula position was tested by 4th intracerebroventricular (ICV) injection of 5-thio-D-glucose, 210ug in 3ul PBS. A greater than 50% rise in blood glucose in response to 5-thio-D-glucose was considered confirmation of correct cannula position. On post-procedure day 7, animals underwent 4uL injection of either artificial CSF, TNF α (700pg), TNF α (700pg = 10⁻⁸M monomer) and SPD304 (2.5 μ g = 10⁻⁴M), or TNF α (700pg) plus FR171113 (1.9ng = 10⁻⁶M). A second dose was administered 20 hours later. Rats were deeply anesthetized and sacrificed 24 hours after the initial dose. Intra-cardiac perfusion, preparation of tissue blocks, and sectioning was performed as described above. The TNF α dose chosen was just above the threshold dose demonstrated to delay gastric transit when administered to directly into the DVC, accounting for distribution within the CSF volume (9). SPD304 and FR171113 doses were chosen to exceed the IC₅₀ based on dose-inhibition studies(10, 11).

Immunofluorescence

Slides were first washed in PBS for 30 minutes, then incubated with a blocking solution (10% normal goat serum, 3% BSA, and 0.2% Triton X-100 in PBS, pH7.4) for 1h at room temperature. Sections were then treated with primary antibody at 4° for 48hrs, washed in PBS, and treated with secondary antibody for 1 hour at room temperature. Finally, slides were incubated with DAPI (1:5000) and mounted with ProLong Gold anti-fade reagent. All antibodies were diluted in blocking solution. Primary antibodies utilized include mouse anti-Hu (1:100), mouse anti-Cd11b (1:100), and rabbit anti-prothrombin (1:50). TRITC-conjugated goat anti-rabbit IgG (1:100) and FITC-conjugated goat anti-mouse IgG (1:100) were used as secondary antibodies. Slides were viewed using a fluorescent microscope (Nikon Eclipse Ti-U). The DMV was identified as described above. Immunofluorescence was calculated using Image J software; background immunofluorescence was subtracted from the mean immunofluorescence within the DMV to yield an estimation of the excess immunofluorescence per unit of area attributable to the antibody in question. This process was performed for both the right and left DMV in each section.

TUNEL Assay

Apoptosis of DMV neurons was determined by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labeling (TUNEL) assay. Rat brain sections prepared as described above were treated for 5 minutes with a permeabilizing solution containing 0.1% Triton X-100 and 0.1% sodium citrate in PBS. Permeabilized sections were incubated with TUNEL reaction mixture in a dark, humidified chamber at 37° for 1 hour, then rinsed in PBS. Negative controls were incubated with labeling solution devoid of terminal transferase; positive controls were treated with recombinant DNase I (300U/mL DNaseI and 1% BSA in 50mM Tris-HCl, pH 7.5) for 10 minutes at room temperature prior to the

TUNEL reaction. Slides were then co-stained with mouse anti-Hu (1:100), FITC-conjugated goat anti-mouse IgG (1:100), and DAPI as described above. TUNEL positivity was quantified using Image J software as described above.

Statistical Methods

Data were analyzed with GraphPad Prism® software from GraphPad Software Inc. (San Diego, CA), using ANOVA and paired or unpaired Student's *t*-tests as appropriate. Bonferroni's test was applied in cases of multiple comparisons. Results are expressed as mean $\pm$ SEM. Significance was accepted at $p < 0.05$.

RESULTS

IBD and elevated TNF α concentrations in serum, CSF, and DMV tissue extract

To confirm elevated circulating TNF α in rats with TNBS colitis, we evaluated serum TNF α concentration by ELISA both before and after induction of colitis. Prior to the administration of saline (control) or TNBS (colitis) enemas, control and colitis groups had similarly low levels of serum TNF α as detected by ELISA (1.6 ± 1.4 vs. 2.0 ± 1.4 pg/ml, $n=10$). After the induction of colitis, the TNBS group exhibited a nearly 5-fold elevation in serum TNF α as compared to the baseline levels (9.8 ± 7.4 vs. 2.0 ± 1.4 pg/ml, $n=10$, Figure 1A), whereas serum TNF α concentration in the control group did not differ from baseline (1.0 ± 0.7 vs. 1.6 ± 1.4 pg/ml, $n=10$).

CSF concentrations of TNF α were four times higher in the TNBS group (3.6 ± 1.6 vs. 0.8 ± 0.6 pg/ml, $n=9$, Figure 1B). Examination of DMV tissue protein extract also revealed higher concentrations of TNF α protein in the colitis group (4.9 vs. 2.0 pg TNF α /ug total protein, $n=9$, Figure 1C). To determine whether the elevated TNF α protein levels might be related to increased local expression, we evaluated TNF α mRNA by quantitative RT-PCR in DMV tissue samples. There was a greater than 15-fold elevation in DMV TNF α transcripts in those animals with colitis (15.7 ± 9.6 vs. 1.0 ± 0.5 , $n=10$, Figure 1D), suggesting that local production may contribute to increased DMV exposure to TNF α .

4V TNF α causes activation of microglia

To investigate the effects of TNF α in the DMV, we administered TNF α directly into the fourth ventricle (4V) of healthy adult male rats. As microglia respond to inflammation and participate in local inflammatory responses, we evaluated activation of microglia by Cd11b immunofluorescence. 4V TNF α caused a significant increase in Cd11b immunofluorescence at 24hrs after injection (1.5 ± 0.2 vs. 1.0 ± 0.1 , $n = 22$, $p<0.05$; Figure 2). Co-administration of TNF α and SPD304, a small molecule inhibitor to TNF α receptor 1 (TNFR1), decreased Cd11b immunofluorescence to control values (1.1 ± 0.1 vs. 1.0 ± 0.1 , $n=22$, $p=NS$). These results indicate that TNF α causes microglial activation via activation of its receptor, TNFR1.

4V TNF α causes production of prothrombin

We previously observed that TNBS colitis is associated with an increase in DMV prothrombin production, and hypothesized that TNF α may contribute to elevated

prothrombin levels within the DMV. We thus evaluated prothrombin immunofluorescence following 4V injection of TNF α (700pg/dose, 2 doses). At 24hrs post-exposure, there was a mean 27% increase in prothrombin immunofluorescence in TNF α treated rats compared to controls (1.3 ± 0.1 vs. 1.0 ± 0.1 , n=8, p<0.05; Figure 3). This increase was prevented by TNFR1 blockade via co-administration of SPD304 (TNF α + SPD304 1.1 ± 0.1 vs. control 1.0 ± 0.1 , n=8, p=NS; Figure 3) suggesting that TNF α can contribute to elevated prothrombin concentrations within the DMV.

4V TNF α causes DMV neuron apoptosis

To determine whether TNF α is capable of triggering DMV neuronal apoptosis in TNBS colitis, we evaluated TUNEL-positivity in Hu-positive DMV neurons following exposure to TNF α via the 4th ventricle. TNF α caused DMV neuron apoptosis at 24hrs post-exposure as measured by TUNEL assay (1.31 ± 0.1 vs. 1.0 ± 0.1 , n= 12, p<0.05; Figure 4). Concurrent TNFR1 blockade with SPD304 prevented DMV TNF α -induced neuronal apoptosis (1.1 ± 0.1 vs. control 1.0 ± 0.1 , n=12, p=NS; Figure 4). To investigate whether thrombin might play a role in TNF α -induced neuronal apoptosis, TNF α was administered in conjunction with the PAR1 antagonist FR171113. PAR1 blockade also prevented TNF α -induced apoptosis (1.1 ± 0.1 vs. control 1.0 ± 0.1 , n=10, p=NS; Figure 4), implicating thrombin and its receptor in this process. A similar pattern was observed when examining activated caspase-3 immunofluorescence in DMV neurons (Figure 5). TNF α -treated animals had a 52% increase in activated caspase-3 immunofluorescence as compared to controls; this increase could be blocked by either TNFR1 blockade or PAR1 blockade.

DISCUSSION

Inflammatory Bowel Disease (IBD) has been associated with dysfunctional regulation of gastrointestinal motility by the autonomic nervous system. As the most significant parasympathetic circuit innervating the GI tract, the vagus nerve is a logical candidate to mediate gastrointestinal dysfunction. Our previous study suggests a mechanism by which IBD may lead to vagal dysfunction: IBD is associated with increased DMV vagal neuron exposure to thrombin; thrombin causes DMV neuronal apoptosis and blunted responses to both excitatory and inhibitory neurotransmitters. In rodents, 4V thrombin-induced vagal dysfunction results in delayed gastrointestinal transit(12). While the effects of thrombin on vagal reflexes have been characterized, the cause of excess DMV thrombin in IBD has not been determined.

The present studies identify TNF α as a potential intermediate in the development of thrombin-related DMV neuronal apoptosis in IBD. The following observations support this hypothesis: 1) TNBS colitis was associated with elevated levels of TNF α in rat serum, CSF, and DMV tissues; 2) TNF α delivered via the 4th ventricle caused increased DMV prothrombin levels; 3) TNF α delivered via the 4th ventricle caused DMV neuronal apoptosis with activation of caspase-3; and 4) TNF α -induced DMV neuronal apoptosis could be prevented by blockade of TNF α Receptor 1 or the thrombin receptor PAR1. These studies suggest that IBD is associated with elevated TNF α in both the systemic circulation and the

central nervous system, and that TNF α exposure can cause DMV neuron apoptosis that is sensitive to thrombin receptor blockade.

The relevance of this study is limited by the fidelity of the model utilized. TNBS colitis in rats has been widely used to study IBD because of the similarity of its colonic and constitutional manifestations to human ulcerative colitis. The degree to which it simulates the CNS effects of IBD in humans is less clear. Additionally, while attempts were made to ensure that the doses of TNF α and the receptor antagonists utilized were physiologically relevant, the concentrations of their endogenous counterparts in states of health and disease are not definitively established, particularly within the CNS.

These observations suggest several potential pathways for the development of DMV neuronal apoptosis in IBD. Activation of microglia and neuronal apoptosis are each observed in the DMV after administration of TNF α . While microglial activation may prompt the production of prothrombin and resultant apoptosis, it is also possible that microglial activation is associated with but not causally related to neuronal apoptosis. While our studies have identified two inflammatory mediators involved in DMV neuronal apoptosis in IBD, the roles of the many other inflammatory cytokines involved in IBD are unknown. Several of these cytokines, including IL-1 β , are known to cause DMV neuronal apoptosis *in vitro*; others cytokines such as IL-6 decrease DMV neuronal proliferation(13). IBD-related autonomic dysfunction may be the net result of the action of many different inflammatory mediators, including thrombin and TNF α , acting on vago-vagal circuits.

The role of TNF α in IBD has been studied in detail, resulting in widespread therapeutic use of anti-TNF α agents. Circulating TNF α concentrations are more than 300-fold higher in patients with IBD than healthy controls, and are also higher in active as opposed to quiescent disease(14). Serum levels of soluble TNF receptors I and II were similarly elevated in IBD patients, particularly in those patients with active Crohn's disease(15). Local expression of TNF α by colon tissue is higher in patients with ulcerative colitis than in controls(1), and elevated TNFRI and II expression has also been reported in rodent models of colitis(16). Colonic TNF α is implicated in disruption of the intestinal epithelial barrier(17) and impaired wound closure(18) at pathologic concentrations.

In addition to its local and systemic effects, there is evidence that TNF α may also affect the central nervous system in IBD. TNF α is readily transported across an intact blood-brain barrier by a saturable transport system(2). States of CNS inflammation, such as autoimmune encephalitis, are associated with upregulation of TNF α transport into the CNS(19). In conditions of systemic inflammation such as sepsis, the blood-brain barrier may be made more permeable by TNF α , but alterations in the transport of TNF α itself have not yet been examined(20). Increased CNS exposure to TNF α may also result from increased local production. In an intestinal ischemia/reperfusion model of systemic inflammation, for example, TNF α expression was upregulated within the brain(21). Our studies confirm increased CNS TNF α in IBD, with elevated CSF [TNF α], and indicate several potential mechanisms of augmented exposure. Elevated concentrations of circulating TNF α in IBD suggest the possibility of increased blood to brain transport; increased DMV expression of TNF α suggests upregulation of local production. Both mechanisms may play a role.

TNF α has global effects on CNS function in IBD. Rats with TNBS colitis exhibit increased intrinsic neuronal excitability, as well as greater propensity for myoclonic seizure activity in response to provocative stimulus. This increase in CNS excitability is dependent upon both TNF α and microglial activation, and indicates that IBD-induced elevations in CNS TNF α can affect neuronal function(22).

In addition to its global CNS effects in IBD, TNF α also has specific effects on the discrete neuronal and glial populations involved in vago-vagal circuits. The net result is impaired gastrointestinal motility. The vagus nerve forms a reflex arc in which sensory information is transmitted from the GI tract to the nucleus of the solitary tract (NTS) in the brainstem. NTS neurons synapse on efferent vagal motor neurons in the adjacent DMV, which then project back to the GI tract to regulate its function. Injection of TNF α directly into this area of the brainstem results in activation of a subpopulation of NTS neurons, and suppression of DMV-mediated gastric tone(23-25). These effects can also be elicited by LPS-induced systemic inflammation, and inhibited by intracranial TNF α -adsorbing agents(25). Our studies demonstrate additional effects of TNF α in the DMV - microglial activation, production of prothrombin, and vagal neuronal apoptosis – all of which may further contribute to autonomic dysfunction in IBD.

Systemic anti-TNF α therapies have proven efficacy in induction of remission for Crohn's disease(26) and ulcerative colitis(27), as well as maintenance of remission in Crohn's disease(28). Treatment is associated with symptomatic and histological improvement as well as decreased need for operation(27). Whether anti-TNF α agents or PAR1 blockade may be effective in treating autonomic dysfunction associated with IBD is uncertain. While our studies address the prevention of TNF α -induced DMV neuron apoptosis by TNFR1 and PAR1 blockade, they are not designed to evaluate a potential therapeutic effect in acute TNF α exposure or in IBD.

The main anti-TNF α agents currently in clinical use do not readily enter the CNS(29). Recently, TNF α inhibitors capable of traversing the blood brain barrier have been engineered for use in treating neuroinflammatory conditions; these have not been tested in IBD(30). In contrast, thalidomide is a small molecule inhibitor of TNF α production that readily traverses the blood-brain barrier. In several small series, thalidomide has proven effective in decreasing intestinal inflammation and patient symptoms associated with IBD, even in patients refractory to conventional anti-TNF α therapy(31-33). These observations raise the question as to whether thalidomide may affect the pathophysiology of IBD within the CNS as well as peripherally.

Although IBD was originally believed to be a process limited to the gastrointestinal tract, the systemic nature of this disease has long been apparent. Still, the effects of IBD on the central nervous system, and the contributions of CNS dysfunction to the pathophysiology of IBD have only recently been investigated. These studies indicate that IBD is associated with increased brainstem exposure to TNF α , resulting in activation of microglia, production of prothrombin, and apoptosis in vagal neurons. This may be one mechanism by which autonomic dysfunction develops in patients with IBD, and may represent a potential target for therapeutic intervention.

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A

B

C

Figure 1. TNF α in IBD

Serum TNF α concentration increased following induction of colitis via TNBS enema, from (A: n=10, p=NS by paired t-test) but did not increase following saline enema. Animals with colitis had elevated concentrations of TNF α in CSF (B: n=9, p=NS by t-test), and DMV tissues (C: n=10, p=N/A). TNF α transcripts were also increased in the DMV tissues of rats with colitis relative to controls (D, n=8, p=NS by t-test).

D

Figure 2. TNF α causes activation of microglia

Following the administration of artificial CSF (A, all images 10X magnification, DMV circled, CC=central canal of the 4th ventricle, AP=area postrema, arrows indicate microglia, **magnified inset provided**), TNF α (B), or TNF α + the TNFR1 inhibitor SPD304 (C) into the 4th ventricle, immunofluorescence was used to examine the DMV for the microglial marker Cd11b (green). Cd11b immunofluorescence was significantly increased at 24hrs in TNF α treated rats, with prevention of this effect by TNFR1 blockade (D: n=22, p<0.05 for TNF α vs. control, p=NS for TNF α +SPD304 vs. control by Bonferroni multiple comparison test).

Figure 3. 4V TNF α causes increased DMV prothrombin

Healthy rats were administered either artificial CSF (A, all images 20X magnification, DMV circled, CC=central canal of the 4th ventricle), TNF α (B), or TNF α + the TNFR1 antagonist SPD304 (C) via indwelling 4V cannula. Prothrombin immunofluorescence (red) was increased in the TNF α group (1.27 ± 0.06 vs. 1.00 ± 0.05 , $n=8$, $p<0.05$), but not the TNF α + SPD304 group (1.07 ± 0.04 vs. 1.00 ± 0.05 , $n=8$, $p=0.33$) as compared to controls (D). The difference between the TNF α group and the TNF + SPD304 group was significant ($n=8$, $p<0.05$, not indicated in the figure), utilizing Bonferroni's multiple comparison test.

Figure 4. TNF α causes DMV neuron apoptosis

Following exposure to artificial CSF, TNF α , TNF α + the TNFR1 inhibitor SPD304, or TNF α + the PAR1 inhibitor FR171113 administered via the 4th ventricle, rats were sacrificed at 24hrs. Neurons were stained with an antibody against the neuronal marker Hu (A, green, all images 60X magnification) to identify neurons (indicated by arrow). TUNEL co-staining (B, red) was then utilized to examine apoptosis in the DMV, and the images merged to confirm localization of TUNEL signal within neurons (C, merged: TUNEL, Hu, DAPI). (D) TNF α -treated animals had a significant increase in TUNEL signal as compared to controls (n=12, p=0.001), whereas animals treated with either SPD304 or FR171113 in addition to TNF α exhibited no significant difference in TUNEL signal compared to controls (n=12 and n=10, respectively, p=NS for each by Bonferroni).

Figure 5. TNF α and activated caspase-3 in DMV neurons

Hu-positive DMV neurons (A, green, all images 60X magnification, neuron indicated by arrow) were evaluated for activated caspase-3 immunofluorescence (B, red; C, merged) following treatment with either artificial CSF, TNF α , TNF α and SPD304, or TNF α and FR171113 via the 4th ventricle. Activated caspase 3 immunofluorescence was significantly increased in TNF α -treated animals compared to controls (n=4, p<0.05). Co-administration of SPD304 or FR171113 resulted in activated caspase-3 immunofluorescence similar to controls (n=4, p=NS in each case by Bonferroni).