Free Radical Biology and Medicine 87 (2015) 312–325

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

MPTP activates ASK1–p38 MAPK signaling pathway through TNF-dependent Trx1 oxidation in parkinsonism mouse model

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ARTICLE INFO

Article history:
Received 6 April 2015
Received in revised form
16 June 2015
Accepted 28 June 2015
Available online 9 July 2015

Keywords:
Thiols
Oxidative stress
Cell death
Graph theory
Neurodegeneration

ABSTRACT

Activation of apoptosis signal-regulating kinase 1 (ASK1)–p38 MAPK death signaling cascade is implicated in the death of dopaminergic neurons in substantia nigra in Parkinson's disease (PD). We investigated upstream activators of ASK1 using an MPTP mouse model of parkinsonism and assessed the temporal cascade of death signaling in ventral midbrain (VMB) and striatum (ST). MPTP selectively activated ASK1 and downstream p38 MAPK in a time-dependent manner in VMB alone. This occurred through selective protein thiol oxidation of the redox-sensitive thiol disulfide oxidoreductase, thioredoxin (Trx1), resulting in release of its inhibitory association with ASK1, while glutathione-*S*-transferase μ 1 (GSTM1) remained in reduced form in association with ASK1. Levels of tumor necrosis factor (TNF), a known activator of ASK1, increased early after MPTP in VMB. Protein covariation network analysis (PCNA) using protein states as nodes revealed TNF to be an important node regulating the ASK1 signaling cascade. In confirmation, blocking MPTP-mediated TNF signaling through intrathecal administration of TNF-neutralizing antibody prevented Trx1 oxidation and downstream ASK1–p38 MAPK activation. Averting an early increase in TNF, which leads to protein thiol oxidation resulting in activation of ASK1–p38 signaling, may be critical for neuroprotection in PD. Importantly, network analysis can help in understanding the cause/effect relationship within protein networks in complex disease states.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by symptoms, such as bradykinesia, rigidity, resting tremors and postural instability. Histopathologically, the disease is characterized by relatively selective loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) in the midbrain and their terminals in the striatum, leading to decreased dopamine levels in the basal ganglia circuitry. Several factors, such as oxidative stress, mitochondrial dysfunction, proteasomal stress and inflammation have been implicated in the pathogenic process [1].

PD associated neurodegeneration has been modeled in rodents using toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), rotenone and paraquat [1]. Interestingly, MPTP, rotenone and paraquat primarily cause mitochondrial dysfunction, which in turn triggers other stressors, such as oxidative stress in neurons. While mitochondrial dysfunction seen as complex I activity loss and oxidative stress manifesting as glutathione (GSH) loss are reported as early deficits in both PD patients and animal models [1–4], how these changes trigger selective degeneration of dopaminergic neurons remain uncertain.

Abbreviations: 6-OHDA, 6-hydroxydopamine; ADAM17, ADAM metallopeptidase domain 17; AMS, 4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid, disodium salt; ASK1, Apoptosis signal-regulating kinase 1; CIB1, Calcium and integrin-binding protein 1; C (in figure), Vehicle (saline injected); DJ1, PARK7; DTT, Dithiothreitol; FADD, Fas-associated protein with death domain; GABA, γ-amino butyric acid; Grx1, Glutaredoxin 1; GSTM1, Glutathione-*S*-transferase μ 1; IC (in figure), Isotype antibody+vehicle injected; IFNγ, Interferon-γ; IL10, Interleukin-10; IL12, Interleukin-12; IL6, Interleukin-6; IM, (in figure), Isotype antibody+MPTP injected; JNK, c-Jun N-terminal kinase; MAP3K, Mitogen-activated protein kinase kinase kinase; MAPK, Mitogen-activated protein kinase; MCP1, Monocyte chemoattractant protein 1; MKK, Mitogen-activated protein kinase kinase; pASK1, Phosphorylated apoptosis signal-regulating kinase 1; PCNA, Protein covariation network analysis; PD, Parkinson's disease; PNS, Post-nuclear supernatant; pp38, Phosphorylated p38; ROS, Reactive oxygen species; SDS, Sodium dodecyl sulfate; SNpc, Substantia nigra pars compacta; ST, Striatum; TC (in figure), TNF neutralizing antibody+vehicle injected; TDOR, Thiol disulfide oxidoreductase; TM (in figure), TNF neutralizing antibody+MPTP injected; TNF, Tumor necrosis factor-α; TNFR, TNF receptor; TRADD, TNFR1-associated death domain; TRAF, TNF receptor associated factor; Trx1, Thioredoxin 1; Tub, β-Tubulin, VMB, Ventral midbrain

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.06.041
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While increased reactive oxygen species (ROS) and late-stage non-specific biomolecular oxidation, such as protein carbonyls have been studied as markers of oxidative stress in PD, early protein thiol oxidation (PTO) has been largely ignored. Such early changes could occur differentially in vulnerable cells in response to oxidative stress and act as switches triggering cell-specific signaling pathways, thereby leading to selective cell loss as seen in PD.

Previous work from our laboratory using MPTP mouse model has shown cell-specific activation of the stress/death signaling mitogen-activated protein kinase (MAPK) phosphorylation cascade [5,6]. While p38 MAPK activation was observed in dopaminergic neurons, c-Jun N-terminal kinase (JNK) MAPK activation was found in microglia. The selective p38 activation resulted in phosphorylation and nuclear translocation of p53 [6], potentially leading to cell death through p53-dependent mechanisms [7,8]. These MAPKs are regulated upstream by the MAPK kinase kinase (MAP3K), apoptosis-signal regulating kinase 1 (ASK1). ASK1 can phosphorylate p38 MAPK and JNK by relaying phosphorylation signals through MKK3/6 and MKK4/7 (MAPK kinase) respectively [9]. Importance of ASK1 in dopaminergic cell death has been demonstrated in PD mouse models, where ASK1 knockout [10] and ASK1 viral-knockdown [11] offered neuroprotection from neurotoxins. Similar effects were also found in *in vitro* neurotoxin models of PD [12,13]. Thus, ASK1-MAPK cascade has emerged as a key cell death pathway in response to oxidative stress [14]. Activation of this pathway stems from PTO-dependent inhibitory association of thioredoxin (Trx1; thiol disulfide oxidoreductase or TDOR) with ASK1. Reduced Trx1 blocks ASK1 activation by autophosphorylation at Thr845 (in mouse; Thr838 in humans) in response to oxidative stimuli [15]. While ASK1 pathway appears to be a critical mediator of cell-specific toxicity, the upstream activators of this pathway have not been clearly identified.

While disruption in cellular signaling has been extensively studied in neurodegenerative models, studies examining signaling networks in a mathematical framework have been limited to protein-protein interactions or transcriptional networks [16]. Thus, studies probing correlation of protein expression between participants of signaling pathways have generally been limited [17,18] and totally lacking in neurodegenerative states. Such analyses give a holistic view of network dysregulation and can potentially help in identifying key nodal proteins, which can then be exploited for therapeutics.

Experimentally, we observe tumor necrosis factor (TNF) to be an early activator of ASK1 pathway acting potentially through Trx1 oxidation and identify it to be the central node in network dysregulation using protein covariation network analysis (PCNA). Finally, we establish a causal association between TNF and ASK1 activation through blocking aberrant network activation by pre-administering TNF-neutralizing antibody intracisternally, which prevents activation of ASK1-signaling cascade.

2. Methods

2.1. Materials

A detailed list of materials is provided in the supplementary document. Rabbit Antiserum to Trx1 was a kind gift from Dr. Gary Merrill (Oregon State University). Ultralow endotoxin level purified rat anti-mouse TNF-neutralizing antibody (MP6-XT22 clone) [19] and its control κ-isotype rat IgG antibody were procured from Biolegend (San Diego, CA, USA).

2.2. Animals

All animal experiments were carried out as per institutional guidelines for the use and care of animals of National Brain Research Centre (protocol NBRC/IAEC/2010/59) and Indian Institute of Science (protocol CAF/Ethics/163/2009). All efforts were taken to minimize animal suffering and number of animals used for experiments. Male C57-BL6/J mice (3–4 months age, 23–30 g) were treated with a single dose of MPTP hydrochloride in 0.9% saline (30 mg/kg body weight sc) and sacrificed 0.5, 1, 4, 8, or 12 h later. The respective control group received vehicle (saline) alone. For subchronic experiments, animals were administered the same dose once daily for 8 consecutive days before sacrificing 24 h after the last dose [5]. For the TNF antibody-mediated protection experiment, 30 min prior to MPTP administration, ~25 μg antibody (IgG protein) or isotype control suspended in 30 μL sterile phosphate-buffered saline (pH 7.4) was injected into mice intrathecally and sacrificed 8 h after MPTP injection. Mice were sacrificed by cervical dislocation and decapitated, and ventral midbrain (VMB) and striatum (ST) dissected out and flash-frozen in liquid nitrogen.

2.3. Processing of tissue

Frozen brain tissue was homogenized in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 2 mM EDTA, protease, and phosphatase inhibitors. The homogenates were centrifuged at 1000*g* for 10 min at 4 °C to obtain post-nuclear supernatant (PNS), which was used for immunoblotting. Cytosol was isolated from PNS by centrifugation at 100,000*g* for 1 h at 4 °C for cytokine analysis. Protein concentration was estimated by the bicinchoninic acid assay (Sigma Aldrich) [20].

2.4. Immunoblotting

PNS from brain regions sacrificed at varying time-points were electrophoresed together using reducing SDS-PAGE. The gels were transferred to nitrocellulose membranes (MDI, India), blocked and incubated with appropriate primary antibody, followed by appropriate horseradish peroxidase-conjugated secondary antibody. Immunostained bands were detected using a chemiluminescence kit (Millipore, Billerica, MA, USA). For densitometry, blots were normalized to the loading control, β-tubulin. No densitometric comparisons were attempted to compare samples across different gels, thereby precluding comparisons between the brain regions studied or protein expression across time-points.

2.5. Immunoprecipitation

Briefly, coimmunoprecipitation studies were carried out using PNS from VMB and ST samples 12 h after MPTP exposure. Antibody binding was carried out using either ASK1 antibody or normal rabbit IgG (for negative control) followed by precipitating the immune complex using protein G-coupled Dynabead suspension (Life Technologies Inc., Carlsbad, CA, USA). Immunoprecipitated complexes were suspended in 2X sample buffer, boiled, and electrophoresed using gradient 8–16% Tris–glycine gels (Life Technologies) and probed with antibodies to ASK1, Trx1, or GSTM1.

2.6. Redox state analysis of proteins

PNS from VMB or ST were reacted with thiol alkylating agent AMS (15 mM) in a denaturing buffer [20 mM Tris (pH 7.5) and 2% sodium dodecyl sulfate (SDS)] (modified from [21]). Excess AMS was quenched with reduced glutathione (30 mM) and boiled with reducing sample buffer before performing SDS-PAGE. Densitometric estimations of band intensities were done using derivatized β-tubulin (AMS-Tub) for normalization. For studying redox-sensitive ASK1 multimer formation after MPTP, PNS samples from VMB or ST from saline and MPTP-treated groups were boiled with reducing (containing dithiothreitol, DTT) or nonreducing sample buffer and separated using SDS-PAGE. ASK1 multimer integrated band intensities in the nonreduced lanes were normalized to the corresponding ASK1 bands from the reduced lanes.

2.7. Cytokine bead array

Mouse inflammatory cytokine 6-bead array kits were used to quantitatively measure cytokine levels in freshly prepared cytosol from VMB or ST as per manufacturer's instructions using FACS Calibur machine (BD Biosciences, Franklin Lakes, NJ, USA).

2.8. Protein covariation network analysis

The relative normalized protein expression data were collated across all animals for each time point and brain region. Protein states, such as phosphorylated protein or reduced protein (AMS-derivatized levels) were treated separately from their corresponding total protein expression levels. Using MATLAB (MathWorks, India), Pearson correlation matrices were drawn for all pairwise protein expression comparisons at each time point and brain region. The correlation coefficients were hard-thresholded to values of either −1, +1, or 0 based on significant negative, positive, or nonsignificant correlation, respectively ($P < 0.05$). Thresholded coefficients were used to determine undirected edges between the protein/protein state nodes, with either −1 or +1 indicating an edge (unweighted). Graphs were visualized and analyzed using NetworkAnalyzer plug-in in Cytoscape 3.1.1 [22,23].

2.9. Data analysis

Parametric statistical analysis of the data was performed using Student's *t*-test for all time point analyses or ANOVA followed by Dunnett's post hoc test for TNF blocking experiments with all pairwise comparisons made against isotype antibody-injected saline group. All graphs shown represent mean ± standard deviation (SD) and '*n*' represents individual animals in each group. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. MPTP activates ASK1 and p38 acutely and selectively in ventral midbrain but not in striatum

ASK1 or p38 was determined as ratio of phospho-protein to total protein signal as described earlier [24] and as phospho-protein signal normalized to the loading control β-tubulin. Change in total protein levels were quantified by normalizing expression to β-tubulin. Significant increase in phosphorylation of both ASK1 and p38 was seen in ventral midbrain (VMB; Fig. 1A and C) 4 h after a single dose of MPTP and was sustained till 12 h. Striatum did not show any

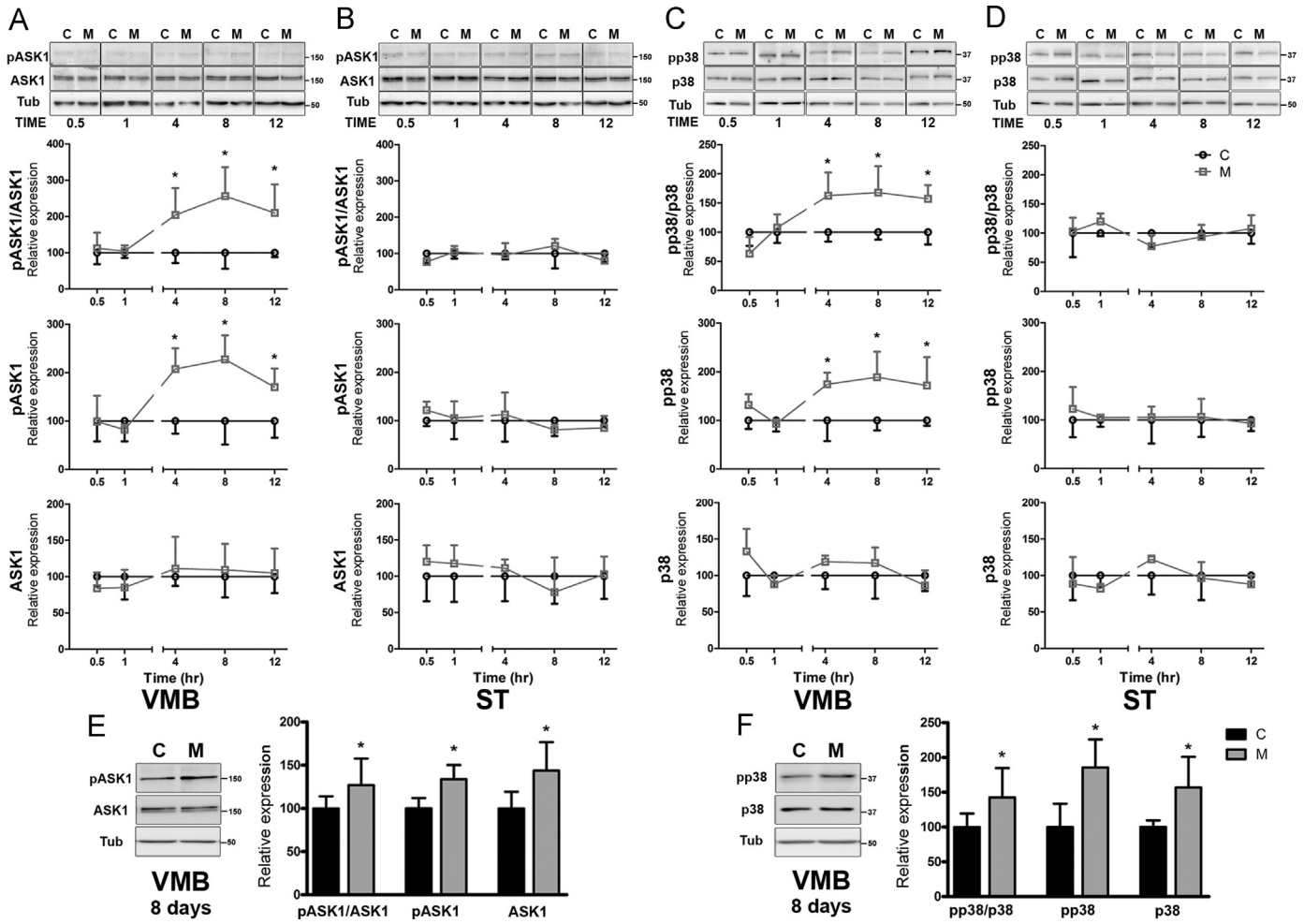

Fig. 1. The ASK1–p38 MAPK pathway is selectively phosphorylated in ventral midbrain after MPTP treatment. Mice were injected with MPTP (30 mg/kg sc) and sacrificed at different time points — 0.5, 1, 4, 8, and 12 h later — or administered the same dose subchronically once daily for 8 days and sacrificed 24 h after the last injection, while corresponding controls received saline. Representative immunoblots depict the expression levels of phospho-ASK1 (Thr845) and ASK1 in ventral midbrain (VMB; A) and striatum (ST; B), and phospho-p38 (Thr180/Tyr182) and p38 in VMB (C) and ST (D), for control ('C') and MPTP-treated ('M') mice and VMB of subchronically administered group (E and F, respectively). Phosphorylation was measured as phospho-protein to total protein ratio (pASK1/ASK1 or pp38/p38) or phospho-protein normalized to loading control (pASK1 or pp38). Total protein was measured after normalization with loading control (ASK1 or p38). Densitometric analyses are shown below the corresponding immunoblots. Three to five animals (*n*) were used per group in the time-course experiment and $n = 7–8$ animals per group for the 8-day experiments. All values represent mean ± SD. Student's *t* test was used to test for statistical significance between the corresponding groups of each time point. * represents $P < 0.05$.

change in phosphorylation of ASK1 and p38 at any of the time points studied (ST; Fig. 1B and D). A significant increase in total ASK1 levels in VMB was not observed as reported before [5], possibly due to differences in mouse colony characteristics or use of polyclonal antibody to ASK1 (No. sc-7931 from Santa Cruz Biotechnology Inc., instead of No. ab45178 monoclonal antibody from Abcam in current work) in the earlier study. Interestingly, both phosphorylation and total protein levels of ASK1 and p38 increased in the subchronic 8-day MPTP model having significant dopaminergic neurodegeneration, indicating that early activation of the pathway is sustained even during the chronic degenerative state (Fig. 1E and F) [5]. While the early changes in ASK1 phosphorylation are larger in magnitude, the increase is comparatively smaller in the subchronic model. This could stem from the death of dopaminergic neurons that contribute to phosphorylation signal of ASK1 in this paradigm, the increases in total ASK1 levels, and homeostatic changes in the MAPK pathway due to repeated exposure to MPTP.

3.2. Increased ASK1 activation in ventral midbrain but not striatum is not due to altered levels of ASK1 inhibiting proteins Trx1 and GSTM1 following MPTP exposure

Cytosolic thioredoxin (Trx1) binds to the N-terminal domain of ASK1 and forms a complex, preventing its oligomerization and autophosphorylation, thus thwarting the initiation of death signaling cascade [15]. Glutathione-*S*-transferase μ 1 (GSTM1) has also been shown to inhibit ASK1 by a similar complex [25]. We hypothesized whether selective ASK1 activation in VMB occurred due to reduction of Trx1 and GSTM1 levels on MPTP exposure. Contrary to expectations, total Trx1 expression increased both in VMB (Fig. 2A) and ST (Fig. 2C) after MPTP exposure. The increase started at 4 h and was maintained till 12 h, similar to ASK1 profile. GSTM1 expression also increased in VMB but not ST at the same time points (Fig. 2B and D). Thus, expressions of ASK1-inhibiting proteins, Trx1, and GSTM1 increase with a temporal profile similar to MAP3K activation in VMB. But, their association with ASK1 may indeed be affected after MPTP exposure.

3.3. Trx1 dissociates from ASK1 in ventral midbrain but not striatal tissue, while GSTM1 interaction remains unchanged

As both Trx1 and GSTM1 inhibit ASK1 activity by forming protein complexes with the MAP3K, we examined the status of these complexes in VMB and ST 12 h after MPTP. Despite the ratio of total available Trx1 to ASK1 protein increasing significantly in VMB, but not in ST (input Trx1/input ASK1; Fig. 3A), coimmunoprecipitation studies revealed decreased association of Trx1 with ASK1 in VMB but not ST (Co-IP Trx1/IP ASK1; Fig. 3C) after treatment with MPTP. Interestingly, GSTM1–ASK1 association was not altered in either brain region (Fig. 3D), while the ratio of total available GSTM1 to ASK1 levels increased similar to that of Trx1 (input GSTM1/input ASK1; Fig. 3B). Negative control data using naive rabbit IgG instead of ASK1 antibody for the coimmunoprecipitation studies showed

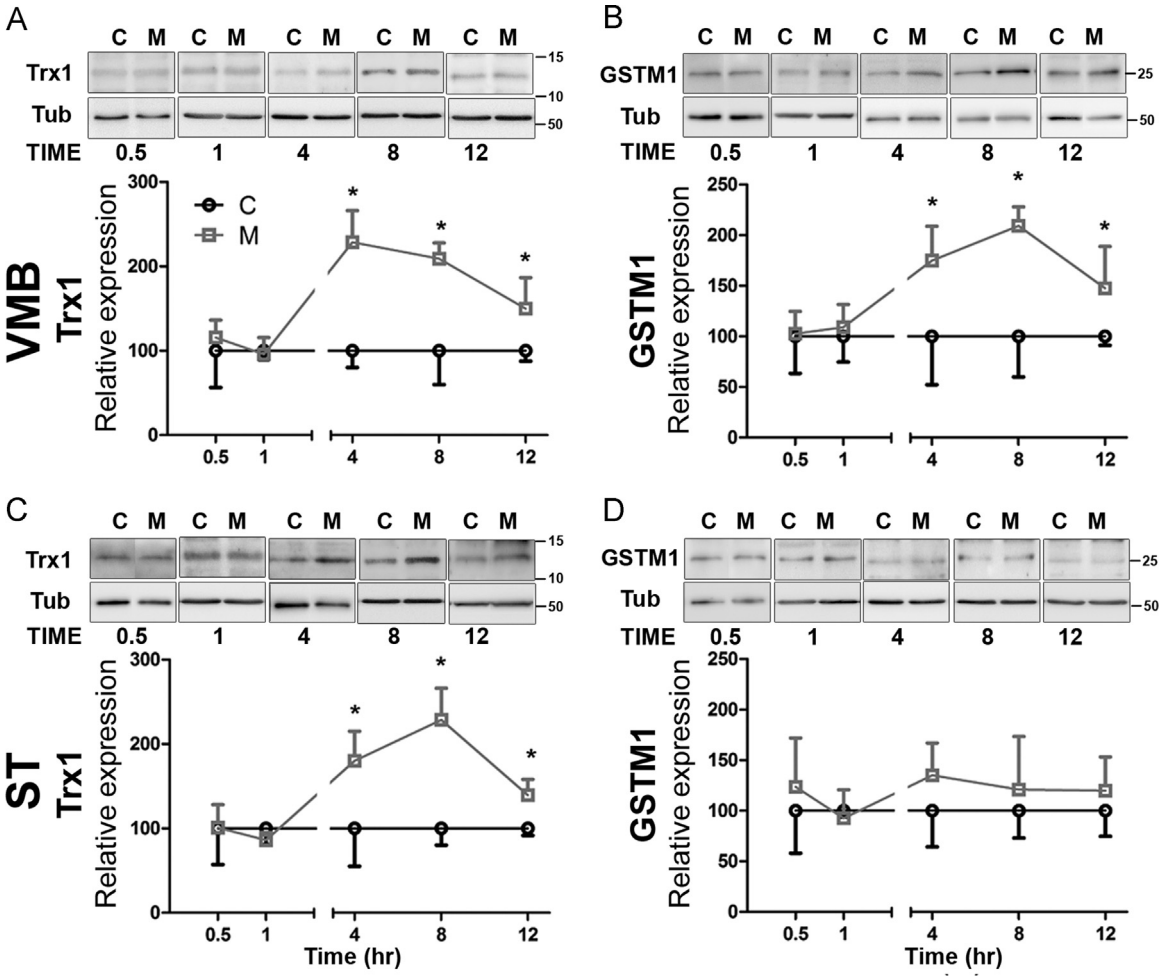

Fig. 2. Total levels of ASK1 inhibiting proteins, Trx1 and GSTM1, increase in ventral midbrain after a single dose of MPTP. Mice were injected with MPTP or saline as noted in Fig. 1. Expression Trx1 levels are depicted in ventral midbrain (VMB; A) and striatum (ST; C), while corresponding GSTM1 profiles are shown for VMB (B) and ST (D). Samples are labeled as control ('C') and MPTP treated ('M'). Values represented in graphs show mean ± SD. Three to five mice (*n*'s) were used at each time point. * represents statistical significance of $P < 0.05$.

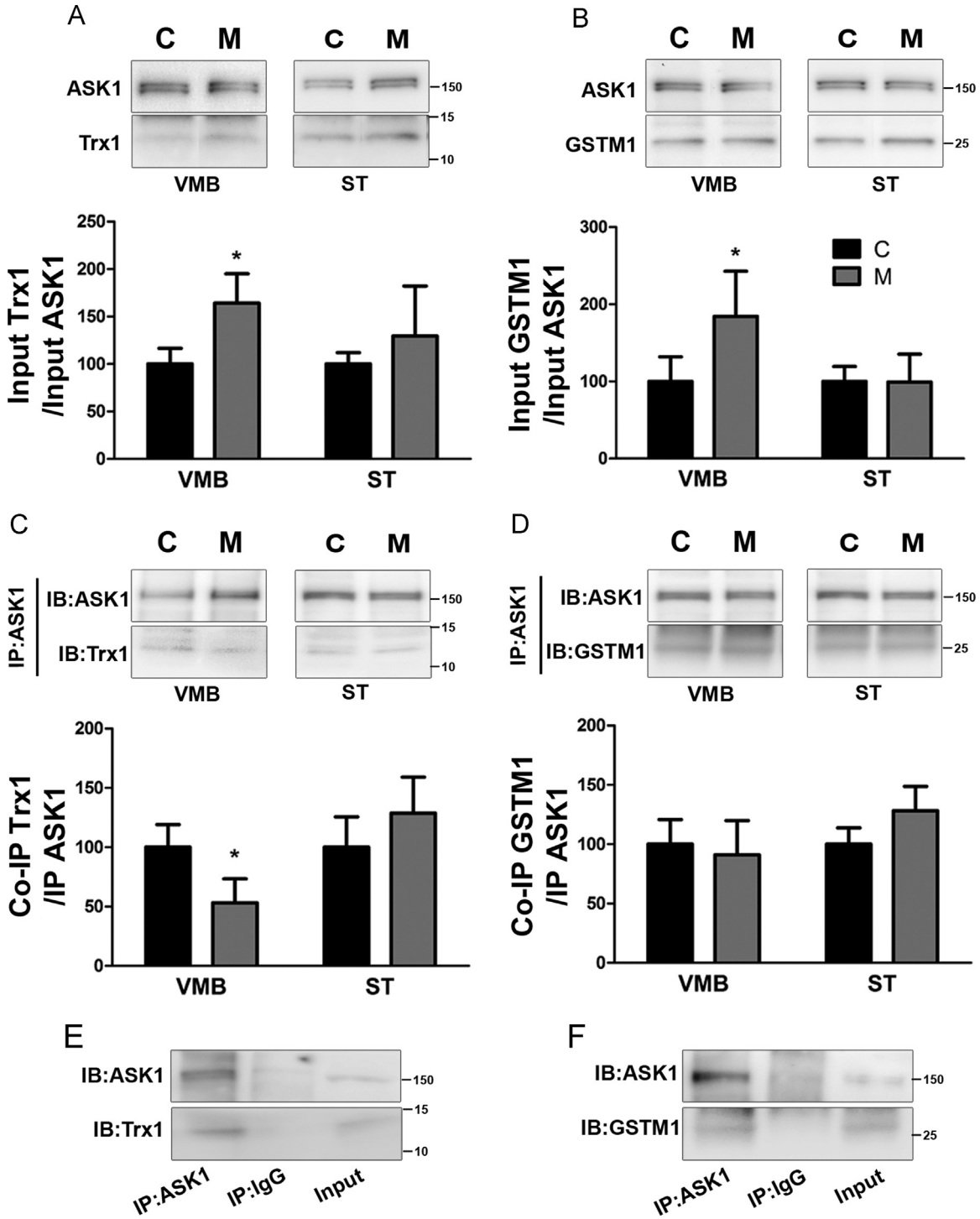

Fig. 3. Trx1 but not GSTM1 selectively dissociates from ASK1 in ventral midbrain after MPTP exposure. Mice were injected with MPTP (30 mg/kg sc) and sacrificed 12 h later, while controls received saline. Coimmunoprecipitation experiments were carried out with protein samples prepared from ventral midbrain (VMB) and striatum (ST). In (A) and (B), representative immunoblots depict the amount of Trx1 (Input Trx1) or GSTM1 (Input GSTM1) relative to ASK1 (Input ASK1), respectively. In (C) and (D), representative immunoblots depict the amount of Trx1 (IB: Trx1) or GSTM1 (IB: GSTM1) coimmunoprecipitated with ASK1 (IP, ASK1; and IB, ASK1), respectively. Samples are labeled as control ('C') and MPTP treated ('M'). (E) and (F) show immunoblots for negative control data for the coimmunoprecipitation studies with Trx1 and GSTM1, respectively. Values in graphs represent mean ± SD. $n=4$ mice and $P<0.05$ was used for determining statistical significance (*).

specificity of detection (Fig. 3E and F). Thus, the two ASK1-interacting partners showed dissimilar behavior and may be differentially involved in ASK1 regulation upon MPTP-induced stress. The dissociation of Trx1 rather than GSTM1 in VMB but not in ST could be responsible for selective ASK1 activation after MPTP exposure (as seen in Fig. 1A and B).

3.4. ASK1 and Trx1 are oxidized in ventral midbrain but not in striatum after acute MPTP exposure

Nadeau et al. demonstrated that Trx1 and ASK1 covalently associate through disulfide linkage and reduced Trx1 is essential for dissociating oxidized ASK1 multimers that undergo autophosphorylation and

activation [26,27]. To test whether ASK1 and its inhibitory proteins Trx1 and GSTM1 potentially get oxidized after MPTP, we performed redox gel analysis wherein protein samples were derivatized with the thiol alkylating agent, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS). AMS covalently reacts with accessible reduced thiols and derivatized proteins have retarded mobility on SDS-PAGE due to electrophoretic mobility shift. ASK1 was increasingly oxidized (measured as reduction in relative intensity of derivatized AMS–ASK1 band normalized to AMS-Tub) in VMB at 4 h and beyond (Fig. 4A) but not in ST (Fig. 4B), consistent with its phosphorylation profile. It is worth noting that underivatized (oxidized) ASK1 bands were also visible below AMS–ASK1, unlike for Trx1 and GSTM1 in our redox gel analysis. To examine whether the decrease in AMS–ASK1 levels in the VMB resulted from increased formation of disulfide-bonded ASK1 multimers as described by Nadeau et al. [27], SDS-PAGE was performed under reducing and nonreducing conditions for VMB and ST samples collected from mice sacrificed 12 h after MPTP exposure. Consistent with the ASK1 phosphorylation status (Fig. 1A and B) and the AMS-derivatization experiment, we found increased formation of high molecular weight ASK1-immunoreactive bands, which could be disulfide-bonded ASK1 multimers, in VMB selectively. The "multimers" ran as a mixture of bands heavier than 500 kDa (as marked on the gel) with immunoreactivity seen in the stacking gel front as well.

Redox status of thiol groups in Trx1 is critical for maintenance of the Trx1–ASK1 complex [28]. Only Trx1 in its reduced form (-SH) is able to bind ASK1, thus keeping it inactive. Since association of Trx1 with ASK1 reduced in VMB but not ST, we examined if increased selective Trx1 oxidation explained this difference. Matching the time profile of ASK1 oxidation and phosphorylation, derivatized Trx1 (or reduced protein) levels steadily decreased in VMB from 4 h onward (AMS–Trx1; Fig. 5A), with ST showing no change after MPTP administration (Fig. 5B).

An earlier study [29] showed that the GSTM1–ASK1 complex is not redox-regulated, unlike Trx1. GSTM1 instead dissociated from ASK1 on heat shock treatment. Accordingly, GSTM1 oxidation did not significantly alter after MPTP treatment in either VMB or ST (AMS–GSTM1; Fig. 5C and D). Thus, increased Trx1 oxidation selectively in VMB after MPTP exposure could potentially lead to ASK1 oxidation and phosphorylation (Fig. 1A) in a temporally coordinated and region-specific manner.

3.5. *Inflammatory response measured as change in cytokine levels could be responsible for ASK1 activation after MPTP*

ASK1 can be activated by cytokines, especially TNF released during inflammatory response. TNF triggers dissociation of Trx1 from ASK1, leading to ASK1 activation [28] and the pathway in turn, regulates cytokine production [30]. To determine if altered cytokine signaling after MPTP exposure lies upstream of ASK1 activation following MPTP, we simultaneously measured cytokine levels using flow cytometry in the two regions at all the five time-

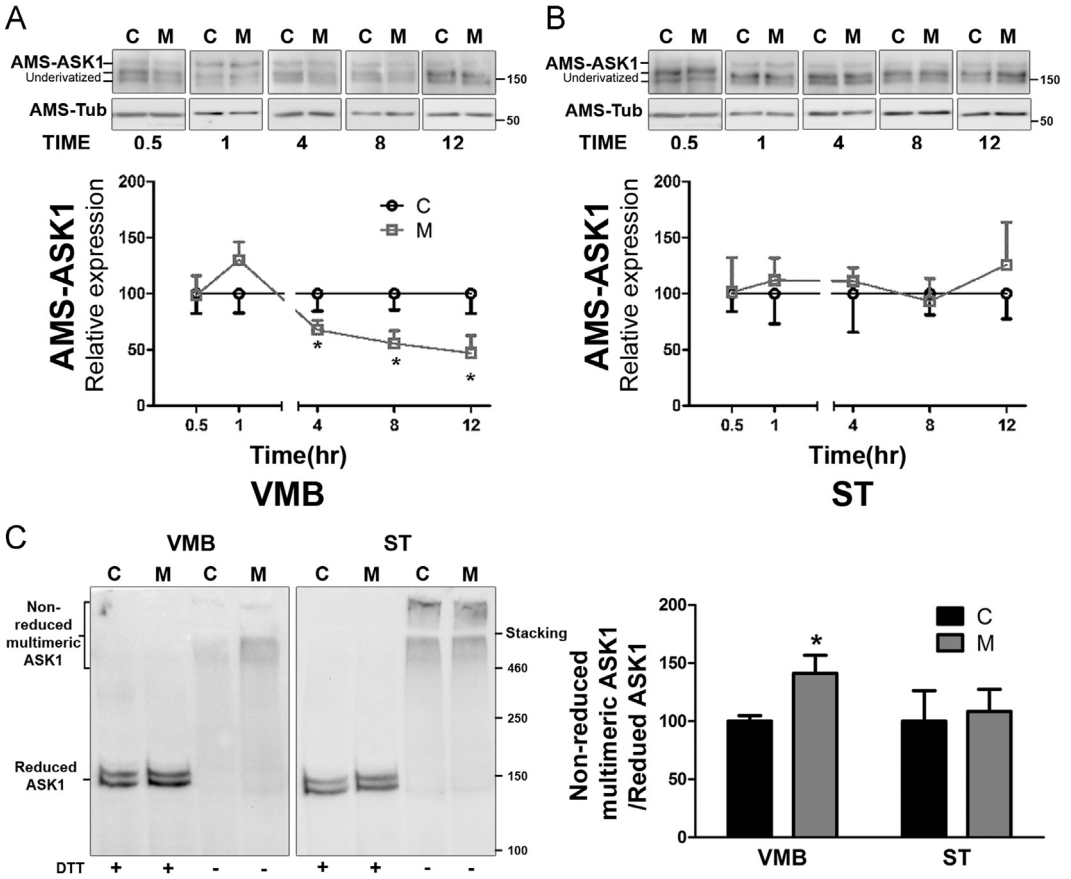

Fig. 4. ASK1 is progressively oxidized and forms high molecular weight multimers in ventral midbrain alone after a single dose of MPTP administration. Mice were injected with MPTP or saline as noted in Fig. 1 for the redox gel analysis using AMS (A and B) or sacrificed 12 h after a single dose of MPTP or saline for examining ASK1 multimer formation (C). For AMS experiments, protein samples were derivatized with the thiol alkylating agent, AMS, such that the reduced proteins show a mobility shift in reducing SDS-PAGE. Blots demonstrate relative expression of AMS-derivatized (reduced) ASK1 (A) from ventral midbrain (VMB) and striatum (ST; B), respectively from control ('C') and MPTP-treated ('M') mice. AMS derivatized ASK1 levels (AMS–ASK1) were normalized with derivatized AMS-β-tubulin as loading control. Four animals (n) were used for either MPTP or saline group at each time point. (C) For detecting the status of redox-sensitive ASK1 multimeric complexes, protein samples were separated from VMB and ST tissues under reducing (DTT: +) and nonreducing (DTT: −) SDS-PAGE. The integrated band densities of nonreduced multimeric ASK1 from nonreducing lanes were normalized to their respective reduced ASK1 band intensities. Five animals were used in each group. Student's t test was used to determine statistically significant differences between the two groups at each time point in the brain regions and indicated with an * ($P < 0.05$). All values in the graphs depict mean ± SD.

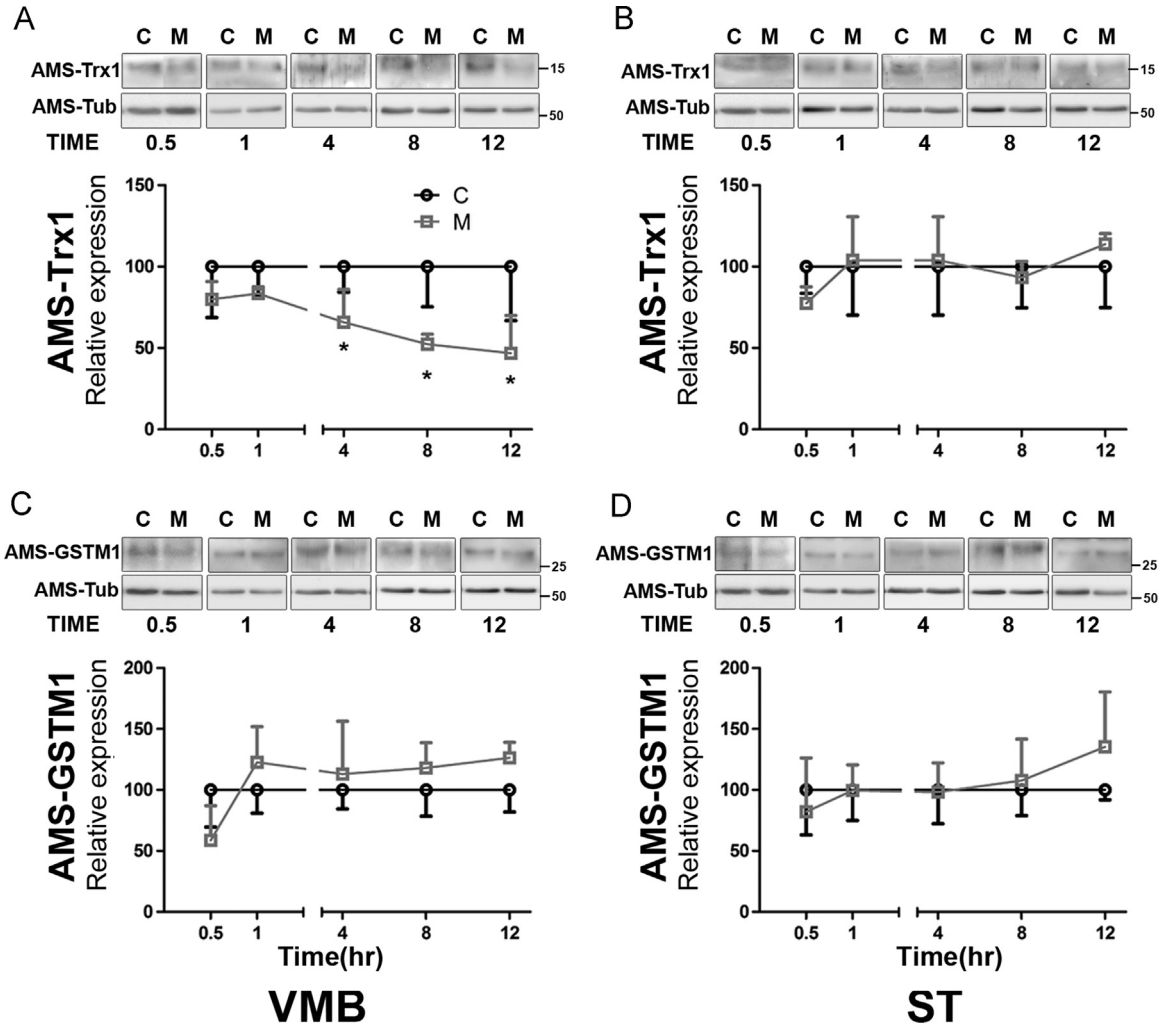

Fig. 5. Trx1 but not GSTM1 oxidation accompanies ASK1 oxidation in ventral midbrain alone after MPTP administration. Mice were injected with MPTP or saline as noted in Fig. 1 and were derivatized with AMS and separated using reducing SDS-PAGE. Blots depict relative expression of AMS-derivatized (reduced) Trx1 (A) and GSTM1 (C) proteins from ventral midbrain (VMB) and striatum (ST; B and D), respectively, from control ('C') and MPTP-treated ('M') mice. AMS derivatized protein (either AMS–Trx1 or AMS–GSTM1) was measured after normalization with derivatized AMS–β-tubulin as loading control. Three to four animals (n) were used for either MPTP or saline group at each time point. Graphs represent mean ± SD. * represents statistical significance ($P < 0.05$).

points following MPTP. Specifically, the cytokines estimated were tumor necrosis factor α (TNF; Fig. 6A), monocyte chemoattractant protein 1 (MCP1; Fig. 6B), interleukin 12 (IL-12; Fig. 6C), interleukin 6 (IL6; Fig. 6D), interleukin 10 (IL10; Fig. 6E) and interferon γ (IFNγ; Fig. 6F). TNF levels significantly increased at 0.5, 1, 8 and 12 h after MPTP exposure in VMB but only at 1 and 8 h in ST. The extent of striatal increase was also lesser than in VMB. Both MCP1 and IL12 levels increased 30 min after MPTP only in VMB and returned to baseline before increasing again at 8 h, possibly indicating a rebound effect. ST showed a smaller but significant increase in MCP1 levels at 8 h alone. Interestingly, the anti-inflammatory cytokine IL10 significantly decreased in VMB but not ST, 8 h after MPTP administration, while IL6 and IFNγ levels remained unchanged.

Thus, there appears to be an early increase in the pro-inflammatory cytokine profile in VMB after MPTP administration. In VMB, TNF levels were enhanced at all but the 4 h time-point, suggesting that it could potentially drive the ASK1 cascade. The other changes in MCP1, IL12 and IL10 could further potentiate TNF signaling and/or have independent downstream roles. Importantly, although ST showed significant increase in MCP1 and TNF, these changes were much smaller in magnitude, were not perturbed in a sustained fashion, unlike in VMB and were not associated with concurrent ASK1 activation (Fig. 1B).

3.6. Protein covariation network analysis reveals ventral midbrain network to be progressively dysregulated after MPTP administration

So far we have reported changes in protein expression after MPTP treatment in VMB and ST at different time-points (Suppl. Fig. 1). As can be seen in Fig. 6 and Suppl. Fig. 1, there are a few molecular changes that precede the activation of cell death signaling mechanisms, namely, induction of pro-inflammatory cytokines — TNF, MCP1 and IL12 — any or all of which can ultimately lead to downstream perturbations. It is difficult to conclude which of these changes, if at all any, could be responsible for ASK1 activation. Further, maximal dysregulation of the proteins was observed in VMB, 4 h after MPTP. While studying individual protein expression changes provides useful information, proteins typically function in large interconnected networks. For example, ASK1 pathway, like any other MAPK pathway works as a cascade of proteins transducing signals from upstream activators to downstream effector proteins.

We employed unweighted undirected protein covariation network analysis (PCNA) to study the network. Fig. 7A-E demonstrate how network connections (significant pairwise correlations in between different proteins/protein state expression) peak at 8 h in VMB, continuing to remain high even at 12 h. On the other hand, ST showed much fewer connections (Fig. 7F-J). Interestingly,

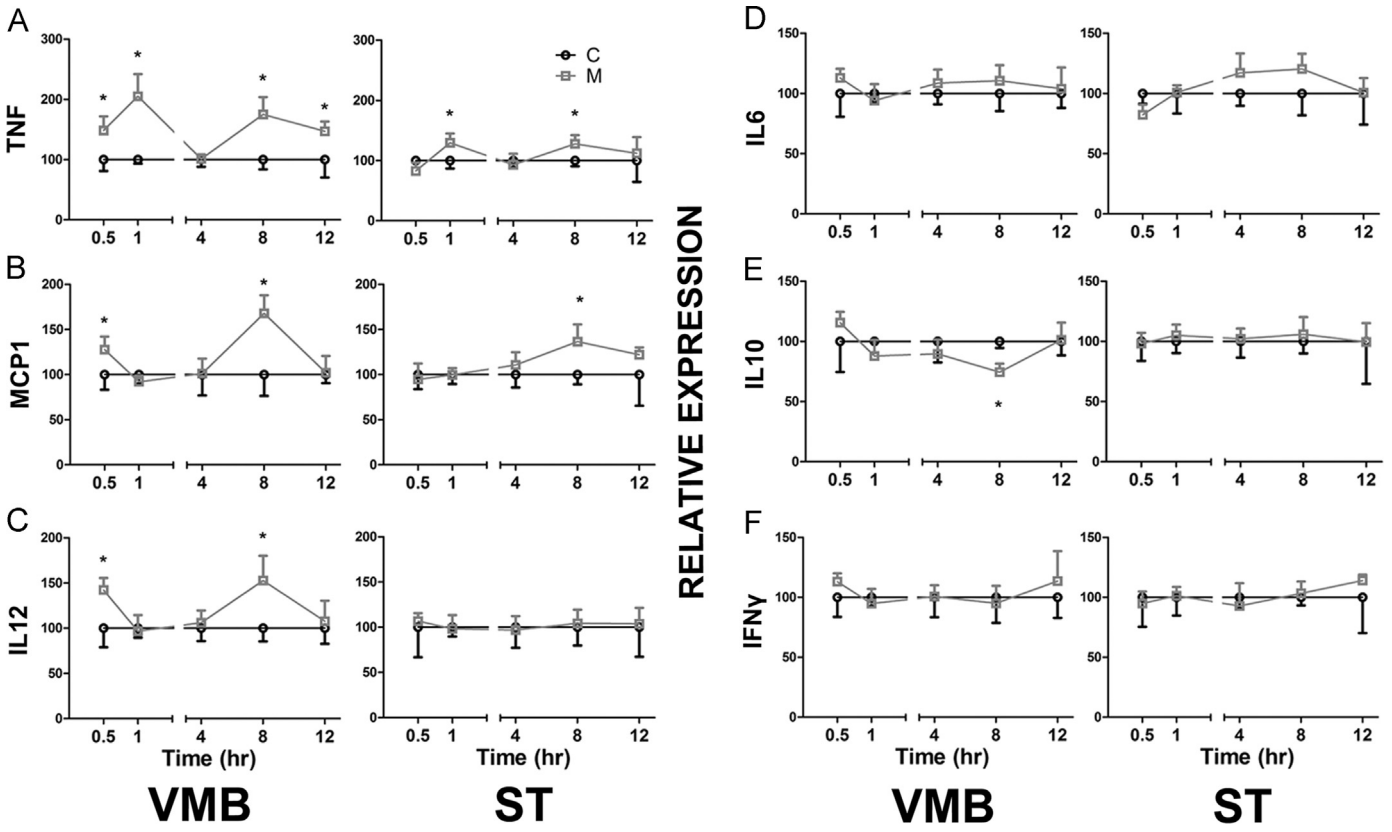

Fig. 6. Cytokine expression analysis reveals large increases in TNF expression in ventral midbrain immediately following MPTP treatment. Mice were injected with MPTP or saline as noted in Fig. 1. Cytokine bead array analysis was carried from cytosolic preparations made from ventral midbrain (VMB) or striatum (ST). Levels of TNF (A), MCP1 (B), IL12 (C), IL6 (D), IL10 (E), and IFNγ (F) in VMB and ST are depicted from vehicle ('C') and MPTP-treated ('M') mice. $n=4$–6 animals. * represents $P<0.05$.

inflammatory cytokine nodes tended to remain interconnected, independent of time-point or brain region examined, possibly due to extensive co-regulation triggered by activation of common transcriptional activator(s) [31]. Also, the different nodes (proteins/protein states) tended to co-vary predictably as per their regulatory mechanism(s) described in literature. For example, pASK1, pp38 and TNF levels appear to correlate positively, while AMS–ASK1 and AMS–Trx1 negatively correlate with pASK1, especially in VMB.

The visualized properties can be mathematically described using network parameters [16,32]. Increase in connections is reflected in the network density and average number of neighbor's parameters (Suppl. Table 1). This increase peaked in VMB at 8 h but remained much lower in ST. The increase in connections also brought nodes together, suggesting formation of "cliques", reflected as increase in average/global clustering coefficient and network centralization and decrease in total number of connected components of the network (Suppl. Table 1).

We also calculated individual node parameters (Suppl. Table 2) and have focused on nodes with high degree in the network. Nodes with high degree (degree ≥ 5) were considered to act as "hubs" [16]. Hub nodes in the 8 h VMB network (network with highest network density) included Trx1, TNF, AMS-ASK1, pp38, MCP1, GSTM1 and pASK1. Of these, Trx1 and TNF had the highest between-ness centrality indicating that these two nodes could potentially act as "hub-bottlenecks" [33], essential for maintaining network structure.

Han et al. differentiated hubs into "party" and "date" hubs based on whether the hubs interacted with other nodes simultaneously (party hubs) or interacted with their partners differentially under different testing conditions (such as different temporal or spatial functional states) of the network. Based on coexpression profiles of the interacting proteins, while party hubs interacted with similar nodes across functional states, date hubs changed partners with whom they connected only in certain functional states. Further, Han et al. observed that date hubs were more critical for global connectivity in the network [34]. On examining the connecting nodes of the hub proteins in the 8 h VMB network, we found that Trx1, AMS–ASK1, pp38, GSTM1, and pASK1 connected to similar nodes even in other networks (mainly the 4 h VMB network), while TNF and MCP1 formed connections with new nodes. Indeed, neither TNF nor MCP1 acted as hubs at any other time point in either of the regions. Applying the "party/date" hub analogy, TNF and MCP1 demonstrated date hub-like features because of their temporally and spatially restricted appearance. However, considering the properties of "hubness," "bottle-neckness," and "date-hubness" together, only TNF possesses all three features among the hub proteins. Thus, it appears to play a central role in the information flow through the dysregulated MAPK pathway. PCNA thus yields an overall picture of the state of the signaling pathway, especially demonstrating when the network is maximally connected, i.e., dysregulated in this context. It can also help in identifying critical nodes that can be targeted to disrupt the network.

3.7. Blocking TNF signaling through pretreatment with TNF-neutralizing antibody abrogates ASK1 activation and downstream p38 activation

As mentioned earlier, TNF signaling has been demonstrated to cause ASK1 phosphorylation [28]. PCNA analysis (Fig. 6, Suppl. Tables 1 and 2) also indicated TNF to play a key role in the network. Based on these results, we examined whether TNF signaling was upstream of ASK1 activation following MPTP exposure by injecting TNF-neutralizing antibody MP6-XT22 [19] or its control isotype antibody, intrathecally in mice 30 min prior to MPTP administration. We sacrificed animals 8 h after MPTP, since maximal network

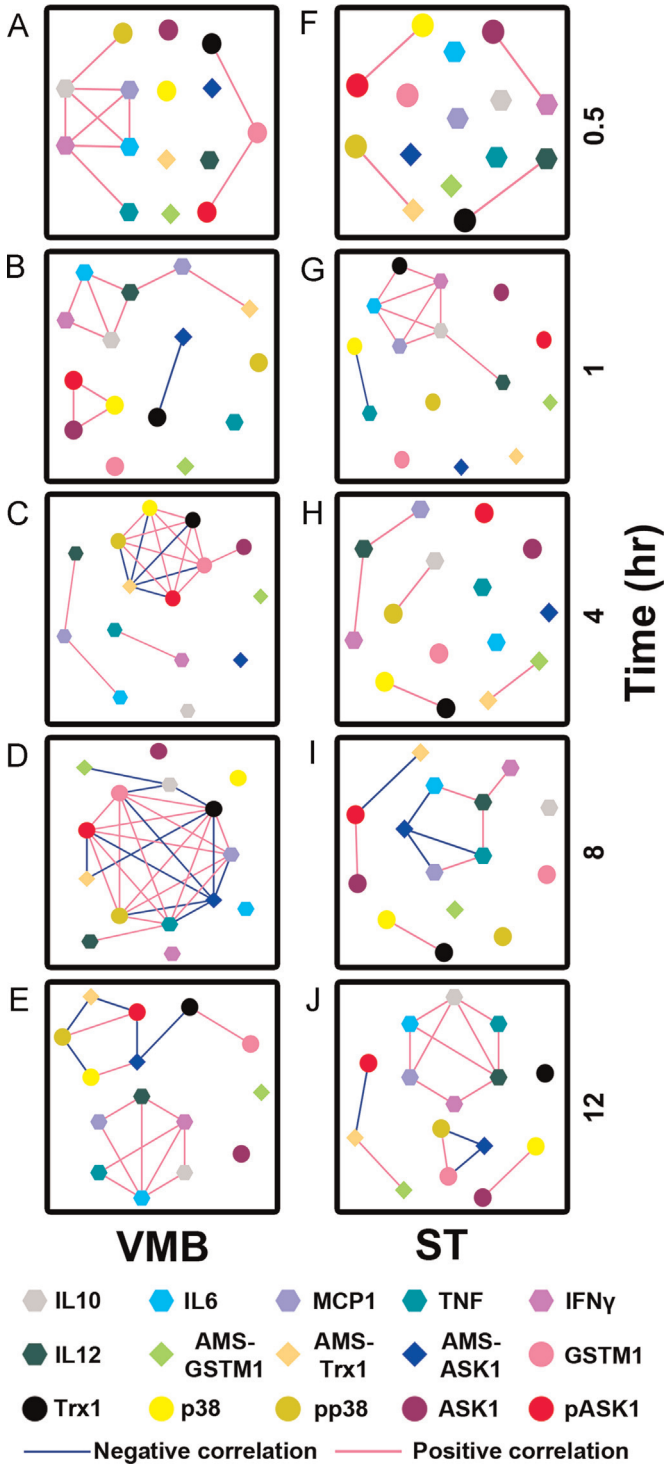

Fig. 7. Protein covariation network analysis reveals maximal perturbation in ventral midbrain at the 8 h time point. Pairwise Pearson correlation coefficient matrices for protein/protein modification states were calculated for all animals at each time point across brain regions. The values were then thresholded to −1, +1 or 0 or +1 based on significantly negative, positive, or nonsignificant correlation, respectively ($P < 0.05$) to create adjacency matrices. Network graphs were visualized and analyzed from adjacency matrices with both −1 and +1 depicting edges between nodes (unweighted network). Different nodes are colored based on identity with edge colors representing −1 (blue) and +1 (pink). Individual panels represent network graphs for the time points 0.5 h (A and F), 1 h (B and G), 4 h (C and H), 8 h (D and I), and 12 h (E and J) for the two regions, VMB (A, B, C, D and E) and ST (F, G, H, I and J), following MPTP treatment.

dysregulation in PCNA was seen at this time (as seen in Fig. 8C). Unfortunately, flow cytometric measurements of cytokines could not be performed with these samples as antibody injection appeared to interfere with detection. Therefore, we examined TNF levels in these samples using western blotting. Neutralizing antibody pre-treatment prevented MPTP-induced TNF increase (Fig. 8A), while the control isotype antibody preserved the activation, similar to that seen in Fig. 6A. Paralleling the effects on TNF protein, we found that ASK1 was only activated (measured as ASK1 phosphorylation) in isotype antibody pre-treated MPTP mice, with no change observed after neutralization (Fig. 8B), with similar effects downstream in terms of p38 phosphorylation (Fig. 8D). ASK1 phosphorylation was also associated with increased ASK1 oxidation in the same group (AMS-ASK1; Fig. 8C). Taken together, these results suggest that following a single dose of MPTP, TNF signaling lies upstream of ASK1 pathway activation in VMB.

3.8. Blocking TNF signaling through TNF-neutralizing antibody pre-treatment prevents loss of reduced Trx1 on MPTP intoxication

We then investigated if TNF-neutralization regulated ASK1-MAPK pathway inhibition occurred through alteration of the Trx1 and/or GSTM1 systems, as seen in Figs. 2 and 5. It is known that TNF signaling can induce production of ROS [35], which can oxidize Trx1 and subsequent ASK1 activation. TNF neutralization prevented total Trx1 and GSTM1 increase after MPTP administration, when compared to control group pretreated with isotype antibody (Fig. 9A and B; compared with Fig. 2A and B). Further, Trx1 oxidation (indirectly measured as AMS derivatized reduced Trx1) was also prevented (Fig. 9C and Fig. 5A), while GSTM1 oxidation was not altered significantly in any group (Fig. 9D and Fig. 5C). Thus, it appears that MPTP-induced TNF production in VMB activates ASK1–MAP3K by oxidizing Trx1 and releasing ASK1 from the inhibitory complex.

4. Discussion

The role of the ASK1 death signaling cascade in selective death of dopaminergic neurons has been reported. However, identifying upstream activator(s) of the ASK1 pathway and indeed other signaling molecules has been hampered by the use of models where neurodegeneration has already begun, thus precluding the discrimination of initial trigger(s) from disrupted secondary signaling pathways. We, therefore, chose to administer a single dose of MPTP to identify potential trigger(s) of the ASK1–p38 MAPK pathway, eventually leading to dopaminergic cell death. Even though the acute model is not linked to any overt neurodegeneration, we demonstrated the perturbation of the very same cascade in the subchronic MPTP model as well (Fig. 1), confirming relevance of the pathway to ongoing cell death. Further, we demonstrated that an early increase in TNF levels after MPTP administration is mainly responsible for Trx1 oxidation, thereby leading to oxidation of ASK1 and activation of the ADK1–p38 MAPK cascade (Figs. 4, 5, 8 and 9). This occurred specifically in the ventral midbrain housing the dopaminergic cell bodies while the striatum containing the dopaminergic terminals remained relatively unaffected.

Different interacting proteins bind to ASK1 and either positively or negatively regulate its activity, such as Trx1, GSTM1, Grx1, DJ1, 14-3-3ζ, CIB1, TRAF2/6, FADD, and Daxx [14]. This gamut of interacting proteins leads to ASK1 being at the apex of a cell stress/death signaling pathway responding to stimuli as diverse as oxidative, heat shock, ultraviolet irradiation, endoplasmic reticulum stress, inflammatory signals, and calcium influx. We primarily focused on two negative regulators of ASK1 — Trx1 and GSTM1. Our attempts to study the interaction of ASK1 with two other redox-sensitive proteins, glutaredoxin (Grx1) and DJ1, were not successful. While a Trx1–ASK1 interaction was negated after MPTP, GSTM1 continued to remain associated with ASK1 (Fig. 3). This discordance putatively arose from the inability of oxidative stress to affect the GSTM1–

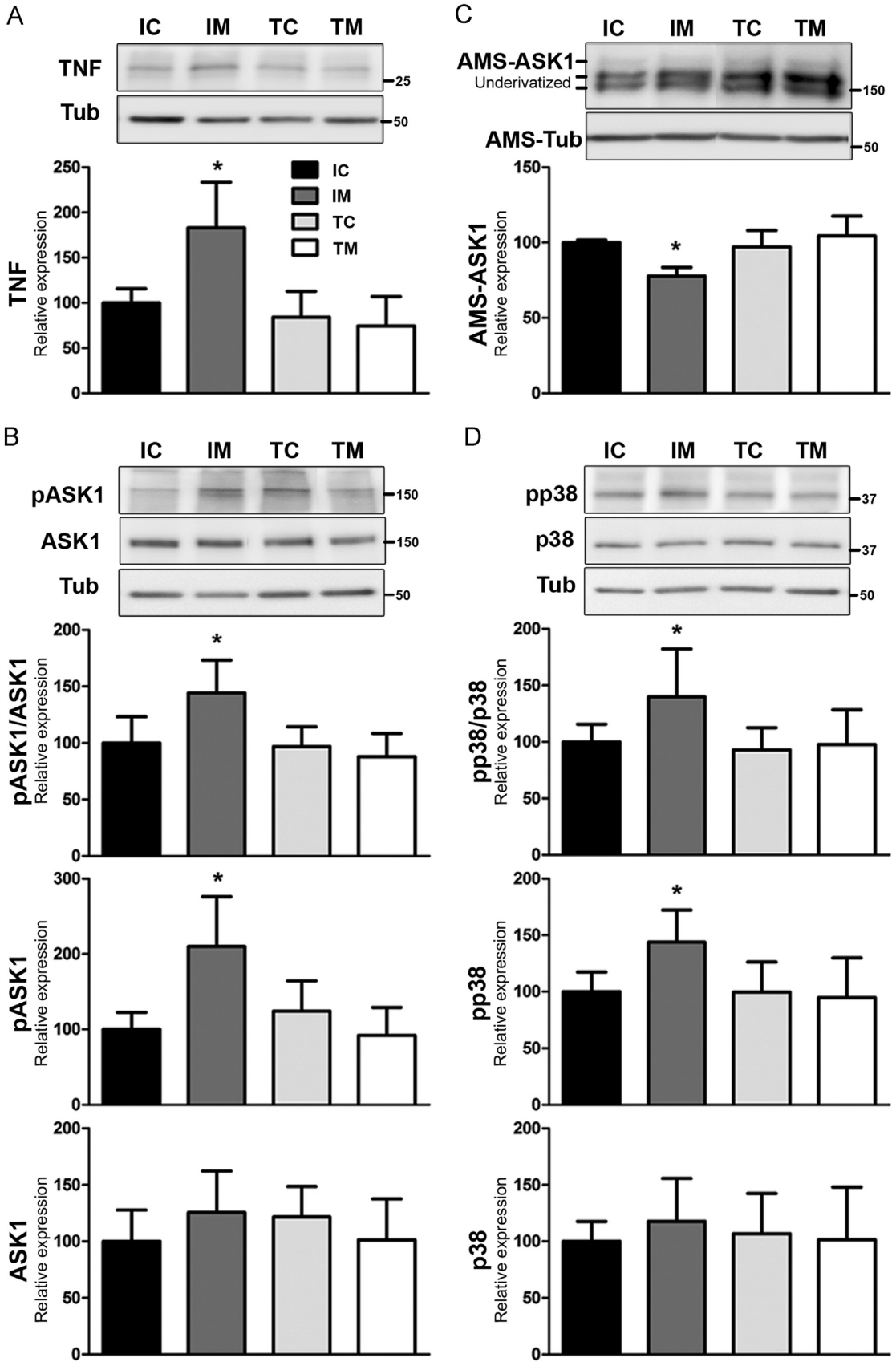

Fig. 8. Neutralizing TNF using prior intrathecal injection of antibody prevents ASK1 oxidation and resultant pathway activation in ventral midbrain. TNF-neutralizing antibody or isotype control was administered intrathecally 30 min prior to MPTP or saline injections and mice were sacrificed 8 h later. Panels demonstrate relative expression of TNF (A), phosphorylation of ASK1 (pASK1/ASK1 and pASK1; B), levels of reduced ASK1 (AMS–ASK1; C), and phosphorylation of p38 MAPK (pp38/p38 and pp38; D) in ventral midbrain. Groups are labeled as Isotype antibody + saline ('IC'), Isotype antibody + MPTP ('IM'), TNF antibody + saline ('TC'), or TNF antibody + MPTP ('TM'). All values represent mean ± SD; $n = 6–7$ mice. ANOVA followed by Dunnett's post hoc test was used to test for statistical significance using IC group as control. * represents statistical significance at $P < 0.05$.

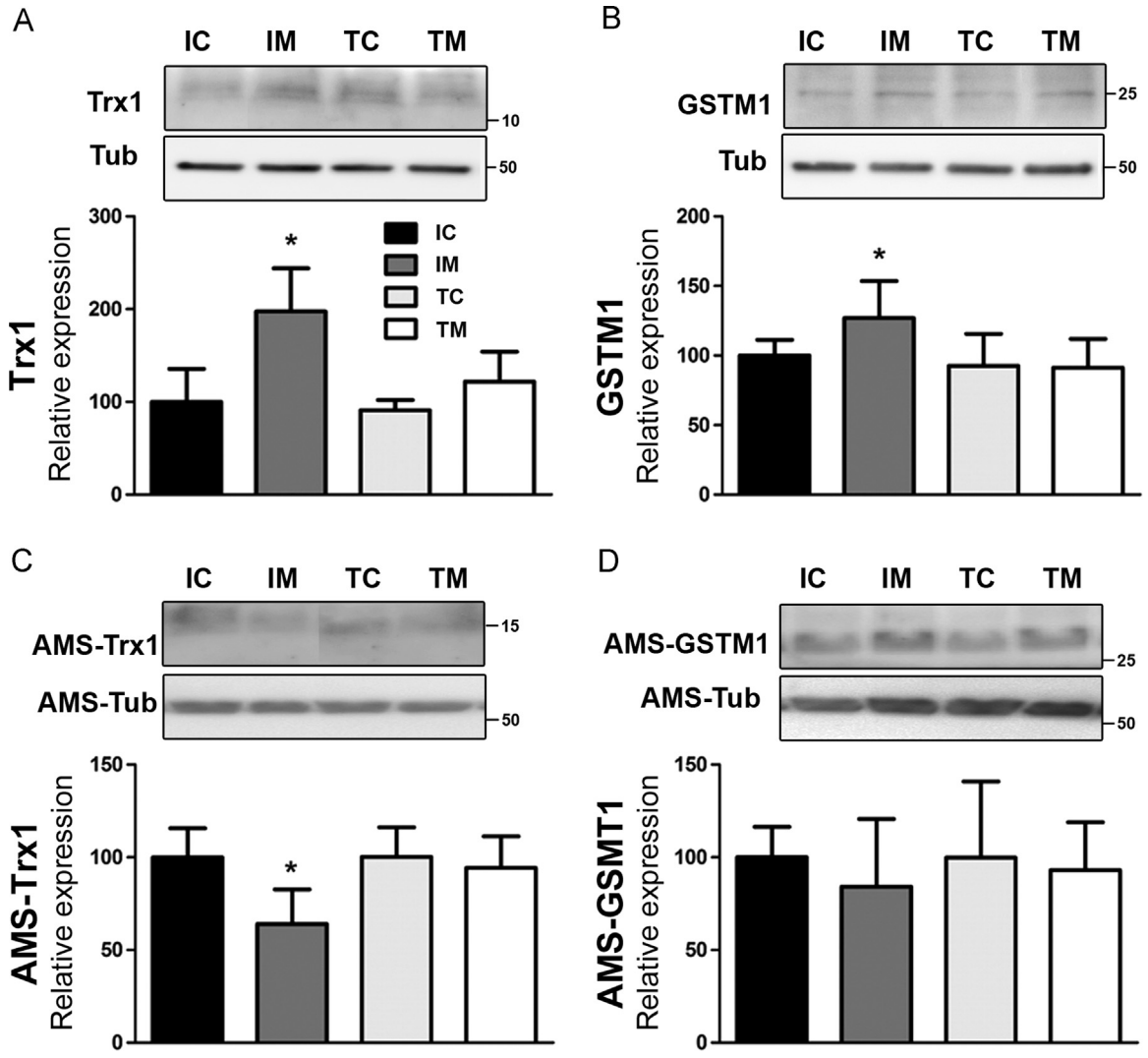

Fig. 9. Neutralizing TNF using prior intrathecal injection of antibody prevents Trx1 oxidation in ventral midbrain. Mice were injected with TNF-neutralizing antibody and MPTP as described in Fig. 8. A and B show levels of total Trx1 and total GSTM1 in ventral midbrain, respectively. Redox gel analysis after AMS derivatization show reduced Trx1 levels (AMS–Trx1; C) and reduced levels of GSTM1 (AMS–GSTM1; D). The four groups are Isotype antibody + saline ('IC'), Isotype antibody + MPTP ('IM'), TNF antibody + saline ('TC'), or TNF antibody + MPTP ('TM'). All graphs represent mean ± SD; $n = 4$-6 mice. ANOVA followed by Dunnett's post hoc test was used to test for statistical significance using IC group as control. * represents $P < 0.05$.

ASK1 complex [29]. It is also possible that GSTM1 may be targeting a different subcellular or cellular ASK1 pool, independently of Trx1.

On investigating the reasons for Trx1 dissociation, our results showed that MPTP exposure leads to PTO in a region- and protein-specific manner (Figs. 4 and 5). We observed that Trx1, but not GSTM1, was oxidized after MPTP treatment with a temporal profile congruent with ASK1 pathway activation (Figs. 1, 4 and 5). Notwithstanding averaging effects of GABAergic neurons on protein expression from dopaminergic terminals in ST, Trx1 could have oxidized preferentially in VMB due to the depletion of already low basal glutathione and increased complex I-mediated ROS in this region following MPTP administration [4,36]. While MPP^+, the active metabolite of MPTP, primarily targets complex I in the inner mitochondrial membrane resulting in generation of mitochondrial ROS, it can also undergo redox cycling in the cytoplasm as well as trigger dopamine leakage into the cytosol from vesicles, resulting in generation of ROS in the cytoplasm as well. Moreover, complex I inhibition can rapidly lead to depletion of cellular ATP and trigger oxidative stress in the cytoplasm [1]. Thus, a combination of such events could directly or indirectly lead to oxidation of the primarily cytoplasmic proteins, Trx1 and ASK1.

In our paradigm, we observed that TNF signaling was responsible for such oxidation since TNF neutralization prevented Trx1 and ASK1 oxidation (Figs. 8 and 9). TNF can increase mitochondrial ROS generation [35] that could potentially oxidize proteins having highly reactive cysteine residues, including Trx1, on account of being a thiol disulfide oxidoreductase (TDOR) [37]. The importance of Trx1 in the neurodegenerative process is further borne out by experiments demonstrating neuroprotection from MPTP in Trx1 overexpressing mice [38]. The redox status of Trx1 is also perturbed in MN9D cells treated with 6-OHDA [11]. Along with Trx1 oxidation, the selective ASK1 oxidation observed in VMB demonstrates that oxidative stress following MPTP exposure can lead to PTO, thereby initiating region-specific signaling cascades.

Amongst the several molecules studied, we found that increases in TNF levels were one of the earliest and consistent perturbations, occurring 30 min after MPTP administration in VMB, and sustaining for all but one time points studied (Fig. 6A). While inflammation has been reported in chronic models of MPTP [39], such early changes with single doses of MPTP have not been shown before. Thus, disruption in cytokine signaling can occur early in the disease process and may have an important role in triggering disease-related cell signaling pathways.

Using graph theory-based PCNA, we demonstrate for the first

time that TNF is a key upstream activator of ASK1 after a single dose of MPTP administration (Fig. 7). We confirmed a causal link by blocking TNF signaling in the brain by injecting TNF-neutralizing antibody (Fig. 8). TNF has been earlier shown to cause ASK1 phosphorylation in cells [28]. TNF acts through binding to its receptors (TNFR1/2), which further recruit the adaptor protein TRADD (TNFR1-associated death domain). TRADD can, in turn, activate the ASK1–MAPK pathway by associating with TRAF2 (TNFR-associated factor). TNF/TRAF2 signaling can activate ASK1 through ROS-dependent release of Trx1 from ASK1 and associating with ASK1 to induce homophilic interactions and autophosphorylation of ASK1 [14].

While the identity of cells responsible for the early TNF release is unclear, dopaminergic neurons are the most likely candidates as they are the first to be targeted by MPTP. They can potentially secrete TNF in a ROS-dependent manner. Indeed, ROS has been displayed to be necessary for activation of ADAM17 (TNF-releasing enzyme) in primary human monocytes [40]. Very early increase in ROS production has also been reported in brain regions in studies with MPTP [4,41]. While early changes in TNF could be due to molecular processes in neurons, alteration in TNF levels in VMB at later time points such as 8 and 12 h after MPTP could be contributed by both neurons and microglia [42]. One such potential mechanism could be p38 phosphorylation-dependent nuclear translocation of the NFκB complex (nuclear factor κ B) triggering transcription of inflammatory cytokines [43]. Other mechanisms could involve sustained oxidative stress, mitochondrial dysfunction, and/or early inflammatory burst [4,42,44].

While our study shows TNF to be responsible for ASK1 pathway activation in the early stages of neurotoxicity, it is uncertain whether TNF is also primarily responsible for the sustenance of the pathway in the neurodegenerative subchronic model as well (Fig. 1C). It is possible that while TNF may activate the pathway acutely, secondary signaling pathways and interacting partners could also get involved in sustenance of ASK1 activity chronically. In this regard, animal studies studying the role of TNF in Parkinsonian dopaminergic cell death have yielded confounding results. While Sriram et al. showed that striatal dopaminergic terminals from TNF receptor double knockout mice are protected from degeneration after MPTP intoxication [45,46], Rousselet et al. did not find any protection of dopaminergic neuronal numbers in SNpc of TNF receptor knockout mice [47]. TNF knockout animals also displayed similar differences in neuroprotection from MPTP [48,49]. Compared to knockout studies, inhibiting TNF in adult animals using virally delivered dominant negative TNF or using systemic inhibitor of soluble TNF offers dopaminergic neuroprotection, at least in the 6-OHDA hemiparkinsonian rat model [50–53]. Although such differences may arise from the choice of drug model, it will be interesting to see if MPTP uses alternate pathways for triggering cell death in knockout animals. Our transient TNF-neutralization approach with anti-TNF antibody (MP6-XT22) binds both membrane-bound and soluble TNF [54] and prevents MPTP-mediated increase without affecting basal levels of TNF (Fig. 8A). It is important to note that there are several molecular players involved in cell death of dopaminergic neurons in PD. The neuroprotection offered by ASK1 knockdown [11] or knockout [10] in neurotoxin-induced PD models is significant, although not complete. Thus, TNF signaling and indeed the subsequent activation of the ASK1 pathway may play an important role in triggering dopaminergic cell death during PD pathogenesis.

Finally, PCNA revealed insights about the network of proteins that we studied, including identification of TNF as an important node in the network to be targeted for disruption (Fig. 7; Suppl. Tables 1 and 2), which was further confirmed by experiments (Figs. 8 and 9). While the activation pattern of signaling molecules after MPTP exposure (Suppl. Fig. 1) and evidence from literature may intuitively suggest the importance of TNF, PCNA analysis confirmed the same in a mathematically rigorous and objective approach. Our analysis of a limited set of nodes known to operate in a well-defined molecular pathway precluded identification of any novel regulatory/co-regulated interactions between the studied proteins in the context of a larger proteomic network, similar to information potentially gleaned from gene covariation network analysis [55] and protein-protein interaction networks [34]. Also, because of the small network size, we could not test whether they have scale-free properties like most biological networks [16]. It will be interesting to study whether such protein expression networks also share these common topological themes. Nevertheless, our analysis reveals the potential value of looking at these changes through a network perspective. Large-scale analyses may indeed be carried out in the future by coupling data from "-omics" platforms, such as phosphoproteomics [56] and redox proteomics [57], to help us understand how post-translational modifications of proteins interact and regulate each other in normal and disease conditions, specifically in brain tissue containing remarkable cellular heterogeneity compared to other tissues.

5. Conclusions

Using the MPTP mouse model of Parkinsonism, we have successfully identified TNF as an early upstream activator of the ASK1-MAPK death signaling pathway, acting through downstream protein thiol oxidation, namely of the thiol disulfide oxidoreductase, Trx1. Thus, studying early changes such as inflammation and disruption of protein thiol homeostasis may play key roles as molecular triggers, initiating the process of neurodegeneration in PD and other diseases. Apart from studying the dysregulation of the pathway biochemically *in vivo*, we have also demonstrated the utility of network analysis approaches in understanding regulation of molecular signaling cascades.

Further, the ASK1 pathway has been shown to be perturbed in other neurodegenerative models as well, including models of Huntington's disease [58], Alzheimer's disease [59] and amyotrophic lateral sclerosis [60]. It will be interesting to study whether common mechanisms exist that lead to early activation of this pathway across diseases. More importantly, how cell-type specific neurotoxicity arises across these diseases is an intriguing question. To conclude, it is essential that we identify early effectors of cellular dysfunction and death process in different neurodegenerative conditions to help us discover disease-modifying strategies.

Acknowledgements

The authors thank Dr. Gary Merrill from Oregon State University, Corvallis, Oregon, USA for providing antiserum to thioredoxin and Dr. Nandini Chatterjee Singh (National Brain Research Centre, India) for providing critical inputs regarding the network analysis. The work was supported by Department of Science and Technology (IRHPA) and National Brain Research Centre. AR received graduate fellowship support from the Council of Scientific and Industrial Research, India. GR and VR acknowledge J. C. Bose fellowship. GR was also supported by the DST Centre for Mathematical Biology and University Grants Commission – Centre for Advanced Studies. The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.06.041.

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