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ORIGINAL ARTICLE

Potential widespread denitrosylation of brain proteins following prolonged restraint: proposed links between stress and central nervous system disease

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Abstract

The biochemical pathways by which aberrant psychophysiological stress promotes neuronal damage and increases the risks for central nervous system diseases are not well understood. In light of previous findings that psychophysiological stress, modeled by animal restraint, can increase the activities and expression levels of nitric oxide synthase isoforms in multiple brain regions, we examined the effects of restraint, for up to 6 h, on levels of S-nitrosylated proteins and NOx (nitrite + nitrate), a marker for high-level nitric oxide generation, in the brains of rats. Results identify functionally-diverse protein targets of S-nitrosylation in the brain, in vivo, and demonstrate the potential for widespread loss of protein nitrosothiols following prolonged restraint despite a concomitant increase in NOx levels. Since physiological levels of protein S-nitrosylation can protect neurons by maintaining redox homeostasis, by limiting excitatory neurotransmission, and by inhibiting apoptotic and inflammatory pathways, we propose that over-activation of protein denitrosylation pathways following sustained or repeated stress may facilitate neural damage and early stages of stress-related central nervous system disease.

Keywords Denitrosylation · S-Nitrosylation · Nitric oxide · Psychophysiological stress

Introduction

The psychophysiological stress response, mediated by activation of the hypothalamus-pituitary-adrenal axis and increased production of adrenal-derived glucocorticoids (GC) and other stress-related hormones, has emerged as a critical link between the environment and central nervous system (CNS) health (Chrousos 2009). Indeed, sustained or repeated stress can induce oxidative stress (Liu et al. 1996), excitotoxicity (Sapolsky 1994), and neuroinflammation (Sorrells et al. 2009) and is recognized increasingly as a risk factor for diverse CNS diseases ranging from psychiatric illnesses (Lucassen et al. 2014) to neurodegenerative disorders (Vyas et al. 2016). The biochemical pathways which underlie both the resilience and sensitivity of the CNS to stress remain to be fully established.

Nitric oxide (NO) has emerged as a possible mediator of stress signaling in the brain (Chen et al. 2015). NO is generated in the brain by constitutively-expressed neuronal and endothelial nitric oxide synthase (NOS) isoforms and by inducible NOS (iNOS), the latter able to generate relatively large amounts of nitric oxide (Hetrick and Schoefisch 2009). Much of NO signaling proceeds by the transfer of nitrosonium ion (NO^+) equivalents to protein thiols, i.e., protein S-nitrosylation (Gould et al. 2013). Deregulation of protein S-nitrosylation has been implicated in aging-related cognitive dysfunction (Zhang et al. 2017) and neurodegeneration (Nakamura et al. 2015). Critically, a baseline level of S-nitrosylation in healthy cells appears to be necessary to prevent inflammation (Marshall et al. 2004) and apoptosis (Haendeler et al. 2002), to limit potentially damaging excitatory neurotransmission (Choi et al. 2000), and to maintain cellular redox homeostasis (Haendeler et al. 2002).

Modeling psychophysiological stress by animal restraint has been reported to increase, in multiple regions from rat brain, the activities and/or expression levels of NOS

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isoforms over a period of hours (Madrigal et al. 2001; Chen et al. 2016; Spiers et al. 2016). In light of these earlier studies, we investigated the effects of restraint on levels of S-nitrosylated proteins from the brains of rats. The objectives of these initial, discovery-based, experiments were (i) to determine if, and following what periods, exposure of animals to restraint may induce changes in protein S-nitrosylation and (ii) to identify proteins showing changes in S-nitrosylation in response to stress.

Materials and methods

Materials

EZ-Link™ HPDP-Biotin, dithiothreitol, Imperial™ Protein Stain, N-ethylmaleimide, Pierce™ Coomassie Plus Protein Assay, and Pierce™ Protease Inhibitor Mini Tablet were purchased from ThermoFisher Scientific. Mini-Protean® TGX™ precast 4–20% gels and Precision Plus Protein™ Dual Color Standards were purchased from Bio-Rad. The Nitrite/Nitrate Colorimetric Assay Kit was purchased from Cayman Chemical. All other chemicals were purchased from Sigma-Aldrich.

Animal study

All procedures were approved by the University Institutional Animal Care and Use Committee. Young adult (6–7 weeks-old) male Sprague-Dawley rats were housed in our animal facility with continual access to standard rodent chow and water. Rooms were programmed for 6 AM/6 PM light/dark cycles and animals were acclimated to the facility for at least 1 week prior to the administration of restraint and subsequent euthanasia. Restraint was applied in a room adjacent to the holding room by an established procedure described by Madrigal et al. (2001) using Decapicone™ restrainers (Braintree Scientific) closed at the tail end with a wire tie. Each of the three stressed groups (2 h, 4 h or 6 h of restraint) was comprised of four animals while the control group contained five animals. Animals from the stressed groups were euthanized by decapitation immediately following the designated restraint period. Control animals were euthanized immediately after being placed in the Decapicones™. Animals from the control and 2 h groups were euthanized on day 8 (post arrival) while animals from the 4 h and 6 h groups were euthanized on day 9 so that all animals in the study could be euthanized between noon and 2 PM. Brains were collected rapidly by dissection, snap-frozen in liquid nitrogen, and stored at −80 °C until use.

Processing of brains

Brains were partially thawed on ice and homogenized, using a 30-mL glass-Teflon homogenizer, with 10 mL of 20 mM acetic acid/acetate buffer pH 4 which contained one predissolved Pierce™ Protease Inhibitor Mini Tablet and had been purged with N_2 for 20 min. Homogenates were immediately diluted 1:1 with Tris experimental buffer (TEB; 500 mM Tris, 10 mM EDTA, 1 mM benzamidine, and 1% *v*/v Triton X-100, pH 7.5) containing 200 mM N-ethylmaleimide (NEM) and allowed to incubate at room temperature while rotating for 30 min to facilitate alkylation of protein thiols. The alkylated homogenates were centrifuged at 100,000 g for 1 h to yield a supernatant containing Triton X-100-soluble protein.

Biotinylation of protein nitrosothiols

S-Nitrosylated protein thiols were biotinylated by the biotin-switch procedure as described by Forrester et al. (2009). Briefly, following removal of unreacted NEM by acetone precipitation and repeated washing of the resulting protein pellets with 70% acetone, pellets were resuspended with a minimal volume of TEB containing 1% Triton X-100 and kept on ice. Samples were incubated for 1 h in the dark following addition of ascorbate (20 mM final) and EZ-Link™ HPDP-Biotin (5 mM) to reduce protein nitrosothiols and biotinylate resulting protein thiols, respectively. Biotin-HPDP links biotin to proteins by a cleavable disulfide bond. Unreacted biotin-HPDP was removed by a second round of acetone precipitation. The protein pellet was resuspended with a minimum volume of TEB containing and kept on ice. Protein concentrations of these reduced and biotinylated protein samples were determined by the Pierce™ Coomassie Plus Protein Assay and diluted with TEB to 1 mg protein/mL.

Capture of biotinylated proteins by immobilized avidin-affinity chromatography

One-half mL of the 1 mg/mL biotinylated protein samples were combined with 0.2 mL of packed and dry immobilized avidin in microspin columns. Following mixing of the immobilized avidin and protein samples, columns were sealed and incubated, while rotating, for 1 h at room temperature. Following collection of unbound protein in the flow-through (FT) fractions by centrifugation, columns were washed 6x with 0.5 mL TEB each to remove residual unbound and weakly-bound protein. The last washes (LW) were saved for analysis. Following centrifugation without addition of TEB to again pack the immobilized avidin to dryness, the columns were resuspended with 0.5 mL of TEB containing 20 mM DTT and incubated for 15 min at room temperature while rotating to cleave the disulfide bonds linking the biotinylated proteins to the avidin. Proteins released by DTT were

collected by centrifugation. The protein concentration of this DTT-eluted fraction was determined by the Pierce™ Coomassie Plus Protein Assay. Pre-column, FT, LW, and DTT-eluted fractions were diluted 1:1 with 80% glycerol (*v*/v) and stored at −20 °C.

Measurement of NOx

Brain homogenates were diluted 1:1 with TEB without NEM and centrifuged at 100,000 g for 65 min. Total NOx, nitrite plus nitrate, was determined in aliquots of the resulting supernatants by the Griess assay (Hetrick and Schoefisch 2009) using the Nitrite/Nitrate Colorimetric Assay Kit from Cayman Chemical. This method has a high, micromolar, limit of detection (Hetrick and Schoefisch 2009) such that measurable increases in Griess-reactive NOx will occur most readily following the induction of iNOS. The protein content of each sample was quantified using the Pierce™ Coomassie Plus Protein Assay.

Analysis and identification of S-nitrosylated proteins

Following biotinylation of S-nitrosylated proteins and fractionation by immobilized avidin-affinity chromatography, proteins in the resulting fractions were resolved by SDS-polyacrylamide gel electrophoresis on Mini-PROTEAN® TGX™ precast 4–20% gels using Precision Plus Protein™ Dual Color Standards as molecular weight markers. Proteins were visualized using Imperial™ Protein Stain.

Representative DTT samples from control and 2 h-stressed groups were run onto gels briefly and unresolved gel bands containing the entire protein compositions of these fractions were excised and shipped to MSBioworks LLC (Ann Arbor, MI) for protein identification by LC-MS/MS.

Statistical analyses

Data are reported as the means ± standard deviations for $N = 4–5$ where each brain and, therefore, each animal represents an $N = 1$. All incubations giving rise to single measured value ($N = 1$) were performed in duplicate. Statistically-significant differences between study groups were examined by unpaired, two-tailed, student t-tests. Statistical significance, specified by P values <0.5, is noted in the figures and explained in the figure legends. P values for non-significant differences are provided in the relevant text.

Reactome pathway analysis

Pathway overrepresentation among the proteins identified in the S-nitrosylated fraction from a 2 h-stressed brain was performed using the Reactome pathway analysis at Reactome.org (Fabregat et al. 2018).

Results and discussion

Protein S-nitrosylation changed in an apparent biphasic manner as a function of the duration of restraint (Fig. 1a). Thus, a relatively short-term restraint of 2 h produced a non-significant increase ($P = 0.16$) in levels of protein S-nitrosylation of 46% above the levels from the brains of unstressed controls while longer periods of restraint yielded lower levels of protein S-nitrosylation. Importantly, 6 h of restraint produced a significant ($P = 0.017$) decrease of 64% in the levels of S-nitrosylated proteins compared to the levels determined following only 2 h of restraint but generated a smaller, and non-significant decrease, of 47% compared to the unstressed

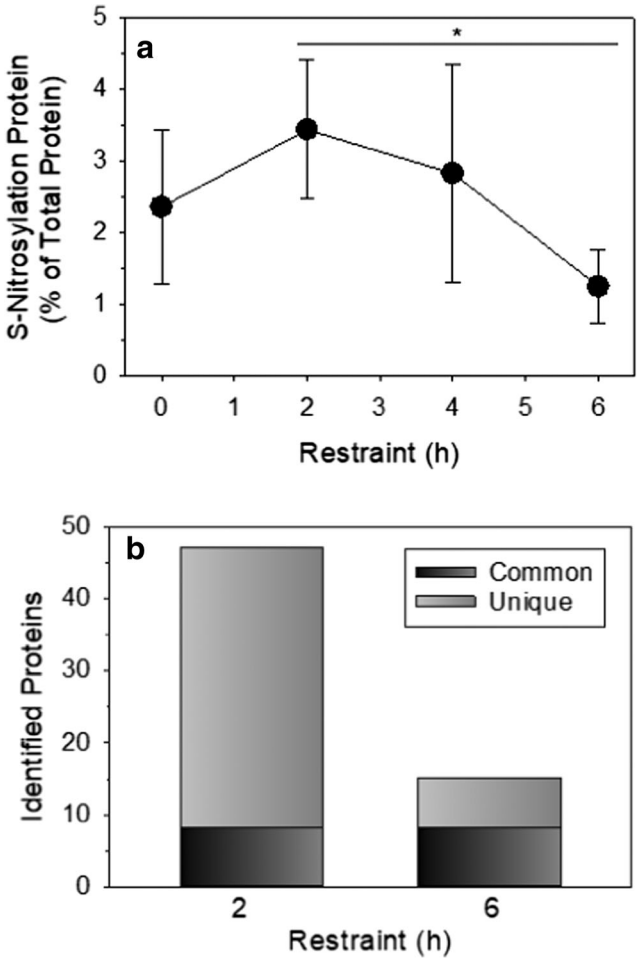

Fig. 1 Restraint induces an apparent biphasic change in protein S-nitrosylation. Alkylated 100,000 g supernatants from the brains under study were subjected to the biotin-switch method to biotinylate protein thiols which had been S-nitrosylated in the brain and resulting biotinylated proteins were isolated by immobilized avidin-affinity chromatography. **a** Amounts of formerly S-nitrosylated proteins, eluted from immobilized avidin by DTT-sensitive cleavage of the biotinyl moiety, were quantified and expressed as the percentages of total proteins applied to the immobilized avidin columns. Data are the means ± SD for $N = 4–5$. $^{*}P < 0.05$ for comparison of values obtained following 2 h and 6 h of restraint. **b** The numbers of proteins identified by LC-MS/MS

controls ($P = 0.12$). While 47 proteins were identified by LC-MS/MS in the S-nitrosylated protein fraction (i.e., the DTT-eluted fractions) from a representative 2 h-stressed animal, only 15 proteins were identified in the S-nitrosylated fraction from a representative 6 h-stressed animal (Fig. 1b, Table 1). Thus, a substantial decrease in both the extent of protein S-nitrosylation and the number of S-nitrosylated proteins was observed following 6 h of restraint compared to only 2 h of restraint.

Changes in NOx levels (Fig. 2), appeared to reciprocate those in protein S-nitrosylation (Fig. 1a). Most notably, NOx

Table 1 Identified S-nitrosylated proteins from 2 h- and 6 h-stressed brains

			2 h stressed			6 h stressed		
Protein	Gene(s)	Accession	SpC	%Cov	%Prob	SpC	%Cov	%Prob
Hemoglobin beta subunits	Hbb2	P11517	18	71	100	nd	nd	nd
	Hbb1	P02091	16	59	100	2	15	100
Actin, cytoplasmic 1	Actb	P60711	17	29	100	6	22	100
Albumin	Alb	P02770	14	24	100	2	3	98
Tubulin beta-2A chain	Tubb2a	P85108	12	18	100	nd	nd	nd
Tubulin alpha-1A chain	Tuba1a	P68370	11	24	100	nd	nd	nd
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	P04797	10	24	100	6	12	100
Myelin basic protein	Mbp	P02688	10	36	100	nd	nd	nd
14–3-3 protein gamma	Ywhag	P61983	10	29	100	nd	nd	nd
Alpha-enolase	Eno1	P04764	8	16	100	nd	nd	nd
Annexin A1	Anxa1	P04764	8	20	100	nd	nd	nd
Heat shock cognate 71 kDa protein	Hspa8	P63018	8	14	100	nd	nd	nd
Fructose-bisphosphate aldolase A	Aldoa	P05065	7	16	100	nd	nd	nd
Heat shock protein HSP 90-beta	Hsp90ab1	P34058	7	8.1	100	nd	nd	nd
Myelin proteolipid protein	Plp1	P60203	7	24	100	nd	nd	nd
Rab GDP dissociation inhibitor alpha	Gdi1	P50398	7	11	100	nd	nd	nd
14–3-3 protein zeta/delta	Ywhaz	P63102	7	26	100	nd	nd	nd
Dihydropyrimidinase-related protein 2	Dpysl2	P47942	6	12	100	nd	nd	nd
Histone H4	Hist1h4b	P62804	6	39	100	nd	nd	nd
L-lactate dehydrogenase B chain	Ldhb	P42123	6	18	100	nd	nd	nd
L-lactate dehydrogenase A chain	Ldha	P04642	6	16	100	nd	nd	nd
Histone H2B type 1	H2b1	Q00715	5	27	100	2	13	94
2′,3′-cyclic-nucleotide 3′-phosphodiesterase	Cnp	P13233	5	10	100	nd	nd	nd
Fructose-bisphosphate aldolase C	Aldoc	P09117	5	10	100	nd	nd	nd
Citrate synthase, mitochondrial	Q8VHF5	4	8.6	100	nd	nd	nd	
Protein-glutamine gamma-glutamyltransferase K	Tgm1	P23606	4	4.6	100	nd	nd	nd
Creatine kinase B-type	Ckb	P07335	4	7.3	100	nd	nd	nd
Gamma-enolase	Eno2	P07323	3	7.6	100	nd	nd	nd
Histone H3.3	H3f3b	P84245	3	15	100	2	10	99
Triosephosphate isomerase	Tpi1	P48500	3	12	100	nd	nd	nd
Peroxiredoxin-2	Prdx2	P35704	3	14	100	2	9	98
Aspartate aminotransferase, cytoplasmic	Got1	P13221	3	7	100	nd	nd	nd
Lysozyme C-1	Lyz1	P00697	3	11	100	nd	nd	nd
Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	Ppp3ca	P63329	3	5.2	100	nd	nd	nd
14–3-3 protein epsilon	Ywhae	P62260	3	9	99	nd	nd	nd
ADP/ATP translocase	Slc25a4	Q05962	2	6	100	nd	nd	nd
Nucleoside diphosphate kinase B	Nme2	P19804	2	12	100	nd	nd	nd
ATP-dependent 6-phosphofructokinase, liver type	Pfkl	P30835	2	2.3	100	nd	nd	nd
Glutathione S-transferase Yb-3	Gstm3	P08009	2	7.8	100	nd	nd	nd
Pyruvate kinase	Pkm	P11980	2	3.8	100	nd	nd	nd
Syntaxin-binding protein 1	Stxbp1	P61765	2	3.4	100	nd	nd	nd
Phosphatidylethanolamine-binding protein 1	Pebp1	P31044	2	8	100	nd	nd	nd
Superoxide dismutase [Cu-Zn]	Sod1	P07632	2	16	100	nd	nd	nd
Polyubiquitin-B	Ubb	P0CG51	2	6.2	100	3	7.1	100
Aspartate aminotransferase, mitochondrial	Got2	P00507	2	3.7	99	nd	nd	nd
Glycogen phosphorylase, brain form	Pygb	P53534	2	1.8	98	nd	nd	nd
Rho GDP-dissociation inhibitor	Arhgdia	Q5XI73	2	11	90	nd	nd	nd
Annexin A2	Anxa2	Q07936	nd	nd	nd	3	7.1	100
Hemoglobin subunit alpha-1/2	Hba1	P01946	nd	nd	nd	3	13	100
Microtubule-associated protein 2	Map2	P15146	nd	nd	nd	3	1	93
Arginase-1	Arg1	P07824	nd	nd	nd	2	5	99
Catalase	Cat	P04762	nd	nd	nd	2	2	95
Serotransferrin	Tf	P12346	nd	nd	nd	2	2	88
Bleomycin hydrolase	Blmh	P70645	nd	nd	nd	2	3	85

DTT-eluted protein fractions from immobilized avidin-affinity columns, containing formerly S-nitrosylated proteins, from representative 2 h and 6 h stressed brains were run onto polyacrylamide gels but not resolved. The unresolved gel bands were excised and shipped to MSBioworks (Ann Arbor, MI) for in-gel digestion and identification by LC-MS/MS. Proteins which gave fewer than 2 spectral counts were excluded. ND = not detected

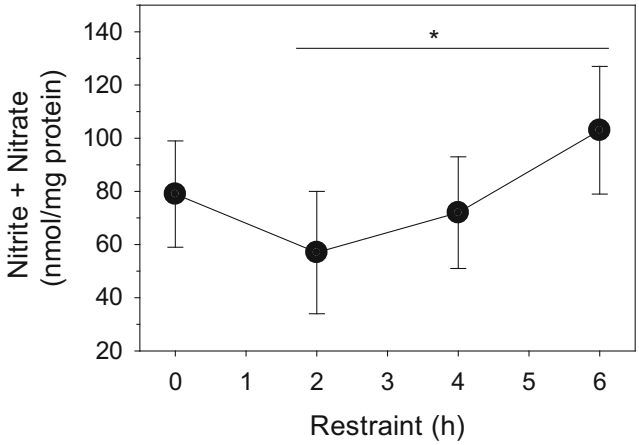

Fig. 2 Prolonged restraint can increase NOx levels in the brain. Levels of total NOx, nitrite plus nitrate ions, were determined by the Griess assay (Hetrick et al. 2009). Data are the means ± SD for $N = 4–5$. $^{*}P < 0.05$ for comparison of values obtained following 2 h and 6 h of restraint

levels increased significantly ($P = 0.013$), and by 84%, following 6 h of restraint compared to levels detected after 2 h of restraint.

Reactome pathway analysis (Fabregat et al. 2018) identified 65 pathways which were significantly overrepresented [false discovery rates (FDR) < 0.05] by the proteins identified in the S-nitrosylated fraction from the 2 h-stressed brain suggesting that the observed protein denitrosylation following prolonged stress might affect numerous neural processes. Table 2 highlights the top ten pathways ranked by FDR. Pathways centered on glucose metabolism were the most significantly overrepresented by these proteins although this result may be biased by the relative abundance of glycolytic enzymes. With the exception of glyceraldehyde-3-phsophate dehydrogenase (GAPDH), which can be inhibited by S-nitrosylation (Molina y Vedia et al. 1992), the significance of S-nitrosylation for the other identified glycolytic enzymes is not well understood. Inhibition of GAPDH activity, and glycolysis in general, can be an important switch, resulting in the rerouting of glucose to the pentose phosphate pathway (PPP) which generates NADPH to support cellular antioxidant defenses (Rasler et al. 2007). Consistent with the role of S-nitrosylation as an intermediate in protein disulfide bond formation (Wolhuter et al. 2018), many of the identified S-nitrosylated proteins- including GAPDH-were found by us previously to also form disulfide bonds in the brain (Foley et al. 2014; Foley et al. 2016).

Table 2 Pathways overrepresented among the proteins identified in the S-nitrosylated fraction from the 2 h-stressed brain

Pathway name	#Entities found	Entities FDR
Glucose metabolism	9	1.93E-07
Gluconeogenesis	7	1.93E-07
Glycolysis	7	8.04E-06
RHO GTPases activate PKNs	5	2.56E-04
Translocation of SLC2A4 (GLUT4) to the plasma membrane	3	5.67E-04
Metabolism of carbohydrates	9	5.78E-04
Chk1/Chk2(Cds1) mediated inactivation of Cyclin B:Cdk1complex	3	9.50E-04
Recycling pathway of L1	4	0.001512
Activation of BAD and translocation to mitochondria	3	0.002049

Pathway overrepresentation analysis was performed using the Data Analyzer at Reactome.org (Fabregat et al. 2018). The top 10 overrepresented pathways ranked by highest probability (lowest false discovery rate, FDR) are shown

The present findings suggest that levels of protein S-nitrosylation in the brain may increase following a relatively short restraint period. Moreover, they demonstrate the potential for a profound and global denitrosylation of brain proteins following prolonged restraint. We suggest that the non-significant increase in protein S-nitrosylation observed following the relatively short restraint period of 2 h may reflect a compensatory response which can prevent neuronal damage during mild stress but which cannot be sustained and gives way to a net and widespread protein denitrosylation. While aberrant protein S-nitrosylation has been implicated in neurodegenerative disease (Nakamura et al. 2015), emerging evidence supports the view that basal S-nitrosylation of proteins may be cytoprotective. Thus, excessive protein denitrosylation can potentially damage neural tissue by over-activating inflammatory signaling (Marshall et al. 2004), excitatory neurotransmission (Choi et al. 2000), and apoptotic pathways (Haendeler et al. 2002) and by deregulating cellular redox homeostasis (Haendeler et al. 2002). In this light, it is noteworthy that prolonged restraint stress can activate neuroinflammatory signaling (Madrigal et al. 2001) and promote oxidative stress (Liu et al. 1996) with time courses coinciding to the onset of protein denitrosylation reported here.

The observation that hemoglobin beta subunits were among the most prominent proteins in the S-nitrosylated fraction from the 2 h-stressed brain, but markedly reduced, or not detected, in the S-nitrosylated fraction from the 6 h-stressed brain (Table 1), raises the possibility that protein denitrosylation in the brain following prolonged stress may extend to the cerebrovascular system. Importantly, nitrosylated beta subunits of hemoglobin (SNO-Hb) serve as a sink of NO^{+} equivalents and transfer these to endothelial protein thiols to dilate blood vessels and couple tissue perfusion to metabolic demand (Allen et al. 2009). In this context, it is noteworthy that cerebral hypoperfusion is linked to the earliest stages of Alzheimer's disease (Daulatzai 2017).

The lack of significant differences in levels of protein S-nitrosylation in the controls from those of either the 2 h or the 6 h restraint groups warrants consideration and may relate, in part, to the use of whole brains rather than brain regions in

this study. Thus, it is plausible that the effects of restraint on nitric oxide metabolism and protein S-nitrosylation may be different, and may occur with distinct kinetics, in different brains regions and that investigation, in future studies, of the impacts of restraint on these measures in discrete brain regions may unmask significant differences from the controls. An alternative explanation for the overlap in the measures of nitric oxide metabolism between the controls and the 6 h-stressed group, in particular, may relate directly to the high baseline inflammation that is well documented in standard laboratory animals due to adlib feeding and a sedentary lifestyle (Martin et al. 2010). Thus, the nuclear translocation and DNA binding of NF-kappaB, which controls the expression of inflammatory mediators (Shih et al. 2015), are blocked by S-nitrosylation of multiple proteins (Marshall et al. 2004). Indeed, the inverse relationship between levels of protein S-nitrosylation and NOx reported here (Figs. 1a and 2) may best be explained by the negative regulation of NF-kappaB-mediated expression of iNOS by protein S-nitrosylation (Park et al. 1997). In this light, the differences in nitric oxide metabolism observed by us are consistent with the possibility that the activities of neuroinflammatory pathways may have been lower in the 2 h restraint group than in the controls. It follows that the differences between the 2 h and 6 h restraint groups may better reflect the potential of prolonged stress to negatively impact inflammation-related nitric oxide metabolism in the brain.

The findings that widespread denitrosylation of proteins occurred following 6 h of restraint compared to levels recorded after 2 h of restraint, in the face of increased nitric oxide production during this same period, argues that denitrosylation likely required upregulation of the activities of one or more protein denitrosylases. Protein denitrosylation is catalyzed mainly by (i) the thioredoxin (Trx) system, comprised of compartment-specific Trx and Trx reductase isoforms, and (ii) the glutathione-dependent nitrosoglutathione reductase system (Benhar et al. 2009) both of which rely on electrons from NADPH. Peroxynitrite, the product of the reaction between nitric oxide and superoxide (Beckman and Koppenol 1996), can produce a rapid increase in NADPH levels in both neurons and astrocytes by activating the glucose-dependent PPP (Garcia-Nogales et al. 2003). Furthermore, in light of findings that stress and GC can promote elevations in blood glucose (Li et al. 2013), it is noteworthy that hyperglycemia was found to induce widespread protein denitrosylation in endothelial cells (Wadham et al. 2007).

In summary, the results described here demonstrate the potential for a widespread denitrosylation of proteins in the brain following prolonged restraint stress. We hypothesize that aberrant protein denitrosylation, perhaps involving increased activity of the PPP and an associated reductive stress, may be a pivotal link between prolonged or repeated psychophysiological stress and early stages of CNS disease.

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