Supplementary Information

Global lipidomics identifies cardiolipin oxidation as a mitochondrial target for redox therapy of acute brain injury

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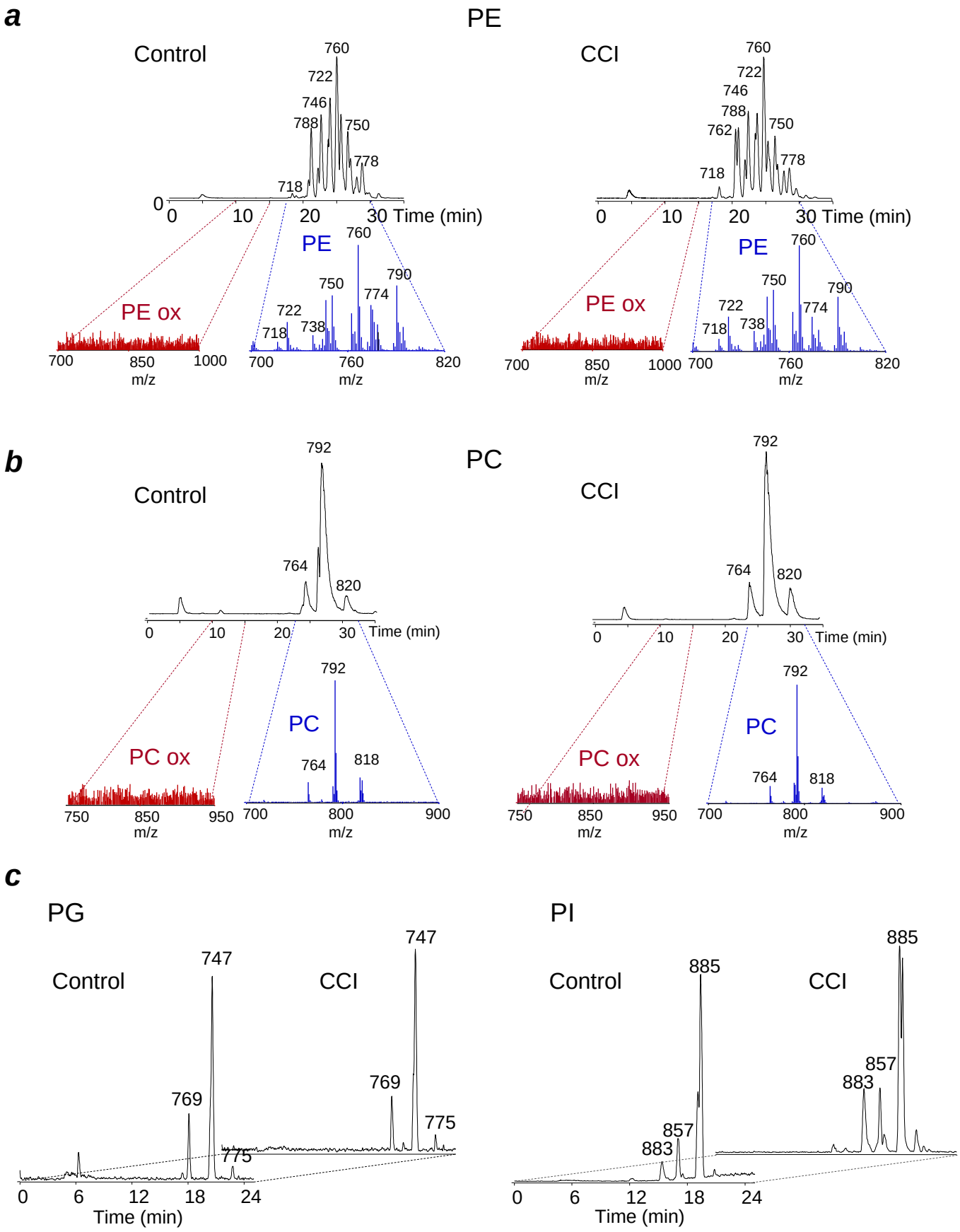
Major CL species undergoing oxidation		Oxygenated CL molecular species			
m/z	Molecular species	m/z	Plus three oxygens	Plus five oxygens	Plus six oxygens
1476	18:1/18:1/18:2/20:4	1524	+		
1476	18:0/18:2/18:2/20:4	1572			+
1500	18:0/18:2/18:2/22:6	1580		+	
1502	18:0/18:1/18:2/22:6	1550	+		
1502	18:0/18:1/18:2/22:6	1582		+	
1504	18:0/18:0/18:2/22:6	1552	+		

Supplementary Table 1. Major CL species that underwent oxidation and oxidation products generated after TBI


a
PE
Control
760
722
746
788
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778
718
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30 Time (min)
PE
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PE ox
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Time (min)
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PC ox
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Time (min)
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PC ox
750
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m/z
700
800
900
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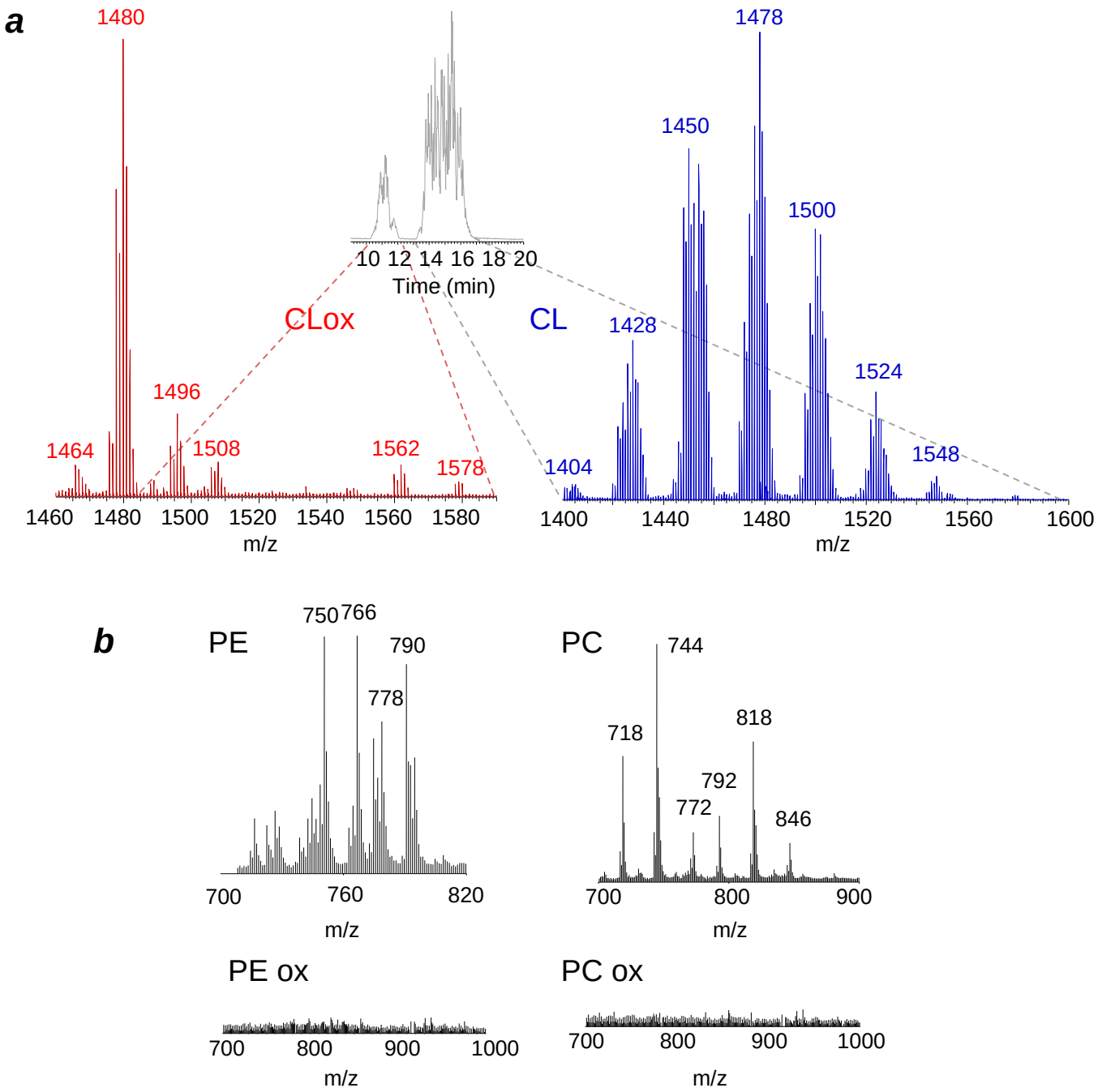
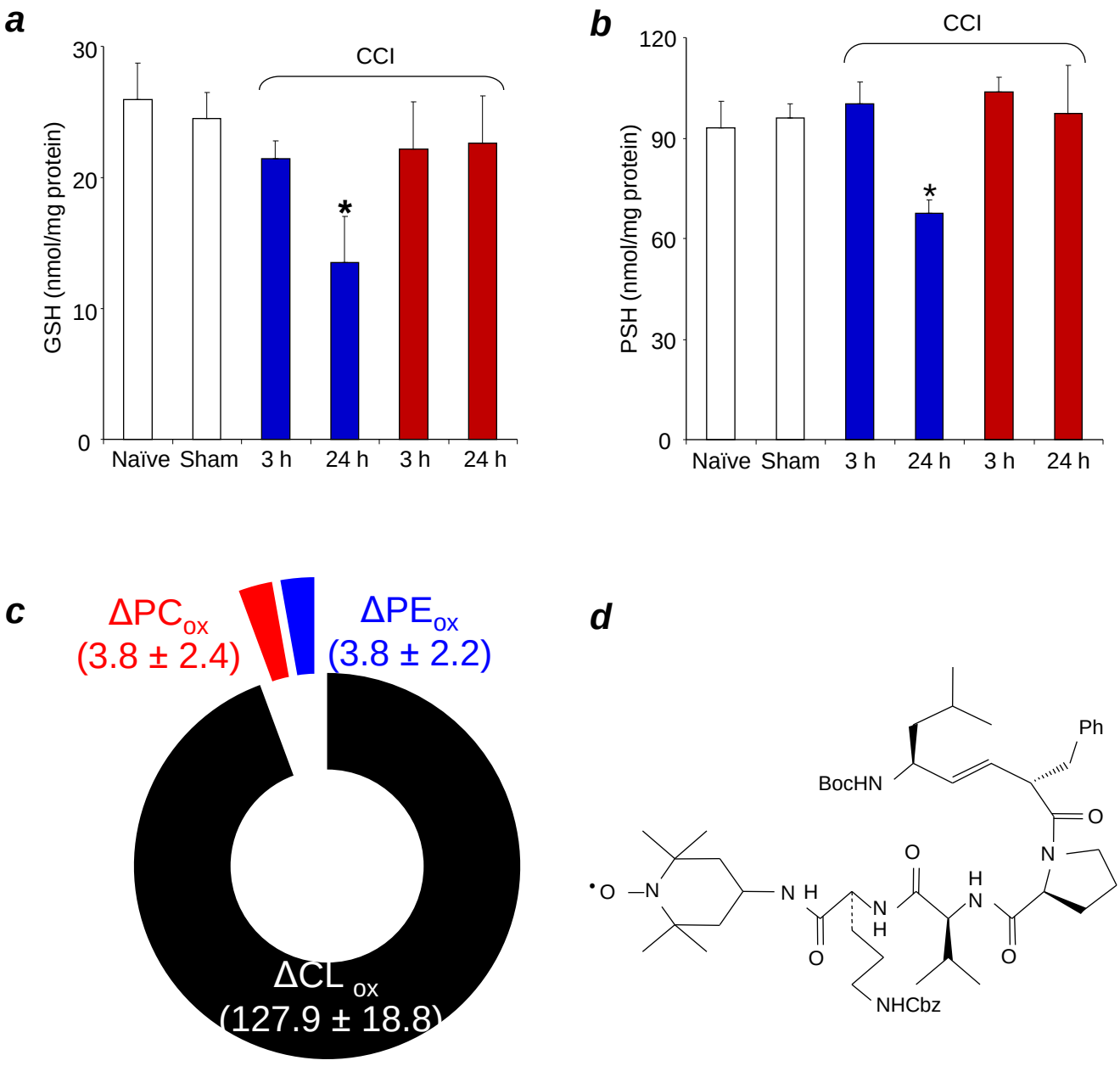
PG
Control
747
769
775
CCI
747
769
775
0
6
12
18
24
Time (min)
PI
Control
885
857
883
CCI
885
857
883
0
6
12
18
24
Time (min)

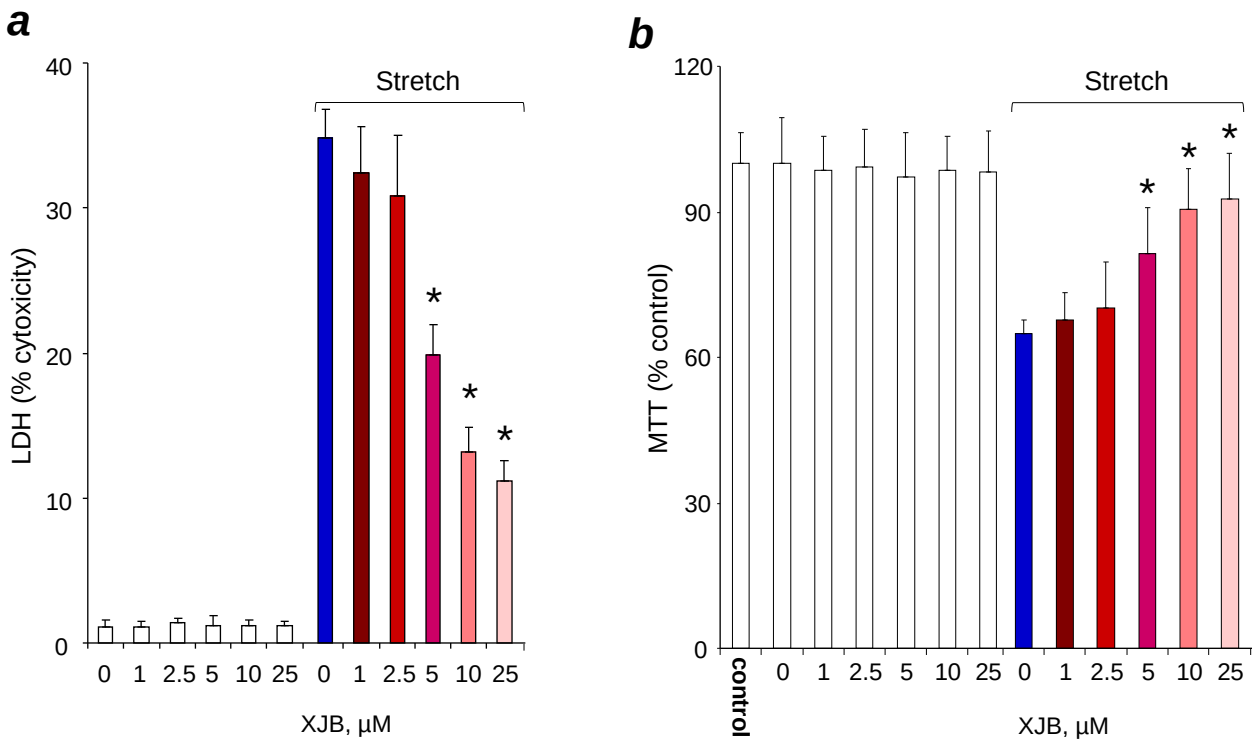
Supplementary Figure 1. 2D-LCMS characterization of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI) and their oxidation products after TBI.
(a, b) Representative reverse phase chromatograms and MS characterization of non-oxidized (shown in blue) and oxidized (shown in red) species of PC and PE in control and TBI brains. There was no accumulation of oxidation products in these two most abundant classes of phospholipids in rat brain after TBI.
(c) Representative reverse phase chromatograms for PG and PI in control and TBI brain. No changes in chromatographic profiles for PG and PI between control and TBI were detectable.



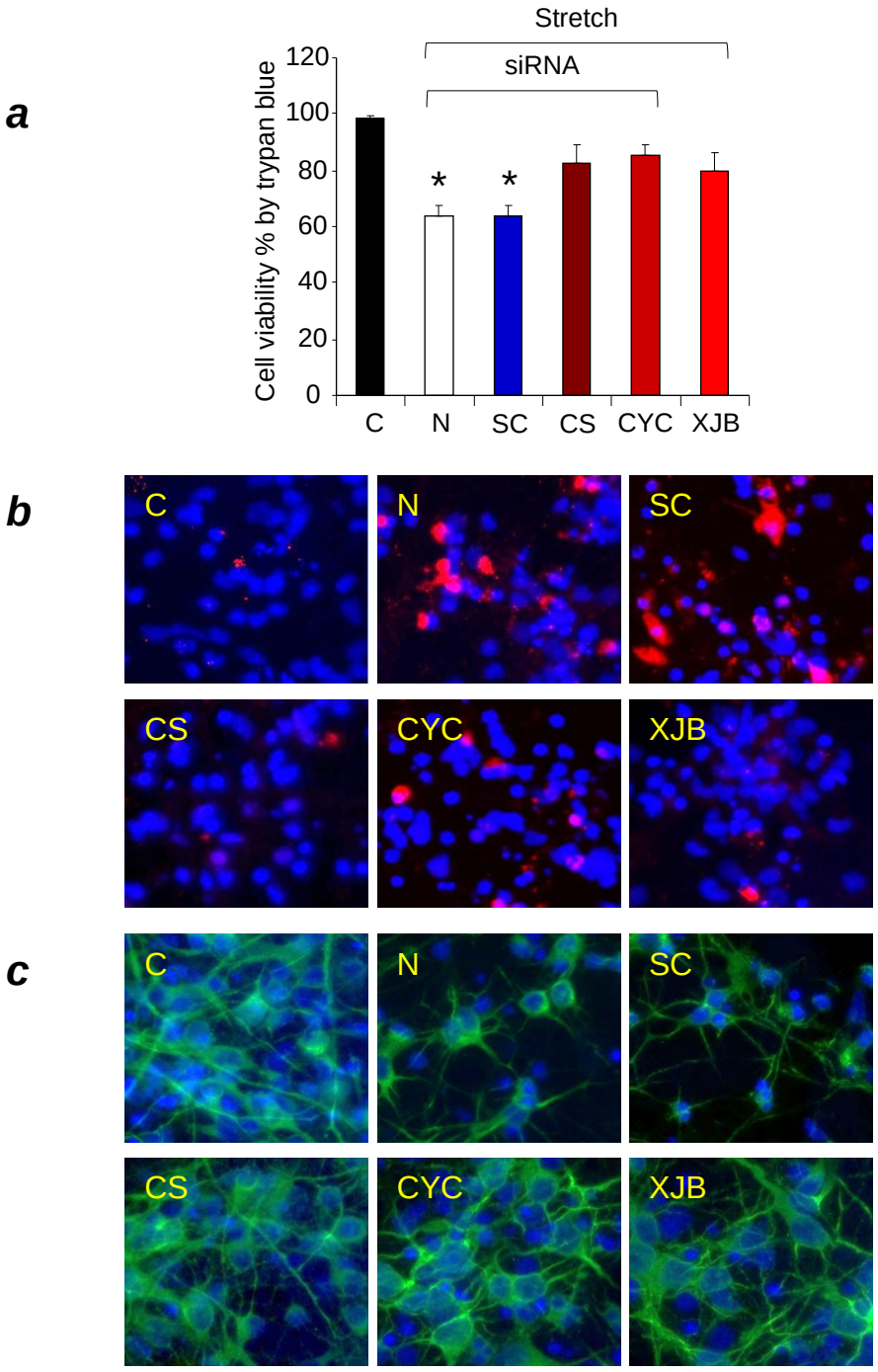
Supplementary Figure 2. 2D-LCMS analysis of phospholipids in the penumbra of contused brain tissue taken from a TBI patient with intractable hypertension. **(a)** Analysis of CL and CLox by 2D-LCMS in human brain tissue after TBI. The tissue was obtained by right temporal lobe resection from a patient with intractable intracranial hypertension in the acute stage following severe TBI. Upper insert: 2nd dimension chromatographic separation of non-oxidized and oxidized CL. The latter eluted during the 10-12 min retention time window. Lower panel demonstrates non-oxidized (blue) and the appearance of numerous oxidized (red) CL (CLox) species after TBI. **(b)** Spectra obtained from oxidized and non-oxidized fractions after reverse phase chromatography for PC and PE in the same brain tissue. In contrast to CL, no oxidation products were detected in PC or PE.



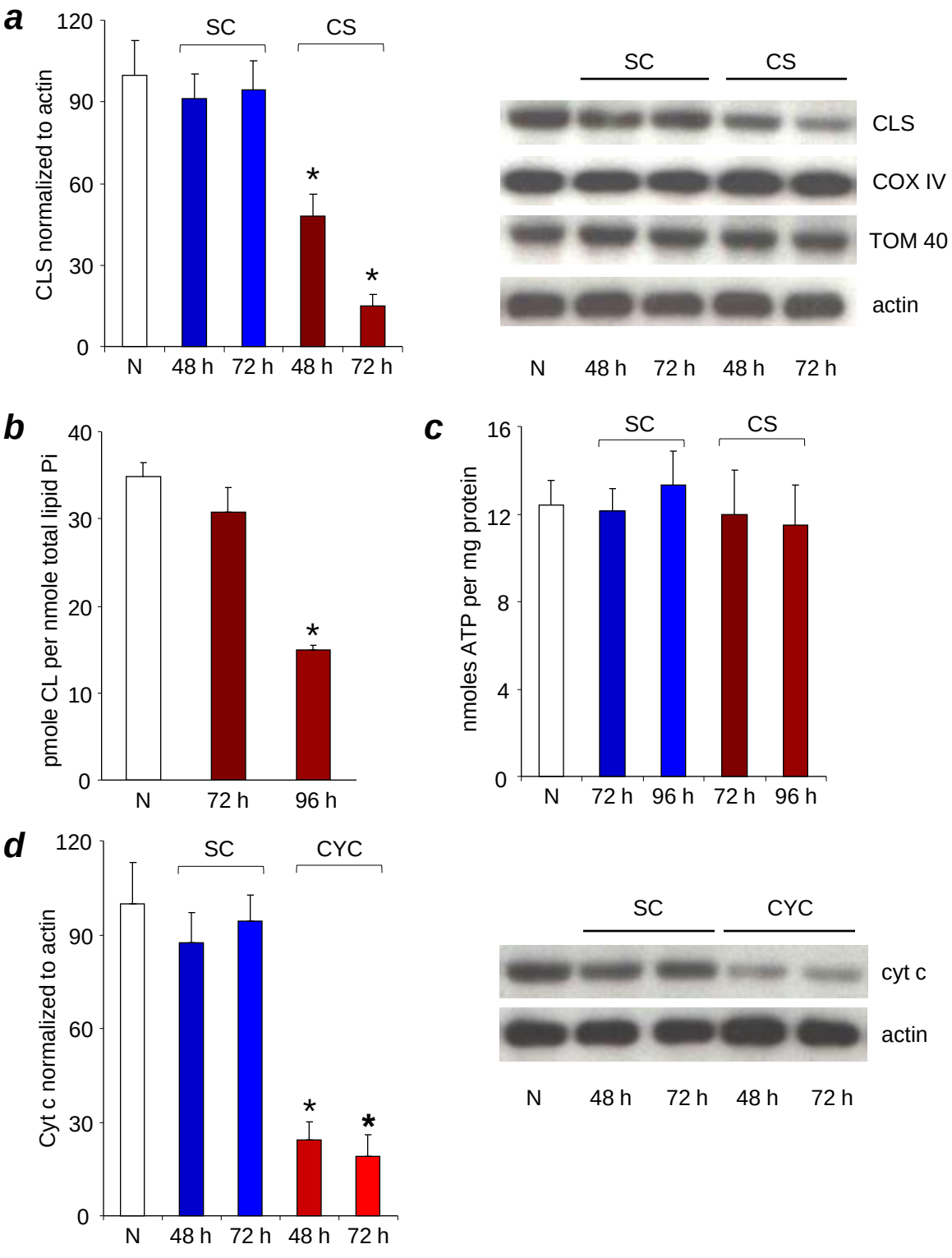
Supplementary Figure 3. Biochemical analysis after TBI and structure of XJB-5-131.Quantification of reduced glutathione (GSH, **a**) and protein sulfhydryl (PSH, **b**) levels in the ipsilateral cortex after CCI . XJB-5-131 (red bars) attenuated CCI-induced GSH and PSH depletion in PND 17 rats (blue bars). $^{*}P < 0.01$ vs. naïve and sham controls, CCI 3h, and CCI (3h and 24h) + XJB-5-131; error bars, standard deviation; n = 4 rats per group. **(c)** Increase in the contents of oxydized cardiolipin (CLox), phosphatidylcholine (PCox) and phosphatidylethanolamine (PEox) in rat cortical neurons 2 hours after stretch assessed by LC-MS. Data are presented as pmol of oxidized phospholipid per nmol of phospholipid; error bars, standard deviation; n = 4 experiments. **(d)** Structure of the hemigramicidin S-peptidyl 4-amino TEMPO conjugate, XJB-5-131.



Supplementary Figure 4. Effects of XJB-5-131 (1-25 μM) on primary rat cortical neurons in culture exposed to *in vitro* trauma by mechanical stretch. Vehicle or XJB-5-131 was added to the medium 10 min before stretch.
(a) Percent cytotoxicity (lactate dehydrogenase (LDH) release relative to Triton exposure (corrected for background LDH). **(b)** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a percentage of normal control conditions. Mechanical stretch induced approximately 35% neuronal death at 24 hours assessed by MTT and LDH. A marked reduction in neuronal death was produced by 5, 10, and 25 μM XJB-5-131 treatments. $^{*}P < 0.01$ vs. stretch only neurons; error bars, standard deviation; data are from three independent experiments and include 8-11 wells for each condition.



Supplementary Figure 5. Response of neurons treated with CL synthase-siRNA or cyt c-siRNA or XJB-5-131 to *in vitro* TBI.
(a) Cell counts after mechanical stretch in cortical neurons transfected with cardiolipin synthase (CS) or Cytochrome c (CYC) or scrambled control (SC) siRNA as well as after treatment with XJB-5-131 were performed using Trypan Blue assay. **(b, c)** Immuno-staining for cleaved caspase 3 **(b)** and neuronal cytoskeleton (microtubule-associated protein 2, MAP-2) **(c)** after mechanical stretch in cortical neurons transfected with cardiolipin synthase (CS) or Cytochrome c (CYC) or scrambled control (SC) siRNA as well as after treatment with XJB-5-131. C: control normal neurons; N: non-transfected neurons. * $P < 0.01$ vs. C, CS, CYC, and XJB; error bars, standard deviation; data are from three independent experiments and include 8-11 wells for each condition.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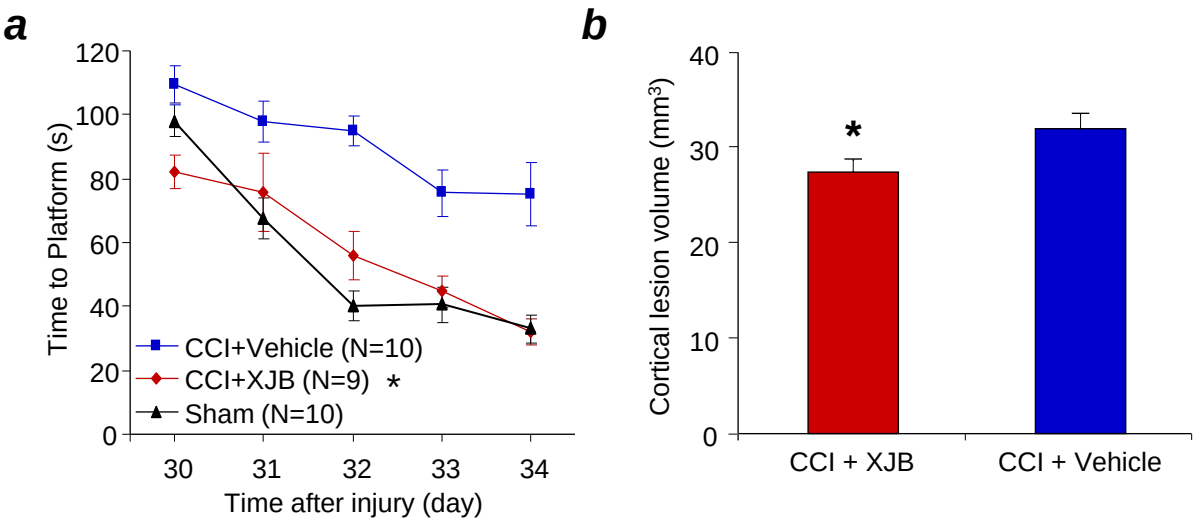
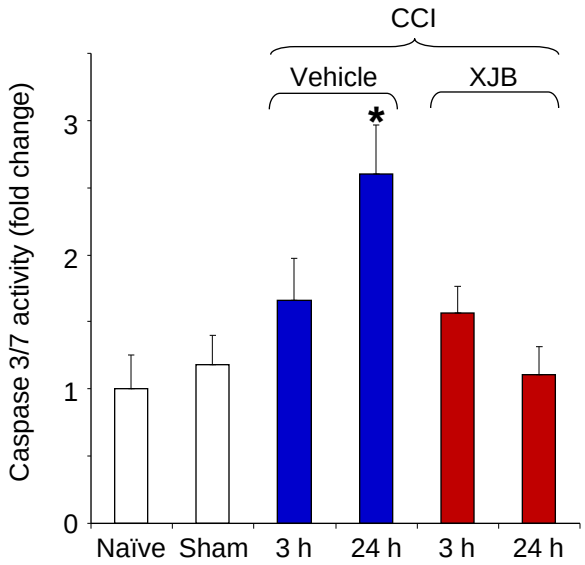
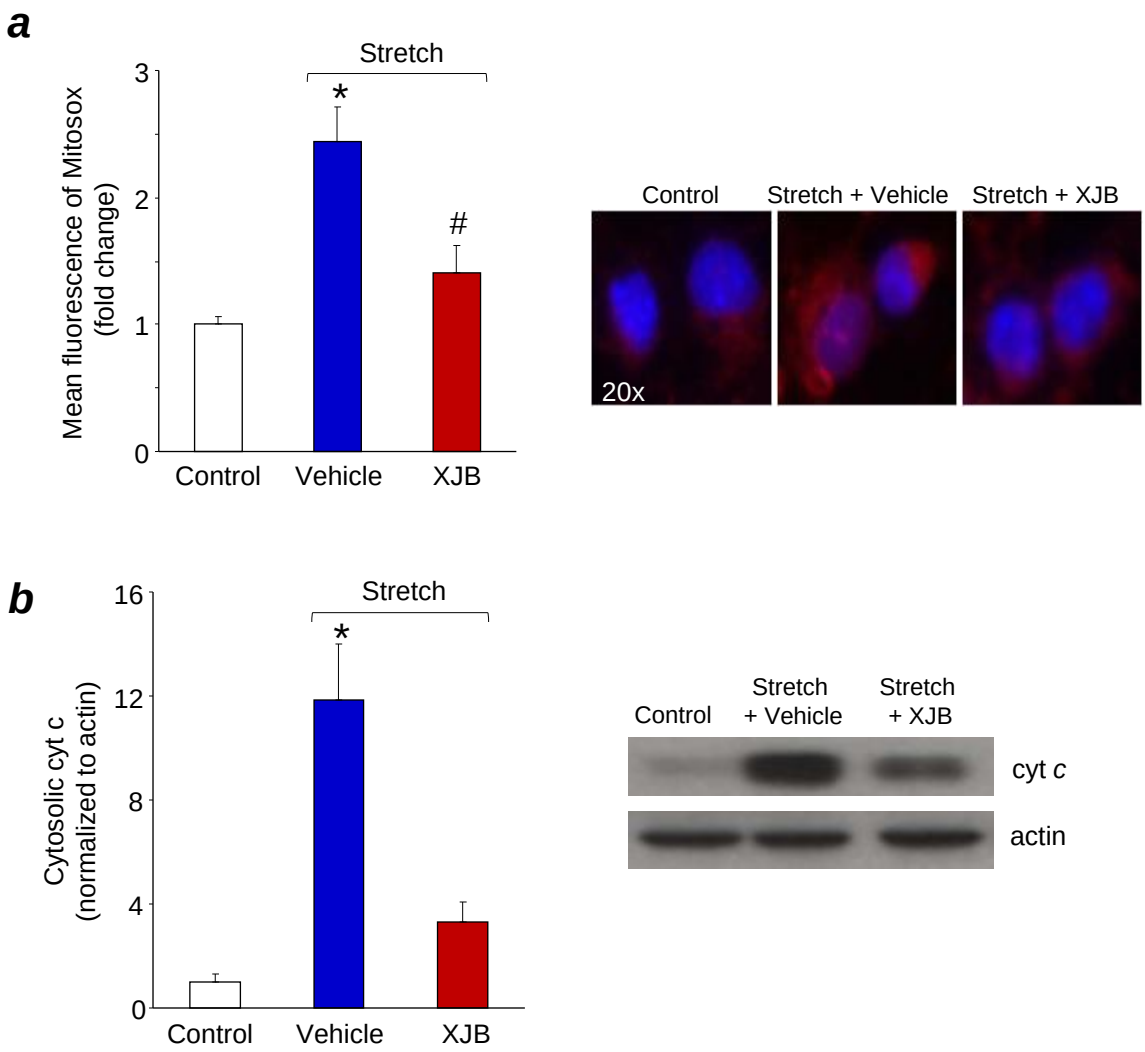
Supplementary Figure 6. Generation of cardiolipin (CL) and cytochrome c (cyt c) deficient neurons.
(a) Primary rat neurons were transfected with CL-synthase (CS) siRNA or a scrambled control (SC) siRNA. Insert: Representative western blot showing effective knockdown of CS at 48h and 72h after siRNA treatment. **(b)** Quantification of total CL content in control and CS knock down neurons by electrospray ionization MS (ESI-MS). Transfection with CS produced ~15% (72 h) and 56% (96 h) decrease in CL content, and did not change the mitochondrial member markers (as assessed by cytochrome c oxidase IV (COX IV) and transporter outer membrane 40 (TOM 40)). **(c)** Assessment of ATP levels in control and CS deficient neurons. CL deficiency did not change cellular ATP levels. **(d)** Primary rat neurons were transfected with Cytochrome c (CYC) or SC siRNA. Insert: Representative western blot showing effective knock down of cyt c at 48 h and 72 h after siRNA treatment. N, nontransfected neurons. $^{*}P < 0.01$ vs. N and SC; error bars, standard deviation; n = 4 experiments.



Supplementary Figure 7. Assessments of sub-chronic neurobehavioral and histological outcome in PND 17 rats treated with XJB-5-131 after TBI.
(a) Morris water maze test at 30-34 days after TBI in PND17 rats. XJB-5-131 enhanced the acquisition of spatial learning and memory vs vehicle-treated controls. A repeated measures ANOVA revealed significant group ($F_{2,26}$ = 19.156, P < 0.001) and day ($F_{4,104}$ = 42.509, P < 0.001) differences, as well as a significant group × day interaction ($F_{8,104}$ = 2.723, P = 0.0091). Bonferroni *post hoc* analyses revealed that the TBI + XJB group performed significantly better than the TBI + vehicle group (P < 0.001; error bars, standard error; n = 9-10 rats per group) and did not differ from the sham controls (P = 0.69). **(b)** Analysis of cortical lesion volume at 36 days after TBI in PND 17 rats. Cortical lesion volume was significantly decreased at 5 weeks in the TBI group treated with XJB -5-131 vs. the TBI group that received vehicle. P = 0.0465, ANOVA, $F_{1,13}$ = 4.840; error bars, standard error; n = 9-10 rats per group.



Supplementary Figure 8. Quantification of caspase 3/7 activity in the ipsilateral cortex. XJB-5-131 attenuated CCI-induced increase in caspase 3/7 activity in PND 17 rats. $^{*}P < 0.01$ vs. naïve and sham controls, CCI 3h + Vehicle, and CCI + XJB-5-131; error bars, standard deviation; n = 4 rats per group.



Supplementary Figure 9. Biochemical effects of XJB-5-131 after *in vitro* TBI. **(a)** Assessment of mitochondrial superoxide generation in primary cortical neurons after mechanical stretch. Left panel shows quantitative analysis of mitochondrial superoxide generation by flow cytometry. Mechanical stretch injury resulted in increased superoxide formation in neuronal mitochondria at 2 hours after stretch injury compared with control. XJB-5-131 attenuated stretch induced mitochondrial superoxide generation. Right panel shows fluorescence microscope assessment of MitoSOX (red) staining in neurons. Co-staining of nuclei with Hoechst 33342 (blue). **(b)** Quantification of cytochrome c (cyt c) release from mitochondria into cytosol after mechanical stretch. Right insert: representative western blots showing control, stretch + vehicle, and stretch + XJB-5-131. XJB-5-131 attenuated stretch-induced cyt c release into the cytosol. $^{*}P < 0.01$ vs. control and stretch + XJB-5-131; $^{\#}P < 0.05$ vs. control; error bars, standard deviation; n = 4 experiments.