Supplementary information

Supplementary Discussion

Interestingly, while the manuscript is under review, another study demonstrated that knockdown of Nogo-A would lead to inhibit hypoxia/reoxygenation-induced activation of mitochondrial-dependent apoptosis in cardiomyocytes[1]. Distinct from the effects of Nogo-A on cortical neurons survival, Sarkey's study in cardiomyocytes suggested that Nogo-A knockdown could markedly attenuate hypoxia/reoxygenation-induced apoptosis depending on the preservation of mitochondrial membrane potential, the inhibition of ROS accumulation, and the improvement of intracellular calcium regulation. We noticed at least the major differences between that study and ours.

First, this study used cultured neonatal rat cardiomyocytes while we used immature rat cortical neurons; second, this study used hypoxia/reoxygenation induced oxidative stress model while we used hydrogen peroxide (H_2O_2)-induced oxidative stress model. In cardiomyocytes, Nogo-A was up-regulated under hypoxia/reoxygenation, while in neurons, Nogo-A was down-regulated under H_2O_2. And hypoxia/reoxygenation induced mitochondrial-dependent apoptosis in cardiomyocytes (Figure 4), and H_2O_2 induced necrotic death in cortical neurons (Figure 2c). The outcome of two cell types and stress types may cause distinct patterns of ROS accumulation which depend on different redox system. In fact, a few proteins show pro- or anti-apoptosis activity in three cell lines or system. Among them, Nogo-B exerts a similar mode. Overexpression of Nogo-B can induce apoptosis in some cancer cell lines[2] , while Nogo-B from PASMCs disrupts the ER-mitochondria unit and suppresses apoptosis in pulmonary arterial hypertension (PAH)[3]. Hence, we hypothesize that the choice between the pro- or anti- oxidative effects of Nogo-A is dictated by the stress type, its intensity and context.

Refs:

1. Sarkey JP, Chu M, McShane M, Bovo E, Mou YA, Zima AV*, et al.* Nogo-A knockdown inhibits hypoxia/reoxygenation-induced activation of mitochondrial-dependent apoptosis in cardiomyocytes. *J Mol Cell Cardiol* 2011 Jun; **50**(6)**:** 1044-1055.
2. Oertle T, Merkler D, Schwab ME. Do cancer cells die because of Nogo-B? *Oncogene* 2003 Mar 6; **22**(9)**:** 1390-1399.
3. Sutendra G, Dromparis P, Wright P, Bonnet S, Haromy A, Hao Z*, et al.* The role of Nogo and the mitochondria-endoplasmic reticulum unit in pulmonary hypertension. *Sci Transl Med* 2011 Jun 22; **3**(88)**:** 88ra55.

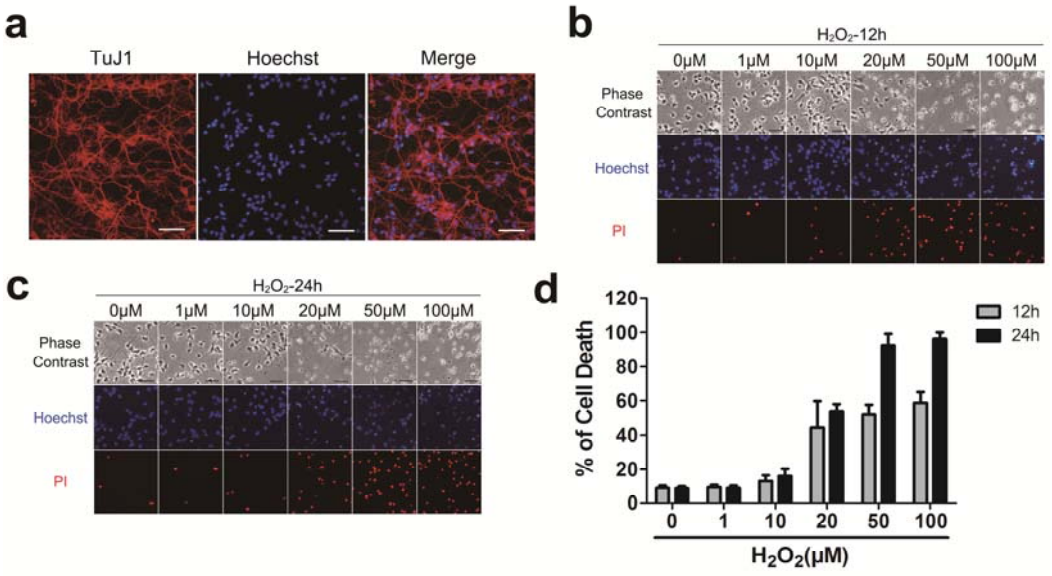
Supplementary Table and Figures

Table 1 RT-PCR primer sequences

Gene name	Primer sequences (from 5' to 3')	Product size (bp)
Nogo-A	F: GATGATTCGATTCCTGAAGTCC	498
	R: AAAGGTCACAGGGCAATTCTAA	
SOD1	F：CATGGGTTCCATGTCCATCA	197
	R：GAGAGTGAGATCACACGAT	
SOD2	F：CAGATCATGCAGCTGCACCAC	231
	R：TGATAGCCTCCAGCAACTCTC	
SOD3	F：GTCTGTCCTGTACTTCACCAGA	189
	R：TATCTTCTCAACCAGGTCAAGC	
Prdx1	F：ACACCCAAGAAACAAGGAGGATT	180
	R：CAACAGGAAGATCATTTATTGTTA	
Prdx2	F：GCGCACATCGGAAAGCCTG	177
	R：GTCCTCAGCGTGGTCGCTA	
Prdx3	F: GGCCACATGAACATCACGCTG	204
	R: CAAACTGGAACGCCTTTACCA	
Prdx4	F: TCCTGTTACAGACTGAAG	180
	R: CAATAAGGTGCTGGCTTG	
Prdx5	F: GAAAGGAGCAGGTTGGGAGTGT	154
	R: GTCCTTGAACAGCTCTGCCAAGT	
Prdx6	F：GCATGTTGGATCCAGCAGAG	232
	R：GTGGGAAGGACCATCACACT	
Catalase	F：TAGCCAGTGCTCTGCAGAT	197
	R：TCAGGGTGGACGTCAGTGA	
Gpx1	F：GACTACACCGAAATGAATGAT	196
	R：ACCATTCACCTCGCACTTCTCA	
Gpx2	F：GAGGAACAACTACCCGGGACTA	215

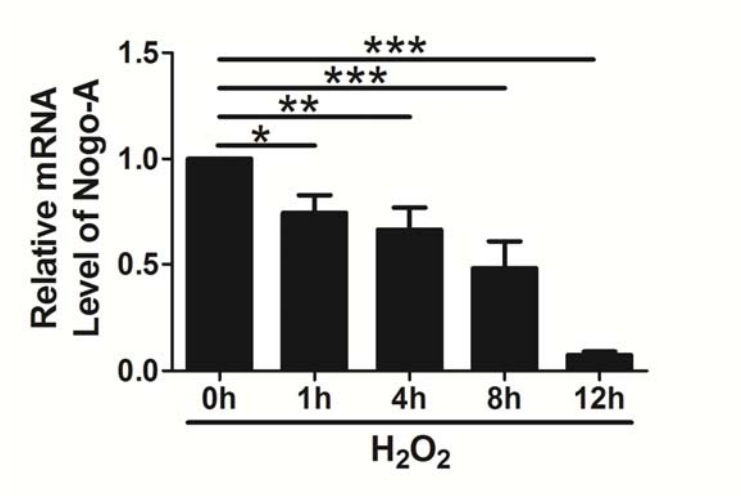
	R: GATTCTGCCCATTGACATCACAC	
Gpx3	F：GCCAGCTACTGAGGTCTGACAGA	169
	R：CCACCCGGTCGAACGTACTTGAG	
Gpx4	F：TATTGAAGCCAGCACTGCTGTG	233
	R：GCAGATCGACTAGCTGAGTGTA	
Trx1	F：GTGAAGCTGATCGAGAGCA	204
	R：CACAGTCTGCAGCAACATC	
Trx2	F：TGCTGGTGGTCTAACTGGAAC	255
	R：GTCTGTGTGATCGTCAATGTC	
Glrx1	F：CAACACCAATGCGATTCAAGA	160
	R：GCAGAGCTCCAATCTGCTTCA	
Glrx2	F：CTGCCTATGGACTCCTGATCA	331
	R：GAGCCTCTTGAAACTGGCTAC	
β-actin	F：GTCCCTCACCCTCCCAAAAG	266
	R：GCTGCCTCAACACCTCAACCC	

Figure S1



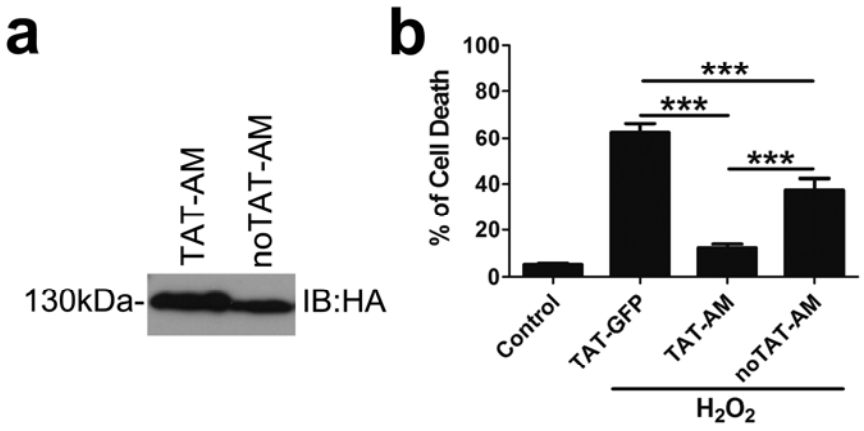
The neuronal cell death model of oxidative stress induced by H_2O_2. (a) Percentage of neurons was determined by double immunostaining with anti-TuJ1 antibody and Hoechst. (b) and (c) H_2O_2 was applied to the neuron cultures at 1, 10, 20, 50, or 100 μM for 12 h or 24 h respectively, and then double stained with Hoechst and PI. Phase contrast image and fluorescence image were presented. (d) The cell death rate was determined by PI (+) / Hoechst (+). Bar = 50 μm. *n* = 4, Mean ± S.D.

Figure S2



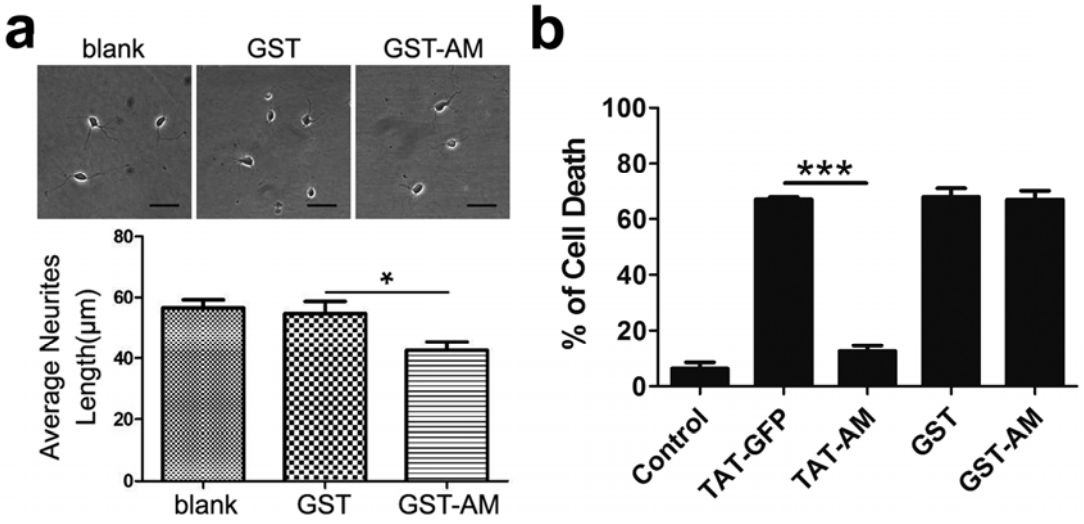
Reduction of Nogo-A mRNA expression exhibits a time-dependent pattern of H_2O_2 treatment. The total RNA was extracted from neurons exposed to 50 μM H_2O_2 for 0, 1, 4, 8 and 12 h and submitted to quantitative real-time PCR. β-actin was selected as an inner standard. *n* = 3, Mean ± S.D., one-way ANOVA, **P*<0.05; ***P*<0.01; ****P*<0.001.

Figure S3



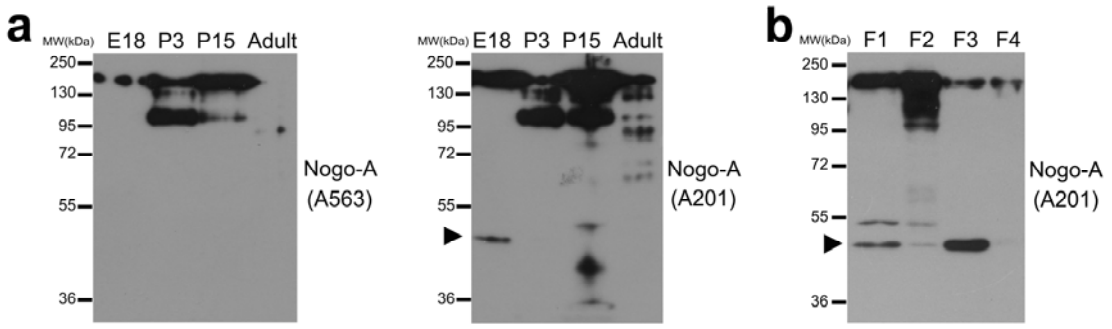
NoTAT-amino-Nogo-A exerts only a partial protective action against oxidative insults. (a) Neurons treated with 0.2 μM TAT or noTAT -AM for 2 h were lysed and immunoblotted with anti-HA antibody. (b) Neurons treated with 0.2 μM TAT- or noTAT- AM 2 h followed by 50 μM H_2O_2 for another 12 h in presence of the proteins, and cell death assay was done as before, TAT-GFP was selected as a control. *n* = 4, Mean ± S.D., one-way ANOVA, ****P*<0.001.

Figure S4



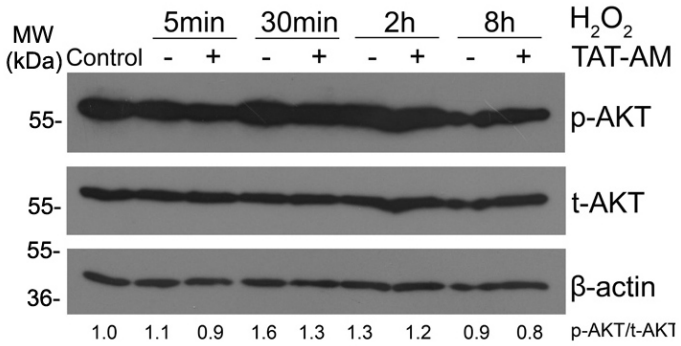
GST-amino-Nogo-A has no protective activity against oxidative insults. (a) GST-AM had an inhibitory activity on neurite outgrowth. 0.2 μM GST or GST-AM was added into neuron cultures for 48h, then phase contrast images were obtained (top panel), and the average neurites length were calculated and analyzed (lower panel). (b) Neurons treated with 0.2 μM TAT-AM or GST- AM for 2 h followed by 50 μM H_2O_2 for another 12 h in presence of the proteins, and cell death assay was done by PI (+) / Hoechst (+), TAT-GFP and GST were selected as the controls for TAT-AM and GST- AM respectively. Bar = 50μm. *n* = 3, Mean ± S.D., one-way ANOVA, **P*<0.05; ****P*<0.001.

Figure S5



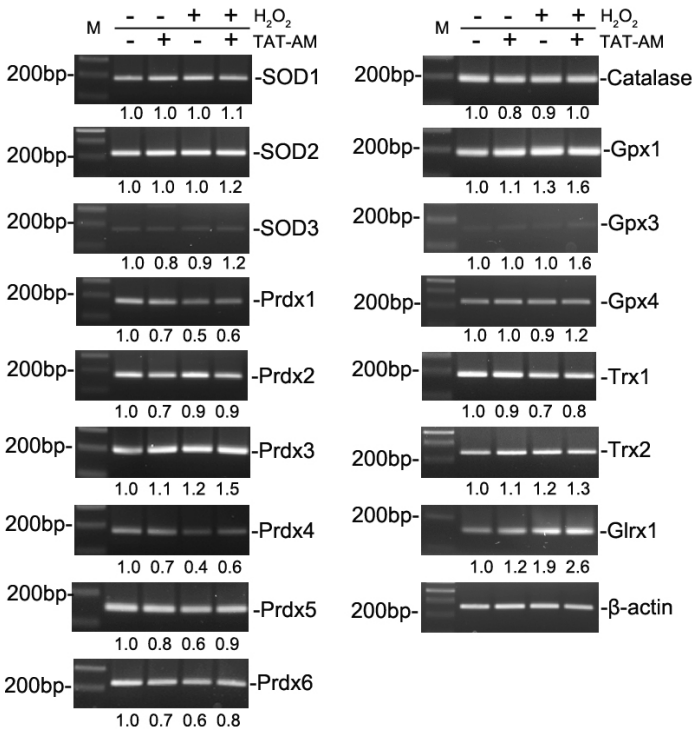
Developmental regulation of Nogo-A expression and proteolytic cleavage in the rat brain. (a) 30 μg total protein of cerebral cortex from E18, P3, P15 and adult rats were extracted and subjected to western blot with A563 and A201 antibodies respectively. (b) The four subcellular fractions of cortical neurons at 1 day in vitro were subjected to western blot by A201 antibody. The 48 kDa band was implicated by arrowhead.

Figure S6



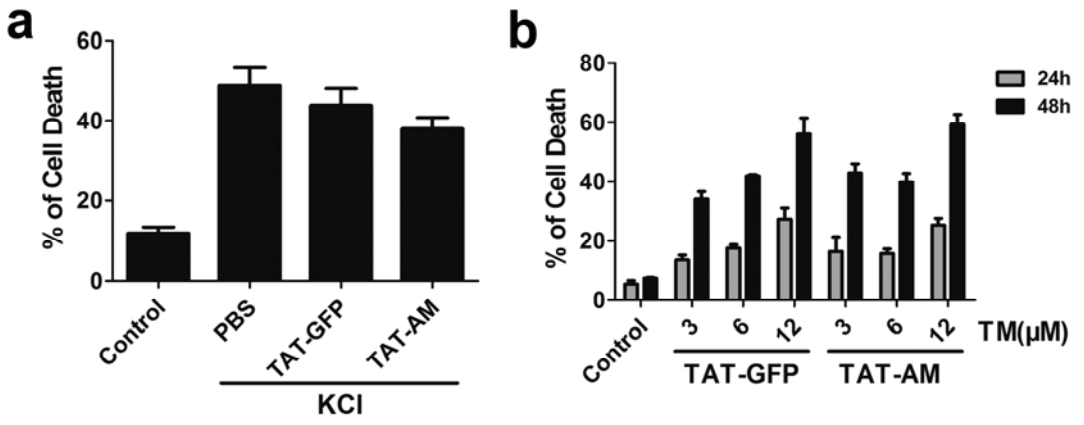
Pretreatment of TAT-amino-Nogo-A does not activate AKT. Neurons were treated with 50 μM H_2O_2 for 5 min, 30 min, 2 h and 8 h with or without pre-incubation of 0.2 μM TAT-AM and then subjected to western blot using antibody against phosphorylated AKT (p-AKT) or total-AKT (t-AKT). β-actin was selected as an inner protein standard. Quantification by densitometric scans was presented by p-AKT/t-AKT.

Figure S7



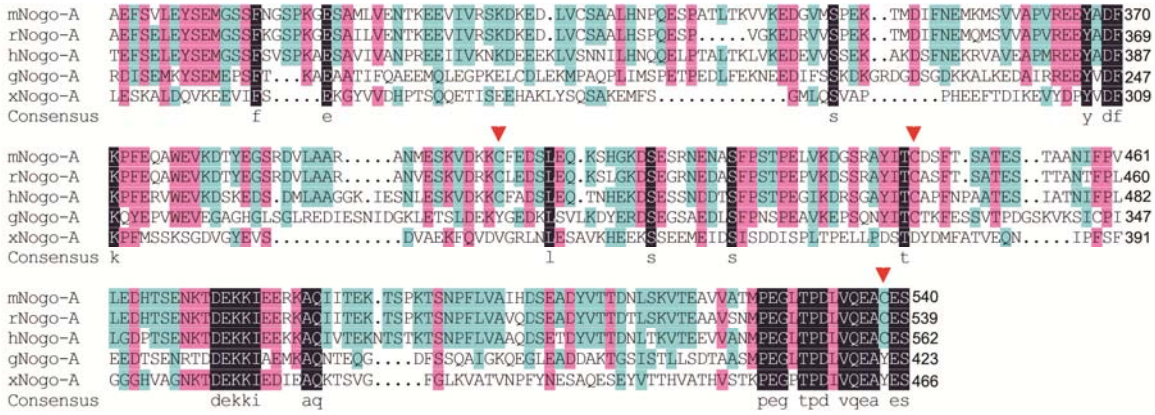
Pretreatment of TAT-amino-Nogo-A does not influence the mRNA level of oxidoreductases. Neurons were treated with 50 μM H_2O_2 for 8 h in presence of 0.2 μM TAT-AM or not, and then performed to RT-PCR. β-actin was selected as an inner standard. PCR products for Gpx2 and Glrx2 were not shown due to little mRNA expression. Quantification by densitometric scans was presented by the ratio vs β-actin.

Figure S8



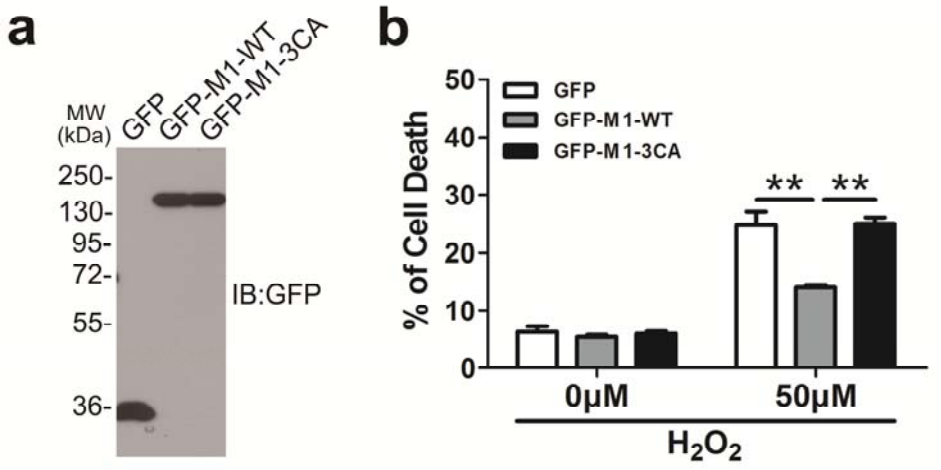
TAT-amino-Nogo-A has no protection on low KCl-induced CGN death and tunicamycin-induced cortical neuron death. (a) CGN were pretreated with 0.2 μM TAT-GFP or TAT-AM for 2 h, and then switched to LK medium (5 mM KCl) in the presence of the TAT-proteins for another 24 h. Control was always in the normal HK medium (25 mM KCl). The cell death rate was calculated by PI (+) / Hoechst (+). (b) Neurons were pretreated with 0.2 μM TAT-GFP or TAT-AM for 2 h followed with addition of 3 μM, 6 μM and 12 μM tunicamycin for another 24 or 48 h. The death rate was calculated as before. TM = tunicamycin. *n* = 4, Mean ± S.D.

Figure S9



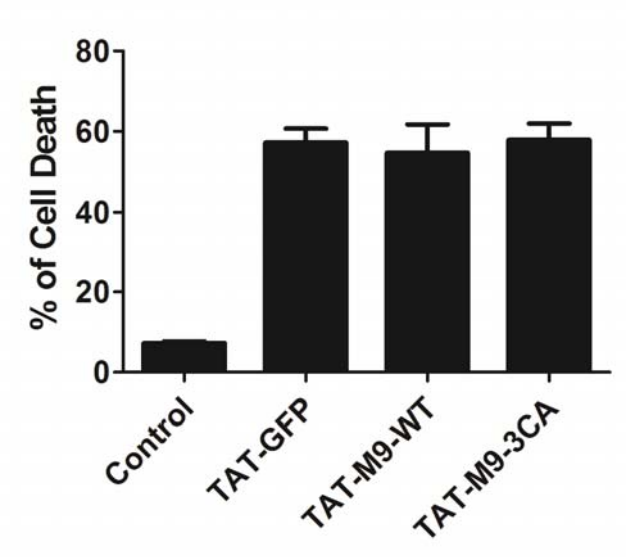
Multiple sequence alignment of 290-562 residues (for human) located in Nogo-A/Rtn4-A protein. The multiple sequence alignment compares Nogo-A protein from mouse (m), rat (r), human (h), gallus (g) and xenopus (x). Amino acid color code: Blue＝100%, purple≥75%, Green≥50%. Conserved Cys residues at 424, 464 and 559 among mouse, rat and human are denoted with “red arrowheads”.

Figure S10



Overexpression of M1-WT but not M1-3CA had a protective effect on Neuro2a cells undergoing oxidative stress. (a) The expression of GFP-tagged M1-WT and M1-CA in eukaryotic cells were confirmed though western blotting with anti-GFP antibody. (b) The plasmids GFP, GFP-M1-WT and GFP-M1-CA were transfected into Neuro2a cells, 36h later, cells were exposed to 50 μM H_2O_2 for 4 h, cell death rate was calculated with PI (+) / GFP (+). *n* = 3, Mean ± S.D., one-way ANOVA, ***P*<0.01.

Figure S11



Amino-Nogo-A can not effectively scavenge H_2O_2 *in vitro*. 0.2 μM TAT-GFP, TAT-M9-WT and TAT-M9-3CA were mixed with 50 μM H_2O_2 for 1 h in cell culture medium without cells respectively, and then the mixture solutions were incubated with Ni-NTA resin to remove the unreacted proteins. After centrifugation, the supernatant were added into cortical neurons for 12 h respectively and cell death assay was done by PI (+) / Hoechst (+) as before. Control neurons were treated with the supernatant from medium only incubated with Ni-NTA resin. *n* = 3, Mean ± S.D.