Supplemental Material

Methods

2-dimensional electrophoresis (2-D)

2-D analysis: Brain tissues samples collected 1 day after injury were washed twice in ice-cold PBS and lysed in sample buffer composed with 7 M urea, 2 M thiourea containing 4% CHAPS, 1% DTT and 2% pharmalyte and 1 mM benzamidine. Proteins were extracted for 1 hour at room temperature with vortexing. After centrifugation at 8,000 *g* for 1 hour at 4°C, insoluble material was discarded and soluble fraction was used for 2-D gel electrophoresis. Protein loading was normalized by Bradford assay. **A.** 2-D PAGE: IPG dry strips were equilibrated for 12-16 hours with 7 M urea, thiourea containing 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-prpanesulfonate (CHAPS, Sigma), 1% dithiothreitol (DTT, Sigma), 1% pharmalyte in 200 μg samples. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham) following manufacturer's instruction. For IEF, the voltage was linearly increased from 150 to 3,500 V during 3 hours for sample entry followed by constant 3,500 V, with focusing complete after 96 kVh. Prior to 2-D, strips were incubated for 10 minutes in equilibration buffer (50 mM Tris-Cl, pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20-24 cm, 10-16%). SDS-PAGE was performed using the Hoefer DALT 2-D system (Amersham) following manufacturer's instructions. 2-D gels were run at 20°C for 1.7 kVh, and then were silver stained as described by Oakley et al [1], but fixing and sensitization step with glutaraldehyde was omitted. **B.** Image analysis: Quantitative analysis of digitized images was carried out using the PDQuest software (BioRad) according to the protocols provided by the manufacturer. The quantity of each spot was normalized by total valid spot intensity. Protein spots were selected for significant expression variation deviated over two fold in expression level compared with control or normal sample.

MALDI-TOF analysis: Enzymatic digestion of protein in-gel spot was enzymatically digested in-gel in a manner similar to that previously described by Shevchenko et al [2] and using modified porcine trypsin. Gel pieces were washed with 50% acetonitrile to remove SDS, salt and staining, then dried to remove solvent, and finally rehydrated with trypsin (8-10 μg/μl) and

incubated 8-10 h at 37°C. The proteolytic reaction was terminated by addition of 5 µl 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. After concentration the peptide mixture was desalted using C18ZipTips (Millipore), and peptides eluted in 1-5 µl of acetonitrile. An aliquot of this solution was mixed with an equal volume of a saturated solution of cyano-4-hydroxycinnamic acid in 50% aqueous acetonitirle, and 1 µl of mixture spotted onto a target plate, and then protein analysis were performed using the MALDI-TOF (Amersham).

Table . Maldi-Tof isolated Hsp70 Tg mouse brain proteins with significantly altered levels after MCAO, identified by mass spectrometry.

Spot identity	Protein identity and accession number	MW (kD) of the matched protein	pI of the matched protein	Protein function
Proteins that show an increase in level				
3501 ↑	**TUBB3 protein (gi 38014278)**	**46**	**4.9**	**Structural component of microtubules**
4067 ↑	**Dihydropyrimidinase-like 2 (gi 4503377)**	**62.78**	**6**	**Developing nervous**
Proteins that show a decrease in level				
5507 ↓	**Heterogeneous nuclear ribonucleoprotein H2 (gi 9624998)**	**46**	**4.9**	**Transcription regulation**
6403 ↓	**Enolase (gi 4503571)**	**47.49**	**7**	**Cytoplasmic glycolytic enzymes**
7810 ↓	**Dynamin (gi 181849)**	**97.79**	**6.9**	**Microtubule bundling and endocytosis**
9508 ↓	**ATP synthase (gi 15030240)**	**59.9**	**9.2**	**Cell cycle progression, cellular differentiation and stress response**

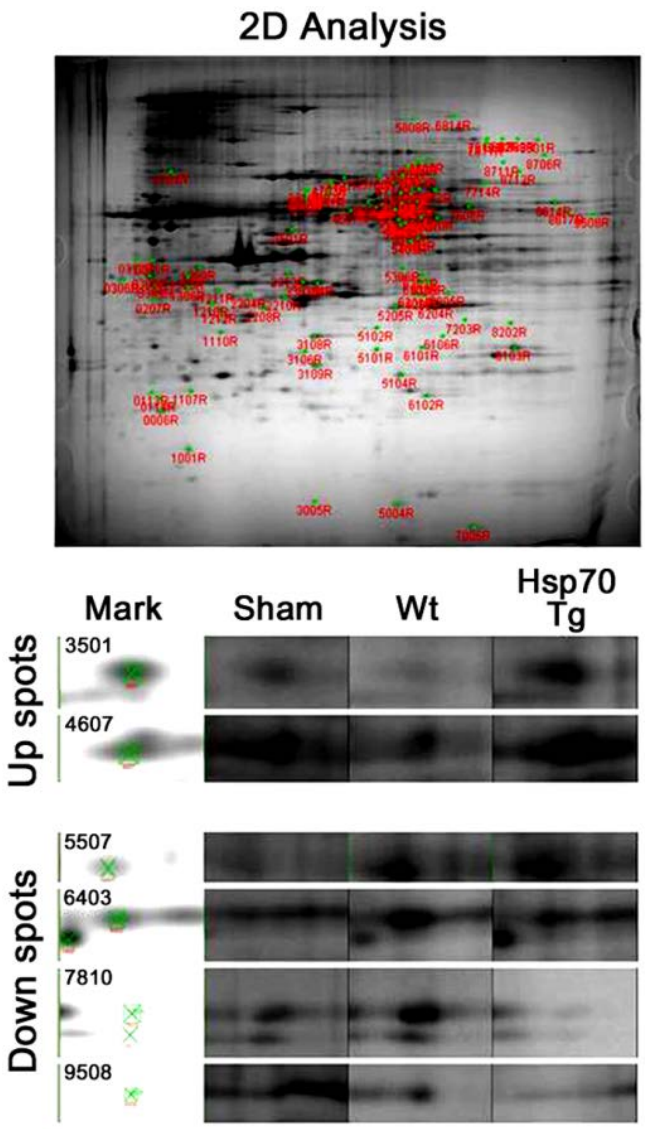

Figure SI. 2-D gel and MALDI-TOF screening of ischemic brain samples. 2-D gel of ischemic brain samples from sham (Sham, no injury), Wt and Hsp70 Tg mice. 2-D gels were generated, stained, and analyzed as described in text. Differentially expressed proteins are depicted by numbers. 6 proteins were found to be significantly different in Hsp70 Tg mice: 2 proteins showed upregulation and 4 proteins showed downregulaiton in 2-D analysis. The 7810 spot (corresponding to dynamin) showed significantly reduced expression in Hsp70 Tg mice compared to Wt mice after experimental stroke.

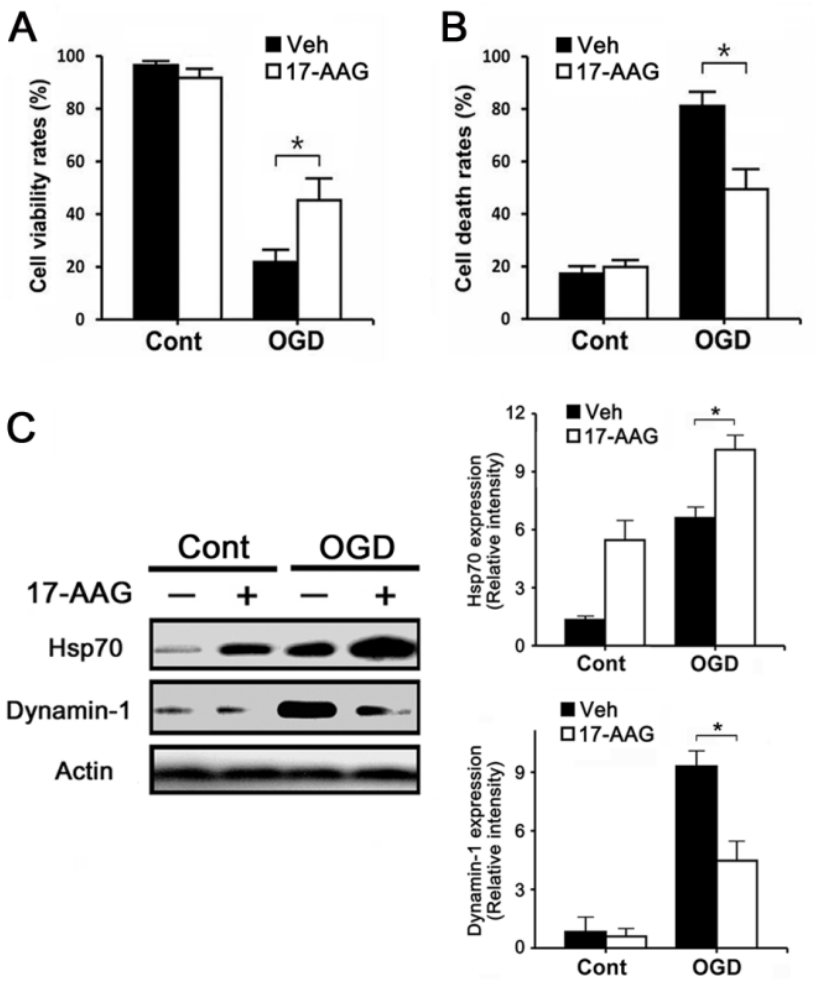

Figure SII. Dynamin-1 expression is increased following experimental stroke, and interacts with Hsp70 and Fas. Neuro2a (N2a) cells were treated with 17-AAG to induce Hsp70. Following OGD, 17-AAG led to neuroprotection in these cells. Cell viability (**A**) and cell death (**B**) assays using MTT and trypan blue, respectively, showed that 17-AAG reduced cell death (n=3/group; *P< 0.01). Immunoblots of Hsp70 and dynamin-1 in N2a cells subjected to 2 hours OGD show that treatment with 17-AAG induced Hsp70, and decreased expression of dynamin-1. **C**, Relative intensities of protein were quantified by NIH Image J soft-ware, and normalized to the intensity of β-actin. Expression of Hsp70 was increased, whereas dynamin-1 was decreased in 17-AAG compared vehicle (Veh) treatment (n=3/group; *P< 0.01).

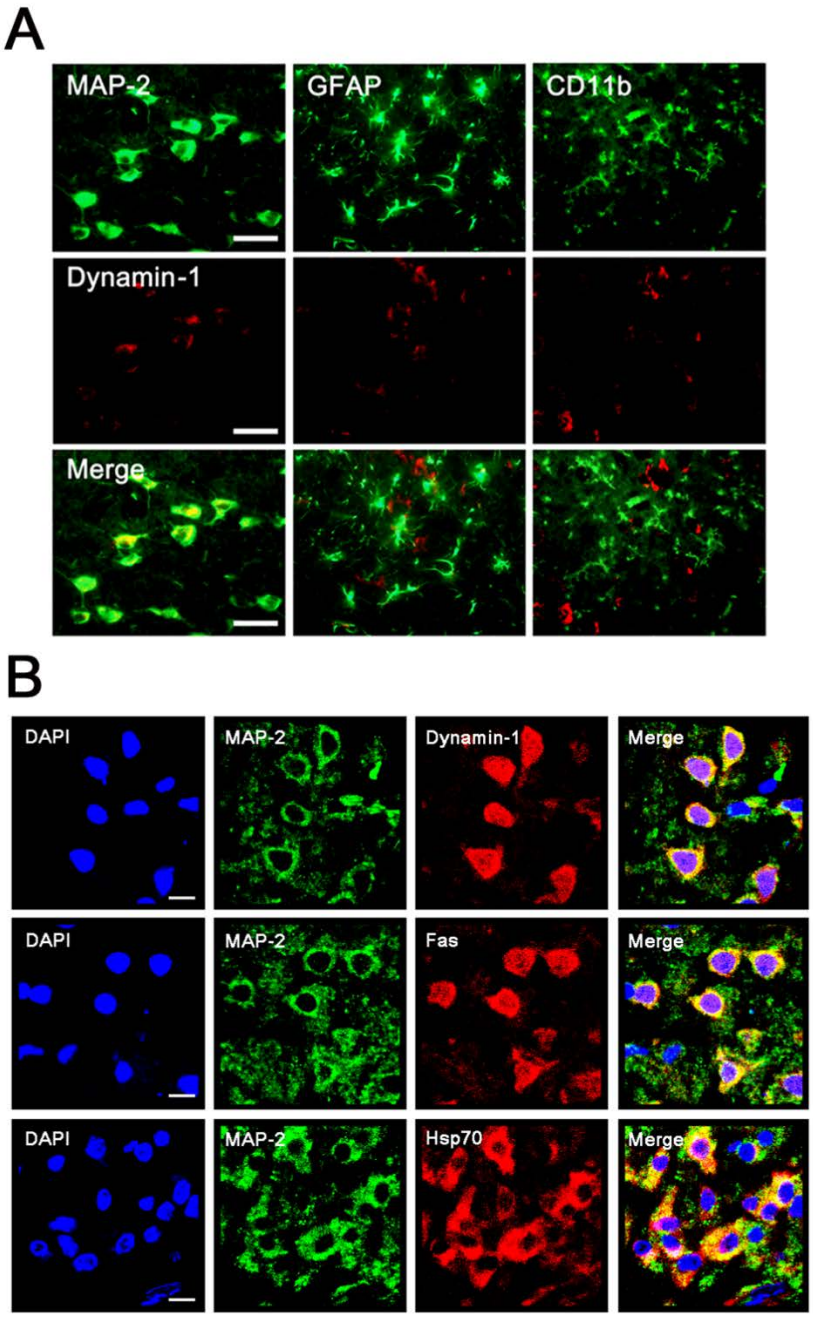

Figure SIII. A, In brains of non-injured mice, MAP-2 and dynamin-1 stains show that dynamin colocalized to the cytosol in neurons, but not to astrocytes (GFAP) or microglia (CD11b) (scale bar=20 μm). B, Co-labeling shows that Dynamin-1, Fas and Hsp70 were all expressed in neurons (MAP-2) after ischemic stroke (scale bar=20 μm).

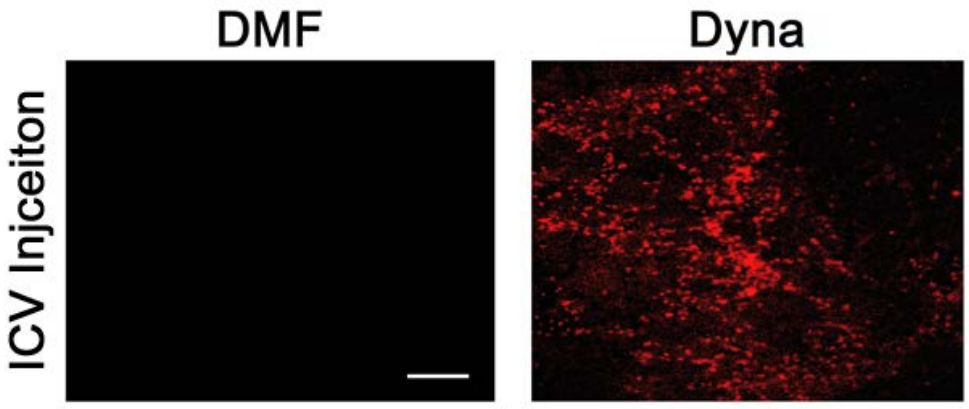

Figure SIV. Intracerebroventricular (ICV) injection of Texas Red conjugated Dyna. Representative brain sections of ICV injection of conjugated Dyna showed positive red fluorescent signals after dMCAO, indicating uptake by cells. This is compared to Veh treated brain, which showed no red signals (n = 3/group).

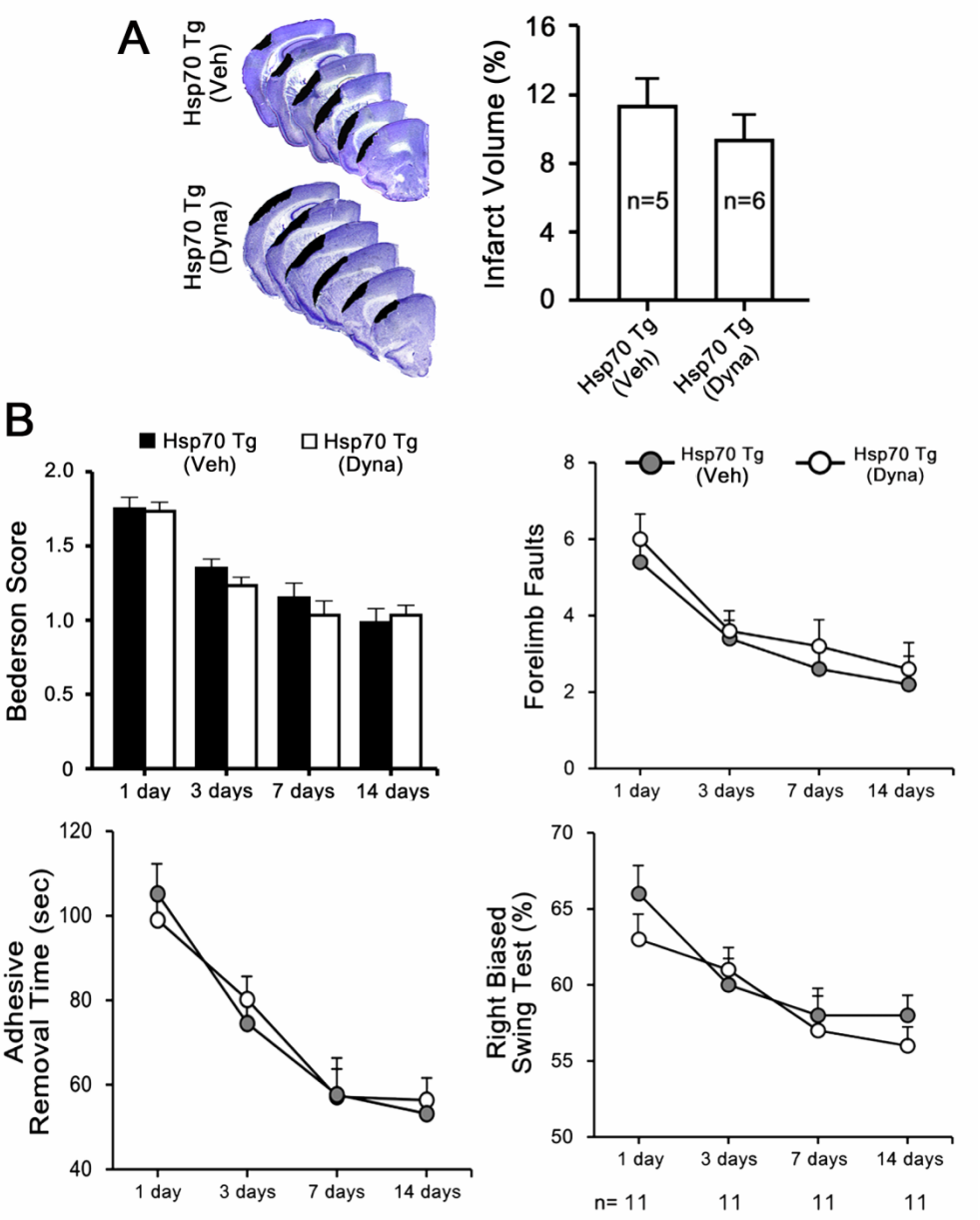

Figure SV. Lack of synergy between Hsp70 overexpression and dynamin inhibition. Dynasore (Dyna) failed to show protection against ischemic brain damage out to 14 days post dMCAO in Hsp70 Tg mice. A, Hsp70 Tg mice with Dyna had no significant effect in reducing infarct volume compared to Hsp70 Tg mice treated with vehicle (Veh; DMF). B, Behavioral tests showed no significant difference between Hsp70 Tg mice treated with Dyna and those treated with DMF (n = 5-6/group).

References

1. Oakley BR, Kirsch DR, Morris NR. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal Biochem*. 1980;105:361-363
2. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem*. 1996;68:850-858