Supporting Information

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SI Materials and Methods

Animals. Eight-week-old male C57BL/6 mice were obtained from the The Jackson Laboratory. After acclimation under a 12:12 light–dark cycle for at least 1 wk, mice were individually housed and were handled once a day for 7–10 d. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas (UTSW) and were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Sleep Deprivation and Recovery Sleep. Singly housed mice were randomly divided into three groups; gentle handling (GH) and cage change (CC) were performed during Zeitgeber time (ZT) 0–6. The control group was allowed ad libitum sleep for 6 h. The GH group was deprived of sleep by gently touching the cages when they started to recline (i.e., huddle) and lower their heads. To eliminate any possibility of additional stress or locomotion, we minimized touching the mice directly. The CC group was deprived of sleep by changing the cages once an hour. As necessary, a minimal GH sleep deprivation (SD) maneuver was additionally performed on each CC mouse. However, the GH was limited to a maximum of 6 min during the 6 h. Subsequently, in a study of the recovery sleep, mice were left to sleep freely during ZT6–8.

Multiple Sleep Latency Test and Delta Power Measurements. The mouse version of the multiple sleep latency test (MSLT) was performed during the sleep recovery period for 3 h from ZT6–9 in the light period. Thus, during this recovery period, all mice received 5 min of sleep disturbance by repetitive 8 s on, 2 s off cycling of an orbital shaker at 100 rpm once every 30 min followed by ad libitum sleep for 25 min (1). The method for forcing wakefulness during MSLT trials was distinct from GH and CC, ensuring that the results were not affected by acclimation of mice to GH or CC maneuvers. Sleep latency times were determined from the onset of sleep, as evaluated by the initial appearance of non-rapid eye movement (NREM) sleep throughout a 20-s epoch during each 30-min trial and used as an index for the degree of arousal. This 30-min trial was repeated six times. During MSLT trials, we measured delta power in the electroencephalography (EEG) during NREM movement sleep. Baseline EEG/electromyography (EMG) recording was monitored before MSLT for 3 d. The data from the third day during ZT6–9 were used as the baseline to normalize EEG power for each mouse. Vigilance state scoring of the EEG/EMG data and fast Fourier transformation (FFT) of the EEG signal to derive the spectral power distribution were conducted as previously described (1). EEG FFT data during NREM sleep in the baseline period were averaged across all epochs in each mouse. The total average EEG power (1–32 Hz) of the baseline period for each mouse was used as a baseline to normalize EEG power. EEG power in NREM sleep during each trial was averaged across epochs and then normalized for each mouse. Delta power was quantified as the sum of the power in the range from 1 to 4 Hz.

Sample Collection. Mice were cervically dislocated at ZT0, -6, or -8, and the diencephalon or whole brain was harvested in ice cold artificial cerebrospinal fluid with added sucrose (254 mM sucrose, 10 mM glucose, 1.25 mM NaH_2PO_4, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, and 3 mM KCl). At ZT6, blood from each mouse was collected into a tube, incubated on ice for 1 h, and centrifuged at 2,300 × *g* for 15 min to obtain serum fruction. All of the samples were stored at −80 °C.

RNA Isolation and RT-PCR. Total RNA was isolated from the whole brain or diencephalon by TRIzol (Invitrogen), purified with the RNeasy Mini Kit (Qiagen), and reverse-transcribed into cDNA using the SuperScript Π system (Invitrogen).

Quantitative Real-Time PCR. For quantitative real-time PCR (qPCR), the mixture contained cDNA, SYBER Green Mix (Applied Biosystems), and the optimal concentration of the corresponding primer pair (Table S1). qPCR reactions were performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems).

Immunoprecipitation. Anti-dynamin (DNM1) monoclonal (Abcam) and anti–*N*-myc downstream regulated gene 2 (NDRG2) antibodies (Santa Cruz) were used for immunoprecipitation. Diencephalon samples were lysed into radio-immunoprecipitation assay buffer containing Complete Mini (Roche) and phosphatase inhibitor mixtures (Sigma-Aldrich). Target proteins were immunoprecipitated from 30- or 100-ug total protein using antibodies with Protein A/G PLUS-Agarose (Santa Cruz).

Western Blot. Anti-DNM1 polyclonal (Abcam), anti-NDRG2 (2), anti-phosphoPKC substrate (Cell Signaling), anti-phosphoAkt substrate (Cell Signaling), anti-phosphotyrosine (4G10), β-actin (Santa Cruz), β-tubulin (Cell Signaling), and histone H3 (Cell Signaling) antibodies were used for Western blots. For 1D Western blots, samples were separated with 10% or 12% SDS/PAGE gels. For 2D Western blots, each sample was purified with the 2D-clean up kit (GE Healthcare), reconstituted with 2D lysis buffer, and separated with immobilin pI 4–7 strip and 10% SDS/PAGE gel. Then, proteins were transferred onto PVDF membranes and incubated with each primary antibody and then corresponding secondary antibodies. Blots were detected with ECL Plus (GE Healthcare); we then quantified the autoradiograph using ImageJ (National Institutes of Health). After antibodies were removed with a stripping buffer [100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris·HCl (pH 6.7)], membranes were repeatedly used for Western blots.

Two-Dimensional Difference Gel Electrophoresis. Reagents were purchased from GE Healthcare. The lysed samples were purified using a 2-D Clean-UP Kit and reconstituted with 2D lysis buffer [7 M urea, 2 M thiourea, 30 mM Tris·HCl (pH 8.5), and 4% CHAPS]. After protein assay (Bio-Rad), reconstituted samples were diluted into 1 μg/μL each with the 2D lysis buffer. Fifty micrograms of samples and the internal standard were prepared and labeled with Cy3, Cy5, and Cy2 (Table S2), according to the manufacturer's instructions and separated with pI 3–10 NL strips and 10–20% acrylamide SDS/PAGE gels. Gel images were scanned with the Typhoon Trio and analyzed with DeDyder 2D Software. Target proteins were selected as those with more than ±20% difference compared with the control sample. In the Biological Variation Analysis (BVA) Module of DeCyder software, protein amount is expressed as standardized abundance calculated using the volume ratio. The standardized abundance was expressed relative to values of the control group at ZT6. Each volume ratio normalized to the internal standard was extracted from Gel data in the BVA Module through XML Toolbox. This volume ratio indicates the corresponding fold changes to the codetected pooled standard spot. Then, each standardized abundance was calculated using the volume ratio (the obtained standardized abundance is relatively

expressed to the codetected spot of the corresponding internal standard), and normalized to the control group.

Protein Identification. For protein identification, 500 μg of the phosphor-protein enriched protein sample was separated by two-dimensional gel electrophoresis and stained with Deep Purple (GE Healthcare). The target spots were picked and identified by nano-liquid chromatography–tandem mass spectrometry operated by the UTSW Protein Chemistry Technology Center. The results were searched against the National Center for Biotechnology Information protein sequence databases using the Mascot search engine (Matrix Science).

Phosphatase Treatment. For protein dephosphorylation treatment, the concentration of reconstituted sample was adjusted at 0.5 μg/μL. Then, 100 μL of the sample was divided into 50 μL each. The 50 μL of sample was mixed with 5 μL of 10% SDS, 10× buffer (New England BioLabs), 5 μL of 10 mM $CaCl_2$, and 345 μL of H_2O with or without 200 units of λ-phosphatase (PPase; New England BioLabs), and incubated at 30 °C for 8 h (3). After being purified with the 2D clean-up kit again, samples were separated by 2DE.

Statistical Analysis. Differences between multiple groups were evaluated by one-way ANOVA after establishing equivalence of variances and normal distribution. Post hoc testing was performed by the Tukey's test. Values of immunoprecipitated samples were normalized with IgG. Values shown are means ± SEM, within $n = 3$–12. Significance was accepted at the 0.05 level. Differences between multiple groups were evaluated by one-way ANOVA after establishing equivalence of variances and normal distribution of data. Post hoc testing was performed by Tukey's test.

1. Sinton CM, Kovakkattu D, Friese RS (2009) Validation of a novel method to interrupt sleep in the mouse. *J Neurosci Methods* 184(1):71–78.
2. Okuda T, Kokame K, Miyata T (2008) Differential expression patterns of NDRG family proteins in the central nervous system. *J Histochem Cytochem* 56(2):175–182.
3. Yamagata A, et al. (2002) Mapping of phosphorylated proteins on two-dimensional polyacrylamide gels using protein phosphatase. *Proteomics* 2(9):1267–1276.

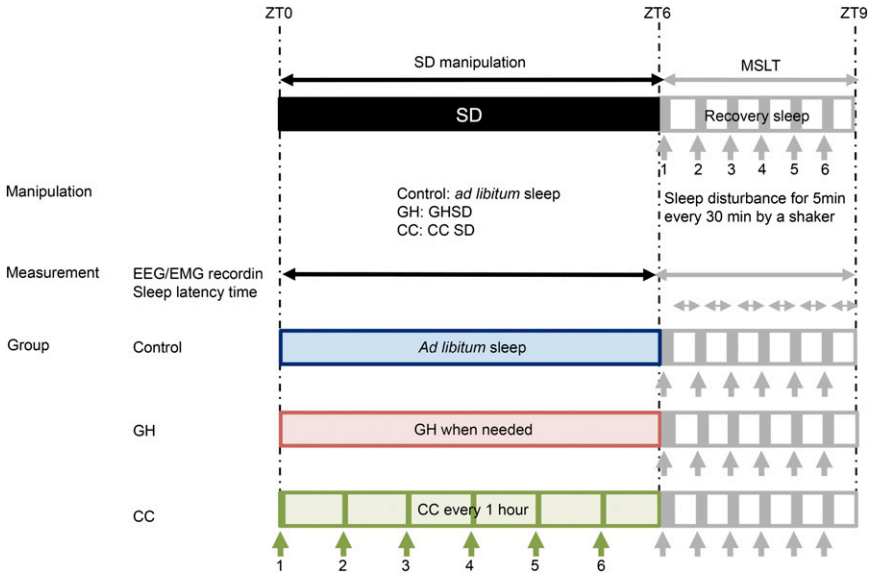

Fig. S1. Experimental timeline of SD followed by MSLT. After different SD manipulations for 6 h, mice were disturbed for 5 min every 30 min by an orbital shaker during sleep recovery period. EEG/EMG signals were monitored throughout ZT0–9. Sleep latency time was measured during the 25-min ad libitum sleep period after each 5-min sleep disturbance.

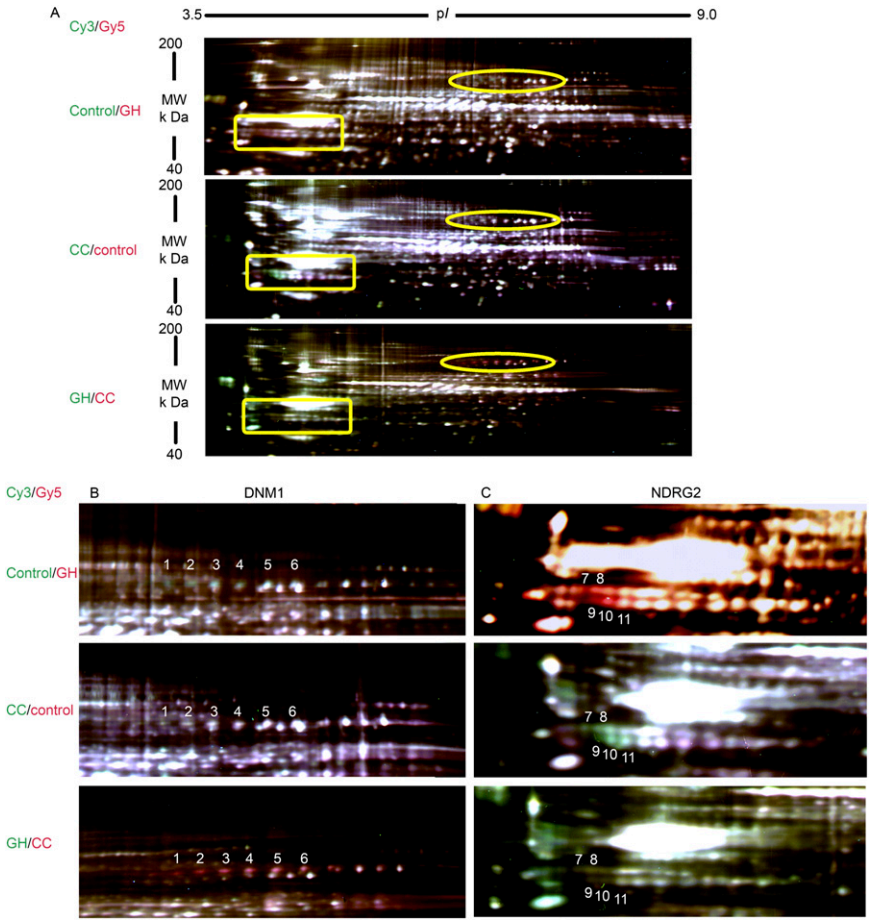

Fig. S2. Screenings for the degree of arousal and homeostatic sleep need biochemical markers assessed by 2D difference gel electrophoresis (2D-DIGE). Phosphorylated proteins were enriched from the diencephalon at ZT6. *Top*, *Middle*, and *Bottom* show 2D-DIGE images as comparisons of the control (green) and GH (red) groups, the CC (green) and control (red) groups, and the GH (green) and CC (red) groups, respectively. The green or red spots (nos. 1–11) represent proteins up-regulated between the paired samples. (*A*) Representative images of 2D-DIGE. Areas enclosed by ellipses and rectangles in *A* are enlarged in *B* and *C*, respectively. (*B*) The candidate spots associated with the sleep latency time and lowered in the GH group compared with the control (green, *Top*) and CC (red, *Bottom*) groups. The levels of the spots were similar in the control and CC groups (white, *Middle*). (*C*) The candidate spots associated with delta power and increased in the GH (red, *Top*) and CC (green, *Middle*) groups compared with the control group. No significant difference of the spots was observed between GH and CC groups (white, *Middle*).

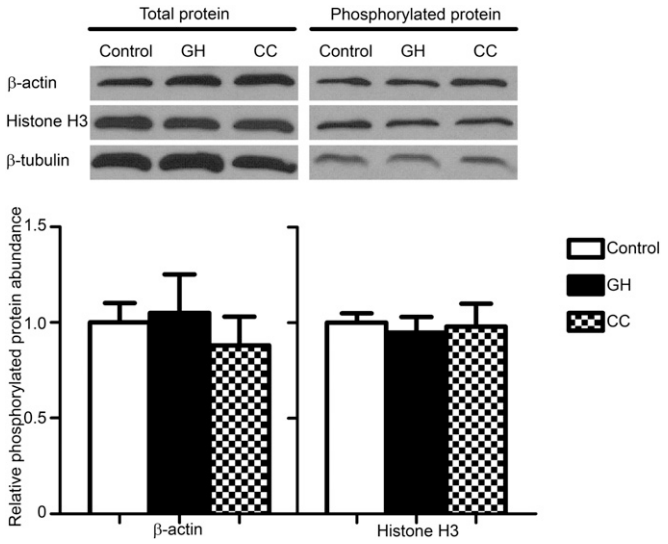

Fig. S3. Quality control for phosphoprotein column. Purification by the phosphoprotein column was verified with Western blot analysis against representative housekeeping genes; β-actin, histone H3, and β-tubulin in both total and phosphorylated protein samples. The phosphorylated protein sample was purified from the total protein sample of the diencephalon at ZT6, using Qiagen phosphoprotein column. After normalization to β-tubulin, the phosphorylated protein amount was renormalized to the total protein amount of corresponding protein and then expressed relative to the control group. Levels of these phosphorylated proteins were similar among all groups, indicating the uniformity of enrichment among the groups.

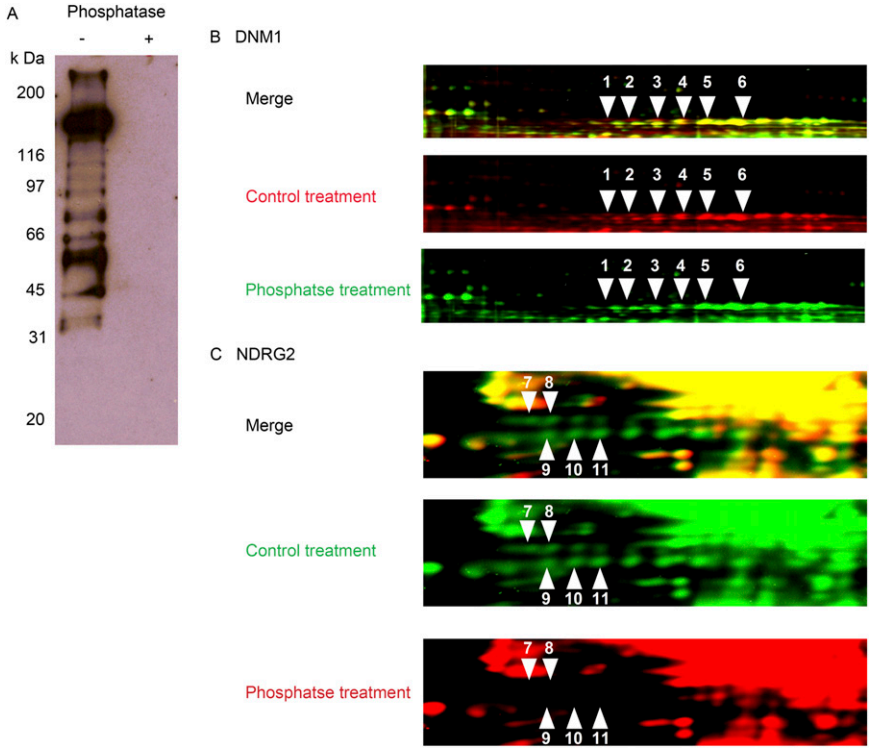

Fig. S4. The 2D-DIGE pattern of target DNM1 and NDRG2 spots after λ-phosphatase treatment. The phosphorylated protein sample was incubated in the absence (control) or presence of λ-phosphatase and then assessed by 1D Western blot (*A*) and 2D-DIGE (*B* and *C*), respectively. The details of the experiment are described in the *SI Materials and Methods*. (*A*) One-dimensional Western blot analysis against anti-phosphotyrosine antibody. Phosphotyrosine signals in the control sample (*Left*) disappeared in the phosphatase treated sample (*Right*), indicating an adequate λ-phosphatase treatment condition. (*B*) The 2D-DIGE pattern of target DNM1 spots with λ-phosphatase treatment. The control and λ-phosphatase–treated samples were labeled with CyDyes (described in Table S2) and assessed by 2D-DIGE. (*Top*) The merged images of the control (red, *Middle*) and λ-phosphatase–treated (green, *Bottom*) samples. Target DNM1 spots numbered 1–4 disappeared in the phosphatase-treated sample and only red spots in the control-treated sample appeared in the merged image (*Top*). Note that the spots eliminated by phosphatase treatment are the fainter ones that are slightly above the more abundant series of unaffected (yellow) spots. This result indicates phosphorylation of our target DNM1 spots numbered 1–4. (*C*) The 2D-DIGE pattern of target NDRG2 spots with λ-phosphatase treatment. Panels show 2D-DIGE images of the control and phosphorylated protein sample and the merged image of those samples. All target NDRG2 spots (numbered 7–11) disappeared in the phosphatase-treated sample (red, *Bottom*) and our target NDRG2 in the control treatment sample (green, *Middle*) were clearly observed in the merged image (green, *Top*). This result indicates phosphorylation of target NDRG2 spots.

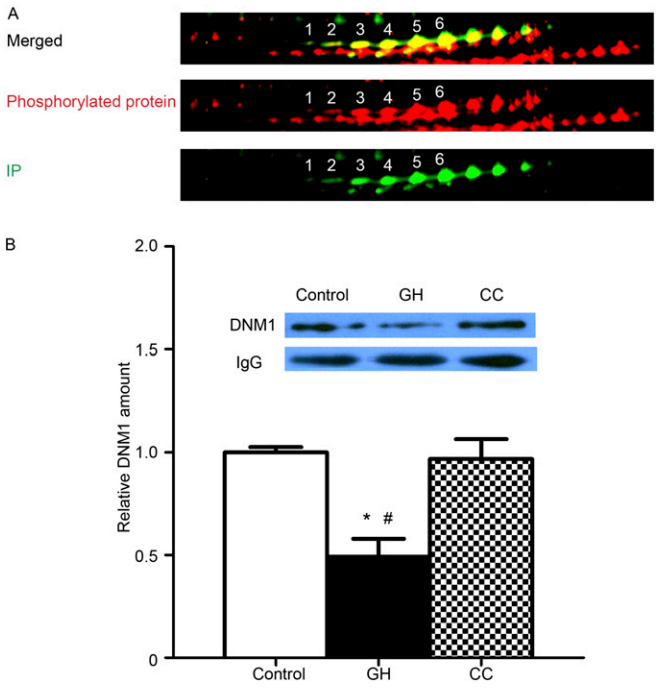

Fig. S5. Confirmation of decreased phosphor-DNM1 levels in the GH group by immunoprecipitation (IP). (*A*) Representative 2D-DIGE images of merged (*Top*), phosphorylated protein (Cy5, red, *Middle*), and immunoprecipitated (Cy3, green, *Bottom*) samples, with annotations indicating target DNM1 spots. All target DNM1 spots are immunoprecipitated and merged with the corresponding spots in the phosphoprotein sample (yellow). (*B*) Quantification of the immunoprecipitated phosphor-DNM1 in 1D Western blot indicating decreased levels in the GH group. Values were normalized by IgG. Data represent means ± SEM $^{*}P < 0.05$ between the control and GH groups, $^{\#}P < 0.05$ between the GH and CC groups by one-way ANOVA with post hoc Tukey's test.

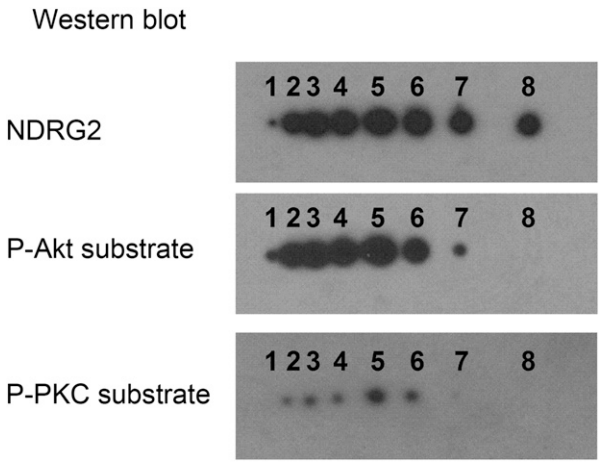

Fig. S6. Target phosoho-NDRG2 spots are detected with antibodies against phosphorylated Akt (P-Akt) substrates and phosphorylated PKC (P-PKC) substrate. NDRG2 was immunoprecipitated from whole protein sample, separated by 2DE, and blotted by anti-NDRG2 (*Top*), P-Akt substrate, and P-PKC substrate antibodies. P-Akt substrate and P-PKC substrate antibodies detect seven and five spots out of eight NDRG2 spots immunoprecipitated, respectively.

Table S1. Sequences of primer pairs for real-time quantitative PCR

Gene	Forward/reverse	Sequence	Accession no.
Homer 1a	Forward	ATGAACTTCCATATTTATCCACCTTACTT	NM_011982.2
	Reverse	GCATTGCCATTTCCACATAGG	
GLUT1	Forward	CCTATGGCCAAGGACACACT	NM_011400.2
	Reverse	CTGGTGTCAGGCAAGGAAAG	
Nur77	Forward	TGATGTTCCCGCCTTTGC	NM_010444.1
	Reverse	GAGCCCGTGTCGATCAGTG	
Arc	Forward	AGCAGCAGACCTGACATCCT	NM_018790.2
	Reverse	GTGATGCCCTTTCCAGACAT	
BDNF	Forward	CCATAAAGGACGCGGACTTGTACA	NM_007540.4
	Reverse	AGACATGTTTGCGGCATCCAG	
c-fos	Forward	CTGTCAACACACAGGACTTTT	NM_010234.2
	Reverse	AGGAGATAGCTGCTCTACTTTG	
Bip (GRP78)	Forward	GCTTCGTGTCTCCTCCTGAC	NM_022310.3
	Reverse	GGAATAGGTGGTCCCCAAGT	
HSP27	Forward	GACAGCTCAGCAGCGGGGTCTC	NM_013560.2
	Reverse	TAAGTGTGCCCTCAGGGGATAGGG	
DNM1	Forward	GCAGAAGGTCCTCAATCAGC	NM_010065
	Reverse	TCGAAGTCCACTGCAAACTG	
NDRG2	Forward	GCACACCATGGAAGTCTCCT	NM_013864.2
	Reverse	ACAATAGTCCCGTGACCCCCGGGAAA	
Cyclophilin B	Forward	GGAGATGGCACAGGAGGAA	NM_011149
	Reverse	GCCCGTAGTGCTTCAGCTT	

Arc, activity-regulated cytoskeletal-associated protein; *BDNF*, brain-derived neurotrophic factor; *Bip (GRP78)*, binding immunoglobulin protein/glucose-regulated protein 78 (*Bip*/*GRP78*); *GLUT1*, glucose transporter 1; *HSP27*, heat shock protein.

Table S2. Combination of CyDye labeling in 2D-DIGE

Figure no.	Cy3	Cy5	Cy2
Fig. S2, *Top*	Control	GH	Internal standard
Fig. S2, *Middle*	CC	Control	Internal standard
Fig. S2, *Bottom*	GH	CC	Internal standard
Fig. S4*B*	Phosphatase treatment	Control treatment	—
Fig. S4*C*	Control treatment	Phosphatase treatment	—
Fig. S5*A*	Immunoprecipitated sample	Phosphorylated protein	—