Enhancement of Stress Resilience through Hdac6-Mediated Regulation of Glucocorticoid Receptor Chaperone Dynamics

Supplemental Information

Supplemental Methods

Animals

Experiments in wild-type mice were conducted in eight week-old C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME). Experiments with Cre-dependent AAV vector to target overexpression of Hsp90 WT or mutant to serotonin neurons were conducted in Pet1-cre mice (1). All mice were 8-12 weeks of age when experiments began and were backcrossed onto C57BL/6J mice for over ten generations. Mice were housed on a 12 hour light (7 AM – 7 PM)/dark cycle with food and water available ad libitum. All studies were conducted according to protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee in accordance with institutional guidelines.

Repeated Social Defeat and Social Interaction Testing

The chronic social defeat stress (CSDS) procedure, consisting of alternate periods of physical contact with a trained CD1 aggressor (5 min) followed by protected sensory contact (24 hours) was repeated over 10 consecutive days, as previously described (2;3). On day 11, social approach/avoidance behavior toward an unfamiliar CD1 social target was assessed in a two-trial social interaction task. TopScan video-tracking software (CleverSys, Reston, VA) was used to measure the time spent in the interaction zone surrounding the target box. Interaction ratios (IRs) were calculated as
the time spent in the interaction zone with the social target present as a percent of the
time spent in the interaction zone with the target absent. Stratification into vulnerable (IR
< 100) and resilient (IR ≥ 100) subgroups was conducted as previously described (4).

Corticosterone Measurements

To measure the corticosterone (CORT) response following CSDS exposure or
corticotropin-releasing factor (CRF)/dexamethasone (DEX) challenge, the tail was
nicked and blood collected in tubes containing 50 mM EDTA. Plasma was separated by
centrifugation at 10,000 x g for 10 minutes at 4°C and stored at -80°C until assayed
using a commercial ELISA kit (AssayPro LLC, St. Charles, MO). Detailed timelines for
CSDS and DEX/CRF test are presented in Figure 1A. In brief, blood was drawn prior to
CSDS exposure and at T30, 90 and 120 minutes to measure CORT response induced
by CSDS. The DEX/CRF test was conducted a week after the end of CSDS to avoid
any potential confounds of acute stress. Blood was first drawn at 3 PM to obtain a
baseline. At 9 AM the following morning, mice were injected with a low dose of DEX,
(0.05 mg/kg, Sigma-Aldrich, St. Louis, MO). Six hours later (3 PM) blood was drawn
again and mice were injected with 0.15 mg/kg CRF (Sigma-Aldrich) with blood drawn
again 30 minutes later.

Measurements of Nuclear GR, Hsp90-GR Association and Hsp90 Acetylation

Two weeks after behavioral testing for social avoidance, mice were subjected to
an additional 10 minutes CSDS exposure. Brains were dissected 30 minutes after the
end of the physical interaction, from animals kept in sensory contact with aggressor.
Punches were taken of the dorsal raphe nucleus (DRN) using a brain matrix. Nuclear
fractions were prepared using a commercially available kit (BioVision, Milpitas, CA). Fractions were separated by gel electrophoresis (4-20% SDS PAGE), transferred to nitrocellulose membranes, and blotted for glucocorticoid receptor (GR) (sc-8992, Santa Cruz Biotechnology, Santa Cruz, CA). Blots were analyzed using the Li-COR Odyssey system and quantified using ImageJ software. The Hsp90-GR complex was immunoprecipitated (IP) from DRN punch homogenates prepared in TE buffer containing sodium molybdate (50 mM NaCl, 10 mM Tris-HCl, 4 mM EDTA, 20 mM NaMoO₄, 10% glycerol), protease and deacetylase inhibitors. 100-500 μg of protein were incubated overnight at 4°C with 2 μg of mouse monoclonal HSP 90α/β F-8 antibody (sc-13119, Santa Cruz Biotechnology). Samples were then incubated with 50 μl protein G beads (Santa Cruz Biotechnology) for 2.5 hours tumbling at 4°C. Beads were washed 4x with sample buffer and eluted by boiling at 95°C for 5 minutes in Western loading buffer. Proteins were separated as described above, and membranes were blotted for GR, Hsp90, or acetyl-K294 Hsp90 antibodies. For detection of acetylated Hsp90, we used an acetyl-specific affinity purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids surrounding K294 of human Hsp90 (600-401-981, Rockland Immunochemicals, Gilbertsville, PA).

In Vivo Drug Treatments

The Hdac6 inhibitor ACY-738 (provided by Acetylon Pharmaceuticals, Boston, MA) was administered at a dose of 5 mg/kg by i.p. injection. Vehicle was 0.75% DMSO/saline in a volume per weight of 10 ml/kg. In the CSDS, treatment started 10 days prior to the beginning of the CSDS procedure and continued throughout the stress period for a total of 20 days (Figure 2B). Injections were then given each day at the
beginning of the sensory contact period with the final injection given 24 hours prior to testing. Data from our laboratory indicate that the IC50 of ACY-738 for Hdac6 is 1.7 nM, and selectivity over Class 1 HDACs is between 60 and 120 fold, depending on the isoform. At the dose used in present study, ACY-738 does not elevate histone acetylation in brain (5).

**Expression Vectors**

Human Hsp90 WT and K294A point mutant were obtained from Len Neckers (National Cancer Institute, Rockville MD). V5-tagged forms of Hsp90 were made by cloning Hsp90 PCR products (Forward primer: CACCCATGCCTGAGGAAACCCAGACC; Reverse primer: GTCTACTTCTTCCATGCGTGATG) into pcDNA Gateway TOPO expression vector (Invitrogen, Grand Island, NY) according to manufacturer’s instructions. GR-green fluorescent protein (GFP) plasmid was obtained from Edwin Sanchez (University of Toledo College of Medicine, Toledo, OH). Flag-tagged human FKBP51 and FKBP52 expression vectors were obtained from Theo Rein (Max Planck Institute for Psychiatry, Munich, Germany).

**Virus Construction and Stereotaxic Surgery**

To overexpress an acetyl-mimic mutant of Hsp90 into serotonin neurons, a Flag-tagged Hsp90 construct containing a lysine to alanine mutation at position 294 (K294A) was cloned into a Cre-dependent AAV2 FLEX backbone (6) under control of the human synapsin promoter (AAV.hSynap.Flex.SV40) and packaged by the University of Pennsylvania Vector Core into AAV 2.9 viral particles. FLEX vectors conditionally
expressing wild type or K294A mutant Flag-Hsp90 or a control construct encoding Td Tomato, were injected into the DRN of mice expressing Cre-recombinase in a serotonin selective manner driven by the Pet1 promoter. Mice were anesthetized with isoflurane and injected bilaterally (coordinates from lambda: +0.03 AP, +0.08 ML, -0.33 DV, at an angle of 15°) with 1 μl of virus (9.04 x 10^{12} GC/ml) per side. Social defeat began 3 weeks post-surgery, to allow time for recovery and viral expression. A timeline of these experiments is presented in Figure 3A.

**Immunohistochemistry**

Mice were perfused with 4% paraformaldehyde (PFA), and brains were processed using standard single or dual immunolabeling methods as previously reported (2). For detection of acetylated Hsp90, we used the same anti acetyl-K294 Hsp90 antibody mentioned above. To validate viral-mediated gene expression, sheep anti-TPH (ab1541, Millipore) and rabbit anti-Flag (F7425, Sigma-Aldrich) primary antibodies were used. Confocal stacks were collected at a slice thickness of 1 μm across the DRN and tryptophan hydroxylase and Flag positive cells, as well as double-labeled cells, were counted for each section.

**Cell-Based Assays**

RN46A-B14 cells, an immortalized rat raphe cell line, were used for all tissue culture assays. For GR translocation cells were seeded in 96 well plates at 25,000 cells/well, grown for 24 hours of in media containing hormone free charcoal-stripped serum, and transfected with GR-GFP using 0.6 μl Lipofectamine 2000 (Invitrogen) per well and co-transfected with 200 ng of Flag-tagged Hsp90 vectors. Twenty-four hours
post-transfection, cells were treated with either vehicle (0.75% DMSO), 2.5 μM tubastatin A (7) (provided by Alan Kozikowski, University of Illinois at Chicago, Chicago, IL), or 2.5 μM ACY-738 (5) for 1 hour, followed by 1 μM dexamethasone (Sigma-Aldrich) or vehicle (2% EtOH) for 30 minutes. Cells were fixed in 4% PFA and immunostained for GFP (Aves, Tigard, OR) overnight using 1 mM Hoechst solution as nuclear stain. Cells were then imaged and ratios of GR-GFP signal inside and outside the nucleus were obtained in a high-throughput manner using the ImageXpress Micro system (Molecular Devices, Sunnyvale, CA), with 4 images per well.

For co-IP, cells were grown in 60 mm dishes, transfected as above with Flag or V5-tagged Hsp90 and FKBPs and harvested 48 hours later, with co-IP and blotting as described above.

**Data Analysis**

All variables were distributed normally and were analyzed using parametric statistics with *t*-test or one-way ANOVA followed by Fisher's PLSD or Tukey *post hoc* tests where appropriate. Correlations between pairs of variables were examined using linear regressions and proportions were compared using the Fisher exact test. Statistical significance was defined as a *P* value < 0.05, and all data is presented as the mean ± SEM.
Figure S1. Time course of corticosterone (CORT) response, Hsp90-GR association and glucocorticoid receptor (GR) translocation following a single social defeat experience. Mice were exposed to a 5 min physical social defeat (SD) by a CD1 aggressor and maintained in sensory contact with the aggressor. Mice were sacrificed at 0, 30, 60, 90, or 120 min following the start of physical defeat. (A) Changes in plasma corticosterone, (B) GR in dorsal raphe nucleus (DRN) nuclear extracts measured by Western blotting, and (C) acetylated Hsp90 (AcHsp90) and GR-Hsp90 association in whole brain lysates. \( n = 10 / \text{group} \) for plasma CORT, 1-2/pooled pairs for GR translocation, and \( n = 3-4 / \text{group} \) for co-IPs. The peak of GR translocation in DRN corresponded with peak of plasma CORT at 30 min. There was a main effect of time post-SD on Hsp90 acetylation, with acetylation peaking at 2 hours, a time-point corresponding to the normalization of CORT levels. (\( F_{4,9} = 4.8, p = 0.02 \), significant main effect of time). (D) A significant negative correlation was observed between Hsp90 acetylation and GR-Hsp90 association, with increased acetylation predicting GR dissociation from Hsp90 (\( R^2 = 0.32, p = 0.007 \)). Altogether, results suggest that Hsp90 acetylation marks stress termination.
Supplemental References


