The Anti-Apoptotic, Glucocorticoid Receptor Cochaperone Protein BAG-1 Is a Long-Term Target for the Actions of Mood Stabilizers

Rulun Zhou,^{1,3} Neil A. Gray,^{1,4} Peixiong Yuan,¹ Xiaoxia Li,³ Jingshan Chen,² Guang Chen,¹ Patricia Damschroder-Williams,¹ Jing Du,¹ Lei Zhang,^{1,3} and Husseini K. Manji^{1,4}

¹Laboratory of Molecular Pathophysiology and ²Clinical Brain Disorders Branch, National Institute of Mental Health, Bethesda, Maryland 20852,

³Department of Psychiatry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814, and ⁴College of Physicians and Surgeons, Columbia University, New York, New York 10032

Increasing data suggest that impairments of cellular plasticity/resilience underlie the pathophysiology of bipolar disorder. A series of microarray studies with validating criteria have recently revealed a common, novel target for the long-term actions of the structurally highly dissimilar mood stabilizers lithium and valproate: BAG-1 [BCL-2 (B-cell CLL/lymphoma 2)-associated athanogene]. Because BAG-1 attenuates glucocorticoid receptor (GR) nuclear translocation, activates ERK (extracellular signal-regulated kinase) MAP (mitogen-activated protein) kinases, and potentiates anti-apoptotic functions of BCL-2, extensive additional studies were undertaken. Chronic administration of both agents at therapeutic doses increased the expression of BAG-1 in rat hippocampus. Furthermore, these findings were validated at the protein level, and the effects were seen in a time frame consistent with therapeutic effects and were specific for mood stabilizers. Functional studies showed that either lithium or valproate, at therapeutically relevant levels, inhibited dexamethasone-induced GR nuclear translocation and inhibited GR transcriptional activity. Furthermore, small interfering RNA studies showed that these inhibitory effects on GR activity were mediated, at least in part, through BAG-1. The observation that BAG-1 inhibits glucocorticoid activation suggests that mood stabilizers may counteract the deleterious effects of hypercortisolemia seen in bipolar disorder by upregulating BAG-1. Additionally, these studies suggest that regulation of GR-mediated plasticity may play a role in the treatment of bipolar disorder and raise the possibility that agents affecting BAG-1 more directly may represent novel therapies for this devastating illness.

Key words: lithium; valproate; bipolar disorder; BAG-1; glucocorticoid; neuroplasticity

Introduction

Despite the devastating impact that bipolar disorder (BD) has on the lives of millions worldwide, there is still a dearth of knowledge concerning its underlying etiology and pathophysiology. Several laboratories have therefore been focusing extensively on elucidating the molecular and cellular mechanisms of action of mood-stabilizing agents, with the expectation that these observations will delineate specific cascades that may be identical to or impinge on those most affected by the disease (Manji and Lenox, 2000a; Birnbaum et al., 2004). However, although acute, *in vitro* effects of mood stabilizers have been identified previously, their therapeutic effects in the treatment of BD are only seen after chronic administration (Quiroz et al., 2004). In addition, chemically dis-

tinct mood stabilizers such as lithium and valproate (VPA), which have fairly distinct primary biochemical targets, show similar (albeit not identical) clinical effects in the treatment of BD. When coupled, these observations suggest that downstream and adaptive changes in cell function, rather than acute actions on direct pharmacological targets, may be of the greatest importance. A series of cDNA microarray studies were undertaken recently to identify changes in gene expression in rat hippocampus brought about by chronic lithium or VPA.

One gene whose expression was upregulated by both chronic lithium and VPA is BAG-1 [BCL-2 (B-cell CLL/lymphoma 2)-associated athanogene]. Athanogene is derived from the Greek word “athanos,” which means “anti-death.” Thus, BAG-1 was initially identified as a protein interacting with and potentiating the anti-apoptotic function of BCL-2 (Takayama et al., 1995).

BAG-1 was one of several genes regulated by both lithium and valproate in both rodent studies and human neuroblastoma cell studies. This gene was selected for extensive additional study because increasing recent data suggest that, in addition to neurochemical alterations, impairments of cellular plasticity and resilience may also underlie the pathophysiology of severe mood disorders (Manji et al., 2000; Duman, 2002; Du et al., 2004). In

Received Nov. 4, 2004; revised March 28, 2005; accepted March 28, 2005.

We acknowledge the support of the Intramural Research Program of the National Institute of Mental Health and the Stanley Medical Research Institute. The statistical analysis of David Luckenbaugh and the editorial assistance of Holly Giesen are very much appreciated.

Correspondence should be addressed to Dr. Husseini K. Manji, Laboratory of Molecular Pathophysiology, National Institute of Mental Health, Porter Neuroscience Research Center, Building 35, Room 1C-917, 35 Convent Drive, Bethesda, MD 20892. E-mail: manji@nih.gov.

DOI:10.1523/JNEUROSCI.4530-04.2005

Copyright © 2005 Society for Neuroscience 0270-6474/05/254493-10\$15.00/0

this context, BAG-1 has three known functions that may be very relevant for the treatment of severe mood disorders: (1) as mentioned, BAG-1 potentiates the function of BCL-2, thereby enhancing cell survival (Takayama et al., 1995); (2) BAG-1 activates extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinases cascades (signaling cascades used by endogenous neurotrophic factors to bring about numerous long-term effects) (Kermer et al., 2002); and (3) BAG-1 is a cochaperone protein involved in modulating the function of the glucocorticoid receptor (GR) (Schneikert et al., 1999). The latter observations are notable, because hypercortisolemia has been postulated to mediate many of the deleterious effects of severe stressors and is a common accompaniment of severe mood disorders (McEwen, 1999; Sapolsky, 2000).

Because the chronic administration of medications brings about numerous effects, investigators have recently developed a series of validating criteria for potential therapeutic relevance (Coyle and Manji, 2002; Coyle and Duman, 2003); we find that BAG-1 fulfills all of these criteria and represents a novel target for the treatment of BD.

Materials and Methods

Animals. All animal treatments, procedures, and care were approved by the National Institute of Mental Health Animal Care and Use Committee and followed the *Guide for the Care and Use of Laboratory Animals*. Male Wistar Kyoto rats (starting weight, 150–200 g) were housed three to four per cage in a 12 h light/dark cycle and had access to water and food *ad libitum*. After a 1 week accommodation period, the rats were treated as follows: for lithium and valproate treatment, drug-containing chow was custom produced by Bio-Serve (Frenchtown, NJ). Drug-containing chow and control chow were identical with the exception of the added drug and were produced at both a low and regular concentration for each drug with concentrations of lithium carbonate (1.2 and 2.4 g/kg) and sodium valproate (10 and 20 g/kg). These doses of lithium and valproate have been used extensively by our group and others and found to lead to serum drug levels similar to those achieved therapeutically in the treatment of bipolar disorder (Chen et al., 1999). As with the clinical situation, rats were initially treated (for 1 week) at the lower dose, followed by 3 weeks of full-dose treatment. The lithium experiment included 12 control and 12 experimental animals, all provided with daily bedding changes to minimize the effects of lithium-induced polyuria (a well known lithium side effect). The valproate experiment also included 12 control and 12 experimental animals.

All rats were weighed and then killed by decapitation during the morning hours. Mean $\pm$ SD for the weights of the animals used for the protein studies of chronic mood-stabilizer treatment were as follows: control, 295.8 $\pm$ 11.8 g; lithium, 271.4 $\pm$ 9.0 g; and VPA, 254.6 $\pm$ 8.9 g. Animal weights in acute treatment were as follows: control, 182.6 $\pm$ 10.0 g; lithium, 178.6 $\pm$ 16.9 g; and VPA, 159.4 $\pm$ 12.7 g. Drug serum levels were performed by Medtox Laboratories (St. Paul, MN) and found to be within the therapeutic range. Only animals with drug levels within the therapeutic concentrations were used for additional studies. Serum blood levels of the animals used for additional analysis were as follows: lithium, 0.5–1.2 mM; VPA, 50–150 μ g/ml.

To test the specificity of the effects for mood stabilizers (explained in detail later), three other psychotropic agents from different classes [amphetamine (a psychostimulant), fluvoxamine (an antidepressant), and haloperidol (an antipsychotic)] were selected to treat rats chronically (3 weeks) through osmotic pumps. Osmotic minipumps (model 2ML4; Alzet, Cupertino, CA) were implanted subcutaneously by aseptic surgery under anesthesia with isoflurane inhalation. The drugs were diluted in 50:50 water/ethanol (v/v) to the concentrations to attain a flow rate of 0.5 μ g $\cdot$ kg^{−1} $\cdot$ d^{−1} (amphetamine), 10 μ g $\cdot$ kg^{−1} $\cdot$ d^{−1} (fluvoxamine), and 0.5 μ g $\cdot$ kg^{−1} $\cdot$ d^{−1} (haloperidol) (the usual doses of the drugs used for chronic studies). Animals were observed daily by the investigators as well as the animal care staff. All rats were weighed and killed. Mean $\pm$ SD weights were as follows: control, 283.88 $\pm$ 14.77 g; amphetamine,

288.71 $\pm$ 10.39 g; fluvoxamine, 276.17 $\pm$ 9.15 g; and haloperidol, 292.00 $\pm$ 29.16 g. Before dissection, the osmotic pumps were taken out to check the remaining drug volumes inside, and delivered drug volumes via the pumps were estimated, which did not exceed 10% difference compared with those calculated by flow rates. All procedures and surgeries were conducted in accordance with the National Institutes of Health *Guidelines for Survival Rodent Surgery*. After the treatments, all animals were killed by decapitation. The rat brains were removed immediately after decapitation and dissected on ice, and hippocampi were rapidly frozen in dry ice and stored at -80°C until additional analyses.

Cell culture. Human SH-SY5Y neuroblastoma cells were cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37°C in DMEM (Invitrogen, Carlsbad, CA) plus 5% fetal bovine serum supplemented with 100 IU/ml penicillin G sodium, 100 μ g/ml streptomycin sulfate, and 0.25 μ g/ml amphotericin B. The medium was changed every 3–4 d. Treatment with 0.05% trypsin and 0.53 mM EDTA was used to split and harvest and to passage the cells. For selected assays, minor modifications were made to the components of the media; these minor modifications are noted in the corresponding sections/figure legends. For the mood-stabilizer (lithium or VPA) treatment, passaged cells were cultured in DMEM plus 0.1% fetal bovine serum without antibiotics.

Immunoblotting (Western blot). Immunoblotting was conducted as described previously (Chen et al., 1998), with minor modifications. In brief, rat hippocampi or SH-SY5Y cells were suspended in ice-cold lysing buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 150 mM NaCl, and 1 mM β -glycerophosphate, as well as the following ingredients, which were added to the buffer immediately before use (final concentration): 5 mM DTT, 1 $\times$ Phosphatase Inhibitor Cocktail I, Phosphatase Inhibitor Cocktail II, and Phosphatase Inhibitor Cocktail (all three phosphatase inhibitors were from Sigma-Aldrich, St. Louis, MO). The brain tissue or SH-SY5Y cells were then homogenized by passing through a 26 gauge needle 10 times and were further sonicated for 1 s 10 times using a VirSonic ultrasonic cell disrupter (VirTis, Gardiner, NY) at setting 2.5; the homogenates were then centrifuged for 15 min at 14,000 $\times$ g to remove insoluble debris. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). Before undertaking the mood stabilizer studies, varying protein concentrations obtained from the hippocampus or SH-SY5Y cells were subjected to Western blotting; subsequent studies were undertaken using hippocampal or SH-SY5Y cell protein concentrations known to be within the linear range for BAG-1 immunoreactivity. Equal amounts of proteins from homogenized hippocampus or SH-SY5Y cells were separated by 14% SDS gradient gel electrophoresis (Invitrogen), transferred to nitrocellulose membranes, and immunoblotted with 1:300 BAG-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies (Amersham Biosciences, Piscataway, NJ) were the secondary antibodies. The immunoreactive bands were visualized by Amersham Biosciences enhanced chemiluminescence (ECL+) and exposed to Kodak Biomax or Biolight film (Eastman Kodak, Rochester, NY). The ECL signal intensities were quantified using the Kodak IS4400CF Image analysis system and standard curve method. An aliquot of pooled “standard” rat brain (hippocampus) or SH-SY5Y cell fraction was run on one lane of each gel. Data were normalized against the standard rat brain or SH-SY5Y fractions to minimize between-blot variability.

Immunohistochemistry. Rats were deeply anesthetized with chloral hydrate and perfused via the ascending aorta with saline until the outflow became clear, followed by 0.1 M PBS, pH 7.4, containing 4% paraformaldehyde for 20 min. The brains were rapidly removed and postfixed in the same fixative plus 20% sucrose at 4°C for 48 h and then rapidly frozen and stored at -80°C . Serial sections (30 μ m/section) were cut coronally using a cryostat and mounted on glass slides. Slides were washed, and nonspecific activity was blocked with 5% normal goat serum in PBS. Brain sections were then incubated with anti-BAG-1 antibody (1:500 in dilution) in 0.1 M PBS containing 1% normal goat serum and 0.3% Triton X-100 overnight. The secondary, biotinylated goat anti-rabbit antibody (1:200) was added for 1 h, followed by an incubation with HRP-avidin-biotin complex (1:50, Vector ABC kit; Vector Laboratories, Burlingame, CA) for 1 additional hour. The color was developed by treatment with 0.05% diaminobenzidine and

0.003% H₂O₂ in PBS. The sections then were dehydrated, cleared, and mounted. To verify specificity of the results, the immunostaining procedure was performed in parallel using anti-BAG-1 antibody preabsorbed with 5–10 μ g/ml synthetic peptide immunogen.

Because lithium has been shown recently to robustly protect against stress-induced CA3 dendritic atrophy (Wood et al., 2004), this region was selected for more detailed investigation. Immunohistograms (three sections from each of the five animals per group) from each of the three treatment groups were analyzed with a Zeiss (Oberkochen, Germany) AxioPlan-2 microscope equipped with a Hitachi (Tokyo, Japan) digital camera and NIH Image 1.62 analysis software by an experimenter blind to the study code. Quantitation for BAG-1 was performed on live acquired images of dimensions 582 $\times$ 228 μ m² in hippocampal CA3 pyramidal neurons. The number of the cells in the analyzed field was obtained by counting individual cells positive for BAG-1.

Transfections. Cells were plated at a density of 1.0×10^5 in 1 ml of DMEM with 0.1% fetal bovine serum and without antibiotics per well on 12-well plates. Cells were treated as described, and media were changed the day before transfection. For each well containing a 1 ml sample, 4 μ l of Lipofectamine 2000 (Invitrogen) was diluted in 50 μ l of Invitrogen Opti-MEM I medium, mixed gently, and incubated for 5 min at room temperature. DNA (plasmid) was diluted in 50 μ l of Opti-MEM I medium and mixed gently. The diluted Lipofectamine and DNA were then mixed gently and incubated for 20 min at room temperature. Lipofectamine 2000 (100 μ l) and DNA complexes were added to each well and gently mixed. Media were replaced 6–12 h after transfection.

Glucocorticoid receptor activity reporter system. The reporter vector used was pGRE-SEAP, which contains a glucocorticoid response element (GRE) as the enhancer and secreted alkaline phosphatase (SEAP) as the reporter. Activated GR binds to GREs and initiates expression of SEAP, the levels of which reflect glucocorticoid activity. The reporter vector pGRE-SEAP also contains a TATA-like promoter named pTAL. To contrast the pure GRE function of pGRE-SEAP, a negative control vector, pTAL-SEAP, a vector derived from pGRE-SEAP with GRE trimmed to test the pure promoter effects, was adopted. As a positive control, pSEAP2 control vector, which can express SEAP in most cell lines, was used to evaluate the transfection and SEAP detection system. Another control vector, pEGFP-Luc, which expresses both enhanced green fluorescent protein (EGFP) and luciferase, was used to evaluate the transfection efficiency (all above-mentioned vectors were from Clontech, Palo Alto, CA). The GR expression vector was pRShGR (American Type Culture Collection, Manassas, VA), which expresses the human GR α . To investigate dexamethasone-induced GR activation, pGRE-SEAP and pRShGR were cotransfected into SH-SY5Y cells for reporter levels, pTAL-SEAP and pRShGR were cotransfected into SH-SY5Y cells to obtain reporter background levels, and the difference was used to determine pure GRE activation. To normalize the values obtained using the SEAP reporter system, for both the pGRE-SEAP/pRShGR combination for GRE activity and the pTAL-SEAP/pRShGR combination for background, the pSV- β -galactosidase control vector (Promega, Madison, WI) was cotransfected, and the levels of cellular β -galactosidase were determined using the Promega β -galactosidase enzyme assay system; the values obtained were used to normalize the levels of the reporter (SEAP).

Glucocorticoid receptor nuclear translocation. SH-SY5Y cells were cultured in 12-well polystyrene plates on the coverslips (Nalge Nunc International, Rochester, NY) preplaced in each well for 6 d. Preliminary dose and time studies were undertaken; the cells were then incubated with dexamethasone (100 nM) for 30 min at 37°C. The cells were then washed once with ice-cold PBS and fixed with PBS containing 4% paraformaldehyde and 120 μ M sucrose for 1 h on ice. After washing the cells with PBS three times, the cells were blocked with PBS containing 10% goat serum, 1% BSA, and 0.2% Triton X-100 for 1 h at room temperature and subsequently incubated with rabbit anti-GR antibody (1:40) in PBS containing 5% goat serum, 0.5% BSA, and 0.2% Triton X-100 at 4°C overnight (120 μ l). The next day, coverslips were washed three times with PBS containing 0.5% BSA and 0.2% Triton X-100. Cyanine 3 (Cy3)-conjugated anti-rabbit IgG secondary antibodies (1:300) were diluted with PBS containing 0.5% BSA and 0.2% Triton X-100. Diluted secondary antibody (120 μ l) was added to each well and incubated for 1 h at

room temperature. Coverslips were then rinsed with PBS containing 0.4% Triton X-100. Nuclei were stained using 300 nM 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) in PBS for 5 min at room temperature. After extensive washing, the coverslips were mounted on glass slides in antifade mounting media (Molecular Probes, Eugene, OR) and observed using the Zeiss LSM 510 laser scanning confocal microscope. For quantitative analysis, a maximum of 100 cells per high-power field (250 $\times$) and 10 fields were counted as one "count"; representative pictures for each group were taken in a blinded manner. The field was moved in a fixed pattern to prevent recounting the same cells and to ensure that cells were sampled equally from the center and edge of the culture slips. Immunopositive cells were examined at 250 $\times$. Scales were calibrated by using a microscope scale bar at the same magnification. Measurements included percentage of positive cells immunostained by the GR antibody (positive cells per total cells) in the nucleus. The results reported for each treatment represent the mean and SE obtained from three independent experiments and six counts.

BAG-1 small interfering RNA studies. To more definitively establish a causal relationship between mood stabilizer-induced changes in GR nuclear translocation/GR-mediated gene expression and BAG-1 upregulation, a small interfering RNA (siRNA) strategy was used. Thus, siRNAs with hairpin structures were designed to target the human BAG-1 coding region, which is shared by all four isoforms of the human BAG-1. Each siRNA contains a fragment of a 19 nt sense sequence and a fragment of a 19 nt antisense sequence joined by a 9 nt loop. We designed three siRNAs for BAG-1 and tested them for their effectiveness in knocking down BAG-1 protein levels. We then selected two of the three according to their effectiveness for additional use in the functional studies. The first one was SiBAG1A, with a sense-strand sequence of 5'-GCACGACCTTCATGT-TACctt-3'; the second one was SiBAG1C, with a sense-strand sequence of 5'-GAGGTTGAAGTAAAGAAGTtt-3'. Their scrambled sequences were used as control. The sense-strand sequence of SiBAG1A scrambled oligo was 5'-GACAAGGCTTCTTCTCCAC-3', and the sense-strand sequence of SiBAG1C scrambled oligo was 5'-GAGTGTAAATGCG-ATGAAAA-3'. Care was taken to ensure that the scrambled sequences did not have nonspecific matches for any genes based on BLAST (basic local alignment search tool) searches in GenBank (<http://www.ncbi.nlm.nih.gov/BLAST/>). The initial step for constructing an siRNA expression vector in our study was to synthesize two 64 bp oligos (two oligo sequences are complimentary to each other except for the terminal four nucleotides), which include both the sense and antisense sequences of the corresponding 19 bp siRNA, the hairpin-forming sequence, and the adapter sequences for cloning. The two 64 bp oligos were annealed to form a double-stranded DNA segment. Because our experiments were geared to investigate longer-term (days) effects of BAG-1 siRNA, we used a strategy whereby the double-stranded DNA segments were then cloned into the pSilencer 3.0-H1 system (Ambion, Austin, TX) according to the guidelines of the manufacturer. The cloning was verified through sequence analysis of the cloned hairpin-siRNA expression DNA segment and adjacent vector sequences. For application of the siRNA, the siRNA expression vector was transfected into cells. The main advantage of the expression-type siRNA is that it can maintain a relatively constant level of siRNA in the cells without fluctuations caused by degradation; this is a particularly important consideration when relatively longer (>24 h) observation periods are needed (as in the studies undertaken here).

Statistical analyses. All data were analyzed in SPSS (release 11.5; SPSS, Chicago, IL) by one-way ANOVA *post hoc* test (for three or more groups) or Student's *t* test (for two groups) and are presented as mean $\pm$ SE (*n* is number of the animals or number of observations). In ANOVA, Q-Q plot was adopted for normal distribution test, and Levene's test was used for the test of equality of group variances. Tukey's *post hoc* test (honestly significant difference) was adopted if the variances between groups were similar.

Results

BAG-1 protein levels are upregulated by both chronic lithium and VPA at therapeutically relevant concentrations in rat hippocampus

Previous microarray studies suggested that chronic (3–4 weeks) treatment with the structurally highly dissimilar mood stabilizers

lithium and valproate both increased BAG-1 expression in rat hippocampus. To confirm that BAG-1 protein was also upregulated, Western blot studies were undertaken. Immunoblot analysis of rat brain tissue showed that chronic treatment with either lithium or VPA significantly upregulated the BAG-1 protein levels in rat hippocampus (Fig. 1*a*). As has been demonstrated previously, three BAG-1 bands with molecular weights of ~50, 36, and 32 kDa were identified in rodent tissue (Fig. 1*a*). Quantitative analysis [optical density (OD)] showed that chronic treatment with lithium or VPA robustly upregulated BAG-1 protein, albeit in a nonidentical manner. Thus, both lithium and VPA produced an ~50% increase in the 32 kDa isoform (relative OD: control, 100 ± 14.23 ; lithium, 159.74 ± 19.63 ; VPA, 155.82 ± 14.22 ; lithium vs control, $p = 0.032$; VPA vs control, $p = 0.048$; lithium vs VPA, $p = 0.983$; $n = 11$). Moreover, lithium and VPA produced ~100% increases in BAG-1 50 and 36 kDa isoforms, respectively (50 kDa, relative OD: control, 100 ± 11.40 ; lithium, 197.64 ± 22.59 ; VPA, 122.64 ± 29.82 ; lithium vs control, $p = 0.021$; VPA vs control, $p = 0.762$; lithium vs VPA, $p = 0.079$; $n = 6$; 36 kDa, relative OD: control, 100 ± 12.89 ; lithium, 133.64 ± 9.16 ; VPA, 209.94 ± 15.50 ; lithium vs control, $p = 0.17$; VPA vs control, $p = 5.00 \times 10^{-6}$; lithium vs VPA, $p = 0.0007$; $n = 10$) (Fig. 1*a*).

Short-term (5–7 d) treatment with lithium or VPA does not affect BAG-1 protein levels in rat hippocampus

To ascribe potential therapeutic relevance, short-term treatment studies were also undertaken (at a time point when clinical effects are generally not observed). To determine whether short-term lithium or valproate treatment has an effect on BAG-1 protein levels, rats were also treated for ~5–7 d. No significant changes in BAG-1 levels were observed with short-term treatment with either drug (50 kDa, relative OD: control, 100 ± 8.60 ; lithium, 104.75 ± 8.50 ; VPA, 109.38 ± 12.13 ; $p = 0.801$; 36 kDa, relative OD: control, 100 ± 8.84 ; lithium, 91.31 ± 8.05 ; VPA, 113.44 ± 10.94 ; $p = 0.264$; 32 kDa, relative OD: control, 100 ± 8.53 ; lithium, 109.33 ± 10.67 ; VPA, 115.41 ± 12.74 ; $p = 0.603$; $n = 8$) (Fig. 1*b*).

Specificity of effects: chronic treatment with non-mood-stabilizer psychotropics does not alter BAG-1 protein levels in rat hippocampus

There is a growing concern in studies of psychotropic drug action that drugs of fairly diverse clinical classes (such as antidepressants, antipsychotics, and antimanics) have been demonstrated to affect certain common targets/processes (such as BDNF expression and hippocampal neurogenesis). For this reason, the demonstration that a particular target is “specific” for a class of drugs has been thought to represent an important validating criteria for the potential therapeutic relevance of the target (Coyle and Manji, 2002; Coyle and Duman, 2003). Thus, to determine whether the lithium- or VPA-induced BAG-1 upregulation was

Figure 1. Effects of psychotropic drugs on BAG-1 protein levels in rat hippocampus. *a*, Chronic mood stabilizers. *b*, Acute mood stabilizers. *c*, Specificity of effects. Male Wistar rats were treated with Li_2CO_3 (2.4 g/kg)- or sodium valproate (20 g/kg)-containing chow for 4 weeks (*a*) or 5–7 d (*b*). The rats were then killed by decapitation, and trunk blood was collected for determination of drug levels of lithium and VPA (*a*, *b*). For studies to delineate the specificity of these effects for mood stabilizers, male Wistar rats were treated with $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ amphetamine (Amp; a psychostimulant), $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ haloperidol (Hal; an antipsychotic agent), or $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ fluvoxamine (Flu; an antidepressant) through an osmotic minipump for 20 d (*c*). The rat brains were removed immediately after decapitation and dissected on ice, and hippocampi were rapidly frozen in dry ice and stored at -80°C until additional analyses. Values showed here are mean $\pm$ SE. C, Control; L, lithium; V, VPA. $n \geq 6$; * $p < 0.05$; ANOVA Tukey's *post hoc* test.

specific for mood stabilizers, the effects of a number of other psychotropic drugs, belonging to different classes, were investigated. For these studies, a psychostimulant, an antidepressant, and an antipsychotic were chosen. Chronic (3 week) treatment with $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ amphetamine (a psychostimulant), $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ haloperidol (an antipsychotic), or $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ fluvoxamine (an antidepressant) was without effect on BAG-1 levels (50 kDa, relative OD: control, 100 ± 11.19 ; amphetamine, 107.31 ± 7.99 ; fluvoxamine, 94.4 ± 8.84 ; haloperidol, 101.01 ± 8.71 ; $p = 0.806$; 36 kDa, relative OD: control, 100 ± 10.75 ; amphetamine, 98.14 ± 4.91 ; fluvoxamine, 98.28 ± 7.02 ; haloperidol, 103.15 ± 6.56 ; $p = 0.963$; 32 kDa, relative OD: control, 100 ± 7.12 ; amphetamine, 111.81 ± 9.08 ; fluvoxamine, 97.68 ± 7.18 ; haloperidol, 97.78 ± 8.16 ; $p = 0.545$; $n = 8$) (Fig. 1*c*).

Together, the results suggest that the regulatory effects of lithium and VPA on BAG-1 require chronic treatment and are specific to these mood stabilizers.

Chronic lithium or VPA increases BAG-1 immunoreactivity in hippocampal CA3 neurons

Chronic restraint stress has been shown to induce a remodeling of apical dendrites on CA3 pyramidal cell neurons, effects that are likely mediated (at least in part) by enhanced glucocorticoid throughput (McEwen, 1999; Sapolsky, 2000). These observations are particularly noteworthy for the purposes of the present series of studies because chronic lithium has been demonstrated recently to prevent the effects of chronic stress on CA3 pyramidal cell neuroarchitecture (Wood et al., 2004). Because BAG-1 exerts an inhibitory effect on GR function, this hippocampal subfield was chosen for more detailed analysis of mood-stabilizer effects on BAG-1 levels. Chronic treatment with both agents was found to significantly increase BAG-1 immunolabeling in hippocampal

Figure 2. Effects of chronic mood stabilizers on BAG-1 protein levels in rat hippocampal CA3 subfield. **A**, Photomicrographs of granule cells of the hippocampal formation. The rectangular boxed area shows the area for amplified observation and counting. **B**, Control. **C**, Lithium treated. **D**, VPA treated. Amplification was at $25\times$ for **B–D**. Arrows in **B–D** indicate samples of positive cells (three sections from each animal, five animals per group). **E**, Effects of chronic lithium or VPA on cell counts of BAG-1-immunoreactive cells in the CA3 subfields of rat hippocampus. The bar graph shows the percentage of control levels in a mean $\pm$ SE pattern. ** $p < 0.01$; ANOVA Tukey's *post hoc* test.

CA3 neurons (average number of the BAG-1-positive cells in the analyzed fields: control, 30.07 ± 0.67 ; lithium, 36.93 ± 0.91 ; VPA, 39.63 ± 0.75 ; lithium vs control, $p = 0.00012$; VPA vs control, $p = 5.03 \times 10^{-6}$; lithium vs VPA, $p = 0.074$) (Fig. 2).

Western blot and immunohistochemistry showed trends of elevated BAG-1 levels in the frontal cortex and striatum, but not as robustly as in the hippocampus.

BAG-1 protein levels are also upregulated by both lithium and VPA at therapeutically relevant concentrations in human neuroblastoma SH-SY5Y cells in a time-dependent pattern

To mechanistically dissect the effects of mood stabilizers on BAG-1 function in detail, *in vitro* systems are necessary. Furthermore, it is important to ascertain whether the effects of mood stabilizers also occur in tissue with a human neuronal phenotype; human neuroblastoma SH-SY5Y cells were therefore used for these mechanistic studies. We detected four isoforms of the human BAG-1 protein in SH-SY5Y cells, corresponding to molecular weights of 50, 46, 36, and 29 kDa, as expected from the human BAG-1 gene structure. The signal of the 29 kDa isoform was very weak and was therefore not included in our quantitative analysis. For time course studies, SH-SY5Y cells were treated for 24 h, 3 d, 6 d, or 8 d with 1.0 mM lithium or VPA (the plasma concentration obtained in humans in the treatment of bipolar disorder). Immunoblot analysis showed that BAG-1 levels were indeed upregulated in these cells by lithium (on day 6, after ODs of controls are adjusted to 1, relative ODs of the three isoforms are as follows: 50 kDa, 1.77 ± 0.44 , $p = 0.033$; 46 kDa, 1.58 ± 0.68 , $p = 0.040$; 36 kDa, 1.21 ± 0.27 , $p = 0.039$) (Fig. 3*a*) or VPA (on day 6, after ODs of controls are adjusted to 1, relative ODs of the three isoforms are as follows: 50 kDa, 1.83 ± 0.23 , $p = 0.003$; 46 kDa, 2.00 ± 0.17 , $p = 0.009$; 36 kDa, 1.39 ± 0.05 , $p = 0.004$) (Fig. 3*b*); even in the cultured system, chronic drug administration was required, and effects were seen beginning at day 6 and lasting through day 8 (the longest time examined).

It should be noted that BAG-1 has different isoforms: in humans, there are four isoforms (50, 46, 36, and 29 kDa) according to gene structure and immunoblotting analyses (Yang et al., 1998). The 29 kDa isoform is very low in abundance, precluding accurate quantitation. In mice, there are two isoforms (50 and 32 kDa) according to gene structure and immunoblotting analyses (Takayama et al., 1998). The gene structure of the rat BAG-1 gene

has not yet been entirely reported, but it is anticipated that rat BAG-1 also has two isoforms. In our Western blot analysis, we found two bands for the short isoform, the same phenomenon also reported by other researchers, and the explanation was phosphorylation (Packham et al., 1997; Hayashi et al., 2000). There is no clear equivalent between BAG-1 isoforms from humans and mice or rats; however, according to gene structure, gene expression, and transgenic functional studies (Takayama et al., 1998; Kermer et al., 2003), murine long-form BAG-1 similar to the human 50 kDa isoform and murine short-form BAG-1 are similar, at least in part, to the three other human isoforms.

Lithium inhibits GR reporter gene activity in a time- and dose-dependent manner

Having established that chronic lithium or VPA upregulates BAG-1 levels in SH-SY5Y cells, these cells were used to investigate the effects of mood stabilizers on BAG-1 regulation of GR function. To establish the glucocorticoid receptor activity reporter system, we tested the transfection at different DNA (plasmid) concentrations and then selected 0.5 $\mu\text{g}/\text{ml}$ based on the transfection efficiencies. For quality control, we tested the background effects of 1.0 mM lithium or 1.0 mM VPA on the expression of pGRE-SEAP, which is a vector reporting glucocorticoid activity through expression of SEAP. Lithium showed no difference compared with control (saline); however, VPA strongly activated pGRE-SEAP and caused a very high expression of the reporter (SEAP). We also tested both mood stabilizers on pTAL-SEAP, a vector identical to pGRE-SEAP but without the GRE, through which pure promoter effects can be tested. The results showed that lithium was not different from saline in its effect on pTAL-SEAP, but VPA strongly activated the vector. In this context, it is noteworthy that Phiel et al. (2001) have demonstrated that VPA is a histone deacetylase inhibitor and that this results in a robust increase in reporter gene expression. The high “basal” (i.e., before the addition of specific agonist) gene expression levels make it difficult to study the more modest effects of a specific agonist (such as glucocorticoid agonist). Because of this robust nonspecific “basal activation” caused by VPA, the GR activity reporter assay using pGRE-SEAP was performed solely using lithium. It should be noted that this is the only assay in which we only investigated the effects of lithium; in all of the other assays, both *in vivo* and *in vitro*, we investigated the effects of both lithium and VPA.

After establishment of the reporter system, we sought to investigate whether lithium-induced BAG-1 upregulation in SH-SY5Y cells (observed at 6 d with 1.0 mM lithium) resulted in an inhibitory effect on GR activity. We found that 6 d of treatment of SH-SY5Y cells with 1.0 mM lithium significantly inhibited GR activity induced by 100 nM dexamethasone (relative SEAP levels: without lithium, 22.72 ± 2.80 ; with 1.0 mM lithium, 16.80 ± 1.30 ; lithium vs control, $p = 0.007$); interestingly, major effects were not seen with lower dexamethasone concentrations (Fig. 4*a*). Studies were also undertaken to determine whether the inhibitory effects of lithium on GR activity were, as postulated, only observed in a time frame in which BAG-1 protein levels were upregulated (Fig. 4*b*). This was indeed the case, with lithium

Figure 3. Effects of mood stabilizers on BAG-1 protein levels in human neuroblastoma SH-SY5Y cells. *a*, Lithium at 1.0 mM. *b*, VPA at 1.0 mM. Western blot analysis detected four isoforms in SH-SY5Y cells with molecular weights of ~50 kDa (BAG-1L), 46 kDa (BAG-1M), 36 kDa (BAG-1), and 29 kDa (p29), respectively; the p29 isoform is too weak to be included in the analyses here. control-L, control-M, and control are controls of the different isoforms. C, Control; L, lithium; V, VPA. $n = 4$; values shown here are mean $\pm$ SE; * $p < 0.05$; t test.

Figure 4. Effects of lithium on GR activity. SH-SY5Y cells were treated with 1.0 mM LiCl (li) for 6 d (*a*) or different times (*b*). Seventy-two hours before the reporter test, cells were transfected with pGRE-SEAP as reporter or pTAL-SEAP as baseline control, together with GR α vector and pSV- β -galactosidase control vector. Twenty-four hours before the reporter test, dexamethasone (dex) was added to different final concentrations (*a*) or to 100 nM (*b*). Values shown here are relative levels of SEAP (mean $\pm$ SE) compared with the background expression levels of pTAL-SEAP, which was used as a control vector. All SEAP levels were normalized by the β -galactosidase activity for transfection efficiency. $n = 6$; ** $p < 0.01$; t test.

exerting significant inhibitory effects on GR activity at 6 or 8 d; no significant effects were observed at 3 d (when BAG-1 levels are not upregulated). Furthermore, for the 6 or 8 d time point, different lithium concentrations (0.3, 0.6, 1.0, and 2.0 mM) were tested. On day 6, the inhibitory effect was observed only for 0.6 mM (relative SEAP levels: saline, 31.02 ± 3.30 ; 0.6 mM lithium, 26.69 ± 3.84 ; lithium vs saline, $p = 0.015$) and 1.0 mM (relative SEAP levels: saline, 31.02 ± 3.30 ; 1.0 mM lithium, 22.98 ± 2.27 ; lithium vs saline, $p = 0.022$), and on day 8, the effect was observed

Figure 5. Effects of BAG-1 siRNA on BAG-1 protein levels. Western blot analysis of BAG-1 protein levels was performed after treatment of BAG-1 siRNAs. SH-SY5Y cells were transfected with SiBAG1A and SiBAG1C together, which are the two siRNAs for human BAG-1 that cloned into pSilencer 3.0-H1 vector. Western blot analysis was performed at 48 and 72 h. Shown here are results of 72 h (*a*) and densitometry analysis of the results of 72 h (*b*). Values are mean $\pm$ SE; $n = 4$; * $p < 0.05$; t test. TOD, Total optical density.

only for 1 mM (relative SEAP levels: saline, 38.10 ± 2.72 ; 1.0 mM lithium, 26.73 ± 2.93 ; lithium vs saline, $p = 0.003$). We therefore conclude that the inhibitory effect of lithium on GR activity was both time and dose dependent.

The inhibitory action of lithium on GR activity is markedly attenuated by BAG-1 siRNA treatment

Although the attenuation of GR activity by chronic lithium is entirely consistent with BAG-1 upregulation, lithium is known to exert a number of biochemical effects (Coyle and Manji, 2002). Thus, to more definitely delineate the role of BAG-1 in mediating the inhibitory effects of lithium on GR activity, BAG-1 siRNA was used to knock down BAG-1 protein levels. For quality control, we first tested the silencing effects of the siRNAs for BAG-1 and also the transfection efficiency. After cotransfection of SiBAG1A and SiBAG1C, significant (compared with the scrambled treated) BAG-1 gene silencing was observed after 72 h (at 72 h, after ODs of controls are adjusted to 1, relative ODs of the three isoforms are as follows: 50 kDa, 0.21 ± 0.19 , $p = 0.030$; 46 kDa, 0.31 ± 0.14 , $p = 0.029$; 36 kDa, 0.42 ± 0.08 , $p = 0.041$) (Fig. 5). We then

Figure 6. Effects of BAG-1 siRNA on lithium-induced attenuation of GR activity. SH-SY5Y cells were treated with 1.0 mM LiCl for 6 d. Seventy-two hours before the reporter gene analysis, cells were transfected with the reporter vector pGRE-SEAP together with GR α vector, pSV- β -galactosidase control vector, either combination of SiBAG1A and SiBAG1C constructs, or vectors for scrambled versions of SiBAG1A and SiBAG1C. Twenty-four hours before testing, dexamethasone was added to a final concentration of 100 nM. All values are relative levels compared with the background expression levels of pTAL-SEAP under saline and are normalized by β -galactosidase activity. Values shown here are mean $\pm$ SE; $n = 5$; * $p < 0.05$; t test.

treated SH-SY5Y cells for 6 d with 1.0 mM lithium, adding the two BAG-1 siRNAs 3 d before harvesting the cells. As predicted, BAG-1 siRNAs significantly attenuated the inhibitory effects of chronic lithium on GR activity (relative SEAP levels, when siRNAs were applied: lithium, 22.25 ± 1.61 ; saline, 24.44 ± 1.24 ; lithium vs saline, $p = 0.45$; when scrambled controls were applied: lithium, 15.04 ± 1.09 ; saline, 22.18 ± 1.64 ; lithium vs saline, $p = 0.048$) (Fig. 6).

Figure 7. Effects of mood stabilizers on agonist-induced GR nuclear translocation. SH-SY5Y cells were treated with 1.0 mM LiCl or 1.0 mM sodium valproate for 6 d. Dexamethasone was added to 100 nM for 30 min before harvesting, and the cells were then fixed and immunostained with 1:40 rabbit anti-GR antibody overnight and then further with 1:300 Cy3-conjugated anti-rabbit IgG antibody for 1 h; finally, the cells were double stained with 300 nM DAPI for 5 min. **a**, Representative microphotographs by a LSM 510 laser scanning confocal microscope under three conditions: saline control (row A), 1.0 mM lithium (row B), and 1.0 mM VPA (row C). **b**, Quantitative analysis based on average values of six counts per test condition, with each count including counting 100 cells per observation field for 10 fields. All values were relative levels compared with saline control and are shown in mean $\pm$ SE format. Li, Lithium; Dex, 100 mM dexamethasone. * $p < 0.05$; ** $p < 0.01$; ANOVA Tukey's *post hoc* test.

Chronic lithium or VPA also inhibits glucocorticoid-induced GR nuclear translocation

As mentioned in Introduction, it is now known that BAG-1 blocks GR-mediated transactivation, at least in part, by blocking GR nuclear translocation (Schneikert et al., 1999). We therefore postulated that lithium- or VPA-induced BAG-1 upregulation would also inhibit GR nuclear translocation. To investigate this, SH-SY5Y cells were treated for 6 d with 1.0 mM lithium or 1.0 mM VPA. The cells were then exposed to 100 nM dexamethasone for 30 min to facilitate GR nuclear translocation. GR nuclear translocation was evaluated by counting (in a blinded manner) after double staining with anti-GR antibodies and DAPI (Fig. 7a). Quantitative analysis showed that 6 d of treatment with either lithium or VPA significantly inhibited dexamethasone-induced GR nuclear translocation (relative levels compared with saline and dexamethasone free: saline plus dexamethasone, 3.52 ± 0.15 ; lithium plus dexamethasone, 1.96 ± 0.26 ; VPA plus dexamethasone, 1.48 ± 0.11 ; lithium plus dexamethasone vs saline plus dexamethasone, $p = 6.30 \times 10^{-5}$; VPA plus dexamethasone vs saline plus dexamethasone, $p = 2.90 \times 10^{-6}$; lithium plus dexamethasone vs VPA plus dexamethasone, $p = 0.188$) (Fig. 7b).

The inhibitory action of lithium or VPA on GR nuclear translocation is markedly attenuated by BAG-1 siRNA treatment

Once again, to more definitely delineate the role of BAG-1 in mediating the inhibitory effects of lithium or VPA on GR nuclear translocation, BAG-1 siRNAs (SiBAG1A and SiBAG1C) were used to knock down BAG-1 protein levels. Knocking down BAG-1 protein levels resulted in a marked attenuation of the inhibitory effects of lithium or VPA on GR nuclear translocation (dexamethasone-induced GR translocation rate in lithium-treated cells: siRNA, 1.72 ± 0.70 ; scrambled, 1.94 ± 0.79 ; siRNA vs scrambled, $p = 0.009$; all values are relative levels compared with the background, under which cells were treated with saline and scrambled and dexamethasone free) (Fig. 8a, representative micrographs, b, quantitative analysis). To further confirm the specificity of this finding, we tested the transfection efficiencies of both siRNA plasmids and scrambled control plasmids cotransfected with pEGFP-Luc. Counting results showed no difference between the two transfection conditions (based on six randomly chosen fields under microscope, $20\times$ lens; transfection ratio of siRNA was 0.431 ± 0.080 ; transfection ratio of scrambled was 0.396 ± 0.083 ; siRNA vs scrambled, t test, $p = 0.567$).

Discussion

In this study, we demonstrated, for the first time, that BAG-1 upregulation may play an important role in the adaptive plasticity underlying the treatment of BD. Thus, we found that the structurally highly dissimilar antimanic agents lithium and valproate have a common effect on robustly upregulating BAG-1 protein levels after chronic treatment with therapeutically relevant concentrations as assessed both *in vitro* and *in vivo*. Furthermore, we found that, completely consistent with the

Figure 8. Effects of BAG-1 siRNA on mood stabilizer-induced attenuation of GR nuclear translocation. SH-SY5Y cells were treated with 1.0 mM LiCl or 1.0 mM sodium valproate for 6 d. Forty-eight hours before harvesting, 0.5 μ g/ml siRNA expression vectors were transfected, and then 30 min before harvesting, dexamethasone was added to 100 nM. Cells were fixed and then immunostained with 1:40 rabbit anti-GR antibody overnight and then further with 1:300 Cy3-conjugated anti-rabbit IgG antibody for 1 h; they were then double stained with 300 nM DAPI for 5 min. **a**, The GR translocation tests in lithium/VPA/saline-treated cells were repeated with either BAG-1 siRNAs (1–3) or scrambles (4–6) added; shown here are representative images of lithium-treated cells. **b**, Quantitative analysis based on average values of six counts per test condition, with each count including counting 100 cells per observation field for 10 fields. All values were relative levels compared with saline plus scramble and are shown in mean $\pm$ SE format. Dex, Dexamethasone at 100 nM; Li, lithium. * p < 0.05; ** p < 0.01; t test.

known effects of BAG-1 as a cochaperone protein for the glucocorticoid receptor, chronic treatment with mood stabilizers attenuated GR nuclear translocation and GR-mediated gene expression. Notably, knocking down BAG-1 protein levels using a BAG-1 siRNA strategy markedly attenuated the effects of mood stabilizers on glucocorticoid-mediated GR nuclear translocation and gene expression; these observations strongly implicate BAG-1 in mediating (at least in part) the effects of mood stabilizers on GR trafficking and GR-mediated gene expression.

Because chronic administration of mood stabilizers brings about numerous biochemical effects, our laboratory (Manji et al., 1999; Manji and Lenox, 2000b) and others (Coyle and Duman, 2003) have established several criteria that findings should meet to maximize the likelihood of their therapeutic importance. First, this effect of mood stabilizers on BAG-1 protein expression and GR trafficking/function is a common effect of the structurally

highly dissimilar antimanic agents lithium, which is a monovalent cation, and valproate, which is an eight-carbon branched fatty acid. Second, this BAG-1 upregulation by lithium and valproate occurs in the hippocampus, a brain region known to be involved in critical affective neuronal circuits. Third, BAG-1 upregulation occurs in the CA3 subfield, a region in which robust protection against stress-induced apical dendritic retraction by lithium (VPA was not examined in that study) has been observed recently (Wood et al., 2004). Fourth, this effect of lithium and valproate on BAG-1 and GR trafficking/function occurs at therapeutic concentrations both *in vivo* and *in vitro*. Fifth, similar to the clinical therapeutic effects, the changes in BAG-1 protein expression and GR trafficking/function were observed only after chronic (and not acute) administration. Finally, the effects were specific for lithium and VPA (mood stabilizers), because chronic administration of an antidepressant, a psychostimulant, or an antipsychotic were all without effect on BAG-1. Although it is impossible to determine whether similar effects on BAG-1 occur in the human brain *in vivo*, our experimental conditions attempt to mimic this situation as closely as possible. Moreover, as we discuss later, indirect evidence suggests that mood stabilizers attenuate GR signaling in humans *in vivo*.

BAG-1 is a family of cochaperones consisting of at least four polypeptides: BAG-1L, BAG-1M/RAP46, BAG-1, and p29 (Yang et al., 1998). These proteins are translated from the same mRNA at alternative translation initiation sites. They possess conserved C-terminal sequences, which enable them to bind and inhibit the action of the molecular chaperone heat shock protein (Hsp70/Hsc70) (Takayama et al., 1999). BAG-1 was originally identified as an anti-apoptotic protein because of its ability to bind and augment the activity of the anti-death protein BCL-2 (Takayama et al., 1995). Since then, other BAG-1 proteins have been identified and shown to interact with several cellular factors, including nuclear receptors, and with some signaling proteins, including the serine/threonine protein kinase Raf-1 (an upstream activator of ERK MAP kinases) (Song et al., 2001). Available data on the negative regulation of GR action by the BAG-1 proteins identify two modes of action: inhibition of the hormone binding activity of the GR and a more direct nuclear action at the level of regulation of the transactivation function of the receptor (Kanelakis et al., 1999; Schneikert et al., 1999).

BAG-1 is now known to exert cytoprotective effects in a variety of paradigms. Thus, overexpression of BAG-1 has been shown to protect against serum deprivation-induced cell death (Kermer et al., 2002), to confer a strong resistance to apoptotic neuronal death (Sourisseau et al., 2001), and similarly, to protect against glucocorticoid-induced apoptosis (Kullmann et al., 1998). These findings are supported by recent studies demonstrating that BAG-1-overexpressing transgenic mice showed decreased mortality compared with their wild-type littermates in a transient middle cerebral artery occlusion model (Kermer et al., 2003). In the only human study of which we are aware, Seidberg et al. (2003) examined injured brain tissue from adult patients that underwent emergent surgical decompression after traumatic brain injury. They found that, similar to the situation with BCL-2, traumatic brain injury was associated with an adaptive increase in BAG-1 isoforms in surviving neurons in the human brain.

The evidence that BAG-1 attenuates GR signaling suggests that the underlying primary pathophysiology of bipolar disorder may also involve abnormalities of this cellular cascade. Indeed, evidence of hypothalamic–pituitary–adrenal axis activation in bipolar disorder is suggested by multiple lines of evidence. These

include lack of dexamethasone suppression of endogenous cortisol levels, increased adrenocorticotrophic hormone (ACTH) response to corticotropin-releasing factor (CRF), and altered responses to the combined dexamethasone/corticotrophin release factor (DEX/CRF) challenge test (Schmider et al., 1995; Rybakowski and Twardowska, 1999). In a recent review, Sirois (2003) found that 75% of patients treated with exogenous corticosteroids exhibited affective symptoms, including mania and depression (Sirois, 2003). In an elegant recent study, using transgenic mice overexpressing GR specifically in forebrain, Wei et al. (2004) showed that GR modulates features of emotional responsiveness generally associated with BD.

In a series of studies, Wood et al. (2004) have demonstrated that chronic lithium treatment robustly prevents the stress-induced decrease in dendritic length, as well as the stress-induced increase in GLT-1 (glial glutamate transporter 1) mRNA expression and the phosphorylation of cAMP-response element binding in the hippocampus. In view of these findings, we specifically investigated the effects of chronic lithium or VPA on BAG-1 levels in CA3 pyramidal neurons of the hippocampus and observed significant increases in this hippocampal subfield. The data suggest that the ability of lithium and VPA to increase BAG-1 expression, and thereby attenuate GR signaling, may play an important role in their therapeutic effects. It is, of course, impossible to obtain longitudinal human data to definitively show that mood stabilizers increase BAG-1 protein levels in the human brain. Nevertheless, indirect evidence has supported this. In the DEX/CRF test, dexamethasone (acting via GR) inhibits the secretion of ACTH and cortisol. Thus, in this test, an attenuation of GR signaling by lithium would be manifested by an enhanced ACTH/cortisol response. This is indeed the case, with chronic lithium treatment resulting in an enhanced cortisol and ACTH response in this test (Bschor et al., 2002, 2003; Kunzel et al., 2003). Together, the data suggest that lithium does attenuate GR signaling in BD patients. It should be noted, however, that lithium and VPA have also been demonstrated to have marked effects on both the hypothalamic and extrahypothalamic CRF systems, precluding a simple interpretation of the clinical findings (Gilmor et al., 2003).

It is noteworthy that, in addition to attenuating GR signaling, chronic lithium or VPA, at therapeutically relevant concentrations, has been demonstrated to activate the ERK MAP kinase cascade (generally used by neurotrophic factors) in rodent brain and in cells with a human neuronal phenotype. Lithium and VPA also robustly upregulate the anti-apoptotic protein BCL-2. The effects of BAG-1 on the ERK MAP pathway and BCL-2 may also play a critical role in the long-term actions of mood stabilizers. In complimentary clinical studies, 4 weeks of lithium treatment produced a significant increase in *N*-acetylaspartate (a putative marker of neuronal viability and function) levels (Moore et al., 2000), which supports the hypothesis that some of the therapeutic actions of mood stabilizers may involve hitherto underappreciated neurotrophic/neuroprotective effects, likely mediated (at least in part) by BAG-1.

In conclusion, we demonstrate, for the first time, that the structurally highly dissimilar antimanic agents lithium and VPA have a common effect on robustly upregulating BAG-1 protein levels after prolonged treatment with therapeutically relevant concentrations as assessed both *in vitro* and *in vivo*. Furthermore, completely consistent with the known effects of BAG-1 as a co-chaperone protein for the glucocorticoid receptor, chronic treatment with mood stabilizers attenuated GR nuclear translocation and GR-mediated gene expression. Also consistent with the ef-

fects of BAG-1 on neurotrophic signaling cascades, lithium and VPA activate ERK MAP kinases and upregulate BCL-2. It is notable that BAG-1 is also included in a large-scale study of the Max Planck Institute of Psychiatry testing associations of single nucleotide polymorphisms of selected genes with treatment response in mood disorders (F. Holsboer, personal communication). Finally, the data suggest that novel BAG-1 activators may represent novel therapeutics for the treatment of severe mood disorders. This raises the exciting possibility that small-molecule “BAG-1 mimetics” could be developed not only for classical degenerative disorders but also for severe mood disorders.

References

- Birnbaum SG, Yuan PX, Wang M, Vijayraghavan S, Bloom AK, Davis DJ, Gobeske KT, Sweatt JD, Manji HK, Arnsten AF (2004) Protein kinase C overactivity impairs prefrontal cortical regulation of working memory. *Science* 306:882–884.
- Bschor T, Adli M, Baethge C, Eichmann U, Ising M, Uhr M, Modell S, Kunzel H, Muller-Oerlinghausen B, Bauer M (2002) Lithium augmentation increases the ACTH and cortisol response in the combined DEX/CRH test in unipolar major depression. *Neuropsychopharmacology* 27:470–478.
- Bschor T, Baethge C, Adli M, Eichmann U, Ising M, Uhr M, Muller-Oerlinghausen B, Bauer M (2003) Lithium augmentation increases post-dexamethasone cortisol in the dexamethasone suppression test in unipolar major depression. *Depress Anxiety* 17:43–48.
- Chen G, Yuan PX, Jiang YM, Huang LD, Manji HK (1998) Lithium increases tyrosine hydroxylase levels both in vivo and in vitro. *J Neurochem* 70:1768–1771.
- Chen G, Zeng WZ, Yuan PX, Huang LD, Jiang YM, Zhao ZH, Manji HK (1999) The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS. *J Neurochem* 72:879–882.
- Coyle JT, Duman RS (2003) Finding the intracellular signaling pathways affected by mood disorder treatments. *Neuron* 38:157–160.
- Coyle JT, Manji HK (2002) Getting balance: drugs for bipolar disorder share target. *Nat Med* 8:557–558.
- Du J, Gray NA, Falke CA, Chen W, Yuan P, Szabo ST, Einat H, Manji HK (2004) Modulation of synaptic plasticity by antimanic agents: the role of AMPA glutamate receptor subunit 1 synaptic expression. *J Neurosci* 24:6578–6589.
- Duman RS (2002) Synaptic plasticity and mood disorders. *Mol Psychiatry* 7 [Suppl 1]:S29–S34.
- Gilmor ML, Skelton KH, Nemeroff CB, Owens MJ (2003) The effects of chronic treatment with the mood stabilizers valproic acid and lithium on corticotropin-releasing factor neuronal systems. *J Pharmacol Exp Ther* 305:434–439.
- Hayashi T, Sakai K, Sasaki C, Itoyama Y, Abe K (2000) Loss of bag-1 immunoreactivity in rat brain after transient middle cerebral artery occlusion. *Brain Res* 852:496–500.
- Kanelakis KC, Morishima Y, Dittmar KD, Galgani MD, Takayama S, Reed JC, Pratt WB (1999) Differential effects of the hsp70-binding protein BAG-1 on glucocorticoid receptor folding by the hsp90-based chaperone machinery. *J Biol Chem* 274:34134–34140.
- Kermer P, Krajewska M, Zapata JM, Takayama S, Mai J, Krajewski S, Reed JC (2002) Bag1 is a regulator and marker of neuronal differentiation. *Cell Death Differ* 9:405–413.
- Kermer P, Digicaylioglu MH, Kaul M, Zapata JM, Krajewska M, Stenner-Liewen F, Takayama S, Krajewski S, Lipton SA, Reed JC (2003) BAG1 over-expression in brain protects against stroke. *Brain Pathol* 13:495–506.
- Kullmann M, Schneikert J, Moll J, Heck S, Zeiner M, Gehring U, Cato AC (1998) RAP46 is a negative regulator of glucocorticoid receptor action and hormone-induced apoptosis. *J Biol Chem* 273:14620–14625.
- Kunzel HE, Binder EB, Nickel T, Ising M, Fuchs B, Majer M, Pfennig A, Ernst G, Kern N, Schmid DA, Uhr M, Holsboer F, Modell S (2003) Pharmacological and nonpharmacological factors influencing hypothalamic-pituitary-adrenocortical axis reactivity in acutely depressed psychiatric in-patients, measured by the Dex-CRH test. *Neuropsychopharmacology* 28:2169–2178.
- Manji HK, Lenox RH (2000a) The nature of bipolar disorder. *J Clin Psychiatry* 61 [Suppl 13]:42–57.

- Manji HK, Lenox RH (2000b) Signaling: cellular insights into the pathophysiology of bipolar disorder. *Biol Psychiatry* 48:518–530.
- Manji HK, Bechuk JM, Moore GJ, Glitz D, Hasanat KA, Chen G (1999) Modulation of CNS signal transduction pathways and gene expression by mood-stabilizing agents: therapeutic implications. *J Clin Psychiatry* 60 [Suppl 2]:27–39.
- Manji HK, Moore GJ, Rajkowska G, Chen G (2000) Neuroplasticity and cellular resilience in mood disorders. *Mol Psychiatry* 5:578–593.
- McEwen BS (1999) Stress and hippocampal plasticity. *Annu Rev Neurosci* 22:105–122.
- Moore GJ, Bechuk JM, Hasanat K, Chen G, Seraji-Bozorgzad N, Wilds IB, Faulk MW, Koch S, Glitz DA, Jolkovsky L, Manji HK (2000) Lithium increases N-acetyl-aspartate in the human brain: in vivo evidence in support of bcl-2's neurotrophic effects? *Biol Psychiatry* 48:1–8.
- Packham G, Brimmell M, Cleveland JL (1997) Mammalian cells express two differently localized Bag-1 isoforms generated by alternative translation initiation. *Biochem J* 328:807–813.
- Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem* 276:36734–36741.
- Quiroz JA, Singh J, Gould TD, Denicoff KD, Zarate CA, Manji HK (2004) Emerging experimental therapeutics for bipolar disorder: clues from the molecular pathophysiology. *Mol Psychiatry* 9:756–776.
- Rybakowski JK, Twardowska K (1999) The dexamethasone/corticotropin-releasing hormone test in depression in bipolar and unipolar affective illness. *J Psychiatr Res* 33:363–370.
- Sapolsky RM (2000) Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch Gen Psychiatry* 57:925–935.
- Schmider J, Lammers CH, Gotthardt U, Dettling M, Holsboer F, Heuser IJ (1995) Combined dexamethasone/corticotropin-releasing hormone test in acute and remitted manic patients, in acute depression, and in normal controls. I. *Biol Psychiatry* 38:797–802.
- Schneikert J, Hubner S, Martin E, Cato AC (1999) A nuclear action of the eukaryotic cochaperone RAP46 in downregulation of glucocorticoid receptor activity. *J Cell Biol* 146:929–940.
- Seidberg NA, Clark RS, Zhang X, Lai Y, Chen M, Graham SH, Kochanek PM, Watkins SC, Marion DW (2003) Alterations in inducible 72-kDa heat shock protein and the chaperone cofactor BAG-1 in human brain after head injury. *J Neurochem* 84:514–521.
- Sirois F (2003) Steroid psychosis: a review. *Gen Hosp Psychiatry* 25:27–33.
- Song J, Takeda M, Morimoto RI (2001) Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. *Nat Cell Biol* 3:276–282.
- Sourisseau T, Desbois C, Debure L, Bowtell DD, Cato AC, Schneikert J, Moyse E, Michel D (2001) Alteration of the stability of Bag-1 protein in the control of olfactory neuronal apoptosis. *J Cell Sci* 114:1409–1416.
- Takayama S, Sato T, Krajewski S, Kochel K, Irie S, Millan JA, Reed JC (1995) Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell* 80:279–284.
- Takayama S, Krajewski S, Krajewska M, Kitada S, Zapata JM, Kochel K, Knee D, Scudiero D, Tudor G, Miller GJ, Miyashita T, Yamada M, Reed JC (1998) Expression and location of Hsp70/Hsc-binding anti-apoptotic protein BAG-1 and its variants in normal tissues and tumor cell lines. *Cancer Res* 58:3116–3131.
- Takayama S, Xie Z, Reed JC (1999) An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. *J Biol Chem* 274:781–786.
- Wei Q, Lu XY, Liu L, Schafer G, Shieh KR, Burke S, Robinson TE, Watson SJ, Seasholtz AF, Akil H (2004) Glucocorticoid receptor overexpression in forebrain: a mouse model of increased emotional lability. *Proc Natl Acad Sci USA* 101:11851–11856.
- Wood GE, Young LT, Reagan LP, Chen B, McEwen BS (2004) Stress-induced structural remodeling in hippocampus: prevention by lithium treatment. *Proc Natl Acad Sci USA* 101:3973–3978.
- Yang X, Chernenko G, Hao Y, Ding Z, Pater MM, Pater A, Tang SC (1998) Human BAG-1/RAP46 protein is generated as four isoforms by alternative translation initiation and overexpressed in cancer cells. *Oncogene* 17:981–989.