Corrections

BIOSCHEMISTRY. For the article “Bioinorganic Chemistry Special Feature: An engineered two-iron superoxide reductase lacking the [Fe(SCys)4] site retains its catalytic properties in vitro and in vivo,” by Joseph P. Emerson, Diane E. Cabelli, and Donald M. Kurtz, Jr., which appeared in issue 7, April 1, 2003, of Proc. Natl. Acad. Sci. USA (100, 3802–3807; First Published March 13, 2003; 10.1073/pnas.0537177100), the authors note that they inadvertently labeled the time axis in Fig. 6 as hours instead of seconds. The corrected figure and its legend appear below. This correction does not affect the conclusions of the article.

MEDICAL SCIENCES. For the article “Asialoerythropoietin is a non-erythropoietic cytokine with broad neuroprotective activity in vivo,” by Serhat Erbayraktar, Giovanni Grasso, Alessandra Sfaceria, Qiao-wen Xie, Thomas Coleman, Mads Kreilgaard, Lars Torup, Thomas Sager, Zubeyde Erbayraktar, Necati Gokmen, Osman Yilmaz, Pietro Ghezzi, Pia Villa, Maddalena Fratelli, Simona Casagrande, Marcel Leist, Lone Helboe, Jens Gerwein, Søren Christensen, Marie Aavang Geist, Lars Østergaard Pedersen, Carla Cerami-Hand, Jean-Paul Wuerth, Anthony Cerami, and Michael Brines, which appeared in issue 11, May 27, 2003, of Proc. Natl. Acad. Sci. USA (100, 6741–6746; First Published May 13, 2003; 10.1073/pnas.1031753100), the author name Jens Gerwein should have appeared as Jens Gerwien. The online version has been corrected. The corrected author line appears below.

NEUROSCIENCE. For the article “Positive and negative regulation of APP amyloidogenesis by sumoylation,” by Yonghong Li, Hui Wang, Su Wang, Diana Quon, Yu-Wang Liu, and Barbara Cordell, which appeared in issue 1, January 7, 2003, of Proc. Natl. Acad. Sci. USA (100, 259–264; First Published December 27, 2002; 10.1073/pnas.0235361100), the authors note that they inadvertently labeled the 95-aa ubiquitin-like protein, which was identified from the library screen (see the first paragraph of Results), as SUMO-3 instead of SUMO-2. SUMO-2 and SUMO-3 are 95% identical at amino acid levels and are considered as a subfamily SUMO-2/3. The antibody used was raised against full-length SUMO-3 but recognizes SUMO-2 as well. The error does not affect the interpretations and conclusions of the paper except that all statements regarding effect of SUMO-3 should be viewed as that of SUMO-2.

Fig. 6. Dependence of NADPH/superoxide oxidoreductase activity on wild-type, C13S, and N-terminal 2Fe-SORs. Rates of NADPH consumption were monitored at room temperature in a 1-ml cuvette as decreases in absorbance at 340 nm (NADPH 6,220 M⁻¹cm⁻¹) in solutions containing (in the added order) 500 M xanthine, 100 M NADPH, 186 units/ml catalase, 1 M Rub, 1 M spinach ferredoxin:NADP⁺ oxidoreductase, and either wild-type or C13S 2Fe-SOR (1 M in [Fe(NHis)4(SCys)] sites) or N-terminal 2Fe-SOR (1 M in iron sites). After recording a “baseline” NADPH consumption rate for 50 sec, a precalibrated amount of xanthine oxidase was added to produce a flux of 12 M superoxide per min, and NADPH consumption was monitored for several minutes. Absorbance spikes caused by the various additions and mixing are omitted, and the traces obtained with each SOR are offset vertically by an arbitrary amount for clarity.
Positive and negative regulation of APP amyloidogenesis by sumoylation

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Amyloid β peptide (Aβ) generated from amyloid precursor protein (APP) is central to Alzheimer’s disease (AD). Signaling pathways affecting APP amyloidogenesis play critical roles in AD pathogenesis and can be exploited for therapeutic intervention. Here, we show that sumoylation, covalent modification of cellular proteins by small ubiquitin-like modifier (SUMO) proteins, regulates Aβ generation. Increased protein sumoylation resulting from overexpression of SUMO-3 dramatically reduces Aβ production. Conversely, reducing endogenous protein sumoylation with dominant-negative SUMO-3 mutants significantly increases Aβ production. We also show that mutant SUMO-3, K11R, which can only be monomerically conjugated to target proteins, has an opposite effect on Aβ generation to that by SUMO-3, which can form polymeric chains on target proteins. In addition, SUMO-3 immunoreactivity is predominantly detected in neurons in brains from AD, Down’s syndrome, and non-demented humans. Therefore, polysumoylation reduces whereas monosumoylation or undersumoylation enhances Aβ generation. These findings provide a regulatory mechanism in APP amyloidogenesis and suggest that components in the sumoylation pathway may be critical in AD onset or progression.

Amyloid precursor protein (APP) is an integral transmembrane glycoprotein of multiple isoforms including the neuronal form of 695 amino acids. Proteolytic cleavage of APP by β-secretase or β-site APP cleavage enzyme (BACE) (1–6) initiates the amyloidogenic pathway with the liberation of N-terminal fragment (β-NTF) and the formation of β-C-terminal fragment, which is then further processed by γ-secretase (7, 8) to release amyloid β peptide (Aβ). A nonamyloidogenic pathway by α-secretase cleavage leads to the secretion of α-NTF and precludes the formation of full-length Aβ (9, 10). Various familial Alzheimer’s disease (AD)-associated mutations in both APP and presenilins cause increased production or deposition of Aβ, which is believed to induce neuronal cell death because of its neurotoxic and/or inflammatory effects (for a review, see ref. 11). These and other observations support that molecular mechanisms involved in regulating APP amyloidogenesis are obligatory players in AD pathogenesis.

Sumoylation has begun to be recognized as an important posttranslational modification capable of influencing protein function (for reviews, see refs. 12–14). Sumoylation is biochemically similar to but functionally distinct from ubiquitination, the latter being understood, but it seems to regulate protein localization and stability, the latter through antagonism of ubiquitin-mediated proteolysis (20–22). Here, we report a previously unknown function for sumoylation in regulating APP processing.

Materials and Methods

Cell Culture. Human embryonic kidney 293T cells and human neuroblastoma SK-N-MC cells were grown in high glucose-containing DMEM supplemented with 10% heat-inactivated FBS.

Normalized Library and Primary Screening. A human fetal brain cDNA library purchased from Life Technologies (Rockville, MD) was normalized by the method of Soares et al. (23). The normalization was confirmed by randomly selecting ~300 clones for sequencing, which showed minimal to no redundancy. The normalized library was plated for single colonies (~100 colonies per plate) and scraped, and DNA preparations were made and assembled in 96-well microtiter plates. For initial screening, the cDNA library was cotransfected with an APP expression plasmid, followed by assays for Aβ, β-NTF, and α-NTF. After comparing the levels of these three APP products with the average of each plate, cDNA pools showing altered patterns of APP processing were deconvoluted and rescreened.

Transfection Studies. For determining the role of SUMO in APP processing, SUMO-3 plasmids were cotransfected with APP695 expression plasmid, followed by ELISAs for Aβ, β-NTF, and α-NTF. Plasmid transfections were performed with Lipofectamine 2000 (Life Technologies) in 24-well plates. For each transfection, 4 × 10⁵ cells were incubated with a total of 800 ng of plasmid DNA combined with 2 μl of Lipofectamine 2000 according to the vendor’s protocol. The total DNA contained 400 ng of APP695wt or 160 ng of APP695sw plasmid and various amounts of other plasmids. The empty vector pcDNA3.1 was supplemented to make up a total of 800 ng of DNA whenever necessary. Each transfection was done in triplicate. Forty-eight hours after transfection, the medium was collected and assayed for Aβ, β-NTF, and α-NTF.

ELISAs. Determinations of Aβ, β-NTF, and α-NTF concentrations in growth medium used standard sandwich ELISA methods with the following antibodies. For total Aβ, the capture antibody was directed to the target protein. The biological function of sumoylation is not fully understood, but it seems to regulate protein localization and stability, the latter through antagonism of ubiquitin-mediated proteolysis (20–22). Here, we report a previously unknown function for sumoylation in regulating APP processing.

Abbreviations: Aβ, amyloid β peptide; AD, Alzheimer’s disease; APP, amyloid precursor protein; BACE, β-site APP cleavage enzyme; NTF, N-terminal fragment; SUMO, small ubiquitin-like modifier.

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was 266.1 raised against Aβ domain residues 13–29, and the detection antibody was biotinylated 3D6 raised against Aβ domain residues 1–5. For β-NTF, the capture antibody was 8E5 raised against residues 444–592 of APP protein, and the detection antibody (specific for free C terminus of β-NTF) was 192wt for wild-type APP and AF20 for Swedish APP, respectively.

Fig. 1. SUMO-3 sumoylation regulates APP processing in 293T cells. (A) Diagram showing APP processing and antibody epitopes used in ELISA: 266.1 and 3D6 for Aβ, 8E5 and AF20 or 192wt for β-NTF, and 8E5 and 2H3 for α-NTF. Drawing not to scale. (B) Dose–effect of SUMO-3 expression on Aβ, β-NTF, and α-NTF generation from cells cotransfected with APP and without or with increasing amounts of SUMO-3 plasmids. All transfections contained 160-ng APP plasmids. The amount of SUMO-3 plasmids used were 0, 40, 160, and 640 ng in transfections corresponding to the columns from left to right. Vector DNAs were supplemented to bring the total amount of DNA to 800 ng for each transfection. A similar dilution scheme was used in other figures as well. The results were expressed as relative to the amount of APP-processing products indicated in each panel to the control. The control was APP transfection alone. (C) Western blot showing dose–effect of SUMO-3 on α-NTF production. Equal amounts of growth media from cells transfected as indicated were probed with 8E5 antibody. (D) Western blot showing SUMO-3 expression and protein sumoylation. Equal amounts of protein from lysates of cells transfected as indicated above were probed with affinity-purified SUMO-3 antibody. Arrows indicate SUMO dimer and trimer. HMW Proteins, high molecular weight proteins. (E) Western blot showing expression and sumoylation/desumoylation of SUMO-3 and mutants. Cells were mock-transfected or transfected with APP and SUMO plasmids, and lysates were probed with anti-SUMO-3 antibody. (F) Effect of SUMO-3 and mutants on Aβ, β-NTF, and α-NTF generation from transfected cells as determined by ELISA. (G) Dose–effect of SUMO-3(11R) on Aβ, β-NTF, and α-NTF generation as determined by ELISA. Each of the above experiments was carried out more than three times with equivalent results as those presented. * Significantly different from APP transfection alone (P < 0.005, ANOVA post hoc tests).
raised against residues 593–596. For α-NTF, the capture antibody was 8E5, and the detection antibody was biotinylated 2H3 raised against Aβ domain residues 1–12.

Standard ELISA procedure was followed. With the appropriate amount of sample, this assay procedure accurately measures concentrations of Aβ, β-NTF, and α-NTF, all compared with purified standards. Final results are expressed relative to controls or APP transfection alone.

**Western Blot Analysis.** Cells were treated according to experimental designs and collected directly in lysis buffer according to the method of Desterro et al. (21). Equal amounts of protein from cell lysates were electrophoretically resolved on Tris-glycine SDS gels and processed by standard method. APP, SUMO-3, and SUMO-1 antibodies were obtained from Zymed, BACE antibody was from Chemicon, and anti-HisG antibody was from Invitrogen.

**Pulse–Chase Experiments.** 293T cells were transfected with APP695 and SUMO-3 plasmids in six-well plates and 48 h later were incubated with serum- and methionine/cysteine-free medium containing 200 μCi (1 Ci = 37 GBq) of Promix L (35S) in vitro cell-labeling mix (Amersham Pharmacia) for 20 min. Medium was replaced, and the cells were then chased in complete nonradioactive medium for up to 240 min. Cell lysates were prepared and immunoprecipitated with an APP C-terminal-specific antibody 369 (24). Immunoprecipitates were subjected to SDS/PAGE and analyzed by radiography.

**Immunohistochemistry.** Brain tissues of AD and Down’s syndrome patients or age-matched nondemented tissues were obtained from NIA/Stanford Brain Bank. Sections (7 μm thick) were preblocked and then probed with anti-SUMO-3 antibody at 1:500 dilution overnight at 4°C. Further processing was done following standard protocol. Tissue sections were finally visualized with DAB or VIP staining. In control staining, nonimmune serum was used in place of the anti-SUMO-3 antibody.

**Results**

**SUMO-3 Sumoylation Regulates APP Processing.** To identify modulators of APP processing, we screened a normalized human brain cDNA expression library consisting of 1,001 pools, each with ~100 plasmids. DNA from each pool was cotransfected with an APP expression plasmid into 293T cells, and 48 h later the growth medium was assayed by ELISA for the level of Aβ, β-NTF, and α-NTF (Fig. 1A). This allowed us to identify a number of plasmid pools that alter patterns of APP processing, including one that down-regulates the level of β-NTF and Aβ but up-regulates that of α-NTF (data not shown). After deconvolution, a single plasmid from this pool was identified capable of altering APP processing as the original pool. Sequence analysis of this clone revealed that it contains a full-length cDNA encoding SUMO-3, the 95-aa ubiquitin-like protein.

We examined the dose-response of SUMO-3 expression on APP processing in 293T cells. The level of β-NTF was significantly lowered with increasing amounts of SUMO-3 plasmid transfected (Fig. 1B). The total level of secreted Aβ was also similarly reduced to ~25% of the control at the highest SUMO-3 condition (Fig. 1B). Conversely, the level of α-NTF was increased in a dose-dependent fashion, which was also evident for endogenous APP (Fig. 1B and C). When the extracts of the transfected cells were subjected to Western blot analysis by using a SUMO-3-specific antibody, both free SUMO-3 and sumoylated proteins were detected in a SUMO-3 plasmid dose-dependent manner (Fig. 1D). The increased sumoylated proteins were detected mainly as high molecular weight species, typical of what has been observed previously (18). Free monomers, dimers, and trimers of SUMO-3 were also evident. Unconjugated multimers of SUMO have been reported in yeast (25). These data indicate that increased SUMO-3 expression and/or sumoylation down-regulate amyloidogenic but up-regulate nonamyloidogenic processing of APP.

We next examined the effect of lowering endogenous SUMO-3 sumoylation on APP processing. Because SUMO-3 sumoylation requires a C-terminal diglycine motif generated through enzymatic removal of the terminal two proximal amino acids, we assessed mutants of the diglycine motif for the ability to act in a dominant negative manner to down-regulate intracellular SUMO-3 sumoylation. Two SUMO-3 variants, SUMO-3(G93A) and SUMO-3(G92AG93A), where one or both of the conserved glycine residues was mutated to alanine, were generated. These mutants were transfected into 293T cells, and the SUMO-3 sumoylation was examined by Western blot analysis. Compared with endogenous levels of sumoylation by SUMO-3 (mock- and vector-transfected controls), both mutants signifi-
Effect of Sumoylation and Desumoylation on APP and BACE Expression.

As a step toward elucidating the mechanistic details for these observations, we examined two obvious targets of sumoylation, APP and BACE. Because sumoylation can stabilize proteins in contrast to ubiquitin-mediated proteolysis, we asked whether APP is directly sumoylated and whether the half-life of APP is affected directly or indirectly by sumoylation. We cotransfected APP and SUMO-3 into 293T cells, and whether the half-life of APP is affected directly or indirectly by sumoylation was assessed on APP processing. When SUMO-3(K11R) mutant was assessed on APP processing. When the immunoprecipitates were subjected to Western blot analysis (Fig. 2C), this effect was somewhat more dramatic in the presence of wild-type SUMO-3 than the mutant. Overexpression of wild-type SUMO-3 also resulted in a faster migration of mature BACE as observed with APP. At high doses of SUMO-3, high molecular weight immunoreactive BACE proteins at ~250 kDa were also increased the steady-state level of APP (Fig. 2B). No additional high molecular weight APP species were observed (data not shown), which is consistent with the above conclusion that APP is not directly sumoylated. The increased total APP by either SUMO-3 or SUMO-3(G93A) may be correlated with overall alterations in cellular metabolism when wild-type or mutant SUMO-3 is expressed at very high levels.

Because SUMO-3 affects APP processing, we next assessed its influence on BACE. BACE levels were unchanged at the lower doses of exogenous SUMO-3. Similarly to APP, BACE levels were slightly elevated when a BACE plasmid was expressed with high levels of exogenous SUMO-3 or SUMO-3(G93A) as detected by Western blot analysis (Fig. 2C). This effect was somewhat more dramatic in the presence of wild-type SUMO-3 than the mutant. Overexpression of wild-type SUMO-3 also resulted in a faster migrating BACE species, which might reflect immature BACE as observed with APP. At high doses of SUMO-3, high molecular weight immunoreactive BACE proteins at ~250 kDa were also

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Fig. 3. Effect of ubiquitination and neddylation on APP processing in 293T cells. (A) Western blot showing expression and conjugation of HisG-tagged ubiquitin and HisG-tagged Nedd-8. Lysates from transfected cells were probed with an anti-HisG antibody. (B) Effect of ubiquitin and Nedd-8 on APP processing as determined by ELISA for Aβ, β-NTF, and α-NTF.
detected on the blot (Fig. 2C). Because SUMO-3(G93A) also increased the level of high molecular weight BACE species (Fig. 2C), it is unlikely that these represent sumoylated BACE. BACE does contain a sequence, LKMD (positions 274–277), which conforms to a sumoylation motif. However, mutation of the acceptor lysine to arginine on BACE did not eliminate or reduce the amount of high molecular weight BACE species when coexpressed with SUMO-3 (data not shown). The level of the high molecular weight BACE seems to be correlated with high BACE expression levels and may simply represent aggregation. Together, these observations indicate that overexpression of both wild-type SUMO-3 and SUMO-3(G93A) increases intracellular APP and BACE levels and that high levels of wild-type SUMO-3 seem to retard or reduce posttranslational modification of APP and BACE. Also, the data show that the effects of sumoylation on APP processing are indirect.

Effect of Ubiquitin and Nedd-8 on APP Processing. To determine whether ubiquitination potentially plays an antagonistic role to sumoylation, we examined whether ubiquitin and Nedd-8 also affect APP processing. Ubiquitin has been implicated in AD, but whether it affects APP processing is unknown. Nedd-8 is a ubiquitin homolog, and conjugation of Nedd-8 to its substrate promotes ubiquitin polymerization (29). Interestingly, the APP binding protein, APP-BP1 (30), is one of two components of the E1 activating enzyme required for Nedd-8 conjugation (31, 32). It is also unclear whether Nedd-8 affects APP processing. We transfected plasmids expressing HisG-tagged ubiquitin or Nedd-8 into 293T cells and examined their expression and substrate conjugation by Western blot analysis. Fig. 3A shows that both free ubiquitin and Nedd-8 and their conjugates were detected. Neither ubiquitination nor Nedd-8 altered APP processing (Fig. 3B), and both were without effect on levels of intracellular APP (data not shown). Therefore, ubiquitination plays no significant role in APP processing.

SUMO-3 in Brain and Neuronal Cells. To determine the physiological relevance of the above observations, we examined whether SUMO-3 protein is present in brain and whether changes in sumoylation also effect amyloidogenesis in neuronal cells. We stained sections of brain from AD, Down’s syndrome, and nondemented individuals with affinity-purified anti-SUMO-3 antibody. Fig. 4A shows that neuronal populations have positive immunoreactivity for SUMO-3. SUMO-3 staining is remarkable...
in hippocampal neurons, a population significantly targeted in AD. Four to five cases were examined for each group. Qualitatively, it seems that a larger percentage of neurons in AD and Down's syndrome brain have a SUMO-3 distribution limited to the neuronal soma in contrast to nondemented brains, where SUMO-3 is both somal and nuclear (Fig. 4A and data not shown). A rigorous analysis with additional cases is needed to confirm this preliminary observation. Nonetheless, we show that SUMO-3 is detectable in brain neurons.

We next examined whether regulation of APP processing by sumoylation is also operative in neuronal cells. An APP expression plasmid was transfected without or with various SUMO constructs into the human neuroblastoma cell line SK-N-MC. APP processing was assessed by ELISA. Fig. 4B shows that the effect of sumoylation on APP processing observed in 293T cells was replicated in SK-N-MC neuronal cells. Therefore, sumoylation plays a role in APP processing in neuronal cells and is likely to influence brain Aβ levels.

Discussion

These results bring a new perspective to the field of AD research, specifically as it pertains to APP processing and biology, which has been extensively characterized over the past decade. The effect of sumoylation on APP biogenesis and processing is multifaceted. We have shown that increased and decreased SUMO-3 sumoylation are correlated with the down- and up-regulation of Aβ peptide production, respectively. Hence, abnormal regulation of APP amyloidogenesis by sumoylation may occur in AD. In support of this notion, SUMO-3 immunoreactivity is present in neuronal populations in brain. Putative SUMO-3 sumoylation disturbances could be caused by a number of factors, including dysfunction of SUMO proteins or the enzymatic and regulatory components involved in protein sumoylation. Sumoylation is a reversible process, employing SUMO-specific proteases to remove SUMO moieties from proteins. Genes encoding enzymes or proteins involved in sumoylation are thus potential risk factors for AD. Environmental factors such as oxidative stress, which has been shown to increase SUMO-3 sumoylation in certain cells (18), may also affect APP amyloidogenesis by effecting sumoylation. From a therapeutic point of view, sumoylation in certain cells (18), may also affect APP amyloidogenesis in this case, on the SUMO chains is a vivid resemblance to ubiquitination. The length of ubiquitin chains can serve different biological activities where polyubiquitination usually designates a protein to proteasome destruction, whereas monoubiquitination regulates other cellular processes such as endocytosis (33). The mechanism governing inhibition of APP amyloidogenesis by SUMO-3 poly-sumoylation warrants further investigation, particularly in light of the fact that both APP and BACE levels are increased by this regulatory process. The latter might be explained by the fact that sumoylation generally affects protein localization and transport, which is consistent with APP and BACE using the same trafficking route and perhaps the same transport compartment (34).

Although the sumoylation substrate(s) relevant to APP processing remains unknown, our observations show that sumoylation plays a critical role in APP amyloidogenesis (schematically summarized in Fig. 4C). Components of the sumoylation pathway should now be further investigated as not only potential AD risk factors but also therapeutic targets.

We thank Drs. J. McCarthy and F. Schimmoller for discussions, A. Lam and J. Miller for technical assistance, Dr. S. Gandy for the 369 antibody, Dr. G. Murphy for brain tissues, and Eli Lilly for funding this research.