Ubiquitinated proteinaceous inclusions are the hallmark of many neurodegenerative diseases. Inefficient proteolysis might lead to the accumulation and ultimate deposition of potentially toxic entities as inclusions within neurons or glial cells. This hypothesis is supported by genetic evidence both from patient populations and from engineered mutations in genes that encode ubiquitin/proteasome components in mice. The appearance of similar inclusions in the brains of elderly individuals of normal and subclinical conditions begs the question of whether there is a general age-related decline in the ability of the ubiquitin/proteasome pathway (UPP) to recognize and eliminate abnormal proteins, and whether such a decline would be reflected by changes in the abundance or activity of some or all components of the UPP. Here we describe alterations in the aging mammalian brain that correlate with a decline in the function of the UPP and review the evidence for age-related changes in specific UPP components. These alterations are discussed within the context of prevalent theories of aging.

Introduction
Theories of human aging must reconcile apparent inevitability with apparent randomness. Barring some as-yet inconceivable intervention, there appears to be an upper limit for human life span, with no documented case exceeding 121 years. The end may be sudden or protracted, and may be marked by the decline of one organ system or many. Somewhere in the system is a stochastic generator breaking the test tubes in Medawar’s* memorable metaphor, and some of us will suffer early and miserably for it. In the end, all will succumb before we get too far into our second century.

Among the more plausible stochastic theories of aging are those involving the loss of quality control; over time, cells fail to eliminate abnormal proteins generated by a lifetime’s worth of environmental damage, and this defect is superimposed on inherent biological sloppiness and any existing genetic predispositions. Whether the source of these abnormal proteins is the surprisingly high “normal” background of translational errors reported in eukaryotic systems (1), elevated oxidation arising from progressive mitochondrial derangement (2), the burden imposed by expanded polyglutamine proteins [reviewed in (3)], or any number of environmental sources, there may ultimately be too much abnormal protein for a cell to deal with, and pathological consequences may ensue. One would expect the issue of abnormal protein accumulation to be particularly acute in a nonrenewing cell population, as typified by neurons in the central nervous system (CNS) of long-lived mammals like ourselves. If presented with a burden of accumulated protein, such cells cannot be replaced and must soldier on. Morphological evidence for this can be found in the form of the age-dependent accumulation of a pigmented granular lipoprotein byproduct of cellular metabolism, lipofuscin, in neurons of the aging nervous system (4). The plight of such cells would be compounded should their proteolytic systems fail, perhaps precipitating a catastrophic deposition of toxic matter. Given that the CNS is the right place to look for such accumulation (see http://sageke.sciencemag.org/literature/overviews/casestudies), we might use neurons as a model system with which to ask: What is the system whose failure underlies such a calamity, and what is the evidence that such a failure might occur?

The Ubiquitin/Proteasome Pathway
Eukaryotic cells have two major systems for the regulated destruction of proteins: the relatively slow, vesicle-dependent lysosomal pathway, and the more rapid ubiquitin/proteasome pathway (UPP), which is operative in the cytosolic and nuclear compartments. The mechanistic separation of these pathways is not complete, with ubiquitin acting as a molecular “tag” for destruction in both. The association of ubiquitin, the lysosomal pathway, and neurodegenerative disease has long been appreciated (5), and the role of ubiquitin in directing traffic within the lysosomal pathway has recently become the focus of considerable research [reviewed in (6)]. The assembly of ubiquitin into chains for targeting substrates to the 26S proteasome (that is, the macromolecular cellular device charged with protein destruction) is better understood [for a recent review see (7)]. To summarize, ubiquitin, a globular 76-amino acid protein, is generally attached to substrate proteins through the formation of covalent isopeptide bonds involving the C-terminal glycine residue of ubiquitin and the side chain of a lysine residue within the substrate. The initial ubiquitin molecule can serve as the target of a second, whose C-terminus is attached to a lysine within the first, and so on until the ubiquitin chain is formed, anchored to the substrate. When this chain reaches a threshold length of four ubiquitin subunits (8), the substrate is directed to the proteasome through affinity between the ubiquitin chain and proteasomal subunits (9). The substrate is unfolded and passes through the interior of the proteasome, where it is degraded by a combination of protease activities; the ubiquitin chain is cleaved from the residual peptide of the substrate, then disassembled to yield the monomeric form. (The enzymatic activities responsible for the cleavage of ubiquitin are discussed in further detail below.) The substrate specificity requirements of the UPP are truly remarkable in that the system must distinguish not only the subset of normal proteins that under certain physiological conditions must be eliminated (short-lived transcription factors,

* http://sageke.sciencemag.org/cgi/content/full/2001/1/oa1

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for example), but must also distinguish pristine and properly folded proteins from potentially toxic damaged or misfolded proteins. The UPP accomplishes this astonishing feat through a hierarchical array of enzymes, including a very small number of ubiquitin-activating enzymes (E1 enzymes), whose job it is to elevate ubiquitin to an energetic state that allows covalent bond formation; a larger set of conjugating enzymes (E2 enzymes) charged with assembling the ubiquitin chains; and an extensive set of ubiquitin ligases (E3 enzymes) that employ protein interaction domains to impart substrate specificity. Countering the construction of ubiquitin chains on substrates are deubiquitinating enzymes (DUBs) that may stabilize substrates by reducing the length of ubiquitin chains below the threshold length or by removing chains from substrates entirely [reviewed in (10)].

Polyubiquitinated proteins are generally targeted and recognized for degradation by the 26S proteasome. Each of these self-compartmentalizing proteases is constructed from a barrel-shaped, 20S core particle in which the proteolytic activity is sequestered, and one or two 19S regulatory particles that cap the ends of the 20S particle. The regulatory particles are believed to recognize, unfold, and translocate ubiquitinated substrates into the central cavity of the 20S proteasome. The UPP is portrayed schematically in Fig. 1.

Age-Related Changes in UPP Proteins

Several investigators have examined the concentrations and activities of UPP components in various cell cultures, tissues from humans, and rodent models as a function of age, and it is fair to say that for many elements of the system, the results have varied widely. It is not our intention to review these findings in detail—that has been done admirably by other authors [for example, by Gaczynska et al. (11)]; rather, the current review will focus on changes that are of relevance to the CNS. There is no compelling evidence for an age-related increase in CNS concentrations of monomeric ubiquitin; no such increase has been observed in the cerebella of mice up to 32 months of age, which is very near the maximum lifespan of laboratory strains (Fig. 2A). A marked increase in high-molecular-weight ubiquitin conjugates with increasing age was observed in our experiments and has been documented by others (12). Where regional differences have been examined (12), the accumulation of conjugates was not uniform but was more dramatic in the cerebellum and brain stem [the regions of the brain responsible for motor functions and vegetative functions (which operate below the level of consciousness), respectively] than in the cerebrum (the site of higher functions, including cognitive processing and sensorimotor integration). The potential significance of such conjugates is discussed below in the context of proteasome activity. No changes in E1 enzyme concentrations or activity have been reported in the aging brain, and we have not detected such changes in the brains of very old mice (Fig. 2B). The literature reveals little about age-related changes in the concentrations and/or activities of the E2 and E3 enzymes. Because of their sheer numbers and widely divergent substrate specificities, any reasonably inclusive analysis of age-related changes in mammalian E2 or E3 enzymes would be an extremely large undertaking.

With regard to the proteasome, a consistent functional decline has been reported in several species and organ systems when samples from elderly rodents were compared to samples from younger organisms. For example, the peptidylglutamyl peptide hydrolyzing (PGPH) activity of the proteasome [an activity resident in the β1 (Y) subunit of the 20S proteasome that cleaves substrates C-terminal to acidic residues (13)] has been shown to decrease by approximately one-half during aging, as evidenced by cleavage of an artificial fluorogenic substrate (14-17). CNS tissues were not included in these studies. In a study by Keller et al., the chymotryptic activity of the proteasome, which is ascribed to the β5 or X subunit (18), was shown to be

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Fig. 1. Schematic representation of ubiquitin-mediated proteolysis. After the generation of a high-energy C-terminal thioester bond by an E1 enzyme, ubiquitin is covalently attached to a substrate protein (either a normal protein destined for degradation or a damaged or abnormally folded protein). Substrate recognition and subsequent chain assembly are mediated through the activities of E3 and E2 enzymes, respectively. Upon reaching the threshold length, ubiquitin chains direct the substrate to the proteasome where it is unfolded (green line) and directed into the central channel of the proteasome. Proteolytic activities lining the inner rings of the proteasome cleave the substrate into short peptides (green bars). DUBs disassemble the ubiquitin chains to yield monomers and may also oppose trafficking to the proteasome by shortening ubiquitin chains or cleaving them off of substrates. A reduction in the efficiency of ubiquitin-mediated proteolysis at any step would lead to an accumulation of substrates, including potentially toxic proteins.

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decreased in the cerebrum but not in the cerebellum of rats (19). In their review of proteasome alterations in the aging brain, Keller and co-workers point out that the loss of activity observed by various laboratories cannot be explained by a loss of subunit expression (20), because where such changes have been noted, the loss of activity precedes gross changes in proteasome components. We have observed that, in the mouse, at least some components of the 20S proteasome core and 19S regulatory caps increase with age (Fig. 2B). It is tempting to speculate that a compensatory mechanism exists wherein the loss of activity leads to an increased abundance of proteasome subunits, an ultimately futile effort to maintain cellular homeostasis. We currently have no evidence to support this hypothesis.

Results from Microarray Studies

The advent of DNA microarray technology has provided a powerful means of surveying the expression of thousands of genes simultaneously. In the context of aging, this methodology has revealed changing patterns of gene expression in the brain that can be clustered into functional systems. Comparing the brains of 5-month-old versus 30-month-old mice, Lee et al. detected increased expression of inflammatory genes indicative of microglial activation and induction of the complement cascade† (21). Also induced in the aged brains were genes that encode lysosomal proteases and genes involved in the stress response, including early response transcription factors and heat shock proteins. In this study, the level of ubiquitin mRNA was found to be elevated, whereas the level of mRNAs encoding other UPP components was decreased. This latter group of components included the ubiquitin ligase Nedd4, the proteasome β-2 subunit, and the deubiquitinating enzyme Usp4 (previously designated Unp). A recent microarray study of gene expression in the aging rat hippocampus also found evidence of increased gene expression consistent with inflammation and identified the proteasome β-2 subunit as being down-regulated at the RNA level (22), but changes in other UPP transcripts were not reported in this study. For the most part, the UPP targets revealed by the microarray approach await validation by Northern and Western blotting or proteomics methodologies, and it would be premature to draw conclusions based on the available evidence, but they do point to age-related dysregulation of the UPP at the transcriptional level. Intriguingly, caloric restriction‡ (at present, the best-characterized means of retarding aging in mammals) was found to at least partially attenuate age-related alterations in gene expression in the rodent brain (21, 23).

The Peculiar Case of Ub+1

It should not be surprising that the assembly of ubiquitin chains through isopeptide bond formation places constraints on the sequence of the C terminus of the protein. Indeed, the entire protein is highly conserved, being identical in rodents and humans and differing by only three conservative amino acid substitutions in the yeast versus the human protein. Van Leeuwen’s group has reported a remarkable perturbation of the C terminus of ubiquitin that may play a role in aging. It has been suggested that molecular misreading by RNA polymerase II of a dinucleotide repeat in the ubiquitin-B gene (UBB, which encodes a stress-activated in-frame fusion of three tandem ubiquitin proteins) results in an aberrant ubiquitin C terminus that is incapable of participating in conjugation. This aberrant form of ubiquitin is referred to as Ub+1. The neopeptipe created by the frameshift event was detected by immunohistochemistry in the brains of Alzheimer’s§ and Down syndrome patients (24) and has also been detected in the brains of non-

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Fig. 2. Western blot analysis of age-related UPP changes in the mouse brain. Lysates were prepared from the brains of female BALB/c mice aged 12 months (12M) to 32 months (32M), obtained from the National Institute on Aging, Bethesda, MD. Lysates were prepared from two mice at each time point (designated a and b). (A) Lysates from cerebella were probed with a polyclonal antibody to ubiquitin (DAKO Diagnostics Canada). The position of substrates conjugated with ubiquitin chains and the position of the ubiquitin monomer are indicated by the schematic diagram on the right (the upper two images represent different exposures of distinct portions of the same membrane). Note the increase in the intensity of high-molecular-weight ubiquitin conjugates in cerebella at 30 and 32 months of age. The membrane was stripped and reprobed with an antibody to β-actin [third panel in (A)] (Sigma). (B) Lysates of cerebra were probed with antibodies to the ubiquitin activating (E1) enzyme (Upstate), the α-1 subunit of the 20S multicatalytic protease-containing proteasome core (Calbiochem), or the S6 subunit of the 19S substrate-binding and unfolding cap structure (Affiniti Research Products). As in (A), the membranes were reprobed with a β-actin antibody (Sigma) to control for loading.

† http://sageke.sciencemag.org/cgi/content/full/2002/29/re3
‡ http://sageke.sciencemag.org/cgi/content/full/sageke;2003/8/re2
§ http://sageke.sciencemag.org/cgi/content/full/2001/1/oa2
demented elderly individuals (25, 26). The possibility exists that the aberrant ubiquitin may itself serve as a substrate for the formation of ubiquitin chains that are destined to remain unanchored to other proteins. Through their affinity for subunits within the 19S proteasome cap structures, such unanchored chains might exert a dominant negative effect on proteasome-mediated degradation of cellular substrates (27). There is also evidence that Ub−1 may be recognized as a ubiquitin fusion degradation substrate (28) with an inherent ability to block the proteasome and induce neurotoxicity (29).

Predicted Consequences of UPP Dysregulation During Aging

It might reasonably be asserted that, for a long-lived nonrenewing cell such as the neuron, an efficient UPP is the key to healthy living; conversely, an inefficient UPP is a recipe for disaster. If an intolerable burden of abnormal protein is presented to the cell, or if one or more components of the UPP are allowed to become scarce, the system is vulnerable to spiraling into a self-amplifying cycle of impairment. For example, the mutant form of the huntingtin protein is inefficiently degraded by the proteasome, and when presented with mutant huntingtin, the proteasome is unable to efficiently degrade other substrates, which will then accumulate (30). If the backlog of ubiquitinated substrates is not cleared from the cell, the polyubiquitin chains on these substrates, through binding to proteasome subunits, have the potential to further inhibit the proteasome’s ability to recognize and eliminate aberrant proteins. Thus, the accumulation of these proteins is likely to wreak further havoc. The triggering insult for the escalating cycle of impairment may come from the mutant protein (the product of germline or sporadic mutation) or from protein damaged by oxidation [reviewed in (31)]. The initial burden might also arise from endogenous neuronal proteins, such as α-synuclein, a protein with unusual folding properties whose accumulation inhibits the proteasome (32). This model is consistent with the molecular evidence reviewed above for the accumulation of polyubiquitin chains and increasing inefficiency of proteasome activity with advancing age. It also predicts dire consequences at the histological level. What is the evidence that such consequences are manifest in the CNS of elderly but nondiseased individuals?

Histological Findings in the Brains of Elderly Humans

Intracellular and extracellular protein aggregates are a unifying histopathological feature of several human neurodegenerative disorders [reviewed in (33)]. These proteins, and by extension the inclusions they form, are ubiquitinated. Accordingly, dysfunction of the ubiquitin/proteasome system has been implicated in the pathogenesis of some of these neurodegenerative disorders, most notably Parkinson’s disease (PD) (see also Andersen Review and Deadly Giveaway!) (34, 35). In some of these diseases, specific neuronal proteins present in the ubiquitinated inclusions are primary candidates for pathogenic mediators. Proteins with polyglutamine expansions are found in ubiquitinated intranuclear inclusions in the CAG trinucleotide repeat disorders [reviewed in (36)]. α-synuclein, convincingly implicated in PD pathogenesis, is a prominent component of Lewy bodies**, which are ubiquitinated intraneuronal inclusions found in dopaminergic neurons of the substantia nigra [reviewed in (37)]. In other neurodegenerative diseases featuring ubiquitinated intraneuronal inclusions, the culprit pathogenic protein remains to be determined. For example, spinal motor neurons in amyotrophic lateral sclerosis†† display intracytoplasmic ubiquitin-positive inclusions of a variety of morphologies (38). The number and identity of ubiquitinated proteins in these structures are unknown.

The molecular mechanisms initiating protein accumulation and aggregation in many of these diseases remain to be defined. However, ubiquitination appears to be a common theme in these disorders, and it is tempting to speculate that altered UPP function plays a role. A substantial proportion of inherited, early-onset, autosomal recessive forms of PD are associated with mutations in the gene encoding parkin††, an E3 ubiquitin ligase (39, 40). Individuals with parkin mutations in only one allele may develop later-onset disease, suggesting that parkin-associated abnormalities of the UPP may be involved in the much more common, later-onset, sporadic form of the disease, also known as idiopathic PD (41).

Ubiquitinated inclusions are not confined to neurodegenerative
disorders, and a variety have been described in specific brain regions as a morphological consequence of apparently normal or “nonpathological” aging. By analogy with the appearance of ubiquitinated inclusions in neurodegenerative disorders, one can speculate that these structures reflect a senescence-associated dysfunction of the UPP. Examples of such inclusions are shown in Fig. 3. Some of these age-associated structures are reminiscent of pathological inclusions in neurodegenerative diseases. Perhaps most instructive in this context is the Marinesco body, a common age-associated, round, eosinophilic intranuclear structure detected most commonly in neurons of the substantia nigra. These structures are ubiquitin-immunoreactive (42), but their biochemical composition is otherwise unknown. Marinesco bodies are thought to be formed in response to cellular stress. Morphologically, they resemble the ubiquitin intranuclear inclusions found in polyglutamine-repeat diseases. In this context, it is interesting that they accumulate nonexpanded ataxin 3 (43), a polyglutamine protein that, when expanded, causes spinocerebellar ataxia type 3.

There are a variety of additional age-associated structural alterations that affect neuronal cell bodies that stain for ubiquitin. These include ubiquitin-immunoreactive cytoplasmic rodlike and skeinlike inclusions found in large (presumably cholinergic) neurons in the neostriatum (a subcortical structure important for modulating motor, cognitive, and emotional information) (44, 45); in eosinophilic, granular inclusion bodies in the cytoplasm of neurons of the inferior olivary nucleus (a cerebellar relay nucleus important for motor coordination) (42); and in “colloid” or “hyaline” inclusions often found in hypoglossal and spinal motor neurons in the elderly (46). These neurons provide motor innervation to the muscles of the tongue and the rest of the body, respectively.

Age-associated ubiquitination is not restricted to inclusions in neuronal nuclei and cell bodies. The development of ubiquitin-positive axonal swellings has long been known to increase in frequency with age in certain parts of the CNS (42), most predominantly in the cuneate and gracile nuclei, which are targets of sensory information arising in the arms and legs, respectively. Similar age-associated changes are seen in sympathetic ganglia and the cerebral cortex. The sympathetic ganglia contain the cell bodies of neurons that control autonomic functions, including the cardiovascular, gastrointestinal, and urinary systems. The cerebral cortex controls higher functions, including cognition and sensorimotor processing. What causes these axonal swellings is unknown. These changes, which may account for some of the neurological decline seen with aging, are referred to as “physiological” neuroaxonal dystrophy, to distinguish them from the changes seen in pathological neuroaxonal dystrophy, in which similar axonal swellings develop throughout the brain and spinal cord as part of a complex neurodegenerative disorder presenting as infantile, late infantile, juvenile, and adult cases (47).

Ubiquitin-immunopositive granular bodies and dotlike structures are also commonly encountered in the aging brain (42, 48). The granular bodies are believed to be located in the distal aspects of dystrophic neurites. They are most commonly encountered in the middle and upper cortical layers, especially layer II of the entorhinal cortex. This is potentially important, because these neurons form a crucial link in the circuit responsible for short-term memory. They are among the first to degenerate in Alzheimer’s disease and show neurofibrillary degenerative changes in normal aging. In contrast, dotlike structures are prominent in the white matter and appear to be located in oligodendroglia and myelin lamellae (42). The significance of these structures is unknown. Most of the inclusions described above are confined to certain regions of the nervous system, suggesting that specific subsets of neurons are differentially susceptible to this process. The factors underlying this regionally selective vulnerability are unknown. This feature recalls the neuropathology of neurodegenerative disorders, wherein ubiquitinated inclusions show an anatomically selective pattern of distribution. Does an age-associated decline in the function of the UPP account for the development of these protein deposits? Does the presence of these inclusions in particular circuits correlate with age-associated decline in the function of these systems, as suggested for physiological neuroaxonal dystrophy? If so, then a delinquent UPP function is a prime suspect with respect to the forces culminating in neurological aging.

Does an Inefficient UPP Contribute to Aging?
The presence of a variety of ubiquitinated inclusions indicates that protein accumulation, aggregation, and ubiquitination occur during aging. By analogy with the involvement of UPP dysfunction in some neurodegenerative diseases that feature ubiquitinated inclusions, it is tempting to speculate that the presence of these inclusions in aging reflects an age-associated progressive failure of the UPP. Indeed, there are a number of nonneuronal diseases in which the UPP may also play a role [reviewed in (49)]; thus, the consequences of age-related changes in the UPP may not be confined to the nervous system. With regard to the nervous system, it must be said that neuropathologists (the sleuths charged with interpreting the pathophysiological significance of ubiquitinated inclusions) are all too aware that in both neurodegenerative disease and normal aging, ubiquitinated inclusions represent mere fingerprints in a crime scene long abandoned by additional culprits and accessories. Taken together, the immunohistochemical and molecular findings implicate a substandard, perhaps deranged, UPP in the neural alterations typical of the aged brain; but, to strain the metaphor, the evidence may be too circumstantial to convict. We expect that more compelling evidence will emerge from targeted disruption of UPP genes in mice. The implications of an acceleration or delay in aging would be profound and might amply reward the investigator with the patience to perform life span studies in mice.

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