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Effect of Ethanol on Protein Quality Control: Implications for Aging and Degenerative Disease

by

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THESIS

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in

Neuroscience

in the

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Abstract

The main protein quality control systems, the ubiquitin-proteasome system and macroautophagy, have been implicated in a diverse range of neurodegenerative disease as well as alcoholic liver disease. Chronic ethanol use increases the load of misfolded and polyubiquitinated proteins in liver tissue and the nervous system, thereby exacerbating the effect of aging and disease. Study of the interplay of age, toxin and disease is a promising avenue for understanding each alone as well as the interplay of the three, an increasingly important topic in our aging society. This paper will discuss the evidence for ethanol's interference with protein quality control and consider the implications for neurodegenerative disease.

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Introduction

Alcoholism is a disease with severe biological consequences. Chronic alcohol abuse ravages the body. The liver is principally susceptible as the main alcohol-metabolizing organ, but no tissue escapes ethanol's toxic effects. The nervous system in particular suffers horrifying consequences. Roughly 10% of chronic alcoholics develop profound neurological diseases such as alcoholic dementia or Wernicke-Korsakoff's syndrome; a greater number, from 50 to 70%, develop subclinical neuronal deficits evident in post-mortem studies. (Martin PR et al., 1986; Eckardt MJ & Martin PR, 1986) Alcohol abuse takes it effects on synaptic processing first, and secondly causes neuronal death compounded by a reduction in neurogenesis (Nixon K & Crews FT, 2002). Both gray and white matter shrink substantially, with noticeable ventricular enlargement. Imaging studies have revealed that the frontal cortex is the first and most severely impacted region, providing a neuroanatomical correlate for the impairment of judgment and reasoning that is a well-known hallmark of addiction (Kubota M et al., 2001; Sullivan E & Pfefferbaum A, 2005). Atrophy is observed in diverse brain regions including not only the frontal lobes but also the diencephalon, basal forebrain, hippocampus, cerebellum, and basal ganglia (Agartz I et al., 1999; Fadda F & Rossetti ZL, 1998; Martin PR et al., 1986).

Ethanol permeates so many different biological membranes and organelles that a full understanding of its toxicity has proven elusive. There are,

however, some hints. One intriguing area of research concerns the impact of ethanol on the two main protein quality control (PQC) systems, the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway. Evidence strongly indicates that ethanol causes damage to the nervous system at least in part through inhibition of PQC and consequent buildup of oxidized and misfolded proteins. An early sign of chronic excessive ethanol consumption (greater than 50-60 grams per day, or 4 to 5 drinks) is liver enlargement and protein accumulation (Zakhari S & Li TK, 2007). Ubiquitin-positive aggregates known as Mallory-Denk Bodies (MDBs) form as a consequence of alcoholic as well as non-alcoholic liver disease; these are seen in the livers of humans as well as hepatocytes from drug-treated mice. MDBs are composed of cytokeratin (CK8 and CK18), ubiquitin, and p62/sequestosome 1 (SQSTM1) (French SW et al., 2010; Bjørkøy G et al., 2005). As the present work is primarily concerned with the nervous system, studies conducted in liver tissue will be consulted as they pertain to nervous tissue. This paper will address both binge and chronic ethanol exposure models, as each recapitulates unique aspects of the drinking behavior of human alcoholics.

The UPS is the main degradation machinery for short-lived or misfolded proteins as well as some longer-lived enzymes. The proteasome is a multicatalytic proteinase that exists as a 26S complex for the breakdown of ubiquitylated proteins or a 20S complex for the digestion of proteins with no ubiquitin tags (Schwartz AL & Ciechanover A, 2009). It is estimated that 75 to

90% of proteins are degraded by the proteasome in a healthy cell (Donohue TM et al., 2007). When a protein is to be degraded, the 8.5 kD ubiquitin protein tag is activated by the ubiquitin activating enzyme E1 in an ATP-dependent manner. The ubiquitin moiety is transferred to a ubiquitin-conjugating enzyme, E2. E2 acts either directly or with the assistance of specific ubiquitin-E3-ligases to recognize and modify the substrate. Degradation of ubiquitylated substrates is performed by the 26S proteasome, which is composed of the ring-shaped 20S proteasome capped by one or two 19S regulatory particles. Three proteolytically active sites reside in the inner chamber of the 20S core: trypsin-like (β1), chymotrypsin (ChT)-like (β5), and peptidylglutamyl peptide-hydrolase (PGPH)-like or caspase-like (β2), while the 19S caps recognize ubiquitylated proteins. In addition to being a cell's first defense against damaged proteins, the UPS is also critical for the regulation of various cellular processes including proliferation, transcription, and apoptosis through its role in protein turnover (Groll M et al., 2005; Szutorisz H et al., 2006; von Mikecz A, 2006; Koch A et al., 2011).

The 20S proteasome can, following stimulation by pro-inflammatory cytokines such as interferon-gamma (INFγ), alternatively bind the 11S (PA28) regulatory subunit to form the immunoproteasome. Immunoproteasome formation also involves replacement of the catalytic β-subunits in the 20S core with three alternative subunits: low molecular weight protein 2 (LMP2) (β1i), LMP7 (β5i), and the multicatalytic endopeptidase complex-like 1 (MECL-1) (β2i) (Aki M et al., 1994). Both the immunoproteasome and the standard proteasome function in

antigen processing and presentation by major histocompatibility complex class-I (MHC class-I) molecules, which are involved in the recognition of foreign pathogen vs. self by T lymphocytes; there are thought to be differences in the efficiency of antigen processing and possibly the type of antigens processed by the two (Kloetzel PM, 2001; Sijts EJAM & Kloetzel PM, 2011). The immunoproteasome may also have a role in T cell proliferation via enhancement of cytokine signaling (Sijts & Kloetzel 2011). There is also a "hybrid" proteasome, i.e. 19S-20S-11S, which may change the pattern of peptides generated from breakdown of specific proteins (Cascio P & Goldberg AL, 2005).

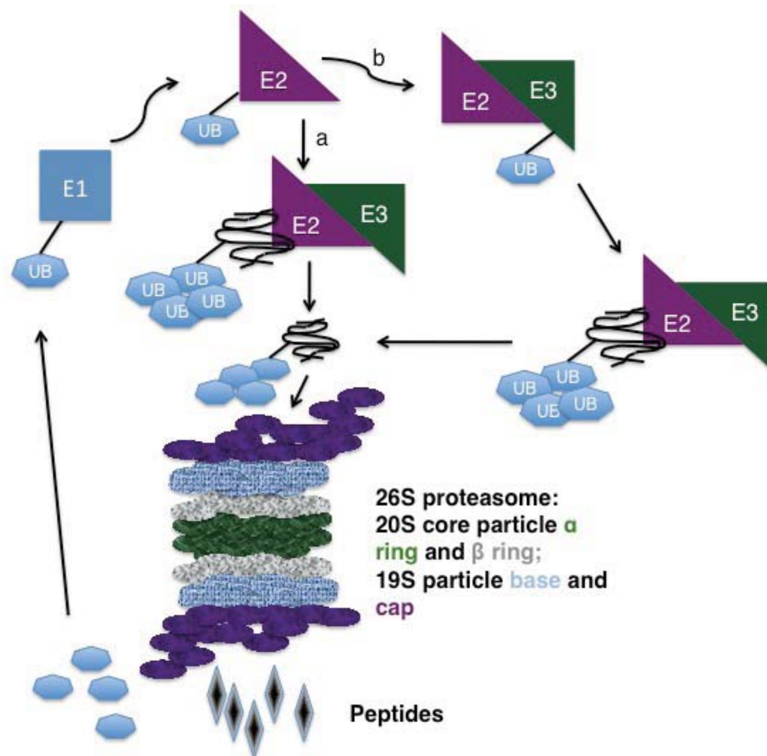


Figure 1: Ubiquitin-proteasome system. UB, ubiquitin. E1, E2, and E3, ubiquitin ligases.

The autophagy-lysosome pathway, in which cytoplasmic material is degraded by lysosomal acidic hydrolases, accomplishes larger-scale digestion such as breakdown of organelles, carbohydrate complexes, and protein aggregates. Its main function in lower organisms is to maintain nutrient homeostasis in the face of changing food supply, and this function persists in mammals (Abeliovich H & Klionsky DJ, 2001; Kuma A et al., 2004). In higher organisms it has evolved functions in diverse processes such as antigen presentation, elimination of foreign organisms, turnover of long-lived proteins, and regulation of development and cell death (Levine B & Klionsky DJ, 2004; Mizushima N, 2005). There are three main types of autophagy in mammalian cells: chaperone-mediated autophagy, in which proteins containing a KFERQ motif are targeted for lysosomal degradation; microautophagy, a less well understood process in which small amounts of cytoplasm are engulfed by invagination and pinching off of the lysosomal lumen; and, finally, macroautophagy (hereafter referred to simply as autophagy), in which larger amounts of cytoplasm containing organelles and protein complexes is engulfed in an autophagosome which then fuses with a lysosome to achieve digestion of the contents. Ubiquitin is a major player in the activation of autophagy as well as the UPS. Autophagy, classically thought of as a cell-death process, is now known to contribute to cellular survival through ridding the cell of unwanted organelles and long-lived proteins as well as extensively damaged proteins (Ravikumar B et al., 2010). Greatly enhanced autophagy will trigger cell death but the role we are

concerned with here with is a protective one (Baehrecke EH, 2005; Shintani T & Klionsky DJ, 2004).

Autophagy is canonically controlled by the mammalian target of rapamycin (mTOR) complexes (mTORC1 and mTORC2) which suppress autophagosome formation unless inactivated directly by nutrient deprivation or indirectly by cellular signals such as growth factors (e.g. AKT/PKB) or cell energy status signals (e.g. AMPK) (Lum JJ et al., 2005). Autophagy is also regulated by such mTOR-independent mechanisms as leucine availability and signaling through *myo*-inositol 1,4,5-trisphosphate receptors (Mordier S et al., 2000; Vicencio JM et al., 2009). Although autophagy was originally thought to be non-specific in its engulfment of cytoplasmic material, recent research has shown this not to be the case. It is now thought that that the adaptor protein p62/SQSTM1 provides a link between ubiquitinated protein aggregates and the autophagy machinery by encasing the entity to be degraded and targeting it to the proteasome (Bjørkøy G et al., 2005, 2006). The regulation and roles of autophagy have been extensively studied and reviewed (see, for example, Mizushima N & Klionsky DJ, 2007; Ravikumar B et al., 2010; Mehrpour M et al., 2010)

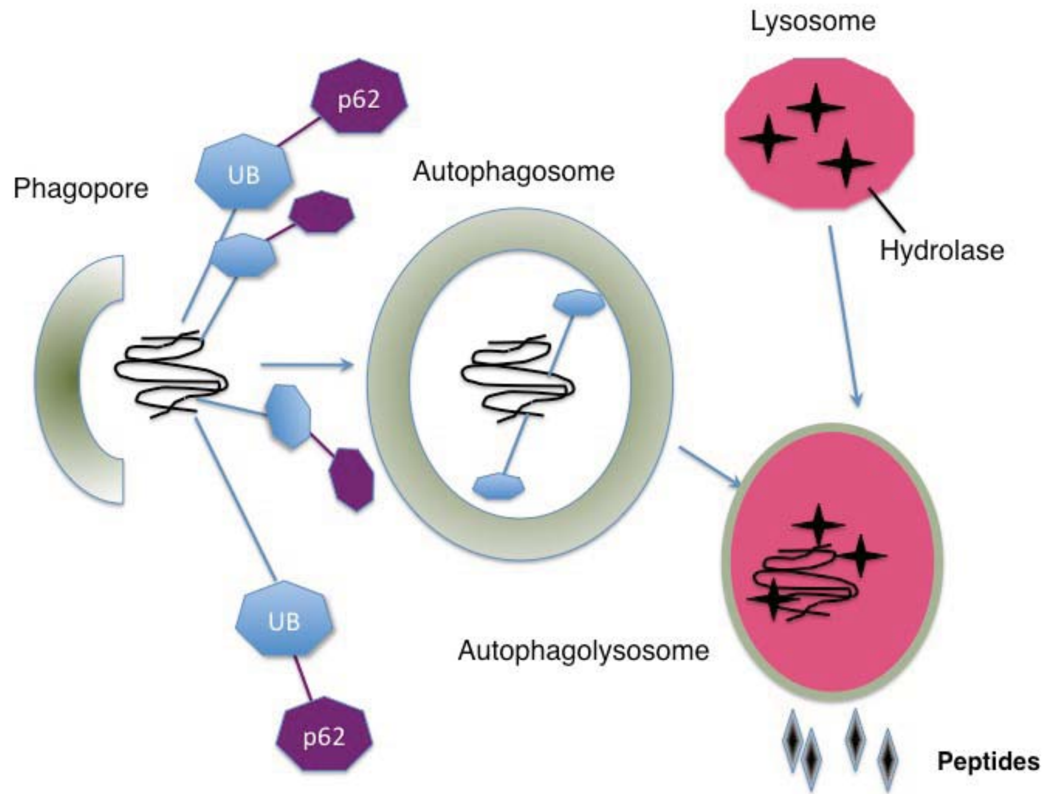


Figure 2: Autophagy-lysosome pathway.

Both the UPS and the autophagy-lysosome pathway are downregulated by ethanol (Donohue TM et al., 1998; Donohue TM, 2009). Sluggish catabolism contributes to a buildup of misfolded proteins as well as to an alteration of numerous cellular signaling pathways and a slowing of protein synthesis triggered by malnourishment of the amino acid pool (Donohue TM & Osna NA, 2003). Ethanol treatment is also thought to induce formation of the immunoproteasome at the expense of the 26S proteasome and interfere with

subunit binding to the 20S core particle, thereby further encouraging the dysfunction of the proteasome system (Bardag-Gorce F, 2010).

The UPS and autophagy are also downregulated with age, even in a healthy organism, and as a consequence oxidized and misfolded proteins build up in aging cells (Carrard G et al., 2002; Farout L & Friguet B, 2006; Lipinski MM et al., 2010). Extensively oxidized proteins are resistant to degradation by autophagic hydrolases, meaning that they persist as cellular “garbage.” As a consequence they slow the turnover of long-lived proteins and organelles, meaning that older, poorly functioning organelles will be doing the cell’s work. Such aggregates will themselves further impair the proteasome (Bence NF et al., 2001). Long-lived postmitotic cells are particularly susceptible to the accumulation of aggregated protein because they cannot asymmetrically mete out indigestible products into daughter cells as dividing cells can. Furthermore, many nondividing cells, cortical neurons among them, have intensive demands for oxygen that result in an increased generation of oxidative radicals and associated protein damage as compared with other cell types (Terman et al. 2010).

The goal of this paper is to discuss the evidence that ethanol interferes with protein quality control through its impacts, direct and indirect, on both the UPS and the autophagy-lysosome pathway, and the implications that interference holds for neurodegenerative disease progression. It is primarily concerned with ethanol’s impact on the nervous system. Much of the work on this

topic has been done in hepatocytes; that work will be discussed as it serves as a model for the nervous system. When considering progressive dementia due to accumulation of misfolded proteins in aging cells one can hardly resist mulling over the effect of PQC in the many neurodegenerative diseases that have proteinaceous aggregates at their cores. This paper will, in its last section, briefly summarize what is known about the interplay of ethanol and degenerative disease and discuss the implications that the combination of age, degenerative disease, and chronic ethanol abuse carry for human health.

Ethanol's Effects on the Ubiquitin-Proteasome System

Ethanol treatment has repeatedly been shown to decrease the chymotrypsin (ChT)-like and trypsin-like activity of the proteasome (Donohue TM et al., 1998; Fataccioli et al., 1999). Dysfunction of the 26S proteasome contributes to the etiology of liver disease (Donohue TM, 2002; French SW et al., 2001). Mallory-Denk Bodies (MDBs) form in part because of the failure of the proteasome to degrade them (Bardag-Gorce F et al., 2002). Ethanol- or drug-primed mice treated with the proteasome inhibitor PS-341 form MDBs while non-primed mice given proteasome inhibitor do not, suggesting that it is the interaction of a drug or toxin with downregulation of the proteasome, or collective damage over time from chronic use of a drug or toxin, that is responsible for MDB formation (Bardag-Gorce F et al., 2004a,b).

It is not ethanol per se but its metabolism that is required for proteasome inhibition; this has been confirmed by experiments rescuing proteasome inhibition by blocking ethanol metabolism with the drug 4-methyl pyrazole (4MP) (Osna NA et al., 2007). Rouach et. al (2005) were the first to definitively show that acetaldehyde affected proteasome activity. Acetaldehyde is the highly reactive oxidation product generated from ethanol by alcohol dehydrogenase (ADH) mainly, and through other means including Cytochrome-P4502E1 (CYP2E1) and catalase in the nervous system (Vasiliou V et al., 2006; Lu Y & Cederbaum AI, 2008). Rouach and others chronically exposed male Wistar rats to ethanol vapor for four weeks and assayed proteasome function using liver extracts. They demonstrate that the proteasome is far less efficient at digesting the protein bovine serum albumin (BSA) when the latter is conjugated to acetaldehyde.

But how is this occurring? This paper will discuss three mechanisms through which ethanol is thought to downregulate the UPS:

1. Reactive species inhibit the proteasome
2. Ethanol alters the interaction of proteasome subunits
3. Ethanol impairs methylation of proteasome subunits

Reactive Species Inhibit the Proteasome

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are oxidant molecules capable of doing cellular damage through the modification of proteins, lipids, and nucleic acids. ROS/RNS are produced naturally in

eukaryotes, consisting of, for example, the reduced forms of electron carriers such as NAD(P)H, or, as in the case of nitric oxide (NO), manufactured by microglia as part of immune defense; they are also produced in response to outside insult, as to X-ray, ultraviolet, or gamma radiation, and, importantly for this work, by the metabolism of ethanol and other toxic compounds (Halliwell B, 2006; Stadtman ER & Levine RL, 2000). The reactive bodies in a healthy cell are balanced by antioxidants; these include proteins such as glutathione (GSH), enzymes such as the superoxide dismutases (SODs) and glutathione-S-transferase (GST), metal chelators, and exogenous nutrients such as polyphenols (Farout L & Friguet B 2006). Sulfer-containing (cysteine and methionine) and aromatic (tryptophan and tyrosine) amino acids are more sensitive to oxidants than others (Stadtman ER & Levine RL 2003). Protein oxidation generally leads to the formation of hydroxyl or carbonyl groups; protein carbonylation is useful as a proxy for investigators assaying for extent of oxidative damage. Lipid peroxidation products such as 4-hydroxy-2-nonenal (4-HNE) also damage proteins, forming adducts that often involve carbonyl groups or cross-linking. Modified proteins tend to be impaired or inactive and may also be more difficult for the cell to break down (Farout L & Friguet B, 2006; Terman A et al., 2010). Oxidized proteins build up over time such that an estimated one-third of all protein in elderly persons is oxidized (Poggioli S et al. 2004; Stadtman ER & Levine RL, 2000).

It has been suggested that certain proteins are particularly susceptible to carbonylation or damage from lipid peroxidation. For example, aconitase, a key component of the citric acid cycle, was found to be significantly more carbonylated than other proteins in aged houseflies (Yan LJ et al., 1997). The set of proteins that is most likely to be carbonylated differs somewhat by species: in 24-month-old mice, albumin and transferrin were specifically carbonylated while in 19-month-old rats it was albumin and the protease inhibitor α1-M and in plasma from 15-year-old rhesus monkeys it was albumin and an unidentified 54-kDa protein (Jana CK et al., 2002). The authors note that amount of protein carbonylation is not simply a function of relative protein abundance. One post-mortem study on proteins in the inferior parietal lobule from human patients diagnosed with either mild cognitive impairment (MCI) or early-stage Alzheimer's Disease (EAD) found, intriguingly, that different sets of proteins were preferentially carbonylated between the two. These were: in MCI, heat shock protein 70 (Hsp70), carbonic anhydrase II (CAII), syntaxin binding protein I (SBPI), and mitogen-activated protein kinase I (MAPKI); for EAD, phosphoglycerate mutase 1 (PM1), fructose bisphosphate aldolase C (FBA-C), and glial fibrillary acidic protein (GFAP) (Sultana R et al., 2010). The issue of specific vs. general susceptibility to carbonylation awaits further clarification. This author is particularly curious whether and how the results of the last paper mentioned are in agreement with one another (i.e., why there is no overlap between the two sets of proteins). It is possible that current diagnoses of MCI

and EAD are erratic such that the two cannot be expected to share cellular characteristics, an influential finding in itself. It is also possible that disease states change the concentration of reactive species in different cellular compartments, resulting in different subsets of proteins being exposed to oxidative radicals in various stages of disease. These findings do not imply that these specific proteins are causative of age-related disease, but with buildup over time and associated inhibition of PQC (to be discussed below) proteins not directly implicated in age-related disease that are very sensitive to oxidation may certainly be contributing or exacerbating factors.

Much has been learned concerning the role of ethanol in enhancing oxidative stress. Ethanol and its metabolites have long been known to produce oxidative stress in a range of tissue types including the brain, heart, and liver (Montoliu C et al., 1995; Knecht KT et al., 1990, 1995; Reinke LA et al., 1987). Levels of reactive species increase in human primary cortical neurons following ethanol treatment (Haorah J et al., 2008) and in the neuronal cell line SK-N-MC (Agudelo M et al., 2011). In an intragastric infusion model of alcoholic liver disease, ethanol was shown to contribute to liver injury via oxidative damage. Alcohol-induced liver injury has been associated with increases in CYP2E1 protein levels, lipid peroxidation, and formation of lipid radicals that depended on a diet rich in polyunsaturated fatty acids (which are especially prone to lipid peroxidation). Those studies also pointed to increases in protein carbonyl formation, formation of the 1-hydroxyethyl radical, and decreases in hepatic

antioxidant defense that could be rescued with administration of antioxidants, notably glutathione precursors, vitamin E, and superoxide dismutase. (Nanji AA et al., 1994; Iimuro Y et al., 2000; Tsukamoto H & Lu SC 2001; Lu Y & Cederbaum AI, 2008). The B vitamin nicotinamide prevents ethanol-induced activation of caspase-3 and release of cytochrome-c and ameliorates cell death in the cingulate cortex, CA1 region of the hippocampus, and lateral dorsal nucleus (LDN) of the thalamus of ethanol-exposed P7 mice (Ieraci A & Herrera DG, 2006). Interestingly, nicotinamide also ameliorated certain behavioral effects: ethanol-induced hyperactivity and loss of fear conditioning were reduced with vitamin treatment. In another mouse model of fetal alcohol syndrome, Vitamin E protected against protein carbonyl formation and CA1 cell death but did not rescue spatial learning deficits in the Morris water maze, indicating, as one might suppose, that although reactive species are vital to ethanol toxicity they are not the only means by which it occurs (Marino MD et al., 2004).

It is relevant to note here that thiamine (vitamin B1) depletion is causative of Wernicke's encephalopathy, a severe nervous system disorder seen in many chronic alcoholics as well as some non-alcoholics such as the severely malnourished. Thiamine absorption is disrupted by chronic alcohol use and is frequently compounded by malnutrition in alcoholics. Thiamine deficiency causes disease largely because the vitamin is a necessary cofactor in three major enzymatic complexes (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and transketolase) (Jhala SS et al., 2011). Thiamine deficiency also increases

oxidative stress by upregulation of NO and other reactive species (Calingasan NY et al., 1998; Langlais PJ et al., 1997). Thiamine deficiency could exacerbate the effects of neurodegenerative diseases by downregulating the activity of the proteasome.

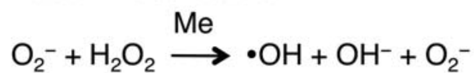
Oxidative stress reduces proteasome activity in cultured neuronal cells. Following application of the reactive molecules paraquat, H_2O_2, or $FeSO_4$ mitochondrial membrane potential drops, proteasome activity declines, and cell death ensues. These effects can be prevented by upregulation of the heat shock protein HDJ-1 (Ding Q & Keller JN, 2001). It is thought that reactive species cause oxidation of proteasome subunits, particularly the S6 ATPase subunit, with consequent reduction in 20S core activity (Donohue TM et al., 2007; Farout L et al., 2006; Ishii T et al., 2005). In hepatocytes and brain tissue, ethanol generates a state of oxidative stress through upregulation of the enzyme Cytochrome-P4502E1 (CYP2E1) as well as through the induction of pro-inflammatory processes and increased reduction of $NADP^+$ to NADPH (Bardag-Gorce et al., 2000; Caro & Cederbaum, 2004; Mandrekar P & Szabo G, 2009)

CYP2E1 metabolizes ethanol to its more reactive byproducts, acetaldehyde and the 1-hydroxyethyl radical (Rao et al., 1996). It is also an independent source of oxidative stress. It is posttranslationally stabilized by ethanol, most likely because its turnover is dependent on the proteasome (Roberts, 1994, 1995; Donohue 2007). All members of the CYT-P450 family catalyze the reduction of O_2 to superoxide anion and to hydrogen peroxide,

leading to generation of the toxic hydroxyl radical in the presence of heavy transition metals such as iron or copper. Metals act as catalysts to produce reactive species via the Fenton reaction or the Haber-Weiss reaction.

Fenton reaction:

Haber-Weiss reaction:



Where Me denotes a heavy transition metal (Persson HL et al., 2005; Dicker E & Cederbaum AI, 1987; for a fascinating and informative discussion of free radical biology see Halliwell B, 2006). Some, like CYP2E1, are greater producers of ROS than others as indicated by a higher rate of oxidase activity when purified and a higher microsomal capacity for NADPH oxidation (Gorsky LD et al., 1984; Caro AA & Cederbaum AI, 2004).

In an in vitro model of alcoholic liver disease, human hepatocyte HepG2 cells expressing CYP2E1 were primed with arachidonic acid (a polyunsaturated fatty acid) and iron and exposed to ethanol for one to two days. This treatment resulted in the formation of 4-HNE adducts and cytokeratin aggregates, coinciding with an inhibition in proteasome function, that were not seen in HepG2 cells lacking CYP2E1. The authors suggest a model in which ethanol induces CYP2E1 which then intensifies the formation of free radicals leading to lipid and protein oxidation. In the model 4-HNE forms adducts with proteasome subunits,

thereby inhibiting the proteasome and leading to buildup of cytokeratin aggregates along with progressively more CYP2E1 protein stabilization (Bardag-Gorce F et al., 2006).

Recently, Haorah J et al (2008) demonstrated that oxidative stress is increased in ethanol-treated cultured human neurons: 17.5 mM EtOH, which the authors note corresponds to a 0.08% blood alcohol content, upregulated CYP2E1 activity and induced ROS and acetaldehyde, itself a potent toxin. They find that NADPH/Xanthine oxidase (NOX/XOX) is induced by acetaldehyde, leading to superoxide production. Expression of the inducible nitric oxide synthase (iNOS) is also upregulated, increasing NO formation; excess NO exacerbates cellular stress through the generation of peroxynitrite radicals ($ONOO^-$) (Sun AY & Sun GY, 2001). These results correlated with an increase in 4-HNE adducts (Haorah J et al., 2008). Similarly, iNOS is induced in the livers of wild-type mice following four weeks of ethanol treatment with concomitant pathological changes such as fatty infiltration, inflammation, focal necrosis, and activation of the inflammatory cytokine tumor necrosis factor-alpha (TNF-α) whereas iNOS knockout mice are largely protected from these effects (McKim SE et al., 2003).

As Haorah et al.'s results highlight, an additional mechanism by which ethanol treatment produces oxidative stress and proteasome inhibition is through induction of pro-inflammatory processes. Chronic ethanol sensitizes Kuppfer cells (liver macrophages) to lipopolysaccharide insult; ethanol-induced ROS triggers a

robust immune response beginning with toll receptor-like 4 (TRL4) signaling that leads to the release of various other pro-inflammatory cytokines including TNFα and INFγ (Pascual M et al., 2011a,b; Mandrekar P & Szabo G, 2009). Inflammatory cytokine signaling and nitric oxide production are upregulated in rat brain following ethanol treatment (Naassila M et al., 1997; Xia J et al., 1999), and mRNA levels of the transcription factors TNFα and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) are increased in the Kupffer cells (liver macrophages) of ethanol-fed rats (Iimuro Y et al., 2000). Upon activation of microglia, oxidants such as superoxide and NO are released (by activation of NADPH and iNOS, respectively) as part of the immune response.

Osna NA et al. (2003) employed HepG2 cells to tease apart the effects of ethanol and CYP2E1 on inflammatory signaling. Those authors found that INFγ upregulated ChT-like proteasome activity in a CYP2E1-dependent manner, most likely by promoting CYP2E1 catalysis. This cytoprotective effect was prevented by ethanol treatment, which blocked INFγ-induced JAK/STAT1 signaling by preventing STAT1 phosphorylation, thereby negating both upregulation of proteasome activity and CYP2E1 catabolism.

More recent experiments suggest that an increase in inflammation mediated by INFγ signaling may cause upregulation of the immunoproteasome at the expense of the 26S proteasome. Evidence in support of this theory comes from a mouse model of drug-induced MDB formation and has yet to be examined specifically in alcoholic liver disease, but principles of MDB formation are

believed to be comparable between the two. Drug (diethyl 1,4-dihydro-2,4,6,-trimethyl-3,5-pyridinedicarboxylate, DDC) feeding for 10 weeks or refeeding after 1 month of withdrawal led to an increase in protein levels of the three immunoproteasome β subunits (LMP2, LMP7, and MECL-1) with a concomitant decrease in the proteasome β5 subunit and a decline in proteasome ChT-like activity in hepatocytes that formed MDBs, with no observable changes in hepatocytes that did not form MDBs. Alterations in subunit protein levels correlated with an increase in TNFα mRNA and both TNFα and INFγ receptors. All DDC-mediated changes were prevented by administering the universal methylation donor S-adenosylmethionine (SAM) with the drug. These findings suggest that the proteasome but not the immunoproteasome has a role in clearing potentially aggregation-prone cytokeratins (Bardag-Gorce F et al., 2010; French SW et al., 2010).

The same group goes on to demonstrate that INFγ applied to Hepa 1-6 mouse hepatocytes induces the expression of immunoproteasome β subunits when serum is absent from the media but not when it is present (i.e., the cells must be undergoing additional stress for the INFγ signal to have an effect). TNFα alone was unable to induce immunoproteasome formation, but it had a synergistic effect when applied with INFγ. In order to deduce which of the numerous INFγ-activated downstream signaling molecules are involved, the authors block NFκB, Jun N-terminal Kinase (JNK) and p38 MAPK signaling separately. They find that blocking NFκB or JNK has no effect on the expression

of immunoproteasome β subunits, but blocking p38 kinase prevents the INFγ-induced upregulation. However, others saw no evidence for p38 MAPK phosphorylation changes downstream of INFγ (Gough DJ et al., 2007; Ramsauer K et al., 2002). Presumably the specific media conditions and cell types account for the discrepancy. These studies also confirm the role of STAT1, noting that INFγ induces STAT1 phosphorylation which is further enhanced by TNFα (Oliva J et al., 2010).

Ethanol alters the interaction of proteasome subunits

Ethanol is believed to impede the binding of proteasome subunits and proteasome interacting proteins (PIPs). Ethanol treatment leads to hyperphosphorylation of the α subunits of the 20S core, thus loosening 20S/19S or 20S/11S association and lowering function of both the proteasome and the immunoproteasome (Bardag-Gorce F et al., 2004; French SW et al., 2010). Ethanol was previously reported to be a phosphatase inhibitor (Higashi K et al, 1996). One month of ethanol feeding causes hyperphosphorylation of rat 26S proteasome α subunits in the 20S core particle with associated decline in ChT-like proteasome activity (Bardag-Gorce F et al., 2004c). In support of this notion, in the livers of ethanol-fed rats Bousquet-Dubouch M-P et al. (2009) found a decrease in 19S and 11S protein in the most active proteasome fraction (that is, associated with the 20S particle) with no change in overall cytosolic protein

levels, suggesting a reduced binding affinity brought on by ethanol treatment. The same study showed effects of ethanol treatment on the PIPs.

There are a large number of PIPs that associate with the 26S proteasome and are essential for efficient proteasome function. They serve various essential roles such as deubiquitination, unfolding, and docking of protein substrate (Bardag-Gorce F et al., 2010). Ethanol treatment interferes with the expression of many PIPs or their binding to the proteasome complex. Notably, all three known mammalian proteasome-associated deubiquitinases (Rpn11, Usp14 and UCHL5/UCH37) were less likely to be pulled down with the proteasome following ethanol treatment, as was the Ecm29 protein that tethers the core particle to the regulatory particle and stabilizes the 26S complex. (Leggett DS et al., 2002; Bousquet-Dubouch M-P et al., 2009).

Ethanol impairs methylation of proteasome subunits

A recent report indicates that the methylation of proteasome subunits is also altered by ethanol treatment (Osna et al. 2010). French and colleagues previously observed that the methylation donor SAM attenuated MDB formation in a mouse model of liver injury. Drug-primed mice form MDBs if re-fed the toxic drug DDC up to four months after withdrawal, whereas mice administered both SAM and DDC do not form MDBs upon re-feeding. These results indicate a role for methylation changes in MDB formation. (Li et al. 2008; Bardag-Gorce et al. 2008). It was also known that ethanol induces changes in methionine synthase

levels in hepatocytes, leading to impaired formation of methionine and an increase of the ratio of endogenous S-adenosylhomocysteine (SAH) to SAM. SAH inhibits methyltransferases through its affinity for the catalytic region of most SAM-dependent methyltransferases (Barak et al. 2003). Increasing SAM in models of liver disease produces a diverse range of protective effects including reducing oxidative stress by increasing available glutathione, decreasing inflammation via the down-regulation of TNF-α and ILK-10, and decreasing the levels of apoptotic normal hepatocytes while stimulating the apoptosis of cancerous hepatocytes (Purohit et al. 2007); increasing the SAH:SAM ratio implies a deleterious loss of these effects.

On the basis of the evidence outlined above, Osna et al. (2010) hypothesized that aberrant methylation may be involved in alcoholic liver disease independently of the methylation of DNA/histone modification. They first confirmed that including SAM in the culture medium of mouse hepatocytes treated with 50 mM EtOH was protective against proteasome inhibition. Ethanol-induced proteasome inhibition could be recapitulated with application of a disease-associated SAM:SAH ratio or with the methylation inhibitor tubercidin; this latter was chosen in order to tease apart effects of oxidative stress from those of protein methylation. They recapitulated these findings with treatment of purified cytosolic fraction (no DNA methylation) as well as with highly purified proteasome, demonstrating with a range SAM:SAH ratios that methylation of the 20S proteasome occurs in a dose-dependent manner.

Consequences of ethanol-induced proteasome inhibition

The downregulation of the proteasome by ethanol is associated with a plethora of negative effects. One of the most interesting, and longest lasting, is that it engenders epigenetic changes. It has been shown to increase acetylation of histone H3 at lysine 9 through the decreased turnover of histone-modifying genes (Bardag-Gorce F et al., 2007; Kim JS & Shukla SD, 2006). Furthermore, proteasome inhibition and ethanol treatment alike are thought to prevent histone remethylation by downregulating remethylation enzymes and interfering with the synthesis of SAM (Pal-Bhadra M et al., 2007; Oliva J et al., 2009). The enzyme that converts methionine to SAM (Methionine adenosyltransferase, MAT1A) is reduced in liver tissue from patients with alcoholic liver disease (Mato JM et al., 1997) The resultant unchecked gene expression has been associated with, for example, an increase in pro-inflammatory NF-κB signaling and a decrease in signaling through pro-survival cAMP response element binding (CREB) family transcription factors (which, interestingly, have been implicated in addiction) (Crews FT & Nixon K 2009; Moonat S et al., 2010).

It is well known that heavy alcohol consumption reduces the function of the immune system. The increased virulence of Hepatitis B and C in alcoholic liver disease is one example of this phenomenon (Szabo G & Mandrekar P 2009; Osna NA et al., 2008, 2009; Gao B, 2002; Nevins CL et al., 1999). Interestingly, chronic ethanol administration also greatly enhances viremia and reduces the

number of circulating $CD8^+$ T lymphocytes, thereby inhibiting the clearance of infected macrophages, in the nervous system of a human immunodeficiency virus encephalitis (HIVE) mouse model (Potula R et al., 2006). Immune system dysfunction is thought to be due in part to a decreased capacity of both the proteasome and the immunoproteasome to produce MHC class I antigens (Osna NA et al. 2007). Furthermore, proteasome inhibition dysregulates the JAK/STAT signaling pathway, thereby interfering with antiviral defense, repair, and inflammation (Gough DJ et al., 2007). Proteasome inhibition and apoptotic signaling can in turn sensitize hepatocytes to TNFα-mediated damage, a finding that was attributed to elevated SAH levels (Barak AJ et al., 2003; Song Z et al., 2004).

As something of an aside, ethanol has other immunomodulatory effects that will exacerbate those stemming from its actions on the proteasome. One example is its upregulation of TNFα-mediated inflammatory signaling. Basal TNFα levels are increased in monocytes from alcoholics (Crews FT et al., 2006). Interestingly, though TNFα induced in the periphery of mice by a single lipopolysaccharide challenge lasted only a few days, TNFα in the brain from that same injection lasted up to 10 months. The continued inflammatory response was attributed to systemic TNFα activating neural TNF receptors, triggering microglial activation which persisted due to a positive feedback loop and the lack of any quenching mechanism in neural tissue. Death of tyrosine hydroxylase-positive neurons in the substantia nigra was seen after some delay, strongly

suggesting that the inflammatory response was neurotoxic (Qin L et al., 2007). Thus, one consequence of chronic alcohol intake may very well be continued TNF-α-mediated neuronal inflammation. TNFα induction and microglia activation are causative of neurodegeneration in vivo and in vitro; microglial activation is involved in the etiology of a range of neurodegenerative diseases (Ros-Bernal F et al., 2011; Hirsch E & Hunot S, 2009). Another example is an ethanol-induced decrease in tripeptidyl-peptidase 2 (TPPII) protein content (Bousquet-Dubouch M-P et al., 2009). TPPII is involved in the preparation of MHC class-I antigens through mechanisms that are somewhat independent of the proteasome. TPPII upregulation can partially compensate for proteasome inhibition (Kloetzel PM, 2004). Stimulation of TNFα and inhibition of TPPII are two of the ways in which chronic ethanol treatment exacerbates the effects of proteasome dysfunction on the immune system.

Protein aggregate formation itself impairs the proteasome, giving rise to a vicious feed-forward loop. Bence et al. (2001) observed that disease-associated huntingtin or cystic fibrosis fragments formed aggregates and inhibited proteasome function, leading to cell cycle arrest, when overexpressed in HEK293 cells. The mechanism by which protein aggregates inhibit the proteasome is not known; the authors postulated that it may be due to saturation of a necessary co-factor(s) or because undegradable proteins could be irretrievably bound to the proteasome itself. This is supported by the knowledge, mentioned above, that highly oxidized proteins are very difficult to degrade (Terman A et al., 2010).

There are known to be positive effects of very low-level ethanol intake (Thun MJ et al., 1997). It is tempting to consider the hormetic effect of small amounts of alcohol to be due to slightly elevated ROS production. Upregulation of the UPS with alcohol taken in moderation could perhaps explain why people who drink one to two drinks per day live longer than those who do not drink at all. It is possible that the oxidative stress from ethanol consumption plays a role in the hormetic effect: in response to proteotoxic stress from oxidative stressors, the transcription factor TCF11/Nrf1 is liberated which activates antioxidant genes such as glutathione-s-synthase (GSH), which in turn protect against further insult (Koch A et al. 2011). It is, however, likely that the largest component of the hormetic effect comes from the antioxidant polyphenols present in red wine and beer. Heavy consumption tips the balance in favor of the pro-oxidants generated by ethanol metabolism (Prickett C et al. 2004). The endpoint of UPS downregulation is apoptotic or necrotic cell death, depending on the presence or absence of ATP (Osna NA & Donohue TM, 2007; Joshi-Barve S et al., 2003).

Ethanol's Effects on the Autophagy-Lysosome Pathway

Autophagy plays an essential role in the elimination of toxic aggregation-prone proteins. For example, mice deficient in the key autophagy (ATG) genes *Atg5* or *Atg7* show deficits in motor function that are accompanied by formation of ubiquitin-positive inclusion bodies in neurons and hepatocytes along with

neurodegeneration, hepatomegaly and shortened lifespan, strongly suggesting that constitutive autophagy is necessary for preventing pathogenic protein accumulation in both the nervous system and the liver (Komatsu M et al., 2005,2006; Hara T et al., 2006). There is also the case of the lysosomal storage disorders, a collection of genetic diseases stemming from primary lysosomal defects that exhibit a range of phenotypes including neurodegeneration from accumulation of waste products and reduced turnover of organelles and long-lived proteins (Nixon R et al., 2008). The bulk of the work on the subject of autophagy and neurodegeneration has been done in studies of adult-onset neurodegenerative disease, to be discussed in the next section.

It is of interest to note here that the UPS and the autophagy-lysosome system do not operate independently of one another. They have certain regulatory signals in common; for example, FoxO3 regulates both systems independently of one another (Mammucari C et al., 2007). It has also been shown that autophagy is upregulated in response to proteasome inhibition, most likely as a cytoprotective mechanism to clear ubiquitinated proteins or replenish amino acid pools (Qing G et al., 2006; Fuertes G et al., 2003). In a *Drosophila* model of the polyglutamine disorder spino-bulbar muscular atrophy (SBMA), a degenerative eye phenotype induced by proteasome impairment was alleviated by histone deacetylase 6 (HDAC6)-mediated stimulation of autophagy. Inhibition of autophagy by knockdown of *atg6 or atg12* enhanced degeneration (Pandey UB et al., 2007). The same principle had previously been demonstrated *in vitro*:

toxic proteins that would ordinarily be broken down by the UPS were diverted into aggresomes for digestion by lysosomes following proteasome inhibition (Iwata A et al., 2005a; Taylor JP et al., 2003). Moreover, administration of the proteasome inhibitor bortezomib to mice overexpressing keratin 8 caused hepatic MDB formation and compensatory upregulation of autophagy. MDB formation was ameliorated by further stimulating autophagy with rapamycin. Thus, proteasome inhibition contributes to MDB formation for which the autophagy-lysosome pathway can compensate (Harada M et al., 2008). By downregulating both systems at once, ethanol provides a double insult.

Ethanol has been shown to inhibit autophagy in cultured murine neuronal progenitor cells. One group exposed GD 12.5-derived neuronal progenitors to ethanol for 72 hours and assayed for the presence of autophagosomes and autolysosomes with the pH-sensitive fluorescent dye monodansylcadaverine (MDC). Those authors observed a decrease in MDC staining in cells treated with relatively high concentrations of EtOH (210 or 333 mg/dL EtOH). They suggest that the observed suppression of autophagy may have more to do with an ability of ethanol to encourage stem-cell to blast-cell maturation rather than a direct effect on autophagy, noting that autophagy is downregulated following cellular differentiation. Therefore, any loss of MDC staining could be a secondary effect of maturation rather than an effect of ethanol directly on autophagy (Prock TL & Miranda RC, 2007).

Far stronger evidence supporting an ability of EtOH to interfere with autophagy comes from studies in hepatocytes (Donohue TM, 2009). Chronic ethanol consumption both slows the catabolism of long-lived proteins in rat liver and suppresses hepatic protein synthesis (Donohue TM et al., 1985, 1989). A reduction in the density of autophagosomes and autolysosomes is observed in these animals (Pösö AR & Hirsimäki P, 1991). Not only are there fewer lysosomes, but the ones that do remain are less acidic and less effective at digestion due to altered trafficking of lysosomal hydrolases (Kharbanda KK et al., 1996). The hepatic lysosomal system is downregulated by low serum ethanol concentrations relative to those that affect the proteasome (6 ± 12 mM for female rats and 27 ± 13 for male rats, vs. > 40 mM for proteasome inhibition in male rats) (Donohue TM et al., 1998, 2007).

In contrast to chronic ethanol exposure, acute exposure under certain conditions induces autophagy (Ding WX et al., 2010). This was shown in mouse liver following a single day on a binge drinking paradigm and was attributed to ROS-mediated inhibition of mTOR signaling. The authors reported increased autophagosome content and increased lipidation of LC3 (a marker of autophagosomes). Further stimulation of autophagy by rapamycin treatment was correlated with reduced lipid droplet formation and reduced hepatocyte apoptosis, whereas pharmacological downregulation of autophagy enhanced these phenotypes. This latter result supports the notion that autophagy is protective against an ethanol insult. Taken together, the chronic and acute results

suggest that autophagy may be upregulated as a protective mechanism against ethanol toxicity but that over time chronic ethanol treatment overwhelms the system.

This paper will discuss five mechanisms through which chronic ethanol use may be interfering with autophagy:

1. Chronic exposure to reactive species damages lysosomes
2. Ethanol interferes with insulin signaling
3. Ethanol upregulates IP3R signaling
4. Ethanol causes ATP depletion
5. Ethanol disrupts protein trafficking

Chronic exposure to reactive species damages lysosomes

As discussed earlier, ethanol and its metabolites increase reactive species through a variety of mechanisms. Lysosomes are particularly sensitive to oxidative damage because they engulf H_2O_2-generating mitochondria and contain high concentrations of metals (primarily iron) from the breakdown of metalloproteins that catalyze the conversion of relatively benign compounds like H_2O_2 into highly reactive oxidants. Reactive agents cause lysosomal damage and lead to inefficiencies in organelle and protein breakdown. Oxidative damage results in lysosomal membrane permeabilization and a decrease in the acidity of the vacuole, rendering catabolism less effective. Lysosomal hydrolases depend on an environment that is about 500 times more acidic than the cytoplasm (pH 4.7 inside the lysosome vs. pH 7.4 in the cytoplasm) (Kharbanda KK et al.,

1997). Ethanol treatment produces lysosomal fragility as assayed by reduced activity of lysosomal acid hydrolases in rat liver tissue from ethanol-fed rats (Donohue TM et al., 1994).

The effect of ROS on autophagy has been demonstrated in studies of the accumulation of the age pigment lipofuscin, one of the clearest examples that oxidative damage affects the lysosomal system and the viability of cells and organisms. Lipofuscin is a nondegradable, yellowish-brown, polymeric compound that slowly accumulates in aging postmitotic cells including neurons. Lipofuscin aggregates associate with lysosomes and are made more numerous by experimentally-induced oxidative damage or treatment with redox-active iron in cultured cells and rats (Zs-Nagy I et al., 1995). Various antioxidants (including vitamin E, selenium, and glutathione) ameliorate lipofuscin aggregation, while vitamin E deficiency increases it. Lipofuscin accumulation is predictive of the life of a cell (for excellent reviews of lipofuscin, see Brunk UT & Terman A, 2002a,b and Kurz T et al, 2010). Studies of lipofuscin provide strong support for the theory that certain oxidized protein complexes are not digestible by the lysosome and can overwhelm the system as the cell attempts to deal with them. Reduced turnover of other cellular components occurs secondary to lipofuscin buildup, as lipofuscin aggregates take up the cofactors and signaling molecules necessary for targeting of other organelles and proteins to the lysosome. Chronic ethanol use may downregulate autophagy by a similar mechanism, that is, by the ROS-

mediated production of damaged protein in such great quantities that eventually it cripples the processes that have evolved to handle it.

It is somewhat counterintuitive that an ethanol-induced ROS onslaught would lead to inhibition of autophagy, given that ROS is known to inhibit mTOR and stimulate autophagy in acute ethanol treatment, in chronic low-dose ethanol treatment, and under starvation conditions (Ding WX et al., 2010; Hagner PR et al., 2009; Scherz-Shouval R et al., 2007). mTOR is a major integration point for various signals that regulate autophagy, including growth factors and nutrient (glucose and amino acid) availability (Kim DH et al., 2002). It has even been postulated that H_2O_2 acts as a necessary signaling molecule for the induction of starvation-induced autophagy (Scherz-Shouval et al., 2007). Only one study has examined the effect of chronic high-dose ethanol on mTOR signaling (Vary TC et al., 2007). This was done using a rat model of alcoholic heart muscle disease, in which myocardial protein content is reduced through long-term alcohol use. The results showed an ethanol-induced decrease in phosphorylation of mTOR at Ser^{2448}, with a reduction in phosphorylation of the downstream effector proteins S6K1 and eIF4G at Thr^{389} and Ser^{1108}, respectively, leading to a reduction in protein synthesis. Activation of mTOR requires phosphorylation at Ser^{2448} among other residues; therefore, in agreement with the findings for acute and low-level alcohol use, mTOR is rendered inactive in this model of chronic high-level alcohol use. The authors did not directly address the impact of these findings on autophagy-lysosome function.

There is most likely a countervailing force that accounts for the observed ethanol-induced inhibition of autophagy with chronic ethanol use despite inactivation of mTOR, though further research is necessary to rule out differences based solely on dosage, time course of administration, and/or tissue type. A simple explanation for the apparent discrepancy between ROS-mediated inhibition of mTOR and ethanol-induced inhibition of autophagy is suggested by studies of lipofuscin: perhaps ROS from chronic ethanol use so damages lysosomal membranes and causes misfolded proteins to accrue in such overwhelming numbers that these factors eventually act as wrenches in the autophagy machinery independently of the cellular signaling pathways discussed above.

Ethanol interferes with insulin signaling

One other intriguing possibility is that ethanol treatment overcomes ROS-induced inhibition of mTOR by interfering with insulin signaling. Insulin inhibits autophagy in the short term through the PI3K signaling pathway. Frequent heavy drinking is a risk factor for type 2 diabetes which is characterized by glucose intolerance, hyperinsulemia, and insulin resistance (Wei M et al., 2000; Zakhari S & Li TK, 2007). Chronic ethanol treatment is causative of hepatic insulin resistance in Long-Evans rats (Derdak Z et al., 2011). Insulin resistance and hyperinsulemia brought on by 8-week administration of a high-fat diet suppresses

autophagy in an insulin-dependent manner in murine liver tissue and cultured hepatocytes (Liu H-Y et al., 2009). That study demonstrates a role for the transcription factor FoxO1 downstream of Akt, in particular for coordination of the *vps34, atg12, and gabarapl1* genes that are essential for autophagosome formation and initiation of autophagy. FoxO transcription factors have also been implicated in regulation of autophagy in *c. elegans, Drosophila melanogaster*, and mammalian skeletal muscle (Melendez A et al., 2003; Juhasz G et al., 2007; Demontis F & Perrimon N, 2010; Mammucari C et al., 2007).

Interestingly, recent evidence from both flies and mammals indicates that disruptions in insulin signaling are one cause of neuronal damage in fetal alcohol syndrome (FAS). In *Drosophila*, developmental ethanol exposure leads to reduced viability (ability to survive to adulthood), delayed development, lower body weight, decreased larval and adult brain size, and defective cell division and proliferation in the larval brain (McClure KD et al., 2011). These phenotypes correlated with dramatic reductions in both *Drosophila* insulin-like peptide 2 (*dilp2*) and the insulin receptor; they were alleviated by overexpression of *dilps* in the whole animal or in the insulin producing cells. The critical period for most of the observed phenotypes was during the second and third larval instar stages, which is a time of rapid cellular remodeling and therefore a time when proper functioning of the autophagy-lysosome system is vital (Di Bartolomeo S et al., 2010). Likewise in mammals, chronic gestational exposure to ethanol lowered the activity of insulin receptor (IR)- and insulin-like growth factor I (IGF-I)- receptor

tyrosine kinase, implying a reduction in insulin signaling. In cultured cerebellar neurons from rat pups exposed to ethanol in utero, the authors describe a reduction in IGF-I and IGF-II expression as well as deficits in glucose uptake and ATP production (De la Monte SM et al., 2005). Taken together these data promote alterations in insulin signaling as one explanation for the ethanol-induced reduction in autophagy.

Ethanol upregulates IP3R signaling

Upregulation of *myo*-inositol 1,4,5-trisphosphate receptor (IP3R) types I and III has been observed in liver epithelial cells of rats treated with ethanol chronically (Bokkala S et al., 1999). The IP3Rs have been shown to regulate autophagy in an mTOR-independent manner through interaction with Beclin-1 (the mammalian homolog of ATG-6), an essential component of autophagosome formation (Vicencio JM et al., 2009). Pharmacological blockade of IP3Rs or downregulation of IP3Rs with siRNAs results in robust stimulation of autophagy (Criollo A et al., 2007a,b). Thus, it is quite plausible that increase in IP3R signaling is driving the reduction in autophagy seen with chronic ethanol use. It is of interest to note that lithium, which depletes cellular inositol and thus inhibits IP3R signaling, ameliorates ethanol-induced toxicity in Drosophila antennal neurons (French R & Heberlein U, 2009; Sarkar S et al., 2005). This effect is due at least in part to inhibition of the GSK-3 homolog *shaggy*, but stimulation of autophagy could also play a part.

Ethanol causes ATP depletion

Chronic ethanol use causes ATP depletion in the liver. This was demonstrated many years ago in isolated mitochondria (Cunningham CC et al., 1990) and has been shown more recently in hepatocytes from rats fed ethanol chronically (Young TA et al., 2006). The defect in ATP production is thought to arise both from ethanol-induced mitochondrial damage that interferes with oxidative phosphorylation and from ethanol-induced depletion of glycogen, the substrate for glycolysis (Van Horn CG & Cunningham CC, 1999; Cunningham CC & Bailey SM, 2001). This phenotype is exacerbated by hypoxic conditions such as those that occur in rat liver tissue after a 4-week chronic ethanol treatment (Young TA et al., 2006; Arteel GE et al., 1997). As the acidification of lysosomes is ATP-dependent, ethanol treatment will impede it (Forgac M, 2007). The 26S proteasome is also ATP-dependent, and will thus be similarly impacted by a depletion of cellular energy stores (Peth A et al., 2010).

Ethanol disrupts protein trafficking

It was recently demonstrated in PC12 neurites that the trafficking of autophagic vacuoles is dependent on microtubules and the kinesin/dynein motors; this had preveiously been shown in hepatocytes. Treatment of PC12 neurites or primary rat hepatocytes with the drug nocodazole, which prevents microtubule polymerization by binding to tubulin subunits, significantly reduced

the movement of autophagic vacuoles and autophagosome-lysosome fusion (Yang Y et al., 2011; Köchl R et al., 2006). Ethanol treatment has been shown to hyper-stabilize microtubules through acetylation of tubulin and formation of acetaldehyde/tubulin adducts, thereby impairing microtubule assembly and especially microtubule regrowth after a challenge (Sherman G et al., 1983; Tuma DJ et al., 1991; Yoon Y et al. 1998; Kannarkat GT et al., 2006). Numerous other negative effects of impaired microtubule network that have little to do with PQC but would certainly exacerbate cellular stress have been documented, including alterations in protein/glycoprotein secretion and receptor-mediated endocytosis (Casey CA et al., 1987; Thiele GM et al., 1999).

Consequences of ethanol-induced reduction in autophagy

Interfering with autophagy contributes to disease through the buildup of toxic material and damaged organelles and an associated reduction in lysosomal function. Inhibition of this system has been implicated in a wide range of ills including neurodegeneration (to be discussed in the next section), immune system failure, cancer, and premature aging.

The same machinery that is used for autophagy is also involved in the targeting and degradation of microorganisms, a process termed xenophagy. Xenophagy removes intracellular and extracellular bacteria and viruses, and it is an important component of innate immune defense in response to pro-inflammatory cytokines such as TNFα and INFγ (Levine B, 2005). It is

additionally necessary for the processing of viral antigens for MHC class II presentation as demonstrated for antigen presentation in lymphoblastoid cells infected with Epstein-Barr virus (Paludan C et al., 2004). Interference with autophagy could very well contribute to the immune system troubles of chronic alcoholics.

Inhibition of autophagy leads to further inhibition of the proteasome as proteasome turnover is autophagy-dependent (Cuervo AM et al., 1995). This is emblematic of a general reduction in organelle turnover and persistence of aggregated proteins and damaged organelles. With reduction in autophagy, organelles and proteins that should undergo autophagic turnover instead persist and accrue ever more oxidative damage, contributing to cellular stress and aging. Aged mitochondria produce less ATP and more oxidative stress the older they become, most likely because of damage to components of the respiratory chain. Complex IV is particularly sensitive to oxidative injury (Tatarková Z et al., 2011). Damage also inhibits mitochondrial fission, leading to the swelling of mitochondria and the appearance of “giant” organelles. This may stem from mutations in the replicative control region (Michikawa Y et al., 1999; Brunk UT & Terman A, 2002a).

The endoplasmic reticulum (ER), too, undergoes increased stress from an excess of unfolded proteins and an inability of autophagy to degrade protein-burdened organelles. Enhanced IP3R signaling, as occurs with chronic alcohol use (discussed above), is one early component of alcohol-induced ER stress.

The unfolded protein response is activated in alcoholic liver disease. Sustained ER stress leads to apoptotic cell death and is particularly deleterious in combination with mitochondrial stress (for an extensive review of ER stress and its implications in liver disease, see Malhi H & Kaufman RJ, 2011).

It is well documented that there is an increased incidence of many cancers in chronic alcoholics (particularly cancers of the liver, mouth, esophagus, pharynx and larynx, colon, stomach, breast and ovaries) (Bagnardi V et al., 2001). Inhibition of autophagy has been linked to liver cancer, breast cancer, leukemia, and lung cancer in human genetic studies and to increased incidence of malignant transformation in model systems (Liang XH et al., 1999; Mortensen M et al., 2011; Takamura A et al., 2011; Jiang ZF et al., 2011). It is reasonable to suppose that reduction in PQC contributes to alcoholic cancers, though more work needs to be done to clarify this issue.

Autophagy serves a dual function: not only does it rid the cell of potentially harmful protein complexes and damaged mitochondria, but it is also vital for providing nutrients when they are not available from an external source. When rats are fasted for 48 hours, they lose 40% of their liver protein and exhibit a significant loss of liver mass which are both rapidly restored upon refeeding; these changes correlate with an induction of autophagy in the fasted state and its nutrient-induced suppression with refeeding (Mortimore GE & Ward WF, 1981; Donohue TM et al., 1994). Newborn mice also exhibit enhanced hepatic autophagy in the first few hours of life when they are adjusting to a novel food

source and are therefore undergoing short-term starvation (Kuma A et al. 2004). Knockout mice lacking crucial ATG genes die prematurely and with evident neurodegeneration (Komatsu M et. al, 2006; Hara T et al., 2006). Autophagy is induced in human hepatocytes between meals, where it aids in the stabilization of nutrient stores (Mizushima N & Klionsky DJ, 2007). Autophagy is therefore a vital source of nutrients in both normal and diseased states. Indeed, lysosomal dysfunction contributes to ethanol-induced hepatomegaly in rats as nutrient stores cannot be efficiently broken down and recycled (Donohue TM et al., 1994).

Conclusion: Implications of chronic alcohol abuse in neurodegenerative disease

It is commonly said that age is the most important risk factor for degenerative disease. This is true in the case of inherited neurodegenerative disorders (familial Alzheimer's Disease, familial Parkinson's Disease, and Huntington's Disease being classic examples): mutant protein is produced from the day of birth, but disease does not manifest until later in life. One can also draw a corollary to the prion disorders, as inherited or introduced prion disease does not make itself known for many years. It is also true for the neurodegeneration brought about by chronic alcohol abuse. Alcoholics, who in general have begun drinking early in life, tend not to show symptoms of nervous system damage until decades later. These thoughts imply that the gradual accumulation of a toxic factor over many years is vital to the pathogenesis of

disease. The interplay of these three processes, natural aging, degenerative disease, and chronic alcohol abuse, may increase the rate and severity of decline.

PQC dysfunction, either of the UPS, autophagy, or both, is almost universal amongst neurodegenerative disorders with intraneuronal inclusion body formation. Whether misfolded proteins cause inhibition of the UPS and autophagy-lysosome system or vice versa is still a matter of debate, but it is clear that upregulation of PQC systems has the potential to ameliorate the effects of neuronal inclusions in many disorders through increased clearance of toxic protein species. Ethanol-induced inhibition of PQC may predispose towards or exacerbate neurodegeneration.

PQC in degenerative disease

The proteasome is the first line of defense against misfolded and aggregate-prone proteins in monomeric form. Malfunction of the proteasome has been implicated in a wide range of degenerative diseases with aggregated protein pathology. For example, proteasome inhibition preferentially increases the accumulation of Huntington's disease-associated huntingtin fragments over that of wild-type huntingtin fragments (Li X et al., 2010). The proteasome is responsible for the degradation of phosphorylated α-Syn causative of PD, prion protein causative of Creutzfeldt-Jakob Disease and other prion disorders, and TDP-43 and tau involved in FTD; all three are also broken down by autophagy

(Machiya Y et al., 2010; Vogiatzi T et al., 2008; Ma J & Lindquist S, 2002; Wang X et al., 2010; Pritchard SM et al., 2011). Interestingly, acute inhibition of the proteasome results in synaptic plasticity defects and defects in learning and memory, indicating a crucial role for protein turnover in memory processes and suggesting a potential mechanism for the memory deficits seen in patients in the early stages of degenerative diseases such as AD (Bingol B & Sheng M, 2011). The same mechanism could theoretically explain the blackouts brought on by binge drinking.

Though the proteasome is essential in homeostatic maintenance, it is likely that mutant aggregate-prone proteins in oligomeric form are cleared by autophagy as the proteasomal pore is too small to admit these bodies. There is abundant evidence highlighting the importance of autophagy in eliminating toxic aggregate-prone proteins and linking downregulation of autophagy to neurodegenerative disease. Mice deficient in Atg5 or Atg7 (both key autophagic genes) show deficits in motor function that are accompanied by an accumulation of cytoplasmic inclusion bodies in neurons, demonstrating that constitutive autophagy is important in preventing protein accumulation in the nervous system (Hara T et al., 2006; Komatsu M et al., 2006). Autophagy is a major degradation pathway for various aggregate-prone neurodegenerative disease-causing proteins including mutant huntingtin, the A53T and A30P point mutants of α-syn causing familial PD, ataxin 3 involved in spinocerebellar ataxia type 3, mutant forms of superoxide dismutase 1 causing familial ALS and mutant forms of tau

causing FTD (Ravikumar B et al., 2002; Ravikumar B et al., 2004; Webb JL et al., 2003; Berger Z et al., 2006; Fornai F et al., 2008). Extensive autophagic vacuolization is seen in neocortical and hippocampal pyramidal neurons in AD brain, suggesting that clearance of these vacuoles is impaired (Nixon RA et al., 2005). In addition, many compounds that upregulate autophagy ameliorate neurodegenerative disease in animal models; these include both compounds inhibiting the negative regulator of autophagy mammalian target of rapamycin (mTOR), such as rapamycin and glucose-6-phosphate, and mTOR-independent compounds such as lithium and carbamazepine (for an extensive review of this subject, see Sarkar S et al., 2009). What is true in neurons is also true in murine liver cells: inhibition of proteasome activity by administration of bortezomib caused MDB-like formation which was prevented by upregulation of autophagy with rapamycin treatment. This indicates that proteasome inhibition contributes to MDB formation and that autophagosomes can digest these aberrant proteins (Harada M et al., 2008). See table 1 for information on inclusion pathology in degenerative disease. A full discussion of the importance of the UPS and autophagy in degenerative disease is beyond the scope of the present work. Many excellent reviews have been published on this subject (Rubinsztein DC, 2006; Nedelsky NB et al., 2008; Nixon RA et al., 2008; Schwartz & Ciechanover, 2009).

Table 1: Selected proteins contributing inclusion pathologies of common degenerative diseases and the PQC systems implicated in disease pathology.

Protein	Disease	PQC pathway	References
Sod-1	ALS	UPS and Autophagy	Ying Z et al., 2009; Fornai et al., 2008
Tau	AD, FTD	UPS and autophagy	Pritchard SM et al., 2011
TDP-43	ALS, FTD	UPS and autophagy	Wang X et al., 2010
Htt	HD	UPS and autophagy	Li X et al., 2010; Ravikumar B et al., 2004
α-Syn	PD	UPS and autophagy	Webb JL et al., 2003; Machiya Y et al., 2010; Vogiatzi T et al., 2008
Pink1	PD	UPS and Autophagy	Shin J-H et al., 2011; Chu CT 2010
Parkin	PD	UPS and Autophagy	Shin J-H et al., 2011; Chu CT 2010
LRRK2	PD	UPS and Autophagy	Lin X et al., 2002; Alegre-Abarrategui J & Wade-Martins R, 2009
PrP	CJD	UPS and Autophagy	Ma J & Lindquist S, 2002; Sikorska B et al., 2004; Lorenz H et al., 2002; Zanusso G et al., 1999
Ataxin-1	SCA1	UPS and Autophagy	Zhai RG et al., 2008; Iwata A et al., 2005b
Ataxin-3	SCA3	UPS and autophagy	Warrick JM et al., 2005; Ying Z et al., 2009; Berger Z et al., 2006.

α-Syn, alpha-synuclein; Htt, huntingtin; LRRK2, leucine-rich repeat kinase 2; PrP, prion protein; Sod-1, superoxide dismutase 1; TDP-43, tar DNA binding protein 43; ALS, amyotropic lateral sclerosis; AD, Alzheimer's Disease; CJD, Creutzfeldt-Jakob disease; HD, Huntington's Disease; PD, Parkinson's Diesease; FTD, frontotemporal dementia; SCA1, spinocerebellar ataxia type 1; SCA3, spinocerebellar ataxia type 3.

Alcohol use and neurodegeneration

Only a handful of studies have attempted to address the question of whether or not chronic alcohol use impacts the progression of degenerative disease, and so, information on this topic is sparse and contradictory at times. Some epidemiological studies suggest that a history of heavy alcohol use contributes to cognitive decline in AD. Ethanol was found to be a risk factor for AD (Fratiglioni L et al., 1993). Conflicting reports, though, indicate that alcohol does not advance the course of disease. The discrepancy is most likely explained by inherent difficulties in population-based studies such as proper control of confounding factors and inaccuracies in self-reporting. Alcoholism did not accelerate plaque formation or muscarinic receptor loss in the cortex of AD patients studied at autopsy (Freund G & Ballinger WE, 1992). One population-based study examining the severity of dementia in abstinent alcoholics with AD indicated that there was no significant effect of past heavy drinking on dementia score, but this population was unique amongst alcoholics: they remained relatively healthy until old age with a very low incidence of malnutrition, head trauma, or liver disease, did not report significant blackout episodes, and did not report significant medical or social problems brought on by drinking. That population would seem to be protected by genetic or environmental factors from ethanol-induced toxicity (Rosen J et al., 1993).

Ethanol induces tau accumulation and cell death in a cell culture tauopathy model (Gendron TF et al., 2008). However, a post-mortem human study found no effect of past heavy alcohol consumption on the concentration or morphology of neurofibrillary tangles (Aho L et al., 2009). Interestingly, there are reports of transient Parkinsonian symptoms incurred during intoxication or ethanol withdrawal (Carlen PL et al., 1981; Luijckx GJ, 1995). In addition, one population-based study links heavy alcohol consumption to an increased risk for developing essential tremor (Louis ED et al., 2009). Our current incomplete understanding of this important subject leaves ample room for further inquiry.

Conclusion

The evidence discussed above suggests that ethanol interferes with the major PQC systems, and a great deal of work shows that neurodegenerative processes and cellular aging act at least in part in the same manner. Attention to the role of PQC systems in the interplay of chronic alcohol abuse, aging, and degenerative disease is warranted as it will aid in understanding these processes both alone and in combination and additionally has the potential to suggest much-needed therapeutic interventions.

Index of abbreviations

4-HNE: 4-hydroxy-2-nonenal

4MP: 4-methyl pyrazole

AD: Alzheimer's Disease

ADH: Alcohol dehydrogenase

AKT/PKB: A serine/threonine protein kinase

AMPK: Adenosine monophosphate-activated protein kinase

ATG: Autophagy

BSA: Bovine serum albumin

CAII: Carbonic anhydrase II

ChT: Chymotrypsin

CK18: Cytokeratin 18

CK8: Cytokeratin 8

CREB: cAMP response element binding (family of transcription factors)

CYP2E1: Cytochrome-P4502E1

CYT-P450: Cytochrome-P450

DDC: diethyl 1,4-dihydro-2,4,6,-trimethyl-3,5-pyridinedicarboxylate

Dilp: *Drosophila* insulin-like peptide

EAD: Early-stage Alzheimer's Disease

EtOH: Ethanol

FAS: Fetal alcohol syndrome

FBA-C: Fructose bisphosphate aldolase C

$FeSO_4$: Iron(II) sulfate

FTD: Frontotemporal dementia

GFAP: Glial fibrillary acidic protein

GSH: Glutathione

GST: Glutathione-S-transferase

H_2O_2: Hydrogen peroxide

HD: Huntington's Disease

HDAC6: Histone deacetylase 6

HEK293: A human embryonic kidney cell line

Hepa 1-6: A murine hepatocyte cell line

HepG2: A human hepatocyte cell line engineered to inducibly express CYP2E1

HIVE: Human immunodeficiency virus encephalitis

Hsp70: Heat-shock protein 70

INFγ: Interferon-gamma

iNOS: Inducible nitric oxide synthase

IP3R: Inositol trisphosphate receptor

JAK/STAT1: Janus kinase/Signal transducer and activator of transcription

JNK: Jun N-terminal Kinase

LDN: Lateral dorsal nucleus of the thalamus

LMP2: Low molecular weight protein 2

LMP7: Low molecular weight protein 7

MAPK1: Mitogen-activated protein kinase I

MAT1A: Methionine adenosyltransferase

MCI: Mild cognitive impairment

MDBs: Mallory-Denk Bodies

MDC: Monodansylcadaverine

MECL-1: Multicatalytic endopeptidase complex-like 1

MHC class-I: Major histocompatibility complex class-I

mTOR: Mammalian target of rapamycin

mTORC1: Mammalian target of rapamycin complex 1

mTORC2: Mammalian target of rapamycin complex 2

NAD(P)H: The reduced form of $NADP^+$

$NADP^+$: Nicotinamide adenine dinucleotide phosphate

NFκB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NO: Nitric oxide

NOX/XOX: NADPH/Xanthine oxidase

$ONOO^-$: Peroxynitrite radical

PA28: 11S regulatory subunit of the immunoproteasome

PD: Parkinson's Disease

PGPH: Peptidylglutamyl peptide-hydrolase

PIPs: Proteasome interacting proteins

PM1: Phosphoglycerate mutase 1

PQC: Protein quality control

PS-341 (Bortezomib): A proteasome inhibitor

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SAH: S-adenosylhomocysteine

SAM: S-adenosylmethionine

SBMA: Spino-bulbar muscular atrophy

SBP1: Syntaxin binding protein I

SK-N-MC: A human neuroblastoma cell line

SODs: Superoxide dismutases

SQSTM1: Sequestosome 1

TNFα: Tumor necrosis factor α

TPPII: Tripeptidyl-peptidase 2

TRL4: Toll receptor-like 4

UB: Ubiquitin

UPS: Ubiquitin proteasome system

β1: Trypsin-like subunit of the proteasome

β1i: LMP2 subunit of the immunoproteasome

β2: PGPH-like (caspase-like) subunit of the proteasome

β2i: MECL-1 subunit of the immunoproteasome

β5: ChT-like subunit of the proteasome

β5i: LMP7 subunit of the immunoproteasome

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