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Diminishing glutathione availability and age-associated decline in neuronal excitability

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ABSTRACT

Oxidative stress is frequently implicated in diminished electrical excitability of aging neurons yet the foundations of this phenomenon are poorly understood. This study explored links between alterations in cellular thiol-redox state and age-associated decline in electrical excitability in identified neurons (right pedal dorsal 1 [RPeD1]) of the gastropod *Lymnaea stagnalis*. Intracellular thiol redox state was modulated with either dithiothreitol or membrane permeable ethyl ester of the antioxidant glutathione (et-GSH). Neuronal antioxidant demand was manipulated through induction of lipid peroxidation with 2,2′-azobis-2-methyl-propanimidamide-dihydrochloride (AAPH). Glutathione synthesis was manipulated with buthionine sulfoximine (BSO). We show that; glutathione content of snail brains declines with age, whereas pyroglutamate content increases; treatment with AAPH and BSO alone aggravated the natural low excitability state of old RPeD1, but only the combination of AAPH + BSO affected electrical excitability of young RPeD1; et-GSH reversed this effect in young RPeD1; et-GSH and dithiothreitol treatment reversed age-associated low excitability of old RPeD1. Together, these data argue for a tight association between glutathione availability and the regulation of neuronal electrical excitability and indicate perturbation of cellular thiol-redox metabolism as a key factor in neuronal functional decline in this gastropod model of biological aging.

1. Introduction

Reactive oxygen and nitrogen species are an integral facet of both the life and demise of cells, tissues, and organisms. Under normal physiological conditions, they serve diverse cellular functions and their biological effects as general oxidizers are kept in check by an extensive array of renewable anti-oxidant defenses that include thiol redox buffers such as the tripeptide y-glutamylcysteinylglycine or glutathione (GSH) as one of its key assets. Many of the cells' building blocks, including peptides and lipids, contain oxidation sensitive elements. Therefore, alterations in cellular redox homeostasis may have far reaching implications for cell function and survival. In fact, oxidative stress, a condition arising from a shift in the global cellular redox balance away from its generally reducing state either because of excessive formation of pro-oxidants, failure of anti-oxidant defenses, or a combination of both, is associated with many (age-associated) disease states such as diabetes and neurodegenerative disorders including Alzheimer's and Parkinson's (Nakamura et al., 2012; Reddy, 2006; Sayre et al., 1997). Moreover, age-related reductions in neuronal excitability and long-term memory acquisition have been linked to elevated levels of lipid (per)-oxidation within the membrane (Watson et al., 2012a, 2013).

A growing body of evidence indicate that the aging brain, whether vertebrate or invertebrate, undergoes a number of physiological changes including a reduction in electrical excitability of its neurons (Burke and Barnes, 2006; Disterhoft and Oh, 2007; Hermann et al., 2007; Klaassen et al., 1998; Oh et al., 2010; Vanfleteren, 1993; Watson et al., 2012a, 2013). The cell biological and electrophysiological foundations of this phenomenon are not entirely clear, but increasing evidence points at involvement of redox-dependent mechanisms of neuronal excitability control (Joksovic et al., 2006; Randall et al., 2012; Ruppersberg et al., 1991; Watson et al., 2012a, 2013). This study focuses on the role of

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various aspects of GSH metabolism in neuronal excitability regulation.

GSH, by means of the thiol group of its cysteine residue, is capable of neutralizing a range of pro-oxidants. Because of its low redox potential and relatively high abundance, the GSH system generally acts as the primary redox buffering system in most cellular compartments. Its oxidation leads to the formation of glutathione disulfide (GSSG) that in turn then can be reduced back into GSH through the activity of glutathione reductase at a metabolic cost of 1 NADPH (Aoyama et al., 2008). Along with NADPH driven regeneration of GSSG, cellular GSH content can be increased by means of de novo synthesis through the γ- glutamyl cycle. GSH synthesis is a self-regulating process incorporating a negative feedback loop, whereby high levels of GSH will inhibit γ-glutamylcysteine synthetase (GCL) the enzyme catalyzing adenosine triphosphate (ATP)-dependent condensation of cysteine and glutamate into the dipeptide γ-glutamylcysteine (Fig. 1) (Chen et al., 2005). This condensation is the rate-limiting step in GSH synthesis and its perturbation has been shown to significantly reduce GSH availability (Genovese et al., 2007). Intriguingly, several studies report an age-related decline in GCL activity in both neuronal and nonneuronal cell types (Stio et al., 1994; Toroser and Sohal, 2005, 2007; Zhu et al., 2006).

In our studies we use the freshwater snail *Lymnaea stagnalis*, a widely used model system in investigations of diverse fundamental neurobiological problems including the study of neuronal aging (e.g., Arundell et al., 2006; Frolkis et al., 1995; Hermann et al., 2007; Janse et al., 1989; Klaassen et al., 1998; Watson et al., 2012a, 2012b, 2013; Wildering et al., 1991). To test the hypothesis that age-associated alterations in GSH metabolism is a factor in declining neuronal electrical excitability, we combined electrophysiological measurements of neuronal excitability of the identified neuron right pedal dorsal 1 (RPeD1), with measurements of GSH and pyroglutamate, an intermediate of GSH metabolism commonly associated with γ-glutamyl cycle perturbation (Njalsson and Norgren, 2005; Pederzolli et al., 2007; Ristoff and Larsson, 2002, 2007), and experimental manipulations of cellular redox states and GSH synthesis rates in brains of differently aged specimens of the pond snail *Lymnaea stagnalis*.

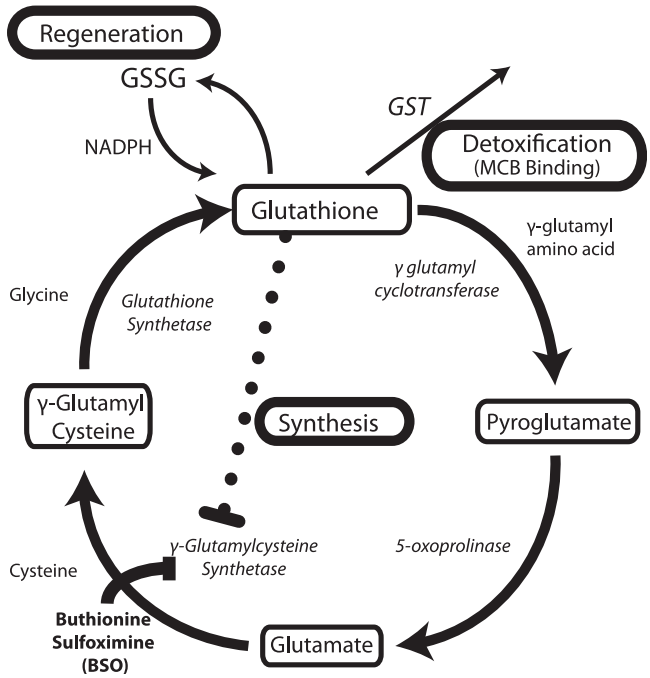

Fig. 1. Simplified glutathione metabolic pathways. Regeneration pathway: glutathione (GSH) donates reducing equivalents, which neutralizes cellular free radicals and causes the formation of glutathione disulfide (GSSG). GSSG can then be regenerated back into GSH through the actions of glutathione reductase at the cost of nicotinamide adenine dinucleotide phosphate (NADPH). The detoxification pathway occurs via the actions of glutathione-S-transferase and will consume GSH through a conjugation reaction. This process is used as a fluorometric means to evaluate GSH levels through the conjugation reaction with the molecular probe monochlorobimane (MCB). Synthesis pathway: GSH can be synthesized within the cytosol through the γ-Glutamyl cycle. The formation of γ-glutamylcysteine from glutamate and cysteine, which is catalyzed by the enzyme γ-glutamylcysteine synthetase (GCL), is the rate-determining step and is inhibited by high levels of GSH or pharmacologically through treatment with buthionine sulphoximine (BSO). A key component of this cycle is pyroglutamate, a metabolite known to build up with perturbation of the γ-glutamyl cycle. Abbreviations: BSO, buthionine sulphoximine; GCL, γ-glutamylcysteine synthetase; GSH, glutathione; GSSG, glutathione disulfide; MCB, monochlorobimane; NADPH, nicotinamide adenine dinucleotide phosphate.

2. Methods

2.1. Experimental animals—culture condition

Experimental animals used in this study were bred and housed in our own facility under previously described conditions (Hermann et al., 2007; Watson et al., 2012a, 2012b, 2013). Briefly, animals were kept under constant environmental conditions at a maximal density of 1.5 animals per liter and fed lettuce and Aquamax-carnivorous grower 400 trout pellets (Purina Mills LLC, St. Louis, MO, USA) *ad libitum*. Water used in the facility was purified through reverse osmosis and reconditioned to a conductivity of ~450 Ω cm via the addition of Instant Ocean salts (1 g per gallon; Aquarium Systems, Mentor, OH, USA). Water temperature ranged between 18 °C–19 °C. $CaCO_3$ was added in powder form and as sterilized cuttlefish bone to keep dissolved calcium at saturating levels.

2.2. Experimental animals—population survival analysis

Population survival characteristics were monitored and evaluated using a 2-parameter Weibull failure model as it has been previously described (Janse et al., 1988; Slob and Janse, 1988). All animals used in the study were sourced from healthy aging populations as defined on the basis of previously established Weibull parameter ranges. The 2 age cohorts used in this study were selected from populations with >99% population survival range (chronological age range 6–10 months) and <25% population survival (chronological age range 22–26 months). These 2 age groups will be referred to as "young" and "old" in the remainder of the text. Animals were taken at random from concurrent populations with

Table 1
Population survival characteristics. Parameter estimates of 2-parameter Weibull failure model fitted to the survival characteristics of *Lymnaea stagnalis* populations used in the study

Population	*a* (error)	*c* (error)	$s \bullet 10^{-2}$ (error)	% Survival at sampling	Age range (mo) at time of sampling
Young	580 (11.8)	3.7 (0.25)	-0.23 ($2.27 \bullet 10^{-2}$)	>95%	6–10
Old 1	615 (10.8)	3.5 (0.22)	-0.20 ($1.90 \bullet 10^{-2}$)	<25%	22–26
Old 2	620 (13.3)	4.0 (0.39)	-0.22 ($1.19 \bullet 10^{-2}$)	<25%	22–26

Note: "*a*" and "*c*" are parameters of the 2-parameter Weibull model ("*a*" is median age; "*c*" is the shape parameter; "*s*" is slope of tangent of survival curve at median age).