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Impact of Oxidative - Nitrosative Stress on Cholinergic Presynaptic Function

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1. Introduction

Cholinergic neurotransmission plays an essential role in a variety of physiological processes in both the central and peripheral nervous systems. Cholinergic neurons use the classical neurotransmitter acetylcholine (ACh) to communicate with their target cells. In the periphery, ACh is the neurotransmitter used at the skeletal neuromuscular junction, at all pre- and postganglionic parasympathetic synapses and at preganglionic sympathetic synapses. In the central nervous system, the actions of ACh are widespread with cholinergic neurotransmission involved in attention, learning and memory, cognition, sleep, wakefulness, and modulation of sensory information (Hasselmo, 2006; Sarter & Parihk, 2005; Woolf & Butcher, 2010). Dysfunction of cholinergic neurotransmission in the central nervous system is apparent in a number of neurological disorders, such as Alzheimer's, Parkinson's, and Huntington's diseases, schizophrenia and amyotrophic lateral sclerosis (Bohnen & Albin, 2010; Mesulam, 2004; Oda, 1999).

Cholinergic neurons innervate almost all areas of the brain, where this can be mediated by either intrinsic interneurons or by extrinsic projection neurons. Cholinergic interneurons localized in the striatum are involved in motor function, cognition, and behavior (Woolf & Butcher, 2010). The basal forebrain, which is comprised of the nucleus basalis of Meynert, medial septum, diagonal band of Broca, the magnocellular preoptic nucleus, and substantia innominata, contains the cell bodies of cholinergic neurons that project to the hippocampus, amygdala, olfactory bulb, and all areas of the cerebral cortex (Woolf & Butcher, 2010). Collectively, basal forebrain cholinergic neuron activity plays a role in attention, learning, memory, perception, and consciousness (Sarter et al., 2003; Woolf, 1998; Woolf & Butcher, 2010). Cholinergic neurons in the mesopontine region (the pedunculopontine and laterodorsal nuclei) project to the thalamus, hypothalamus, basal forebrain, medial frontal cortex, brainstem and spinal cord (Woolf & Butcher, 2010). Descending cholinergic projections from the mesopontine area decrease muscle tone during rapid eye movement sleep while ascending cholinergic projections are involved in cognitive functions and consciousness (Woolf & Butcher, 2010). Cholinergic projections to the interpeduncular nucleus originate from neurons with cell bodies in the medial habenula; these neurons regulate electroencephalogram patterns and rapid eye movement sleep (Woolf & Butcher, 2010).

The cycle of ACh synthesis, storage, release and degradation has been well-characterized at the cellular and molecular levels and is depicted in Figure 1. ACh is synthesized in the

cytoplasm of cholinergic neurons from the precursors choline and acetylCoenzyme-A by the enzyme choline acetyltransferase (ChAT), and it is then taken up into synaptic vesicles for storage by the vesicular acetylcholine transporter (VAChT) (Prado et al., 2002). Depolarization of the nerve terminal causes exocytotic fusion of synaptic vesicles with the presynaptic membrane at specialized release sites called active zones (Garner et al., 2000); this is a calcium-dependent process that involves the coordinated actions of many presynaptic proteins such as SNARE and Rab proteins (Sudhof, 2008). When vesicles fuse with the presynaptic membrane, ACh diffuses into the synaptic cleft where it can bind to nicotinic and muscarinic receptors located on both pre- and postsynaptic cells (Gotti et al., 2009; Nathanson, 2008). ACh signalling is terminated by its diffusion away from the synaptic cleft and by its rapid hydrolysis into choline and acetate by acetylcholinesterase (AChE) (Lane et al., 2006). After fusion, synaptic vesicles recycle (S.M. Smith et al., 2008) and are re-filled with neurotransmitter in preparation for another round of depolarization-induced release. The choline derived from ACh hydrolysis is recycled into the presynaptic terminal by the sodium-dependent, high-affinity choline transporter CHT for re-synthesis of ACh (Birks & MacIntosh, 1961; Collier & MacIntosh, 1969; Collier & Katz, 1974; Haga, 1971; Okuda et al., 2000; Yamamura & Snyder, 1972). Mechanistic details of the molecular regulation of these processes in both health and disease are lacking.

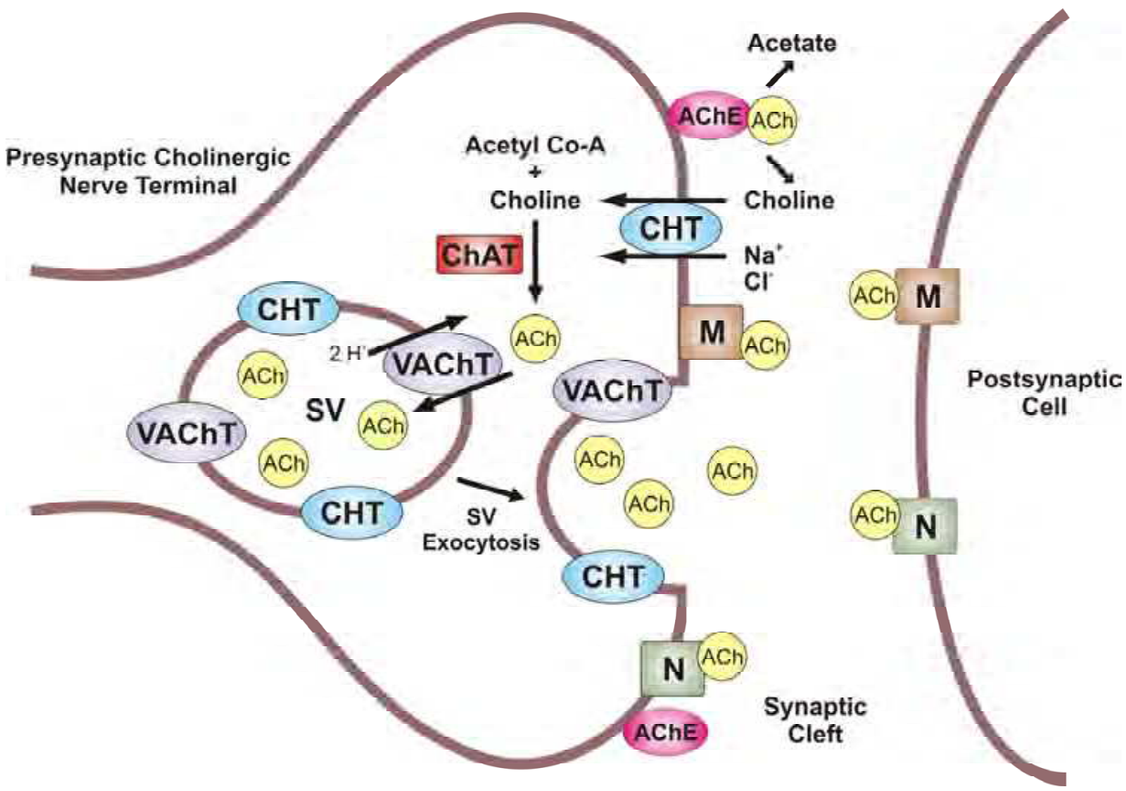


Fig. 1. Mechanisms involved in the synthesis, storage, release and degradation of ACh at the cholinergic synapse. Abbreviations: Acetyl Co-A, acetylCoenzyme-A; ACh, acetylcholine; AChE, acetylcholinesterase; ChAT, choline acetyltransferase; CHT, sodium-dependent, high-affinity choline transporter; M, muscarinic receptor; N, nicotinic receptor; SV, synaptic vesicle; VAChT, vesicular ACh transporter.

Age-related changes in brain that lead to neuronal and vascular pathology frequently compromise synaptic transmission. Many reports associate neurological and cardiovascular diseases with cholinergic neuron dysfunction, and draw parallels between the reduction in cholinergic neurotransmission and cognitive deficits. However, little is known about the actual mechanisms by which these changes impact cholinergic neuron function or the extent to which this can be prevented or reversed. Some of these changes are initiated by oxidative and nitrosative stress which then increases as the pathology progresses. There is also a relationship with altered amyloid precursor protein (APP) metabolism and β-amyloid peptide (Aβ) production that is likely due to changes in the expression or activity of enzymes directing APP into an amyloidogenic pathway. Modified APP/Aβ metabolism is a hallmark of Alzheimer's disease, but is also a result of cardiovascular disease including ischemia (Bennett et al., 2000; Lee et al., 2006; Nihashi et al., 2001; Shi et al., 2000), stroke (Petcu et al., 2008) and hypertension (Gentile et al., 2009; Skoog et al., 1996; Skoog et al., 2006). Age-related increases in soluble oligomeric Aβ acutely modulate synaptic communication at concentrations far below those causing neurodegeneration, and play a central role in Alzheimer's-related synaptic changes (Marcello et al., 2008). The tissue stress response and the generation of reactive oxygen (ROS) and nitrogen species (RNS) in aging brain and early stages of vascular and neuronal disease are attributed partly to increasing Aβ (De Felice et al., 2007). Increased ROS-RNS can initiate changes that result in both reversible and irreversible alterations in protein structure and function. This Chapter focuses on studies of ChAT and CHT structure and function in conditions with altered exposure to ROS-RNS, and the consequences for cholinergic neuron communication related to Alzheimer's disease pathology and potential outcomes related to neuroprotection.

2. Cholinergic neuron function in aging brain

A large literature addresses the role of cholinergic neurons in cognition and attentional processes, and their involvement in Alzheimer's disease (Sarter & Bruno, 1997; Sarter & Bruno, 2004; Schliebs & Arendt, 2006). The current opinion is that while dysfunction of cholinergic neurons is not the cause of this disease, loss of their function is central to changes that occur in brain with normal aging and in a spectrum of disorders that includes mild cognitive impairment (MCI) (Mesulam, 2004). Studies in aging humans and animal models show that some cholinergic pathways have diminished function compared to younger groups. Cognitive decline correlates with elevated oxidative stress in a cohort of normal aging human subjects (Foster, 2006). Also, in aging rats, spatial learning deficits are associated with elevated markers of oxidative stress (Nicolle et al., 2001). However, the small loss of forebrain cholinergic neurons does not relate strongly to cognitive status (McKinney & Jacksonville, 2005) providing compelling evidence that other dynamic cholinergic processes must be affected that are not detected simply by counting the number of neurons.

A comparison of two cholinergic pathways in aged rat brain suggests that neurons in different brain areas can respond differentially to age-related stressors. Basal forebrain cholinergic neurons lose the activity or expression of essential proteins that maintain chemical transmission and their numbers are reduced, whereas brainstem cholinergic neurons in the pontine nuclei are preserved (Baskerville et al., 2006). This is reinforced by the observation that cultured pontine neurons are resistant to ROS-RNS whereas basal forebrain cholinergic neurons are vulnerable (Fass et al., 2000; McKinney et al., 2004). The reasons for this are not known, but age-related oxidative stress in the cortex (Nicolle et al.,

2001) may elevate basal forebrain neuron metabolism. Oxidatively-stressed aged cortex is less responsive to ACh and may compensate for this by requiring enhanced input from basal forebrain neurons thereby causing increased neuron firing rates and/or changes such as increased expression of genes that are involved in energy production (Baskerville et al., 2008; Ongwijitwat et al., 2006; Yang et al., 2006). Alternatively, up-regulation of expression of metabolic genes may be normal and an "adaptive" consequence of aging in basal forebrain neurons that precedes their degeneration (Baskerville et al., 2008). Elevated metabolic activity may also precede increased ROS-RNS levels and, along with pathology such as increased Aβ levels, may mediate their selective vulnerability. It is important to note that basal forebrain cholinergic neurons project to regions with Aβ deposits, whereas pontine cholinergic neurons do not.

A shift in thinking about the timing and nature of changes in cholinergic neuron function in aging and disease came about with reports that ChAT activity is unchanged or even increased in the hippocampus and cortex of subjects with MCI (DeKosky et al., 2002; Ikonomovic et al., 2003; Mufson et al., 2003). This is in sharp contrast to reports of large decreases in cholinergic neuron markers and numbers in Alzheimer's disease (Davies, 1979; Francis et al., 1985; Perry et al., 1977; Rylett et al., 1982; Sims et al., 1983). Mechanisms underlying this apparent increase in cholinergic neuron function are unknown, but it was suggested that basal forebrain cholinergic neurons undergo sprouting in an attempt to maintain neurotransmission or to repair entorhinal cortex damage (Ikonomovic et al., 2003). This has stimulated studies on the functional status of cholinergic neurons in MCI and early Alzheimer's disease; a pivotal study used pharmacological-functional magnetic resonance imaging (fMRI) to document that cholinergic neurotransmission is compromised (Goekoop et al., 2006). It is important to note that most of the studies reported to date have monitored postsynaptic events for insight into the status of cholinergic neurotransmission and to reveal alterations that would result in decreased cholinergic synaptic function and neuron responsiveness in MCI and Alzheimer's disease (Grön et al., 2006). There is a large gap in knowledge about the effects that perturbations such as ROS-RNS, Aβ or altered antioxidant defense mechanisms have on cholinergic presynaptic functions, and the role that this has in compromised neurotransmission. Moreover, most studies have assayed static measures such as neuron numbers or enzyme levels that do not accurately reflect the more dynamic aspects of neurotransmission (Mesulam, 2004), and this may give an incorrect assessment as they do not reveal changes in the capacity for neurotransmitter synthesis and release.

3. Cholinergic neurons have a role in regulation of APP metabolism

A critical link has emerged between cholinergic neurons and APP processing. These neurons are particularly susceptible to the adverse effects of Aβ, and this may partially underlie their vulnerability in amyloidogenic diseases (Auld et al., 1998; Tran el al., 2002). Of note however, cholinergic neurons are also integral in the development of amyloid-based pathology (Auld et al., 1998; Francis et al., 1999; Tran et al., 2002) as they can regulate APP processing and, in turn, Aβ can decrease ACh synthesis and release (Heinitz et al., 2006; Hoshi et al., 1996, 1997; Kar et al., 1996, 1998; Pederson et al., 1996; Pederson & Blusztajn, 1997; Satoh et al., 2001). The stimulation of either nicotinic or muscarinic ACh receptors on cholinoreceptive neurons can promote non-amyloidogenic APP cleavage, thereby potentially decreasing the production of toxic Aβ (Isacson et al., 2002; Seo et al., 2001; Tran et

al., 2002; Unger et al., 2005; Verhoeff, 2005). Thus, the activation of either α7- or α4β2-nicotinic receptors (Lahiri et al., 2002; Shimohama & Kihara, 2001; Zamani & Allen, 2001) can decrease Aβ toxicity by protecting neurons through activation of PI3-kinase and increasing levels of Bcl-2 and Bcl-x50. Taken together, the loss of cholinergic neuron innervation may promote Aβ-based pathology, and therapies that would enable cholinergic neurotransmission may indirectly decrease neurodegeneration by reducing Aβ production.

Aβ can assume various physical conformations that may differ in their biological actions. These various Aβ peptides are produced both intracellularly and extracellularly and appear in brain tissue as soluble oligomers and diffusible ligands (Gong et al., 2003; Klein, 2002; Klein et al., 2004) that can aggregate to form insoluble plaques (Kuo et al., 1996; Lue et al., 1999; Walsh et al., 2002a, 2002b; J. Wang et al., 1999). The increased levels of soluble Aβ correlate with synaptic loss and cognitive impairment, and it is now known that synaptic neurotransmission is impacted directly by soluble Aβ with this leading to decreased memory (Klein, 2002). Aβ can bind to a number of different cell surface receptors, modulate synaptic events, lead to generation of ROS-RNS or the release of inflammatory mediators, and disrupt cellular calcium homeostasis (W.W. Smith et al., 2006; Tran et al., 2002). In animal models having chronic delivery of Aβ into rat brain there is increased iNOS expression and disruption of hippocampal cholinergic transmission and memory impairment (Tran et al., 2001).

4. Aβ promotes oxidative stress that can affect neuron function

The inside of cells is normally a reducing environment that maintains proteins in their native reduced states. However, even under these "reducing" conditions some proteins do exist in their *S*-glutathionylated forms (Klatt & Lamas, 2000). Moreover, if ROS-RNS generation exceeds the antioxidant capacity of the cell, then oxidative-nitrosative stress can occur with modification of cellular constituents causing loss of their function. Aβ, particularly the toxic Aβ(1-42), can be involved both directly (Butterfield & Bush, 2004) and indirectly (Lahiri & Greig, 2004) in the generation of ROS-RNS in the brain, thus causing oxidation of lipids and proteins (Qi et al., 2005) and nitration of tyrosine residues in proteins. As it is critical that the oxidative balance inside of the cell is tightly regulated, compensatory mechanisms become upregulated in normal cells that have been exposed to oxidant stress. These adaptive processes are found in the brain, but they can become dysregulated during aging (Mattson & Magnus, 2006) and in pathological states (Mattson, 2004; Zhu et al., 2004). Mitochondria are a source of hydrogen peroxide (H_2O_2), and Aβ in the presence of redox-active ions such as iron and copper can lead to the generation of excess H_2O_2 (Pedersen & Blusztajn, 1997). This ROS may induce cellular damage, particularly by the generation of highly-reactive superoxide radical anion ($\bullet O2^-$) or hydroxyl radical ($\bullet OH$), the latter being formed in the Fenton reaction in the presence of redox-active iron. However, under some conditions protective mechanisms such as the activation of stress-activated protein kinase (SAPK) pathways are engaged with the induction of downstream antioxidant enzymes (Tamagno et al., 2003). Thus, Aβ alone or in combination with other constituents can lead to the generation of ROS-RNS that can damage macromolecules and result in the modulation of neurotransmission and cause neurotoxicity.

Cholinergic presynaptic function is affected by low levels of Aβ (pM-nM), with the result being decreased pyruvate dehydrogenase activity (Hoshi et al., 1996, 1997), ACh synthesis (Hoshi et al., 1996, 1997; Pederson et al., 1996; Pederson & Blusztajn, 1997) and release (Heinitz et al., 2006; Kar et al., 1996, 1998; Satoh et al., 2001). However, there are conflicting reports on the effects of Aβ on the functions of both ChAT and CHT proteins. Hippocampal ChAT activity is decreased after several days of Aβ administration into rat brain (Nitta et al., 1994, 1997), but this may be due to the loss of neurons rather than to a direct effect on the enzyme. The acute addition of Aβ to neural cells can either reduce ChAT activity (McMillian et al., 1995; Pederson et al., 1996) or have no effect (Hoshi et al., 1996, 1997; Kar et al., 1998); it is difficult to interpret these studies as the Aβ conformation was not determined and it is increasingly clear that the oligomeric, fibrillar or other forms of Aβ have quite different cellular effects. Experiments that involve the use of a variety of model systems indicate that the high-affinity choline uptake activity of CHT can be modulated by Aβ, but the results are also variable. Some reports show that Aβ can actually increase high-affinity choline uptake activity (Bales et al., 2006; Kristofikova et al., 2006), whereas others suggest that Aβ impairs choline uptake (Apelt et al., 2002; Kar et al., 1998; Klingner et al., 2003; Kristofikova et al., 2001, 2008; Opazo et al., 2006; Payette et al., 2007) or that Aβ has no effect on high-affinity uptake of choline (Forgon et al., 1998; Hartmann et al., 2004; Melo et al., 2002). It is known that cholinergic neurochemical function is affected directly by Aβ in a manner that is not related to neuron degeneration, but it is not known to what extent these effects are due to the oxidant stress engaged by increasing Aβ. Unfortunately, for the most part these results are descriptive in nature rather than being mechanistic and do not offer insight into the potential reversibility of the functional changes or information that would be useful for the development of approaches to support cholinergic neuron function.

5. ROS – RNS cause reversible and irreversible modification of proteins

Reactive cysteine (Cys) thiol groups are ionized at physiological pH, and proteins having reactive Cys thiols are found in cells in multiple forms as they are modified by ROS and RNS or form homo- and hetero-protein complexes. Figure 2 shows common modifications that reactive Cys thiols can undergo using ChAT as the model because it is an outstanding example of a protein that has an unusually high number of Cys residues. The modification of reactive Cys thiols during oxidative or nitrosative stress is a key regulator of protein activity (Klatt & Lamas, 2000), and can result in either loss- or gain-of-function (Barrett et al., 1999; Borges et al., 2002; Fukuda et al., 2005; Humphries et al., 2002; Ishii et al., 2005; Jaffrey et al., 2001; Lind et al., 2002; J. Wang et al., 2001). There are cellular mechanisms that protect proteins from irreversible inactivation by oxidation that involve the reversible addition of antioxidant peptides, particularly reduced glutathione (GSH) (Townsend, 2007). The formation of protein-GSH mixed disulphide conjugates can protect vulnerable thiol groups in proteins from further damage during transient oxidative stress (Borges et al., 2002; Jaffreyet al., 2001). A critical point is that protein-GSH mixed disulphide formation is reversible, with the native protein being regenerated as the interior of the cell returns to a more reduced state. However, the more highly oxidized forms of proteins, sulphinic ($Pr\text{-}SO_2H$) and sulphonic ($Pr\text{-}SO_3H$) acids, are not reduced in cells with this leading to irreversible loss-of-function of the target protein. Brain and other tissues can take a double

hit during aging by increased ROS–RNS levels and decreased antioxidant mechanisms, with this characterized by a decreased ratio of reduced to oxidized GSH [GSH:GSSG]. This is illustrated in necropsy brain fractions from subjects that were neurologically normal or that had MCI or Alzheimer's disease. The effects of oxidative stress were localized mostly to synaptic regions, with the largest changes seen in presynaptic [synaptosome] fractions as significant decreases in antioxidant levels and increases in markers of oxidative damage (Ansari & Scheff, 2010). These changes occurred early in the disease and the free radical burden increased as an active persistent process in aging and disease (Ansari & Scheff, 2010). Similar findings are seen in aging rodents (Gilmer et al., 2010).

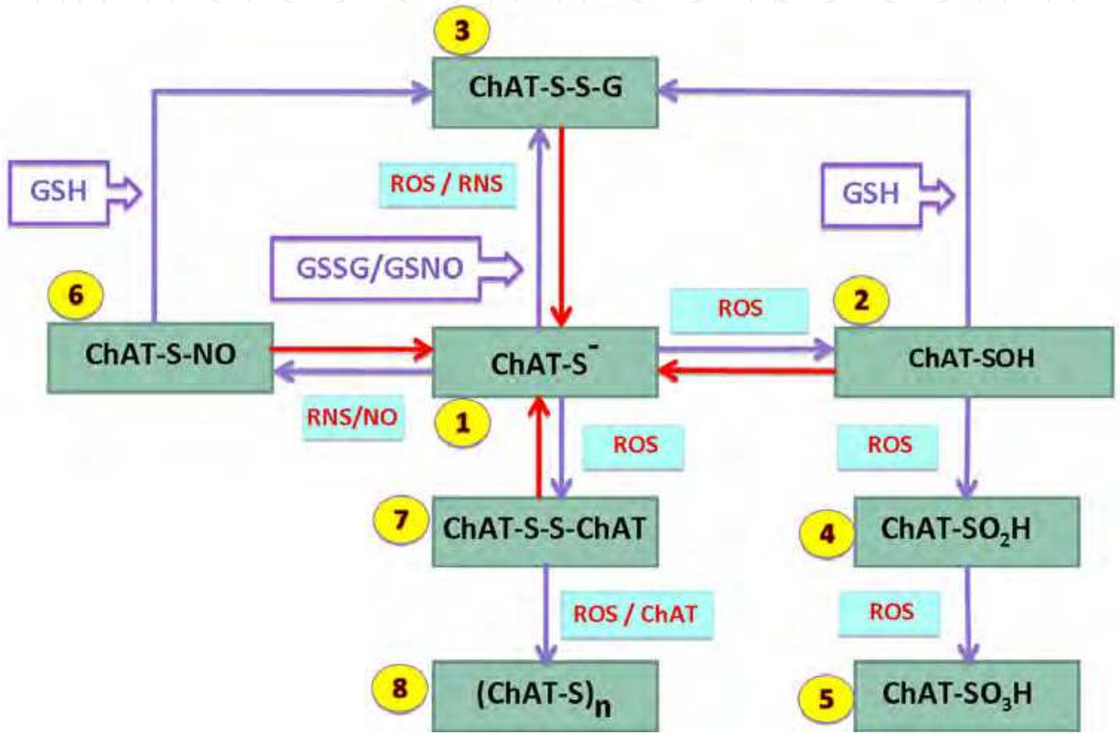


Fig. 2. ROS–RNS can cause reversible and irreversible modification of proteins at reactive cysteine thiol residues. The common modifications that reactive Cys thiols can undergo are shown using ChAT as an example to illustrate that the protein can be found in cells in multiple forms that are modified by ROS and RNS with the potential formation of homo- and hetero-protein complexes. Unmodified ChAT-S$^-$ (1) is oxidized by low levels of H_2O_2 to its sulphenic acid, ChAT-S-OH (2); ChAT-S-OH reacts non-enzymatically with glutathione (GSH) to form ChAT-GSH mixed disulphide, ChAT-S-S-G (3) that protects Cys from further oxidation. ChAT-S-OH (2) is further oxidized by excess ROS to inactive sulphinic ChAT-SO_2H (4) and sulphonic ChAT-SO_3H (5) acids when GSH is depleted. Also, ChAT-S$^-$ (1) can react with NO to form *S*-nitrosylated ChAT, ChAT-S-NO (6) by reaction at reactive Cys thiols. ChAT-S-NO (6) can also react with GSH to give ChAT-S-S-G (3). Alternatively, ChAT-S$^-$ (1) can be converted to ChAT-S-S-G (3) by reaction with *S*-nitrosoglutathione (GSNO). In the absence of GSH, ChAT-S$^-$ (1) can be converted by ROS to intermolecular dimers ChAT-S-S-ChAT (7) or higher-order oligomers (8) that may precipitate from the cytoplasm. [*Adapted from Giustarini et al., 2004*].

6. Effects of oxidative and nitrosative stress alter ChAT activity

It is striking that ChAT contains twenty Cys residues, which is a substantially greater proportion of Cys (3.2%) than is normally found in intracellular proteins (~ 1%). This could make ChAT particularly vulnerable to oxidative-nitrosative stress. Indeed, ChAT activity is decreased by acute exposure of either the purified protein (Guermonprez et al., 2001; Kim et al., 2006; Morris, 1967; Roskoski, 1973) or brain fractions or cells (Liu et al., 1999) to ROS-RNS agents, with some protection being given by the addition of thioreductants (Liu et al., 1999; Morris, 1967). We found that the activity of purified human ChAT is rapidly lost in the absence of reducing agents and that its activity is partially recovered by the addition of fresh reducing agents (Kim et al., 2005). Nitric oxide (NO) donors can also inactivate ChAT with this effect being partially reversed by GSH (Liu et al., 1999). A common site for post-translational modification and redox regulation of proteins is at reactive Cys thiols with this leading to protein *S*-glutathionylation or *S*-nitrosylation. Reactive Cys thiols ($Cys\text{-}S^-$) are ionized at physiological pH and are more easily oxidized than are fully-reduced Cys (Cys-SH). ROS-RNS could also cause both reversible and irreversible changes to ChAT resulting in it having altered structure and function. The functional consequences of the oxidation of reactive Cys thiols in ChAT could include regulation or inactivation of catalysis, homo- or hetero-dimerization or oligomerization by disulphide bond formation, altered binding to cellular protein partners, or altered protein stability.

Structural reorganization of a protein can also serve to protect vulnerable reversibly-oxidized Cys residues during acute oxidative stress by the formation of intramolecular disulphide bonds (Cumming & Schubert, 2005). An example of this is seen in protein tyrosine phosphatases where a reactive catalytic Cys residue undergoes reversible oxidation to sulphenic acid, and then participates in the formation of an intramolecular disulphide bond with one of two "backdoor" Cys residues in the catalytic site to protect it from further oxidation to irreversible sulphinic or sulphonic acid species (Chen et al., 2009). The disulphide bond formed is resistant to further oxidation by low levels of ROS and can be re-reduced by GSH. Re-reduction of the catalytic Cys thiolate drives oxidation of the two backdoor Cys causing them to form a disulphide and facilitates regeneration of the active phosphatase. This model is significant with regard to ChAT as there are five Cys residues in its catalytic site, and at least four of these are arranged in a configuration that disulphide bonds may be able to form with only minor changes in side chain torsion angles (Kim et al., 2006).

7. Subcellular trafficking of CHT proteins, an important mode of regulation of high-affinity choline uptake, is altered by nitrosative stress

In many experimental paradigms, high-affinity choline uptake by CHT proteins is the rate-limiting step in the production of ACh, and it is modulated by neuronal activity and dependent upon the sodium electrochemical gradient (Birks & MacIntosh, 1961; Guyenet et al., 1973; Haga, 1971; Simon & Kuhar, 1975; Yamamura & Snyder, 1972). With molecular tools becoming available since the cloning of the CHT gene in the year 2000 (Okuda et al., 2000), the importance of the subcellular trafficking of CHT proteins as a regulatory mechanism for choline uptake, and thus for ACh production, has emerged. CHT proteins are distributed between the plasma membrane and subcellular compartments such as endosomes and synaptic vesicles (Ferguson et al., 2003; Nakata et al., 2004; Ribeiro et al., 2003). It was discovered that CHT proteins are internalized from the plasma membrane to

endocytic vesicles via clathrin-mediated endocytosis (Ribeiro et al., 2003), a process that is dependent on a dileucine-like internalization motif located in the cytosolic carboxyl-terminal tail of CHT (Ribeiro et al., 2005); dileucine internalization motifs interact with the adaptor protein 2 complex of the clathrin-mediated endocytosis machinery (Traub, 2009). A subsequent study showed that internalized CHT undergoes constitutive recycling to the plasma membrane; importantly, potassium-mediated depolarization increases the rate of CHT recycling in neural cells, but not in non-neural cells (Ribeiro et al., 2007b). In addition, a recent report indicates that preventing CHT entry into the endosomal compartment using a selective endosomal ablation strategy decreases both CHT levels at the cell surface and choline uptake activity in a time-dependent manner (Ivy et al., 2010).

Since subcellular trafficking of CHT proteins is such a crucial mode of regulation of high-affinity choline uptake, it is important to address the question of how oxidative/nitrosative stress affects the proteins involved in vesicle trafficking. It has been demonstrated by a number of groups that oxidative stress perturbs the dynamic endocytosis of cell surface proteins such as receptors and transporters, with this having key roles in regulating their trafficking and activity. This may involve either the modification of specific amino acids in cargo proteins or be related to changes in the interaction of these receptors or transporters with other cellular proteins involved in their trafficking. For example, the dopamine transporter is inactivated by the RNS peroxynitrite by an action on a Cys residue in its third intracellular loop, with this resulting in toxicity to dopamine neurons (Park et al., 2002). This is prevented, but not reversed, by GSH and some reducing agents. Other studies reveal that the effects of ROS-RNS agents on cell surface proteins can be mediated indirectly by an action on components of the protein trafficking machinery, and this can likely involve changes in both clathrin-mediated and clathrin-independent pathways (Ozawa et al., 2008). Interestingly, this does not always involve inhibition of function of the protein; NO can regulate endocytosis by *S*-nitrosylation of dynamin which then affects endocytic vesicle budding thus facilitating internalization of some membrane proteins (G. Wang et al., 2006). This gain-of-function example illustrates the physiological role for low levels of NO. It has also been demonstrated that peroxynitrite stimulates synaptic vesicle exocytosis and induces the nitration of tyrosine residues in SNARE complex proteins (Di Stasi et al., 2002). However, another recent report showed that the fusion of synaptic vesicles with the presynaptic plasma membrane is impaired by oxidative stress (Arai et al., 2011).

Guermonprez and coworkers (2001) determined that peroxynitrite decreases CHT activity in synaptosomes from *Torpedo marmorata*. We have undertaken studies to identify the mechanisms by which oxidative-nitrosative stress alter choline uptake activity thereby interfering with cholinergic neurotransmission. Thus, we found that peroxynitrite causes a rapid, dose-dependent inhibition of CHT activity that is attenuated specifically by scavengers of peroxynitrite (Pinthong et al., 2008). Other oxidants such as H_2O_2 have no effect on CHT activity (Guermonprez et al., 2001; Pinthong et al., 2008). Importantly, doses of SIN-1, a peroxynitrite-generating molecule, that significantly decrease CHT activity do not compromise membrane integrity or alter cellular membrane potential (Pinthong et al., 2008). The SIN-1-induced decreases in choline uptake activity correlate with decreased CHT cell surface levels that result from accelerated endocytosis of CHT proteins by a clathrin-dependent mechanism (Pinthong et al., 2008).

Based on the important role that neuronal activity has in regulating the levels of CHT that are available at the plasma membrane to take up choline as substrate for ACh synthesis, it is predicted that the reduction in synaptic efficacy that is associated with elevated oligomeric

Aβ levels (Selkoe, 2002) would decrease the movement of CHT to the cell surface as constituents of synaptic vesicles. Thus, under conditions of oxidative-nitrosative stress, this effect of reduced synaptic efficacy associated with increasing oligomeric Aβ could further negatively impact CHT protein levels at the plasma membrane. This in turn would lead to diminished cholinergic signalling, a process found to be crucial in maintaining the cholinergic phenotype and regulating APP metabolism (Isacson et al., 2002; Seo et al., 2001; Tran et al., 2002; Unger et al., 2005; Verhoeff, 2005).

8. ROS-RNS may affect post-translational modifications and protein-protein interactions that regulate CHT and ChAT activities

Post-translational modifications can greatly influence both the function and subcellular localization of proteins. Phosphorylation, the reversible addition of a phosphate group to serine, threonine or tyrosine residues, is an important signalling event that has been shown to regulate both ChAT and CHT proteins. The activities of both ChAT and CHT are altered by treatments that modulate protein kinases or change the levels of protein phosphorylation in the cell (Black et al., 2010; Breer & Knipper, 1990; Cancela et al., 1995; Cooke & Rylett, 1997; Dobransky & Rylett, 2005; Ford et al., 1999; Gates et al., 2004; Guermonprez et al., 2002; Issa et al., 1996; Ivy et al., 2001; Knipper et al., 1992; Vogelsberg et al., 1997). Both ChAT and CHT proteins are phosphorylated in neural cells (Bruce & Hersh 1989; Dobransky et al., 2000; Gates et al., 2004; Habert et al. 1992; Schmidt & Rylett 1993). ChAT is a substrate for protein kinase C (PKC), casein kinase 2 and calcium/calmodulin-dependent kinase II (Dobransky et al., 2000), and CHT activity is modulated by activation of PKC (Black et al., 2010; Gates et al., 2004). Thus, pathological changes in protein kinase levels or functions could affect the functions of ChAT and/or CHT. For example, diminished PKC protein levels and signalling are seen in Alzheimer's disease (Alkon et al., 2007). However, ROS can lead to enhanced PKC activity (Zhao et al., 2011). Since PKC plays an important role in regulation of both ChAT and CHT function, it will be interesting to determine how oxidative stress alters phosphorylation-dependent regulation of ChAT, CHT and ACh synthesis, and the role that this plays in the failure of cholinergic neurotransmission in Alzheimer's disease and related disorders.

Protein-protein interactions are another molecular mode of regulation of protein function and/or subcellular localization, and some protein binding partners for ChAT and CHT have now been identified (Bales et al., 2006; Dobransky et al. 2003; Ribeiro et al., 2007a; B. Wang et al., 2007; Xie & Guo, 2004). In general, the interaction of CHT with other cellular proteins regulates it subcellular distribution. Notably, it has been determined that CHT interacts with the carboxyl-terminus of amyloid precursor protein (APP) family members (B. Wang et al., 2007) and with Aβ peptide (Bales et al., 2006). Mice lacking both APP and APP-like protein 2 have reduced levels of CHT protein at nerve terminals and this is seen as decreased high-affinity choline uptake activity; APP appears to be a modulator of both presynaptic localization and endocytosis of CHT proteins (B. Wang et al., 2007). Recent data indicate that modulation of APP metabolism or processing is an early cellular response to oxidative stress (Recuero et al., 2010). Based on the intimate relationship between oxidative/nitrosative stress and Aβ production, ROS-RNS generation could impact CHT activity by altering the interactions of this transporter with APP or Aβ. However, the mechanisms by which oxidative/nitrosative stress directly affects the interactions of ChAT and CHT with their protein binding partners have not been investigated.

9. Conclusion

The regulation of cellular proteins related to their reactions with ROS and RNS are described by the nitroso- and disulphide proteomes, and this has emerged as a critical modulator of protein function in both physiological and pathological situations (Ghezzi & Benetto, 2003; Lopez-Sanchez et al., 2009; Torta et al., 2008; Yano et al., 2002). A crucial point is that cellular mechanisms of limited capacity are normally in place, such as the formation of protein mixed-disulphides with glutathione (GSH), that transiently protect proteins from detrimental effects of ROS-RNS until redox balance is restored in the cell. *S*-glutathionylation at critical reactive Cys residues may even result in a gain-of-function for some proteins. However, during aging and disease, GSH levels are decreased and this can lead to irreversible oxidation and loss-of-function of vulnerable proteins. Investigations that are combining a range of proteomic, cell biology and *in vivo* experimental approaches have begun to characterize the dynamic responses of cholinergic neurons to the changes in brain that are induced by oxidative-nitrosative stress. A critical outcome of these studies is the identification of conditions that determine if the structural and functional modifications to ChAT and CHT that occur with tissue stress have the potential to be reversible or whether they are persistent. Future studies should focus on defining changes that are consistent with healthy and successful brain aging compared to changes that are associated with the onset of age-related disorders such as Alzheimer's disease. This will give insight into changes that are potentially reversible and that are amenable to intervention, and assist with the identification of therapeutic targets for protection of these proteins in normal aging and in diseases that may involve Aβ-induced / oxidative stress-induced changes.

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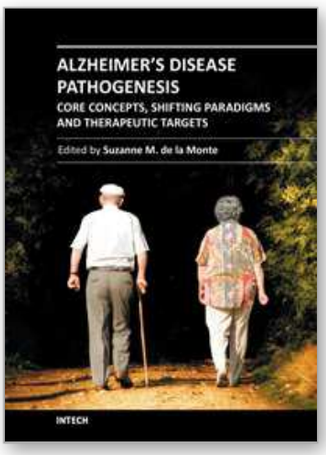
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Alzheimer's Disease Pathogenesis: Core Concepts, Shifting Paradigms, and Therapeutic Targets, delivers the concepts embodied within its title. This exciting book presents the full array of theories about the causes of Alzheimer's, including fresh concepts that have gained ground among both professionals and the lay public. Acknowledged experts provide highly informative yet critical reviews of the factors that most likely contribute to Alzheimer's, including genetics, metabolic deficiencies, oxidative stress, and possibly environmental exposures. Evidence that Alzheimer's resembles a brain form of diabetes is discussed from different perspectives, ranging from disease mechanisms to therapeutics. This book is further energized by discussions of how neurotransmitter deficits, neuro-inflammation, and oxidative stress impair neuronal plasticity and contribute to Alzheimer's neurodegeneration. The diversity of topics presented in just the right depth will interest clinicians and researchers alike. This book inspires confidence that effective treatments could be developed based upon the expanding list of potential therapeutic targets.

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