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Alzheimer's Disease: Making Sense of The Stress

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ABSTRACT

To facilitate a deep understanding of the mechanisms involved in neurodegeneration and Alzheimer's disease fundamental knowledge is required about the action and function of enzymes in the brain that not only metabolise arginine (neuronal nitric oxide synthase) but are closely associated with oxidative (superoxide dismutase; catalase; glutathione peroxidase) and/or nitrosative stress. In particular the focus extends towards enzymes that contribute to amyloid peptide aggregation and senile plaquedeposits (fibrillogenesis). Of special importance are the glycine zipper regions within these amyloid peptides, especially $A\beta_{25-29}$ and $A\beta_{29-33}$ (that contains two isoleucine residues) and the pentapeptide $A\beta_{17-21}$ (that contains two phenylalanines), each generated by enzymatic cleavage of the intramembrane amyloid precursor protein. Use of antisense-sense technology has identified regions in each enzyme that are capable of binding with the amyloid peptides. After an initial inhibition of each enzyme there is an oligomerisation into soluble fibrils which accumulate and eventually precipitate. The use of nanoparticles do not just prevent but reverse the formation of these fibrils either by disrupting the binary adduct –enzyme-Aβ-peptide- or by reaction with, and therefore deplete, Aβ-monomers in solution and so block potential aggregation sites on the enzyme itself. Future therapy towards Alzheimer's disease should target the C-terminal region of the amyloid precursor protein and substitute hydrophobic residues for the glycine amino acids within the glycine zipper region.

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FIBRILLOGENESIS

Even though there is overwhelming evidence of deposits of hyper phosphorylated tau proteins, neuro fibrillary tangles and aggregated β-amyloid (Aβ) senile plaques in the astrocytes (neuroglial cells) of the human brain as being classic towards neuropathology of Alzheimer's Disease **(AD)** [1-4] limited information is available on their formation and/or participation. This progressively fatal disease, of growing concern in dementia, contributes to the slow destruction of neurons, concomitant loss in cognitive thought, memory and reasoning, and neuropsychiatric behaviour [5]. In order to develop early diagnostic markers for the disease, it is necessary to elucidate the mechanisms of AD-related degenerative pathways within the brain and to provide a medical and scientific basis for understanding the disease [6].

Precursors to these aggregated fibril deposits are amyloid peptides, formed *in vivo* from secretase enzymes acting upon a membrane bound Amyloid Precursor Protein **(APP)**. BACE (β-APP-cleaving-enzyme) (β-secretase) an aspartic protease and γ-secretase cuts APP to release [$A\beta_{1-40/1-42}$; and/or $A\beta_{11-40/11-42}$] amyloidogenic peptides while α- and γ-secretase cuts APP to release [$A\beta_{17-40/17-42}$] amyloid peptide [7] [Figure 1].α-Secretase is related to the ADAM (a disintegrin/ metalloproteinase) family of transmembrane and metalloendopeptidases that contains a prodomain, a metalloprotease, a disintegrin, a cysteine-rich peptide, an epidermal growth factor, a transmembrane domain and a C-terminal cytoplasmic tail [8]. The multiprotein complex (γ-secretase) composes five subunits – Presenilin 1 **(PS1)**, Presenilin 2 **(PS2)**, a Glycoprotein Nicastrin **(Nct)**, Aph-1 and Pen-2. Detailed functional mechanisms of the various components of α-, β- and γ-secretase complexes are reported elsewhere [9-15].

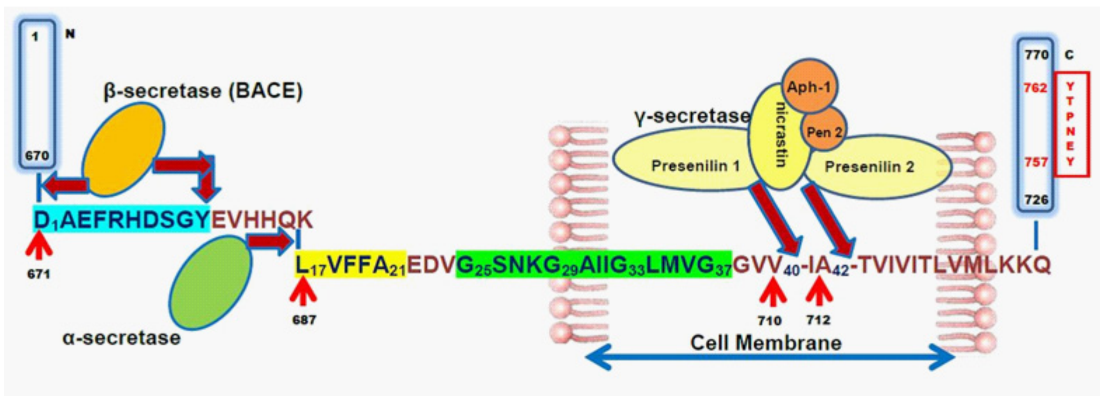

Figure 1: Map of amino acid residues for Amyloid Precursor Protein **(APP)** indicating cleavage points for α-, β-, γ-secretase enzymes. The transmembrane segment and the C-terminal YENPTY domain are indicated. The glycine zipper motifs ($Gly_{25}SNKGly_{29}AIIGly_{33}LMVG_{37}$) are illustrated in green; the hydrophobic pentapeptide with two phenylalanines (Phe_{19}; Phe_{20}) is illustrated in yellow and the decapeptide at the N-terminal region of $A\beta_{1-40/1-42}$ in light blue.

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Despite the facts that research towards AD pathophysiology is driven by Aβ deposits and hyperphosphorylated tau proteins this approach does not answer critical anomalies: failure of APP transgenic animal models to reproduce a full spectrum for AD disease nor the correlation of levels of senile plaques to cognitive behavior [16]. This suggests that APP may have other physiological functions manifest towards AD including cell adhesion, neuronal migration, axonal transport, synaptogenesis and/or transcription [17]. Critical to this idea is the hexapeptide motif [YENPTY; Tyr^{757}-Glu-Asn-Pro-Thr-Tyr^{762}] found in the final segment of the C-terminal of APP [Figure 1] which appears crucial, not only to the binding of cytosolic adapter proteins and endocytosis mediated by clathrin, but in the pathophysiology of AD. It is, unfortunately, beyond the scope of the present review to discuss the role of this motif along with its potential phosphorylation sites [Tyr^{757}; Thr^{761}; Tyr^{762}] though certain research articles appear in the literature [18,19].

Any decrease in Aβ catabolism is responsible for the accumulation of these peptides in the brain and their subsequent aggregation [20]. Consequently the greater the initial concentration of Aβ *in vivo*, the greater the probability for their aggregation. Such aggregation, otherwise termed fibrillogenesis is, therefore, central to the pathogenicity [21] of AD and occurs when the peptides undergo a self-induced conformational change, via hydrophobic-hydrophobic and π-π interactions of relevant amino acid side chains from a α-helical structure to an oligomeric β-sheet. The ability of a peptide to facilitate such a transition is controlled by amino acid residues that can adopt both a β-sheet as well as an α-helical structure within its native conformation [22]. Strong evidence [23-28] suggests that Aβ-peptides $A\beta_{1\text{-}40/1\text{-}42}$, the hydrophobic pentapeptides $A\beta_{17\text{-}21}$, $A\beta_{29\text{-}33}$and the neurotoxic fragment $A\beta_{25\text{-}37}$, have certain structural criteria that initiate fibrillogenesis [28] and/or the formation of neurotoxic oligomers [29]. The first 10 residues [Asp^{1} – Tyr^{10}] [Figure 1] initiates the α- to β- transition while the amino acids Leu^{17} – Ala^{21} that includes Phe^{19} and Phe^{20} contributes, through the π-π interactions, towards the major β-sheet region and the formation of aggregated fibrils [30,31]. Intermediate hydrophobic-hydrophobic interactions and hydrogen bonding also promote a β-sheet structure [20,21]. Disagreement is still strong, however, whether the α-helix or β-sheet or, indeed, both are the toxic elements [32]. Reports on the fibrillogenetic mechanism suggest an equilibrium exists between the monomeric free peptide and Aβ-peptide concentration dependent 'nucleii-micelles' [33]. Initially there is kinetic association of the peptides without any aggregation taking place (lag-phase) followed by the formation of critical nuclei and hydrophobic-hydrophobic interactions (nucleation phase) [34]. Finally a fibril-aggregate association-dissociation equilibrium occurs between monomers. It is well known that the primary causative agents of AD are the soluble Aβ-peptides and not the inert, protected insoluble ones [35,36]. Furthermore it is also known that Aβ fibrils increase monomer concentration (via nucleation) and speed up their assembly into toxic oligomers [37]. The presence of a rapid growth/elongation phase and an equilibrium phase during fibrillogenesis points towards a nucleated-polymerization model.

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ARGININE METABOLISING ENZYMES

The astrocytes in the diseased brain are not only surrounded by insoluble amyloid plaques [38] but function to store the amino acid arginine and this justifies any argument to study arginine metabolising enzymes with respect to the etiology and pathogenetic mechanism of AD. Added to this is the fact that, in a diseased brain and cerebrospinal fluid, there are elevated levels of arginine even though it is uncertain whether these levels are as a result, or a cause, of the disorder [3]. Consequently it follows that a therapeutic tool for studying AD is to investigate these enzymes and their intimate association with amyloid peptides. Arginine metabolizing enzymes [39,40], in conjunction with amyloid peptides, assist in fibril formation by stimulating fibril elongation or increasing 'seeds' necessary for the nucleation step. After free monomeric Aβ-peptides bind to an enzyme it forms a nucleus, initiates aggregation and becomes directly associated with aggregated monomers already present to elongate the fibril. The nucleus forms after the lag phase and in a 'supersaturated' solution of fibrils which eventually exceed a critical concentration of amyloid peptide [41,42]. Over time an equilibrium takes place between soluble Aβ-monomers and micelles to provide nuclei for new fibril growth [43,44]. Eventually, as the concentration of fibrils increases, more micelles are formed until a point of saturation when insoluble fibrils precipitate.

It is also necessary to find the critically important amino acids, within the peptide fragments, that are mechanistically responsible; not only in fibrillogenesis, but also for the reason they inhibit the enzymes. Without enzyme there is no fibril formation – soluble or insoluble – which supports evidence that, indeed, the enzymes are catalytic towards fibrillogenesis. The amyloid peptides undergo time-dependent hydrophobic-hydrophobic associations then dissociate from the enzymes suggesting that the amyloid peptides are converted into a form that can no longer bind [45,46].

THE GLYCINE ZIPPER

A critical prominent feature, within $A\beta_{1-40,1-42}$, is the triple glycine zipper motif – [Gly^{25}-Ser^{26}-Asn^{27}-Lys^{28}-Gly^{29}-Ala^{30}-Ile^{31}-Ile^{32}-Gly^{33}-Leu^{34}-Met^{35}-Val^{36}-Gly^{37}] [Figure 1] that, not only spans the membrane section of the APP precursor protein, but is instrumental in the pathogenicity of amyloid peptides[47-50]. An understanding of the mechanism for this phenomenon arises from other reports in which the three individual glycine zippers [G-X-X-X-G] within $A\beta_{25-37}$ and the hydrophobic fragment $A\beta_{17-21}$[Leu^{17}-Val^{18}-Phe^{19}-Phe^{20}-Ala^{21}] [Figure 1] not only initiate fibrillogenesis but inhibit the arginine metabolising enzyme - Neuronal Nitric Oxide Synthase **(nNOS)** [45-49]. Several researchers [51-53] imply that the Aβ-toxicity arises through oligomeric amyloid peptides. Fonte and co-workers [54] established that a Gly^{37}Leu mutation within the Aβ-peptide dramatically decreased Aβ-toxicity in *Caenorhabditis* **elegans** [55]. These workers also examined this toxicity with second-site substitutions and mutations of Asn^{27}, Ile^{31} and Met^{35} into glycine. Their reasons behind this arose from the packing of α-helical structural models between two 'back-to-back' glycine zippers whereby Gly^{25}, Gly^{33} and Gly^{37} from one zipper helix would

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make close contact with Asn^{27}, Ile^{31} and Met^{35} of the second zipper helix. Furthermore if the Aβ-toxicity is due to this Gly^{37}Leu mutation then substituting a smaller glycine residue for Asn^{27}, Ile^{31} and/or Met^{35} compensates in equal terms to restore Aβ-toxicity.

Controversy exists over the structure of the Aβ-peptide that is toxic to neurons. Some researchers [56,57] found that when Gly^{25}, Gly^{29}, Gly^{33} and $Gly^{37}$are mutated to leucine and/or alanine and/or isoleucine there is substantial decrease in Aβ-toxicity especially with Gly^{33} and Gly^{37}. At the same time this decrease in toxicity is not only mirrored to a decrease in formation of *in vitro* Aβ-oligomers but to an increase in formation of insoluble fibrils as determined by Thioflavin-T assay. This kind of substitution adds fuel to an earlier concept that Aβ-toxicity is due to an increase in hydrophobicity of the amino acid residues within the glycine zipper region. A Leu^{17}Pro mutation not only interferes with β-strand formation blocking *in vivo* amyloid formation [58] but also is equal in toxicity to wild type Aβ-peptides. Nevertheless even though a Gly^{37}Leu substitution disrupts β-strand formation the process of forming these β-strands by Aβ-peptides does not necessarily decrease Aβ-toxicity. Any mutation at Met^{35} eliminates toxicity.

OXIDATIVE/NITROSATIVE STRESS

The pathogenicity of neurodegenerative disorder, including AD, is also manifested through both oxidative and nitrosative stress [59-67]. The former is defined as an over-production of Reactive Oxygen Species **(ROS)**, generated from a combination of readily available oxygen and free radicals, (either internally or externally) such that normal biological processes for their removal are inadequate. Free radical toxicity [Figure 2] including superoxide and peroxide cause extensive damage to lipid membranes and cellular tissues [66,68]. The brain has limited defence against this kind of damage [69] and relies upon antioxidants and the Enzymes Glutathione Peroxidase **(GPx)**, catalase and Superoxide Dismutase **(SOD)** [Figure 2] to ensure 'safe' levels. Nitrosative stress is, in part, a syndrome that arises from a series of molecules (Reactive Nitrogen Species; **RNS**) that originate from the reaction of Nitric Oxide **(NO)** with Superoxide (O_2^-) to yield Peroxynitrite **($ONOO^-$)** [70,71] [Figure 2].This highly reactive species can, in turn, oxidise a multitude of biological targets such as haemproteins (cytochromes, myoglobins, haemoglobins) and/or amino acids especially sulphur bearing (cysteine, methionine). In so doing there is obvious compromise to protein structure and function and cell signalling with, inevitable, cell apoptosis and necrosis [72,73]. The small neurotransmitter molecule - nitric oxide–is an important signalling molecule in the brain. Apart from modulating different intracellular pathways associated with AD NO is regarded as a 'janus' molecule in cell survival and/or cell death since in high concentrations it is neurotoxic while at low levels it is neuroprotective [74].

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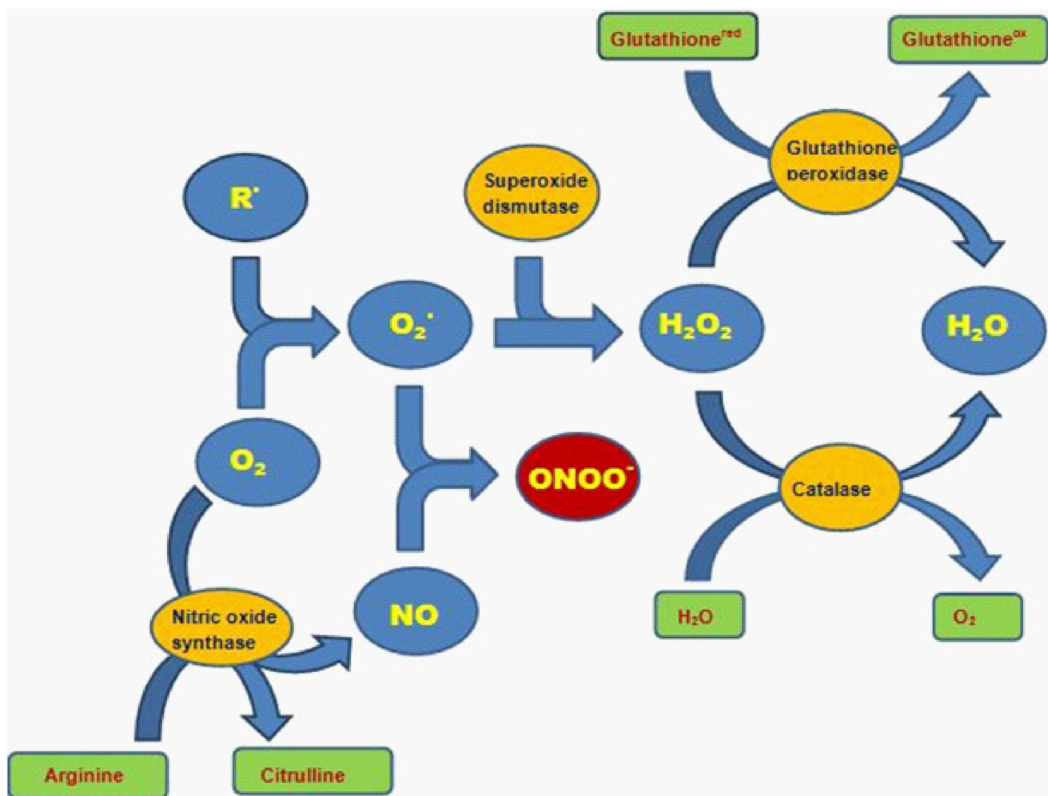

Figure 2: Interelationships for free radical toxicity with respect to nitric oxide synthase and antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase) in the brain.

The toxicity of the Aβ-peptide fragments [$A\beta_{1-40}$, $A\beta_{1-42}$, $A\beta_{25-29}$, $A\beta_{25-33,}$ $A\beta_{25-37}$, $A\beta_{17-21}$, $A\beta_{29-33,}$ $A\beta_{29-37}$, $A\beta_{33-37}$] and pseudo Aβ-peptides $A\beta_{21-17}$ [AFFVL]; $A\beta_{17-21p}$ [LVEEA];$A\beta_{33-29}$ [GIIAG]; $A\beta_{29-33p}$[GAEEG] [Figure 1] is reflected in the total number of glycine zipper motifs. This toxicity is also studied through their interactions with the specific enzymes identified as being significant in AD. Various biophysical techniques, such as kinetic, thermodynamic, spectrofluorimetric analysis, resonance energy transfer and antisense-sense technology are employed. Kinetic parameters (V_{max},K_m,K_i) and affinity constants (K_d) are found by kinetic analysis while the binding constants for the formation of soluble/insoluble fibrils (fibrillogenesis) are estimated from fluorescence quenching [45,48]. The mechanisms for aggregation and degree of spontaneity may be determined from thermodynamic assessments of enthalpies **(ΔH)**, entropies **(ΔS)** and Gibbs free energy **(ΔG)** profiles at different temperatures [45,49] while Fluorescence Resonance Energy Transfer **(FRET)** [45,48,49] determines orientations, positions, molecular recognition and conformational structural changes of the enzyme-Aβ-peptides interactions. The principle of antisense technology refers to peptides synthesised from the non-coding DNA/RNA strand that can interact and/or act as antagonists to the normal coding strand peptide (sense) and thereby interfere with transcription and/or translation [75] of the parent molecule.

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NEURONAL NITRIC OXIDE SYNTHASE (NNOS)

Nitric Oxide Synthase **(NOS)** [EC. 1.14.13.39] oxidises L-arginine to L-citrulline and Nitric Oxide **(NO)** [Figure 3]. Structurally nNOS binds a cofactor, tetrahydrobiopterin (H_4B), arginine and heme at an N-terminal oxygenase domain that is linked via Calmodulin **(CaM)** to a C-terminal reductase domain which houses the cofactors, reduced Nicotinamide Adenine Dinucleotide Phosphate **(NADPH)**, Flavin Adenine Dinucleotide **(FAD)** and Flavin Mononucleotide **(FMN)** [Figure 3]. A three-electron flow, mediated by Ca^{++} within the calmodulin [76-80], from the reductase domain oxidises NADPH to $NADP^+$ through FAD and FMN to the H_4B and Fe-hemecentre in the oxygenase domain. The accepted distance for effective electron transfer to occur is around 15 Å and since the distance between the FMN cofactor and haem-Fe is > 25 Å there is evidence to suggest that a major structural shuffle takes place. Calmodulin facilitates a large conformational change within nNOS by swinging the FMN cofactor about 12 Å from its environment with FAD towards the haem [81]. The overall oxidation, with molecular oxygen, of arginine occurs from the proximal orientation to form N-hydroxyarginine that eventually collapses to citrulline and NO. Cys^{415}, a crucial amino acid for binding of the haem to the enzyme, makes close contact with the Fe haem atom from a distal orientation thereby not interfereing with the binding of oxygen to nNOS.

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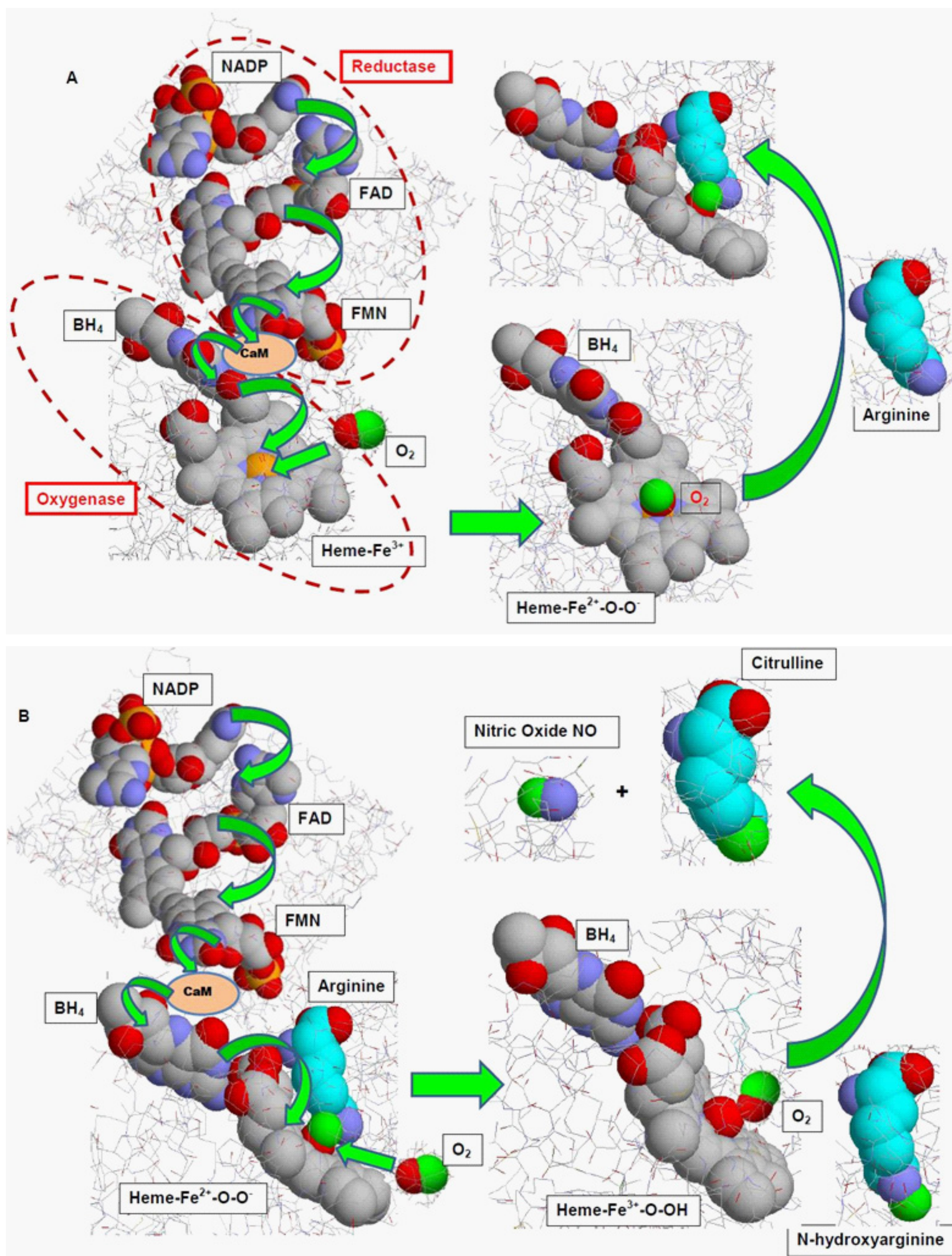

Figure 3: Mechanism of neuronal nitric oxide synthase in the conversion of arginine into citrulline and Nitric Oxide **(NO)** via N-hydroxyarginine. A three electron-shuttle, mediated by calmodulin **(CaM)** occurs between cofactors NADPH, FAD and FMN (reductase domain) to tetrahydropterin cofactor and Fe-haem centre (oxygenase domain); **A:** Oxidation of arginine with O_2 from a proximal orientation to form N-hydroxyarginine; **B:** Further oxidation and collapse of N-hydroxyarginine into citrulline and NO.

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Non-competitive inhibition of nNOS by each Aβ-peptide with one binding site affords an initial decrease in enzyme activity. Careful scrutiny of the amino acid sequences of these Aβ-peptides along with their respective K_i values indicates that the glycine zipper motifs $Aβ_{17-21}$ [LVFFA]; $Aβ_{25-29}$ [GSNKG]; $Aβ_{29-33}$ [GAIIG] and $Aβ_{33-37}$[GLMVG] are critical [45-49]. When the enzyme is incubated with four synthesised pseudo-peptides – two with a reversed sequence[$Aβ_{21-17}$ - AFFVL; $Aβ_{33-29}$ - GIIAG] and two with both phenylalanines and both isoleucines substituted with polar glutamic acid residues [$Aβ_{17-21p}$ - LVEEA; $Aβ_{29-33p}$ - GAEEG] – increase in K_i values are noted. This reinforces that the two phenylalanines, [Phe^{19}; Phe^{20}] with their π-π interactions, and two isoleucines [Ile^{31}; Ile^{32}] in $Aβ_{17-21}$ and $Aβ_{29-33}$ are essential for initial inhibition with nNOS [Figure 4] [46]. It is realized that while the inhibitor constants (K_i) increases 2-3 fold for each of the pseudo-peptides when compared with the normal peptides the dissociation constant K_d increases between 20 and 50 fold [45,49]. Since inhibition occurs over the first few minutes - before complete activity is restored - the incubation of nNOS with the five amyloid peptide fragments [$Aβ_{17-21}$; $Aβ_{25-29}$; $Aβ_{29-33}$; $Aβ_{33-37}$; $Aβ_{25-37}$] catalyzes the formation of fibrils – first soluble then insoluble. The decrease in concentrations of soluble fibrils with respect to time is mirrored by the increase in concentration of insoluble fibrils [Figure 5]. It is evident that the hydrophobic nature of $Aβ_{17-21}$, the glycine zipper peptides [$Aβ_{25-29}$; $Aβ_{29-33}$; $Aβ_{33-37}$ and $Aβ_{25-37}$] are all triggers in the formation of fibrils and a force critical in the association of the peptides with the enzyme.

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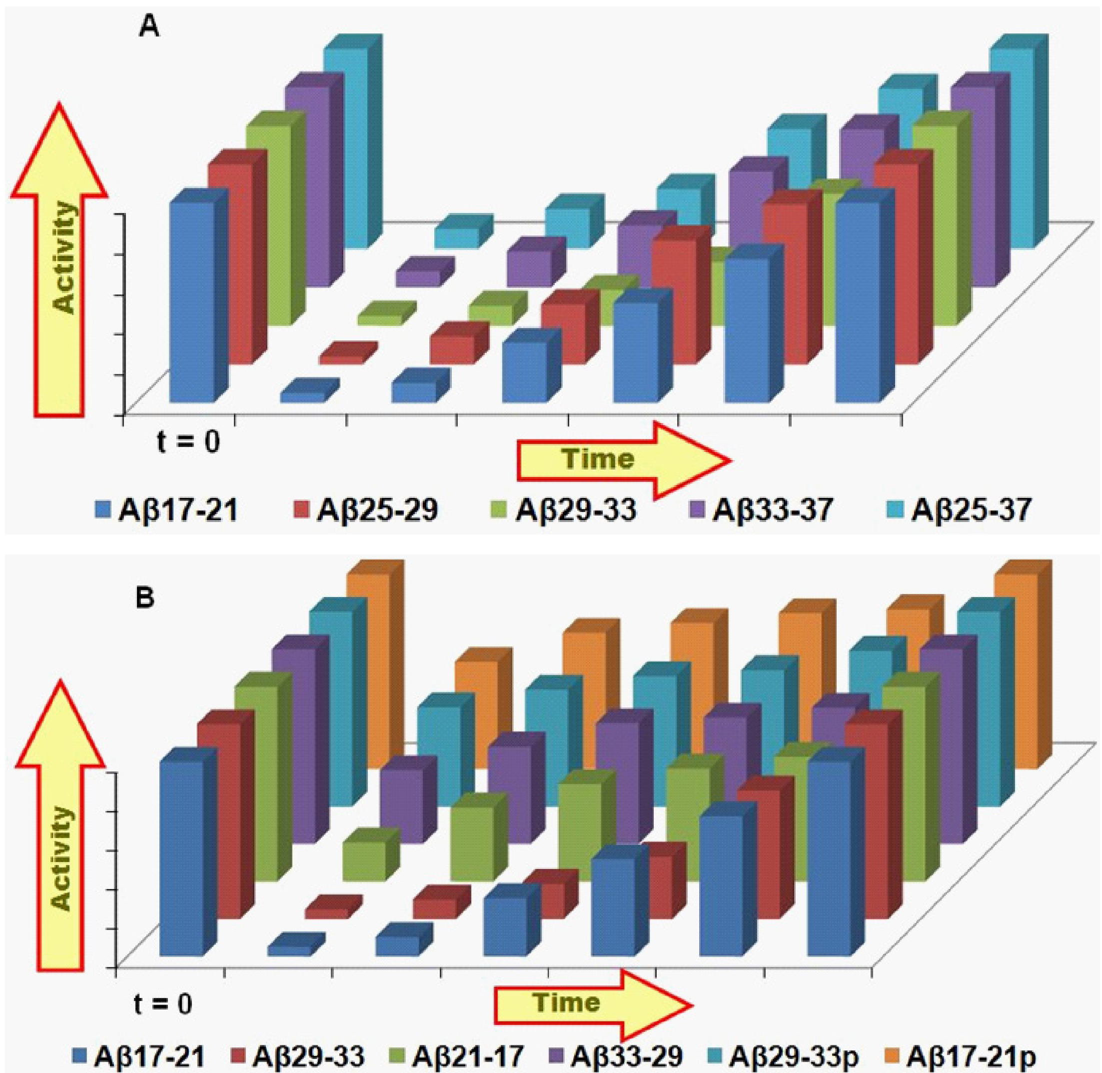

Figure 4: Interaction of neuronal nitric oxide synthase with several amyloid peptide fragments with respect to time. **A:** Interaction with pentapeptide $A\beta_{17-21}$ and various glycine zipper fragments; **B:** Interaction with certain pseudo-peptides (see text for details) with $A\beta_{17-21}$ and $A\beta_{29-33}$ inserted for a comparison. Note a rapid decrease in activity from t = 0 followed by a gradual recovery to 100 % activity over time.

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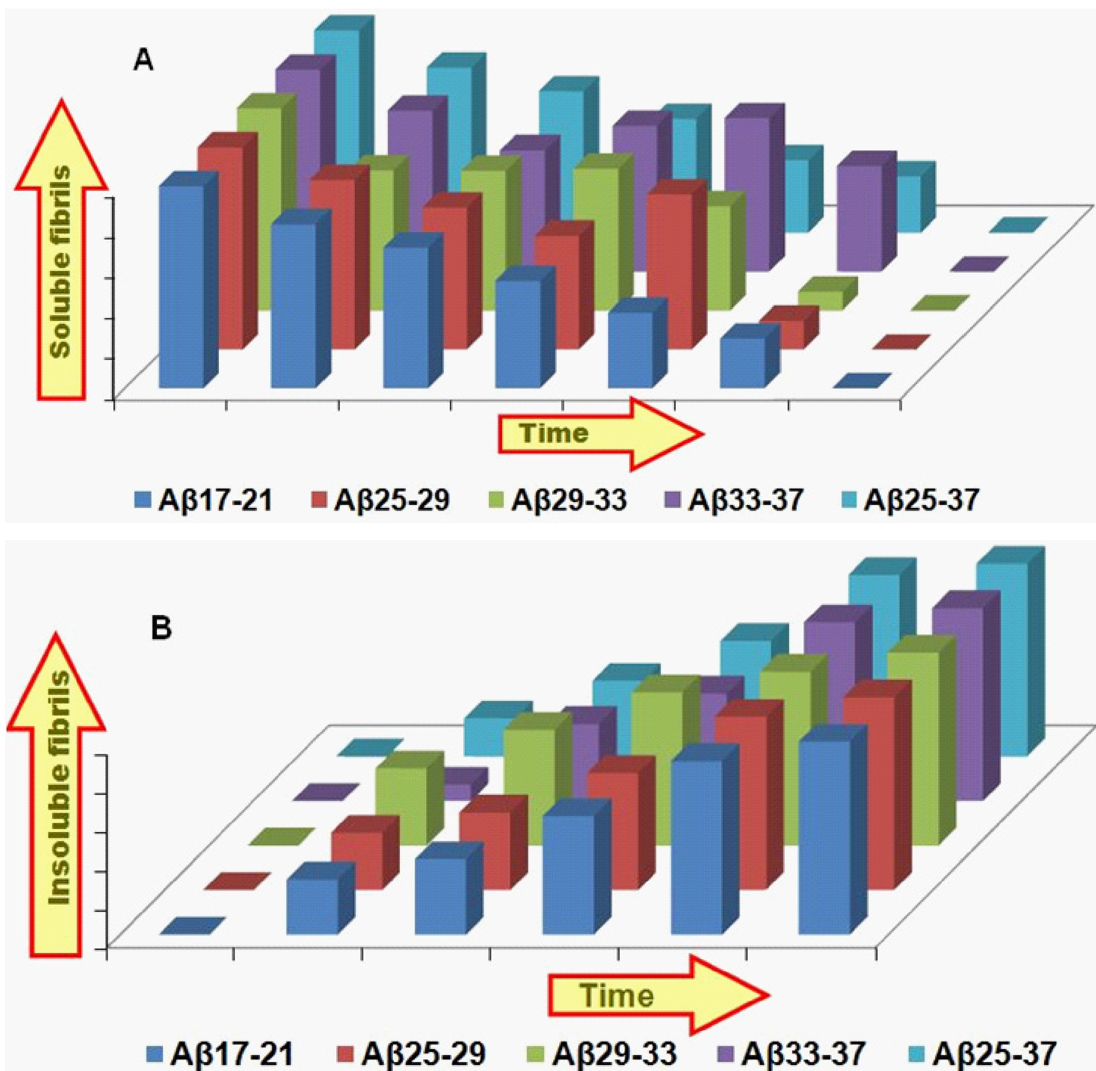

Figure 5: A: Soluble fibrils **B:** Insoluble fibrils produced with respect to time by the action of various amyloid peptides with nNOS.

Despite extreme controversy about the merit, of antisense – sense technology, against accepted biotechnological dogma [75] each Aβ-peptide is presented, antagonistically, to the various enzymes. $Aβ_{25-29}$ and $Aβ_{29-33}$possess five-residue sequences (antisense) to Ala^{995}-Arg^{996}-Leu^{997}-Leu^{998}-Ser^{999} [ARLLS] and Pro^{1172}-Arg^{1173}-Tyr^{1174}-Tyr^{1175}-Ser^{1176} [PRYYS] from nNOS[PDB; 1TLL]that can actually interact with the peptides[Figure 6]. It is obvious that peptide Gly^{29}-Ala-Ile-Ile-Gly^{33} (GAIIG) [antisense PRYYS] binds closer (± 1.5 Å), and therefore tighter, to the FAD-FMN interface than peptide Gly^{25}-Ser-Asn-Lys-Gly^{29} (GSNKG) [antisense ARLLS] that is ± 13 Å away. $Aβ_{33-37}$, on the other hand, has a weaker association for nNOS with only a four-residue sequence [Gln^{1026}-Tyr^{1027}-Gln^{1028}-Pro^{1029}] that interacts at a distance of ± 21 Å. Further support of

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this comes from our group [45] (and mentioned earlier)that two consecutive phenylalanines and/or isoleucines are crucial to inhibition and fibrillogenesis; the non-polar glycine zipper ($A\beta_{33-37}$) afforded very mild inhibition and weak association with over 50 % soluble fibrils remaining after 96 hours of incubation [45,49]. Even though $A\beta_{17-21}$ has several four-residue sequence antisense motifs available, [His^{988}-Lys^{989}-Lys^{990}-Arg^{991}] is in close proximity to $A\beta_{25-29}$ and to its antisense Ala^{995}-Arg^{996}-Leu^{997}-Leu^{998}-Ser^{999} to make it a preferred motif with respect to inhibition and fibrillogenesis. The reverse sequence 'pseudo' peptides – $A\beta_{21-17}$ [AFFVL] and $A\beta_{29-33p}$ experience a four-residue and five-residue antisense motif respectively with the latter sharing the same antisense segment for Gly^{25}-Ser-Asn-Lys-Gly^{29} [Ala^{995}-Arg^{996}-Leu^{997}-Leu^{998}-Ser^{999}; ARLLS] [Figure 6]. As far as can be ascertained the 'pseudo' peptide $A\beta_{17-21p}$ [LVEEA] offers no binding and/or interactions with nNOS whatsoever while $A\beta_{33-29p}$ 'pseudo' peptide [GIIAG], with a five-residue antisense motif, offers an extremely weak association with nNOS at Ser^{959}-Asn^{960}-Asp^{961}-Arg^{962}-Ser^{963} at a remote distance of 36 Å. It is interesting that peptide GAII (and a 'recognition element' LVFF) also interfere with fibrillogenesis lending credence to the role of Leu^{17}-Val^{18}-Phe^{19}-Phe^{20}-Ala^{21} [LVFFA] in the fibrillogenetic mechanism [82,83].

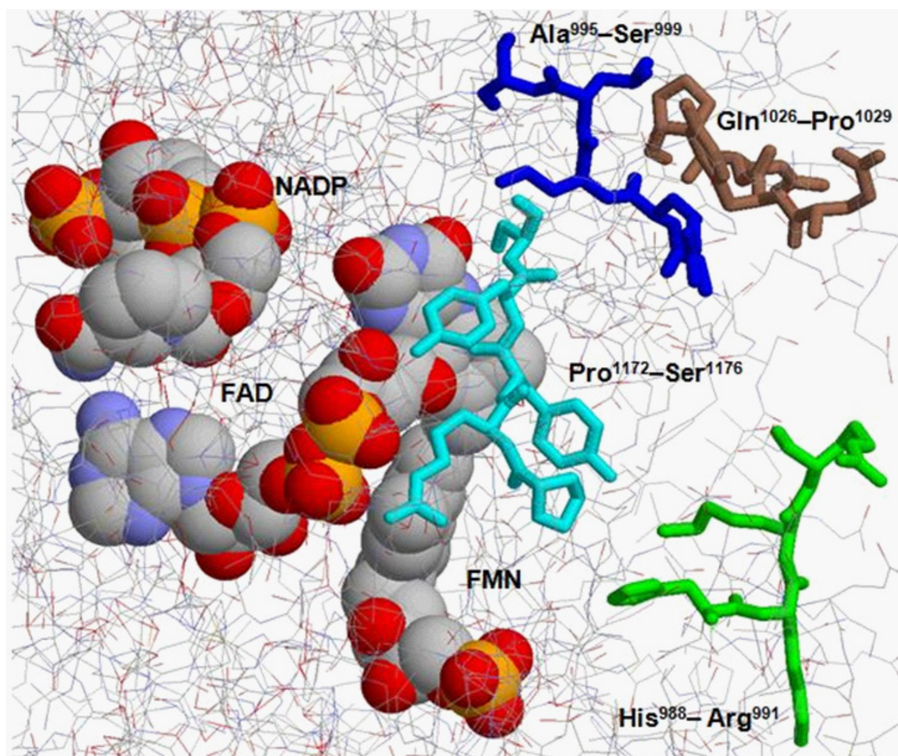

Figure 6: Antisense-sense technology illustrating possible binding of amyloid peptides with the reductase domain cofactors within neuronal nitric oxide synthase. His^{988} – Arg^{991} is antisense peptide for $A\beta_{17-21}$; Pro^{1172} – Ser^{1176} is antisense peptide for $A\beta_{29-33}$; Ala^{995} – Ser^{999} is antisense peptide for $A\beta_{25-29}$; Gln^{1026} – $Pro^{1029}$is antisense peptide for $A\beta_{33-37.}$

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The interactive forces between the bound Aβ-amyloid peptides and nNOS are also determined from temperature-dependent thermodynamic parameters – enthalpy **(ΔH)**, entropy **(ΔS)** and Gibbs free energy **(ΔG)**. Since these values are all positive it shows that not only are hydrophobic forces in operation when the Aβ-peptide fragments interact with nNOS but the interactions are non-spontaneous [45,49]. There is also a quenching of the intrinsic fluorescence of the enzyme. As temperature increases the binding constants, determined by Stern-Volmer analysis, increase reflecting that the interaction of the amyloid peptides with nNOS is endothermic and the quenching is dynamic. Stern-Volmer fluorescence quenching constants (K_{SV}) for $A\beta_{17\text{-}21p}$ and $A\beta_{29\text{-}33p}$ are 2-3 folds lower than the corresponding $A\beta_{21\text{-}17}$; $A\beta_{33\text{-}29}$. FRET analysis of the interaction of $A\beta_{17\text{-}21}$ and the three glycine zipper motifs $A\beta_{25\text{-}29}$; $A\beta_{29\text{-}33}$; $A\beta_{33\text{-}37}$; with nNOS leads to substantial quenching of the fluorescence by $A\beta_{17\text{-}21}$ and $A\beta_{29\text{-}33}$ supporting evidence that these fragment peptides are critical in fibrillogenesis [45,48,49]. Reverse sequenced 'pseudo' amyloid peptides fragments $A\beta_{21\text{-}17}$; $A\beta_{33\text{-}29}$ [AFFVL; GIIAG] and the polar substituted ones [$A\beta_{17\text{-}21p}$; $A\beta_{29\text{-}33p}$] [LVEEA; GAEEG] show insubstantial fluorescence quenching with a more restricted influence on the surface tryptophan fluors. There are six tryptophan residues on the surface of nNOS [Trp_{306}, Trp_{421}, Trp_{510}, Trp_{625}, Trp_{678} and Trp_{716}] yetTrp_{716} [46] is the only one involved in fluorescence quenching by the Aβ-peptides. The normal substrate (arginine) binds near the active region about 2.25 nm from Trp_{716} [40] then, after interaction with an Aβ-peptide ($A\beta_{17\text{-}21}$, $A\beta_{25\text{-}29}$ and $A\beta_{29\text{-}33}$) the distance becomes between 2.83 and 2.97 nm while for the pseudo-peptides $A\beta_{21\text{-}17}$,$A\beta_{17\text{-}21p}$,$A\beta_{33\text{-}29}$ and $A\beta_{29\text{-}33p}$ [AFFVL; LVEEA; GIIAG; GAEEG] it extends to between 3.08 – 3.74 nm [40]. These fluctuations in distance, fluorescent intensity and transfer efficiency illustrate an increase in interaction energy for the pseudo-peptides with nNOS lending support for the strategic position of the Phe_{19}, Phe_{20}, Ile_{31} and Ile_{32} in the original peptides not only for inhibition of the nNOS but for initiation of fibrillogenesis [45,49]. Furthermore, from an antisense-sense strategy and, in view of the locus of the binding of the Aβ-peptides to nNOS, the mechanism for this non-competitive inhibition is by interruption of electron transfer between FMN cofactor and the haem-Fe. Nevertheless, with respect to all evidence herewith presented $A\beta_{17\text{-}21}$ and $A\beta_{29\text{-}33}$ remains the two most effective peptides for inhibition and fibrillogenesis.

CU-ZN SUPEROXIDE DISMUTASE

Brain superoxide dismutase (Cu-Zn SOD) [E.C. 1.15.1.1], is also closely associated with various neurodegenerative disorders including Alzheimers diseaseis homodimeric and catalyzes the dismutation of superoxide to molecular oxygen and hydrogen peroxide [84-88] [Figure 7]. Each monomeric subunit contains a structurally important and stabilising zinc atom surrounded by His^{71}, His^{80} and Asp^{83} and bridged by another His^{63} to a catalytic copper atom attached to a further three histidines: His^{46}, His^{48} and His^{120}. An additional water molecule completes the metallic cluster active region. Mechanistically there is a sequential reduction/oxidation of a metal Center **(Cu)** with a concomitant oxidation/reduction of superoxide radicals. As the superoxide radical interacts with Cu^{2+} it is reduced to Cu^{+} and the water molecule moves out of the active site. The Cu^{+} shifts in position, releases O_2 then combines with a second superoxide radical to lose its coordination with His^{63} before a final oxidation of Cu^{+} to Cu^{2+} and a release of H_2O_2 [Figure 7].

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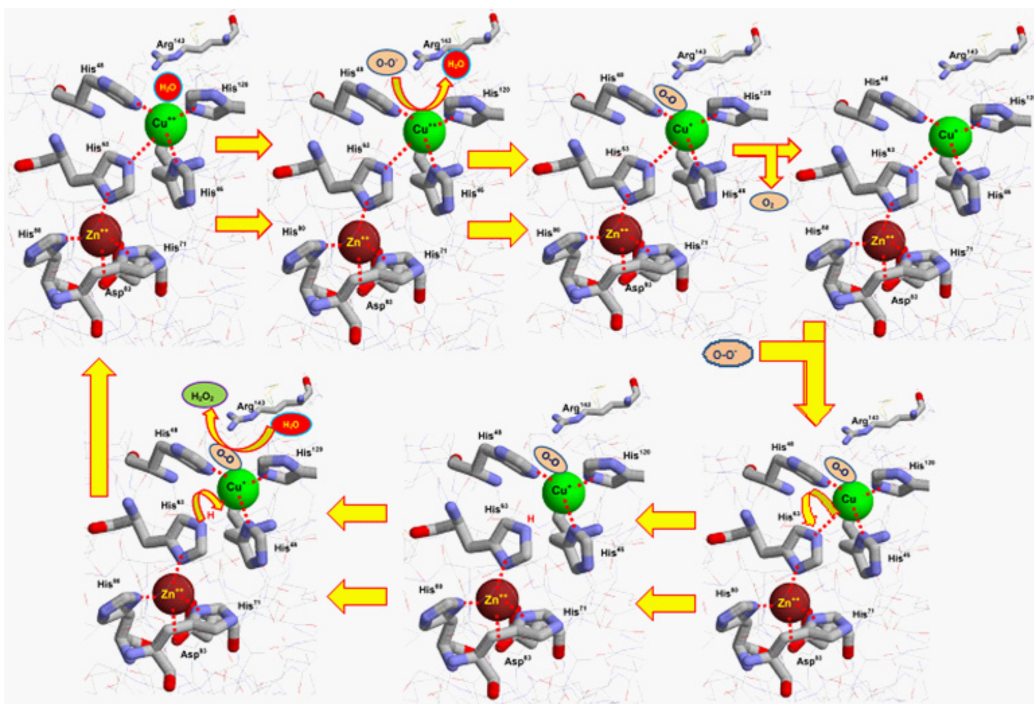

Figure 7: Mechanism for the dismutation of superoxide into molecular oxygen and hydrogen peroxideby Cu-Zn superoxide dismutase. Each subunit contains zinc with His^{71}, His^{80} and Asp^{83} and bridged by another His^{63} to a catalytic copper atom attached to His^{46}, His^{48} and His^{120}; a water molecule completes the active region. A sequential reduction/oxidation of the Cu and a concomitant oxidation/reduction of superoxide radicals occur. The water molecule moves out of the active site and the Cu^{+} releases O_2 then combine with a second superoxide radical to form Cu^{2+} and the release of H_2O_2.

It is obvious that any inhibition of any key antioxidant enzyme in the cell leads to increased oxidative stress in the cell, which is known to cause neurodegeneration and memory deficits [89,90]. With respect to Aβ-peptide interaction with SOD it is realized, though the difference in structure of the peptides appears insignificant the inhibition is quite substantial [86,89] with a consequence of enhanced oxidative stress. Since aggregation of Aβ-peptides is mediated by Zn and Cu and that the β-site cleavage enzyme BACE1 [Figure 1] binds Cu^{2+}there is significant support for the role of SOD in the pathogenicity of AD [91-94]. $A\beta_{29\text{-}33}$ that contains a single glycine zipper motif and $A\beta_{25\text{-}37}$ containing the triple glycine zipper afforded similar inhibition (K_i = 6-7μM) [86]. There are reports [95] that substantial fibril formation occurs only after a critical disulphide bond in the enzyme is broken thereby lowering its structural stability and exposing fibril-forming core regions that interact with each other initiating fibrillogenesis. Contradictory to this is the report of negligible fibrillogenesis with this enzyme and amyloid peptides [86]. There is only one surface tryptophan per subunit [Trp^{32}] that is 2.33 nm away from the structurally important Zn^{2+}

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and 2.17 nm to the Cu^{2+} catalytic centre. No detailed FRET analysis, however, is reported [86]. Antisense-sense technology for glycine zipper-SOD interactions reveals no extensive binding of the Aβ-peptides occurs reinforcing the notion that this type of interaction does not support fibrillogenesis [86].

CATALASE

One enzyme that detoxifies H_2O_2 into H_2O and O_2 and effectively removes potential highly reactive hydroxyl radical is the ubiquitous catalase [Figure 8A; 8B] [96-99] [E.C. 1.11.1.6].

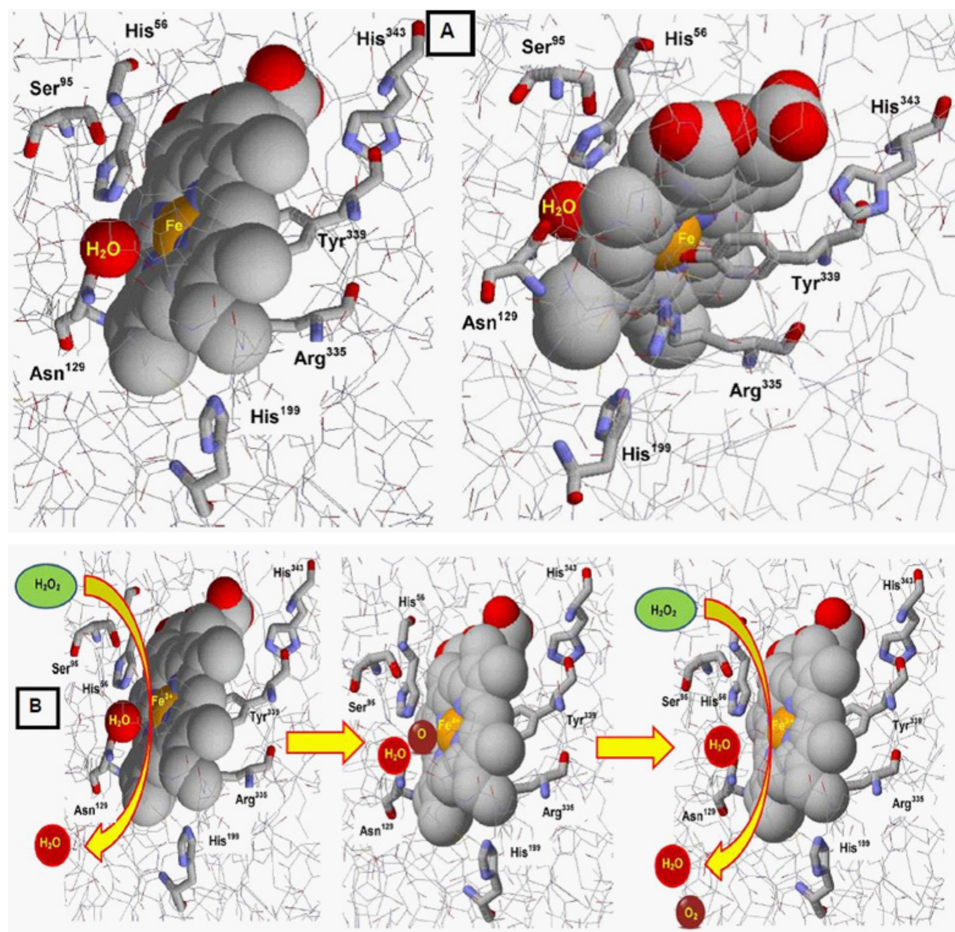

Figure 8A: Proximal and distal surfaces of the active centre haem of catalase showing all relevant amino acid residues involved in the mechanism of the enzyme. **Figure 8B:** Mechanism for detoxification of H_2O_2 into H_2O and O_2 by the ubiquitous catalase. A two-electron redox reaction oh the Fe^{3+}-haemwith cleavage of O-O bond of hydrogen peroxide to form a ferryl-oxo species (Fe^{4+}=O) followed by oxidation of a second hydrogen peroxide molecule into two molecules of water. Transfer of associated protons is facilitated by active site His^{56}, Ser^{95} and Asn^{129} while oxidation spin states for the haem Fe are maintained by Tyr^{329}.

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Mechanistically there is a two-electron redox reaction involving a tetrapyrrole complex (Fe^{3+}-haem) in which O-O bond of hydrogen peroxide is cleaved releasing water and forming a ferryl-oxo species (Fe^{4+}=O). Finally this oxygen becomes available to oxidise a second hydrogen peroxide molecule into two molecules of water. Transfer of associated protons is facilitated by active site His^{56}, Ser^{95} and Asn^{129} while oxidation spin states for the haem Fe are maintained by Tyr^{329}[Figure 8].

It is well known that the enzyme catalase also associates with several Aβ-peptides and senile plaques and the ability of this enzyme to breakdown H_2O_2 is regarded as being an inherent mechanism for cell protection [100-103]. These workers are also responsible for extensive work on the amyloid binding properties of catalase and, indeed, they have proposed that Pro^{401}-Asn^{402}-Tyr^{403}-Tyr^{404}-Pro^{405}contains a sequence to the Aβ-peptide anti-sense [GLMVG] that actually binds Aβ-peptides [104]. It seems surprising that this peptide interferes with the enzyme due to the distance (32.7 Å) from this peptide to the haem reactive centre [Figures 8B; 9]. Furthermore a second sequence that is anti-sense peptide to GLMVG and may interact with the enzyme is Ala^{383}-Asn^{384}-Tyr^{385}-Gln^{386}-Arg^{387}. Once again, however, the distance from this peptide to the haem centre (22.8 Å) sheds doubt on any effect that this Aβ-peptide has on the process of fibrillogenesis and senile plaque formation. Two other sense sequences, more appealing for interaction with the enzyme and much closer to the reactive haemcentre, are Val^{145}-Gly^{146}-Asn^{147}-Asn^{148}-Thr^{149}, antisense to Aβ-peptide Gly^{29}Ala-Ile-Ile-IIG^{33} and Gly^{352}-Arg^{353}-Leu^{354}-Phe^{355}-Ala^{356}, antisense to Aβ-peptide Gly^{25}Ser-Asn-Lys-Gly^{29} [Figure 9]. It is notable that the guanidinium side-chain from Arg^{353} is juxtaposed to the Fe atom at the haem centre. Catalase is inhibited by Aβ-peptides by an oxidation process [105] while at the same time there is neutralization of the toxicity of the Aβ-peptides , through a binding at its cytotoxic domain [106]. Such an inhibition, however, reflects an increase in the physiological levels of reactive oxygen species. As a paradox, therefore, it is essential to develop inhibitors of these harmful Aβ-peptide-catalase interactions in order to minimize Aβ-peptide induced oxidative stress [107]. Furthermore there is significant physiological implications that NO, the reactive nitrosative species, is a competitive inhibitor towards catalase [108]. A structural and kinetic analysis for the interaction of NO with catalase is reported [109].

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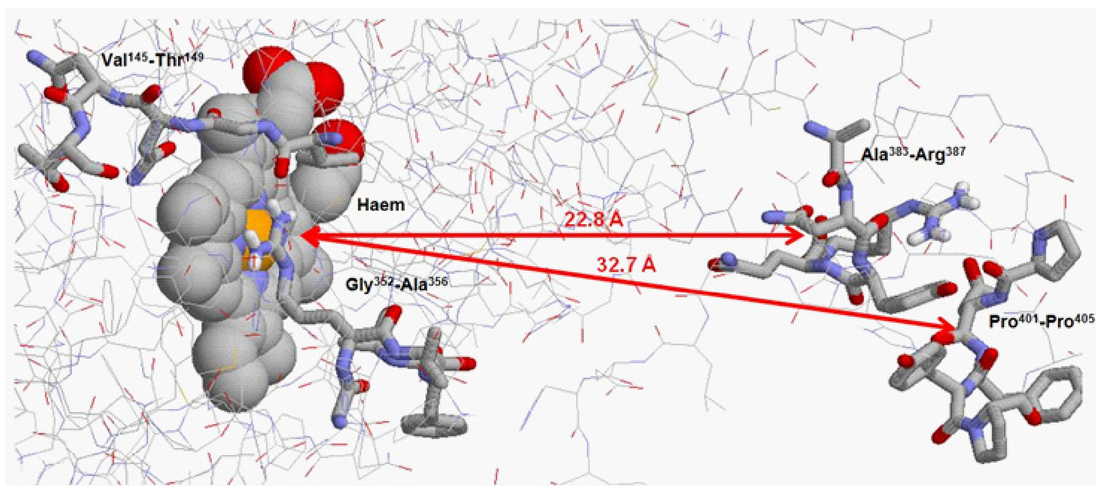

Figure 9: Antisense-sense technology illustrating possible binding of amyloid peptides with catalase. Val^{145}-Gly^{146}-Asn^{147}-Asn^{148}-Thr^{149} is antisense to $Aβ_{29-33}$ and Gly^{352}-Arg^{353}-Leu^{354}-Phe^{355}-Ala^{356} is antisense to $Aβ_{25-29.}$Note the relative position of the Fe haem centre to the guanidinium side-chain from Arg^{353}. The two sequences Pro^{401}-Asn^{402}-Tyr^{403}-Tyr^{404}-$Pro^{405}$and Ala^{383}-Asn^{384}-Tyr^{385}-Gln^{386}-Arg^{387}appear to be too distant from the active Fe-haem to be of any consequence.

GLUTATHIONE PEROXIDASE

A second enzyme that has its reputation as an anti-oxidant as well as converting H_2O_2 into H_2O is glutathione peroxidase [110-112] [E.C. 1.11.1.9] of which there are eight different tissue-specific isoforms identified in humans. The initial reaction is with H_2O_2 to form a selenenic intermediate [Se-OH] [Figure 10] at the active site before interaction with reduced glutathione [GSH] leading to the generation of a glutathiolatedselenol [Se-SG] [113-115]. Reaction with a second molecule of reduced glutathione yields oxidised glutathione [G-S-S-G] and restores the active site. A subsequent return of G-S-S-G into reduced glutathione involves NADPH-dependent glutathione reductase with $NADP^+$/NADPH links to GSH pathway by glucose-6-phosphate dehydrogenase and the pentose-phosphate cycle. The fact that the accumulation of amyloid peptides, neuronal degeneration and toxicity are mediated by reactive oxygen species supports an interaction of these peptides with glutathione peroxidase. Indeed it is found that $Aβ_{25-35}$ decreases its activity *in vitro* [116]. Furthermore, since glutathione is the most prevalent antioxidant in the brain, elevated ratios of oxidized to reduced glutathione is used as a measure of intensity of oxidative stress and consequently is implicated in AD [117]. Removal of free radicals by antioxidant scavengers or enzymes protects neuronal cells from Aβ-toxicity.

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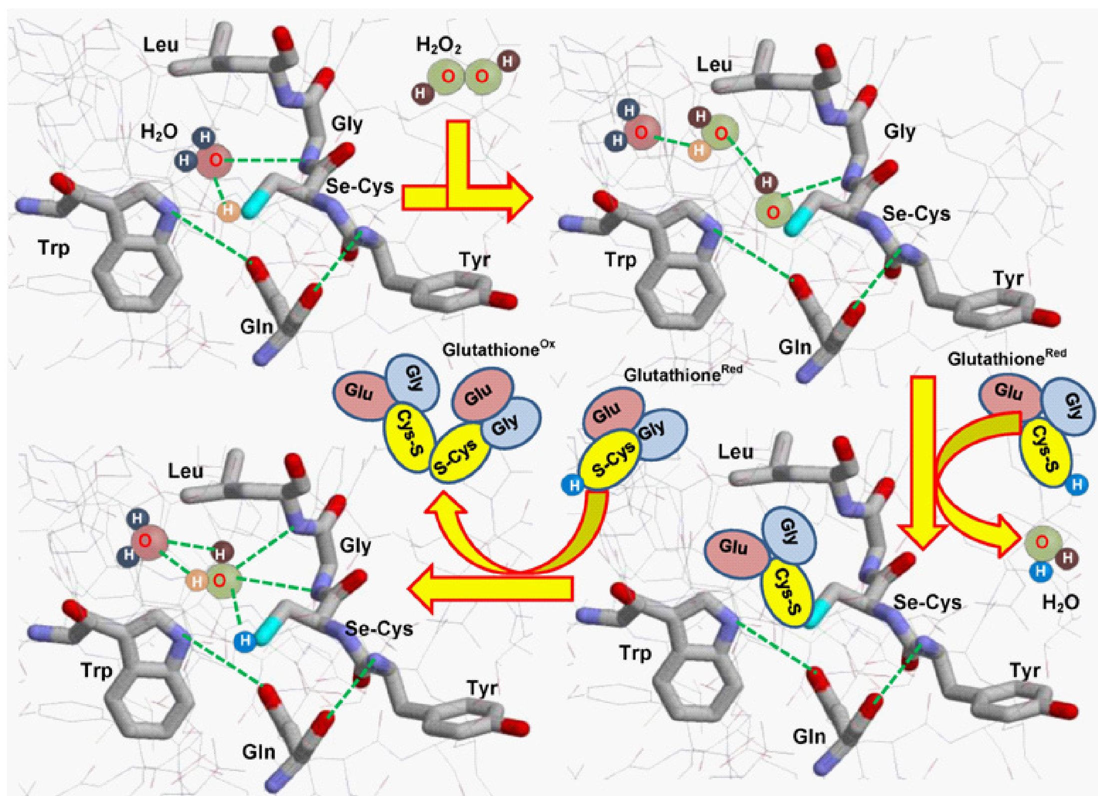

Figure 10: Mechanism for detoxifying H_2O_2 into H_2O by glutathione peroxidase. The initial reaction with H_2O_2forms a selenenic intermediate [Se-OH] at the active site before interaction with reduced glutathione [GSH] to generate a glutathiolated selenol [Se-SG].Reaction with a second molecule of reduced glutathione yields oxidised glutathione [G-S-S-G] and restores the active site.

Amyloid binding properties of glutathione peroxidase is manifested by considering antisense-sense technology. The glycine zipper motif - Gly^{33}-Leu-Met-Val-Gly^{37}- does not bind to the enzyme. Two peptide sequences: Gly^{25}-Ser^{26}-Asn^{27}-Lys^{28}-Gly^{29}and Leu^{17}- Val^{18}-Phe^{19}-Phe^{20}-Ala^{21} bind with the enzyme (antisense) about 25 – 29 Å from the active selenium centrethrough Glu^{191}-Ala^{192}-Leu^{193}-Leu^{194}-Ser^{195}and Lys^{86}-Asn^{87}-Glu^{88}-Glu^{89} –Ile^{90}respectively. This is considered a weak association when compared to the peptide Gly^{29}-Ala^{30}-Ile^{31}-Ile^{32}-Gly^{33} which interacts with the enzyme (antisense) in two domains: one quite distant (12.5Å) from the selenium atom at Pro^{134}-Ser^{135}-Asp^{136}-Asp^{137}-Ala^{138} motif [Figure 11] and the other in closerproximity (< 4.8 Å) atVal^{51}-Arg^{52}-Asp^{53}-Tyr^{54}-Thr^{55}. It is interesting and significant that the selenium atom from one subunit active centre is 7.5 Å from Lys^{86} of the other sub unit and 7.1 Å from Arg^{52} of the same sub unit.

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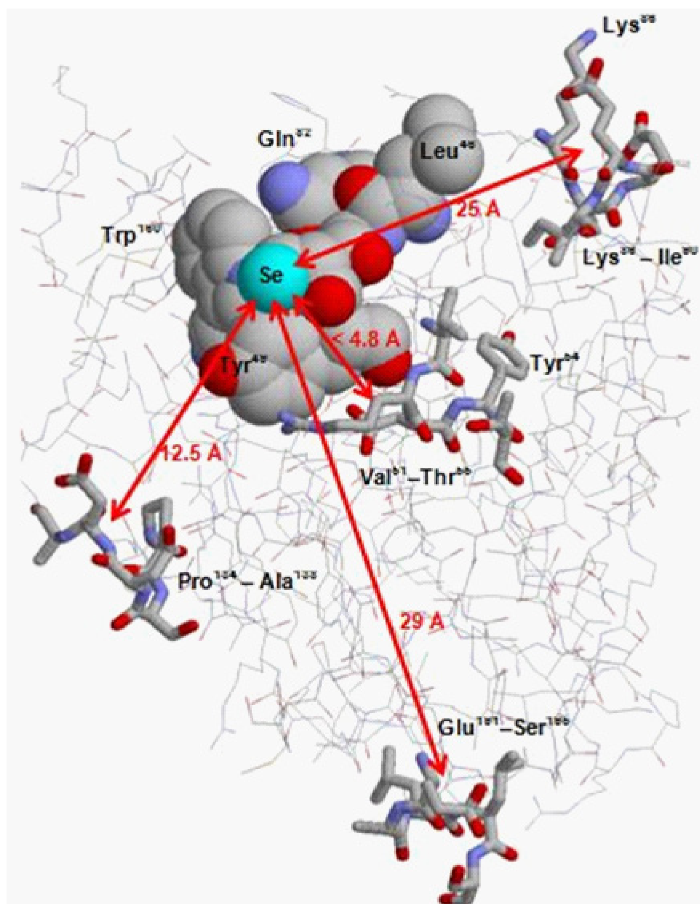

Figure 11: Antisense-sense technology illustrating possible binding of amyloid peptides with glutathione peroxidase. Two peptide sequences: Gly^{25}-Ser^{26}-Asn^{27}-Lys^{28}-Gly^{29} and Leu^{17}- Val^{18}-Phe^{19}-Phe^{20}-Ala^{21} bind with the enzyme (antisense) about 25 – 29 Å from the active selenium centre through Glu^{191}-Ala^{192}-Leu^{193}-Leu^{194}-Ser^{195} and Lys^{86}-Asn^{87}-Glu^{88}-Glu^{89} –Ile^{90} respectively. This is a weak association when compared to the peptide Gly^{29}-Ala^{30}-Ile^{31}-Ile^{32}-Gly^{33} which interacts with the enzyme (antisense) in two domains: one 12.5Å from the selenium atom at Pro^{134}-Ser^{135}-Asp^{136}-Asp^{137}-Ala^{138} motif and the other in closer proximity (< 4.8 Å) at Val^{51}-Arg^{52}-Asp^{53}-Tyr^{54}-Thr^{55}.

REVERSE FIBRILLOGENESIS

All of the reactions between the enzymes and amyloid peptides occur in the brain astrocytes and consequently any understanding of their action to either serve as biomarkers or to induce unfolding and aggregation of the amyloid peptides [118-122] into senile plaques may contribute, overall, to an understanding of nitrosative and oxidative stress in neurodegenerative disorders. Metabolites that inhibit progression of AD require an understanding of the molecular causes underlying the neurodegenerative processes. It is clear that substances that only block final stages

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of fibrillogenesis (Aβ deposition) are less effective than those that prevent the initial stages of formation (Aβ nucleation) [33,34]. Furthermore any form of Aβ-peptide that cannot interact with an enzyme must be an aggregated fibril, initially soluble and identified by Congo Red assay [123] then becoming insoluble, identified by thioflavin-T fluorescence [124-127]. The rate of formation of soluble/insoluble fibrils is dependent on both the structure of amyloid peptide as well as the enzyme [39,45-49].

Any selective molecule that inhibits the interaction of amyloid peptides with monomers will prevent the toxic formation of oligomers and their inherent toxicity [128], would prevent fibrillogenesis, suppress fibril dependent neurotoxicity and consequently slow the progress of AD [20,24,35,36]. Reversing fibril formation will, in turn, reverse oligomer formation and consequently reverse fibrillogenesis. The medical literature on amyloid-related disease [129-135] abounds with reports on anti-amyloidogenic/anti-aggregation agents [136-140].

NANOTECHNOLOGY

There is little doubt that nanotechnology/nanomedicine - terms that characterises, synthesises and apply functional units on the nanoscale (10^{-9}m) -have been exploited extensively to reveal a myriad of new opportunities in health care [141-155]. Consequently the prospect to use nanoparticles to reverse fibrillogenesis is indeed exciting. The preparation of nanoparticles bybiological processes through the bioreduction of metal salts appears to be favorableto, cost-effective and eco-friendly [156-158]. A key to understanding an Aβ-peptide-nanoparticle complex arises from considering the 'corona dynamic layer effect' [159-165] in which a hydrophobic environment is created by the peptides surrounding the nanoparticle [40]. This depletes monomeric peptide concentration, preventing the lag phase, preventing the initial association phase, preventing the formation of critical nuclei and preventing fibril initiation/elongation. Inconclusive results have otherwise been reported on the effect of nanoparticles on fibrillogenes is [166,167].

The effect of nanoparticles, on the interaction of Aβ-peptides ($Aβ_{17-21}$, $Aβ_{25-29}$, $Aβ_{29-33}$, $Aβ_{33-37}$, $Aβ_{25-37}$) with nNOS is of special interest as this opens up further insights into the mechanisms of fibrillogenesis. When gold/silver nanoparticles are incubated with induced fibrils (generated from nNOS and Aβ-peptides) there is an immediate rapid decrease in fibril concentration to zero [46] [Figure 12]. No fibril formation occurs if either nanoparticle-Aβ or nanoparticle-nNOS are incubated together. If, however, nNOS or Aβ-peptide is added respectively Aβ fibrils rapidly form but to a lesser extent [Figure 12] suggesting that the nanoparticles do not just prevent but reverse the formation of fibrils. In other words the nanoparticles are capable of either disrupting the binary adduct – nNOS-Aβ-peptides or react with, and therefore deplete, Aβ-monomers in solution and so block potential aggregation sites on the nNOS molecule.

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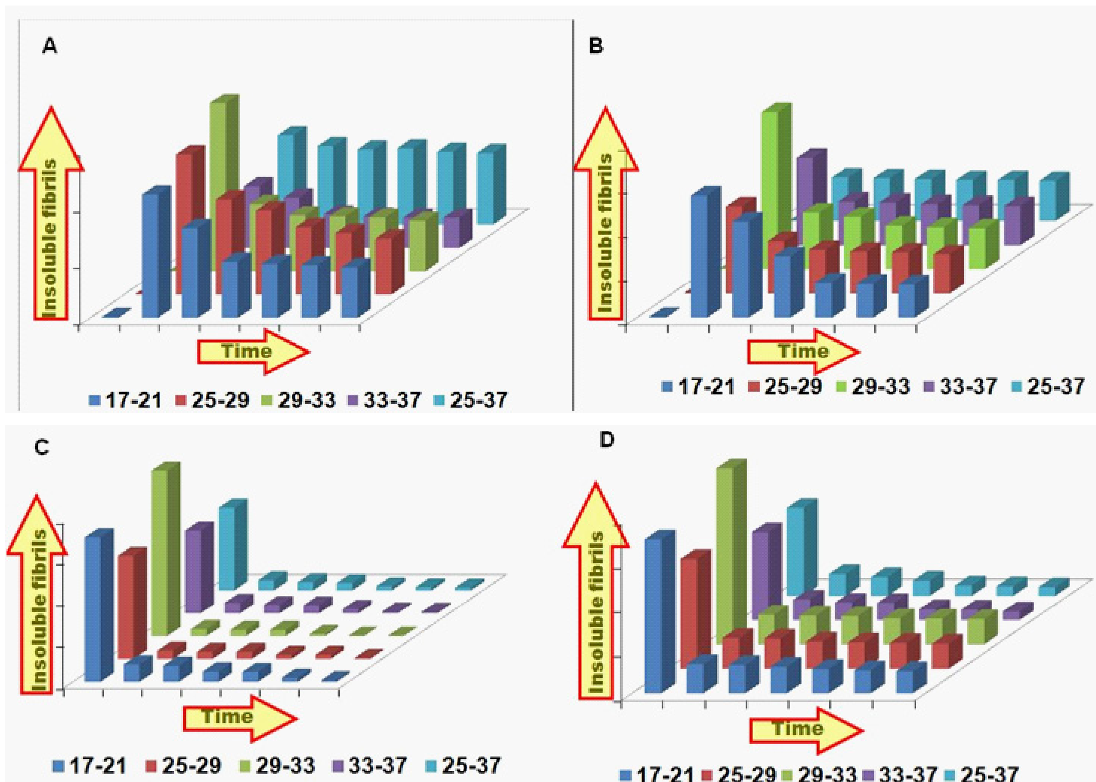

Figure 12: Insoluble fibrils (measured by Th-T fluorescence) produced with respect to time by the action of various amyloid peptides with nNOS in the presence of Au and/or Ag nanoparticles. **A:** nNOS is incubated with Ag- and/or Au-nanoparticles prior to addition of Aβ-peptides. **B:** Aβ-peptides are incubated with Ag- and/or Au-nanoparticles prior to addition of nNOS. **C:** nNOS is incubated with Aβ-peptide prior to addition of AgNP. **D:** nNOS is incubated with Aβ-peptide prior to addition of Au nanoparticles.

CONCLUDING COMMENTS

The affinity of peptide fragments $A\beta_{17-21}$, $A\beta_{25-29}$, $A\beta_{29-33}$, $A\beta_{33-37}$ and $A\beta_{25-37}$with various enzymes is measured by correlation of kinetic, thermodynamic, fluorescent and antisense/sense peptide binding analysis to support a three-stage process. First a rapid inhibition of the enzymes; second the formation of soluble fibrils (quantified by Congo Red); third complete conversion into insoluble fibrils (indicated by Thioflavin T fluorescence). This three-step scenario supports the following facts: a) fibrils are not formed from the Aβ-peptides in the absence of enzyme; b) rate of formation of insoluble fibrils is mirrored by rate of decrease of soluble fibrils; c) generated fibrils do not interfere with enzymes active site; d) there is only one binding site for the Aβ-peptide on each enzyme; e) fluorescence quenching reveal a formation of Aβ-enzyme complex; f) one tryptophan residue becomes exposed on the enzyme surface; g) this tryptophan is between 2.8 to 3.7 nm from the bound Aβ-peptide; h) peptides bind with enzymes by hydrophobic forces and

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non-spontaneous; i) critical residues within the Aβ-peptides are two phenylalanine [Phe_{19}, Phe_{20}] and two isoleucine [Ile_{31}, Ile_{32}]; j) monomeric Aβ-peptides form nucleuii on binding to enzymes then aggregate to an elongated fibril; k) Ag/Au nanoparticles reverse fibrillogenes is by depleting amyloid peptide concentration and preventing nucleii formation.

There is no general agreement about the toxic oligomer component for Aβ-amyloid fibrils though the soluble structure predominates in these arguments. It is clear, however, from both *in vivo* and *in vitro* studies [54] that the APP C-terminal glycine zipper region [Gly^{25}-Ser^{26}-Asn^{27}-Lys^{28}-Gly^{29}-Ala^{30}-Ile^{31}-Ile^{32}-Gly^{33}-Leu^{34}-Met^{35}-Val^{36}-Gly^{37}] is a critical component. Furthermore it is the disruption of the glycine zipper that decreases Aβ-toxicity.

How does the transmembrane bound glycine zipper transform into a toxic oligomer in the brain? As pointed out from other studies [168] and from discussions above the Aβ-peptide exists as a β-strand conformation while in the soluble cytoplasmic environment and then transforms to the membrane-associated α-helix structure. It is more than likely that the relatively small glycine substituted wild type Aβ-peptides form amyloid fibrils quicker than any other residue present in the crucial positions mentioned above. Furthermore it is suggested that mutant oligomerised Gly^{37}Leu Aβ-peptides may compete with wild type oligomers for the relevant binding sites. A Gly^{37}Leu substitution interferes with Aβ-channel formation [50], and decreases toxicity [54] while a dysfunction of endoplasmic reticulum and mitochondria is associated with amyloid accumulation [169-171]. Second site mutations [Ile^{31}Gly; Met^{35}Gly] all increase the toxicity by accumulative effects reflecting, not only that toxicity is directly related to the number of glycine residues but the capability of restoring a packed α-helical structure. Compounds that interfere, specifically, with glycine zipper formation will interfere with Aβ-toxicity.

Both Aβ-toxicity and amyloid precursor protein processing are the two cornerstones towards dementia in the elderly. Unfortunately interactions of any drug with APP is not without consequence. If BACE is eliminated there is emotional cognitive changes and abnormalities in myelination; if PS1 is affected then this results in memory impairment and/or synaptic plasticity; interference with nicastrin lead to skin tumours. The active site of BACE is large suggesting that any potential inhibitor would be too large to negotiate through the blood-brain barrier. Even though a decrease in γ-secretase may appear advantageous the down-side is that this enzyme is required for processing several transmembrane proteins and T- and B-cell maturation [172].

A fundamental understanding of the action and function of enzymes closely associated with oxidative and/or nitrosative stress and arginine metabolising enzymes with respect to amyloid peptide aggregation and therefore senile plaque formation facilitates a deeper understanding of neurodegeneration in Alzheimer disease. Future AD therapy must target an inhibition of glycine zipper formation that, in turn, points towards the generation of a modified C-terminal region in the APP to include other hydrophobic residues substituted in place of glycine.

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