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## A $\beta$ peptide interactions with isoflurane, propofol, thiopental and combined thiopental with halothane: A NMR study <sup>☆</sup>

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### ABSTRACT

A $\beta$  peptide is the major component of senile plaques (SP) which accumulates in AD (Alzheimer's disease) brain. Reports from different laboratories indicate that anesthetics interact with A $\beta$  peptide and induce A $\beta$  oligomerization. The molecular mechanism of A $\beta$  peptide interactions with these anesthetics was not determined. We report molecular details for the interactions of uniformly <sup>15</sup>N labeled A $\beta$ 40 with different anesthetics using 2D nuclear magnetic resonance (NMR) experiments. At high concentrations both isoflurane and propofol perturb critical amino acid residues (G29, A30 and I31) of A $\beta$  peptide located in the hinge region leading to A $\beta$  oligomerization. In contrast, these three specific residues do not interact with thiopental and subsequently no A $\beta$  oligomerization was observed. However, studies with combined anesthetics (thiopental and halothane), showed perturbation of these residues (G29, A30 and I31) and subsequently A $\beta$  oligomerization was found. Perturbation of these specific A $\beta$  residues (G29, A30 and I31) by different anesthetics could play an important role to induce A $\beta$  oligomerization.

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### 1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease affecting millions of elderly people worldwide and AD is characterized by amyloid deposits in the brain. Although AD was discovered one century ago, the molecular cause of AD is not yet known. Researchers have discovered several risk factors associated with AD, but at present there is no cure. Amyloid deposits consist of either the 40-residue containing A $\beta$ 40 and/or the 42-residue containing A $\beta$ 42 peptide. A $\beta$ 42 has a higher aggregation tendency compared to A $\beta$ 40 [1] and A $\beta$ 42 peptide is more pathogenic than A $\beta$ 40 [1,2]. The structural alteration of A $\beta$  leading to oligomerization is believed to play an important role in AD [3].

At clinically relevant concentrations of halothane and isoflurane, A $\beta$  peptide oligomerization and cytotoxicity was reported in rat pheochromocytoma cells [4]. Biophysical studies involving size-exclusion chromatography, analytical ultracentrifugation and photo-induced cross-linking experiments indicated the inhaled anesthetics halothane and isoflurane facilitate intermediate A $\beta$  oligomer formation [5]. Halothane induced more amyloid plaque in transgenic mice with AD pathology [6]. It is also reported that A $\beta$  oligomers generate *in situ* altered neuronal function [7]. However, the specific binding site of these anesthetics as well as molecular mechanism of anesthetic-induced A $\beta$  oligomerization is unknown.

We previously reported that halothane interacts with amino acid residues (G29, A30 and I31) in the critical hinge region of A $\beta$ 40 and A $\beta$ 42 using nuclear magnetic resonance (NMR) spectroscopic studies conducted in a membrane mimic environment consisting of sodium dodecyl sulphate (SDS) [8]. The oligomerization propensity of A $\beta$ 42 is much higher than A $\beta$ 40. Hence A $\beta$ 40 is better suitable than A $\beta$ 42 for time dependence NMR studies.

In this report we investigate the specificity of other anesthetics (both inhaled and intravenous) to interact with A $\beta$ 40 peptide in a membrane mimic environment. We also want to investigate whether the halothane–A $\beta$  interaction is affected by the presence of other anesthetic with different physiochemical characteristics. We wish to address the following questions:

- Does the popularly used inhaled anesthetic, isoflurane, interact with A $\beta$ 40?
- Do intravenous anesthetics, propofol and thiopental interact with A $\beta$ 40?
- Does thiopental influence A $\beta$ –halothane interactions?
- Can we derive a molecular mechanism of A $\beta$  peptide oligomerization due to the influence of these popularly used anesthetics?

### 2. Materials and methods

To understand these important questions, we have conducted a series of NMR experiments to investigate A $\beta$ 40 peptide interactions with isoflurane, propofol, thiopental and thiopental combined with halothane.

<sup>☆</sup> Part of the results was presented at Biophysical Society meeting, 2007.

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## 2.1. Materials

Deuterated sodium dodecyl sulphate (SDS<sub>D25</sub>) (Cambridge Isotope Laboratories), halothane (Sigma-Aldrich), isoflurane (Lancaster Synthesis Inc., USA), propofol (Sigma-Aldrich, USA), and thiopental (Abbott Laboratories, Chicago, USA) were procured. We purchased uniformly <sup>15</sup>N labeled Aβ40 from Recombinant Peptide Technologies (Atlanta, GA, USA). We used a gas tight micro syringe (SGE LE Syringe, Australia) for the addition of halothane and isoflurane. For anesthetic (halothane and isoflurane) concentration determination, two coaxial NMR tubes (5 mm and 3 mm) were used [9]. The chemical structure of four anesthetics under study, i.e., halothane, isoflurane, propofol, and thiopental is shown in Fig. 1. Reported molecular volumes for halothane, isoflurane, propofol are 110 Å<sup>3</sup> [10], 144 Å<sup>3</sup> [10] and 191 Å<sup>3</sup> [11], respectively. The molecular volume of thiopental is higher than propofol. Halothane and isoflurane both are inhaled anesthetics with similar physicochemical characteristics. Both halothane and isoflurane are liquid at room temperature, similar boiling points and hydrophobic in nature. Halothane belongs to haloalkane category and isoflurane belongs to haloether category. Halothane is smaller sized than isoflurane. On the other hand, propofol and thiopental are intravenous anesthetics and both belong to non-opioid category. Both propofol and thiopental have similar physicochemical characteristics as propofol is rapidly redistributed like the thiopental. Propofol is a substituted phenol and thiopental contains cyclic ring with electronegative oxygen and sulphur atoms. Thiopental is bulkier than propofol.

## 2.2. Preparation of Aβ peptide solution in SDS

SDS<sub>D25</sub> powder was added in 200 μl PBS buffer (pH 7.2), mixed slowly and sonicated for a few seconds. One milligram uniformly <sup>15</sup>N labeled lyophilized powder of Aβ40 was added to SDS<sub>D25</sub> solution and gently mixed. A sodium 3-trimethylsilyl-[2,2,3,3-<sup>2</sup>H<sub>4</sub>]-1-propionate (TSP) solution in D<sub>2</sub>O (50 μl) was added to the Aβ40 solution. TSP served as a chemical shift reference and D<sub>2</sub>O provided the field/frequency lock for the NMR spectrometer. The final volume of each NMR solution containing Aβ40 was 500 μl and the concentration of <sup>15</sup>N labeled Aβ40 peptide was 0.45 mM. The pH of the Aβ peptide solution was adjusted and measured at 7.2 for all NMR studies before addition of anesthetics.

## 2.3. Anesthetic addition to the Aβ peptide solution

For isoflurane, we added total 10 μl neat isoflurane consecutively (3 μl, 3 μl and 4 μl respectively) to the Aβ peptide solution using an air tight micro syringe. For propofol, 3 μl neat propofol solution was

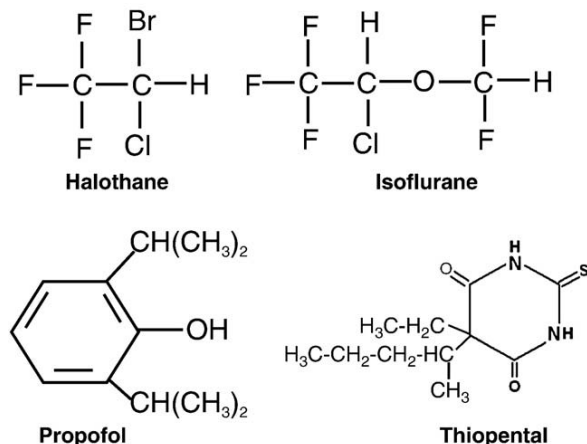


Fig. 1. The structure of four different anesthetics under study.

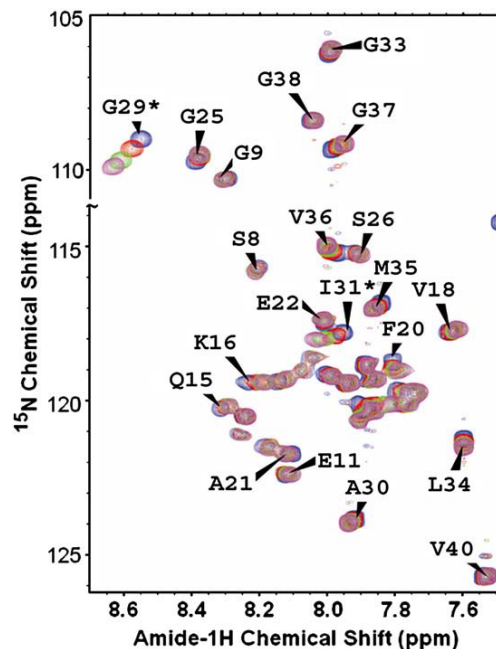


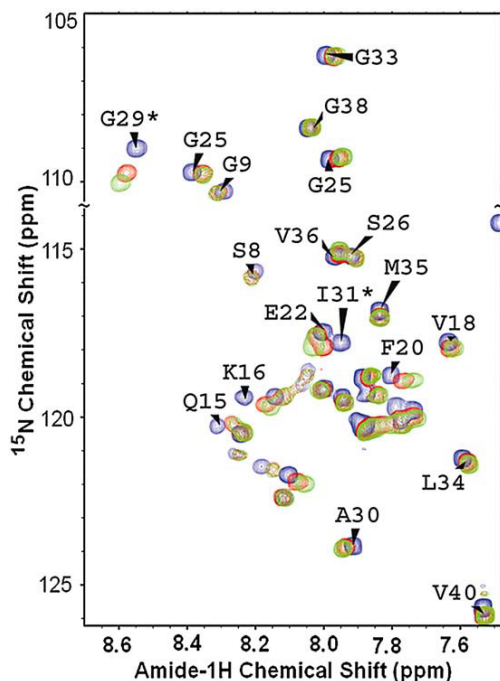
Fig. 2. An overlay of <sup>15</sup>N/H HSQC spectra of uniform labeled Aβ40 in SDS micelles with/without isoflurane is presented. Each peak in the HSQC spectra corresponds to the amide proton of Aβ40. The Y-axis represents the chemical shift position of <sup>15</sup>N of the amide proton and X-axis represents the <sup>1</sup>H chemical shift of the amide protons. The sequence specific assignments are performed based on different NMR experiments as mentioned in the text. The color-coding of the amide protons represents different conditions: (1) Only Aβ40 peptide (blue); (2) Aβ40+3 μl isoflurane (red); (3) Aβ40+6 μl isoflurane (green) and Aβ40+10 μl isoflurane (magenta), respectively. Critical residues involved in Aβ oligomerization are indicated by asterisks (\*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

added directly to the Aβ peptide solution followed by the addition of another 3 μl neat propofol solution. The final propofol concentration was 63.98 (~64) mM. For thiopental (MW 264.321), addition of 6 μl stock solution (400 mM) to 500 ml Aβ40 solution yields 4.8 mM thiopental concentration. The final thiopental solution was 7 mM with the addition of 3 μl thiopental solution (400 mM).

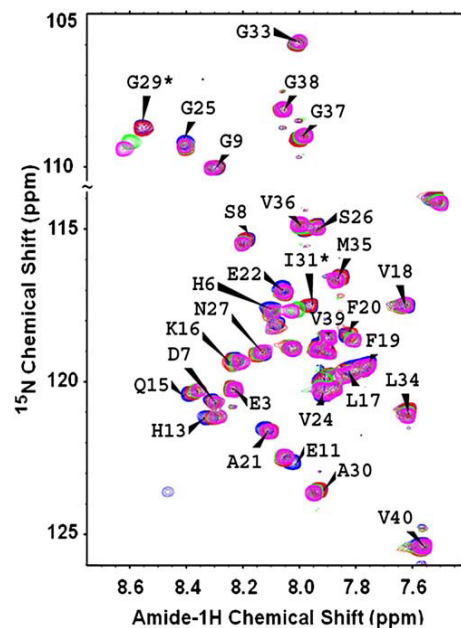
In the combined thiopental and halothane experiments, 5 μl thiopental stock solution (400 mM) was added first to the Aβ peptide solution (thiopental concentration 4 mM) and HSQC experiment was performed. Then 4 μl halothane was added to that solution and a HSQC experiment was performed. An additional 2 μl halothane was further added followed by another HSQC experiment. Finally another 2 μl halothane was added, thus the solution contains 4 mM thiopental plus 8 μl halothane and HSQC experiments performed at different time points.

## 2.4. NMR experimental details and data analysis

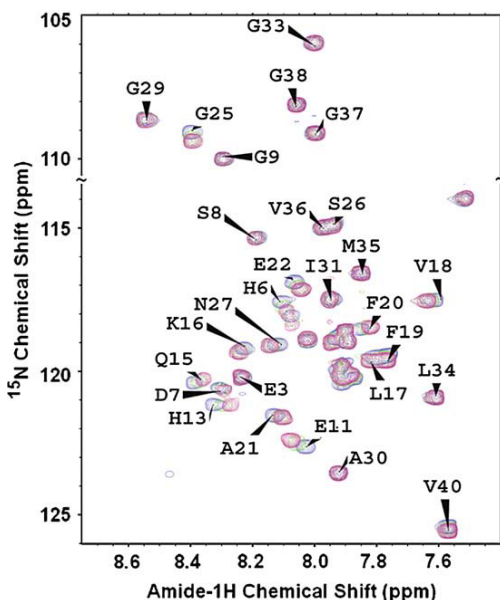
We performed all NMR experiments using a Bruker DRX spectrometer operating at a proton frequency of 500.132 MHz using a 5 mM TXI probe (Bruker, Germany). The sequence specific assignments of all amide protons of Aβ40 at pH 7.2 were performed earlier. For each anesthetic, first we prepared the Aβ40 peptide solution at pH 7.2 and added the respective anesthetic mixed gently and left anesthetic to equilibrate followed by a HSQC experiment. All NMR data from the Bruker instrument were converted into nmrPipe format and processed using nmrPipe programs [12] using an Octane silicon graphics computer. Data were analyzed using PIPP [13] and SPARKY [14] programs using in-house scripts. Halothane and isoflurane concentrations were determined using our modified experimental technique consisting of two coaxial NMR tubes (5 mm and 3 mm) [9].



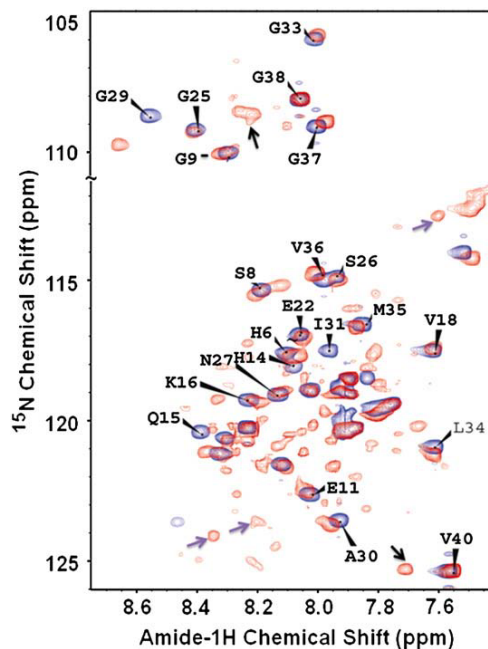
**Fig. 3.** Overlay of  $^{15}\text{N}/\text{H}$  HSQC spectra of uniform labeled  $\text{A}\beta_{40}$  in SDS micelles with/without propofol is presented. Each peak in the HSQC spectra corresponds to the amide proton of  $\text{A}\beta_{40}$ . The Y-axis represents the chemical shift position of  $^{15}\text{N}$  of the amide nitrogen and X-axis represents the  $^1\text{H}$  chemical shift of the amide protons. The sequence specific assignments are performed based on different NMR experiments as mentioned in the text. The color-coding of the amide protons represents different conditions: (1) Only  $\text{A}\beta_{40}$  peptide (blue); (2)  $\text{A}\beta_{40}+32$  mM propofol (red); (3)  $\text{A}\beta_{40}+64$  mM propofol (green), respectively. Critical residues involved in  $\text{A}\beta$  oligomerization are indicated by asterisks (\*) sign. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 5.** Overlay of  $^{15}\text{N}/\text{H}$  HSQC spectra of uniformly  $^{15}\text{N}$  labeled  $\text{A}\beta_{40}$  in SDS micelles in four different conditions: (1)  $\text{A}\beta_{40}$  only (blue); (2)  $\text{A}\beta_{40}+4$  mM thiopental (red); (3) 4 mM thiopental+4  $\mu\text{l}$  halothane (green); and 4) 4 mM thiopental+6  $\mu\text{l}$  halothane (magenta). The Y-axis represents the chemical shift positions of the amide nitrogens ( $^{15}\text{N}$ ) and the X-axis represents the chemical shift positions of the amide protons ( $^1\text{H}$ ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** Overlay of  $^{15}\text{N}/\text{H}$  HSQC spectra of uniform labeled  $\text{A}\beta_{40}$  in SDS micelles with/without thiopental is presented. Each peak in the HSQC spectra corresponds to the amide proton of  $\text{A}\beta_{40}$ . The Y-axis represents the chemical shift position of  $^{15}\text{N}$  of the amide nitrogen and X-axis represents the  $^1\text{H}$  chemical shift of the amide protons. The sequence specific assignments are performed based on different NMR experiments as mentioned in the text. The color-coding of the amide protons represents different conditions: (1) Only  $\text{A}\beta_{40}$  peptide (blue); (2)  $\text{A}\beta_{40}+4.7$  mM thiopental (green); (3)  $\text{A}\beta_{40}+7$  mM thiopental (magenta), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 6.** Overlay of  $^{15}\text{N}/\text{H}$  HSQC spectra of uniformly  $^{15}\text{N}$  labeled  $\text{A}\beta_{40}$  in SDS micelles in two different conditions: (1)  $\text{A}\beta_{40}$  only (blue); (2) 4 mM thiopental+8  $\mu\text{l}$  halothane after 28 h (red). The Y-axis represents the chemical shift positions of the amide nitrogens ( $^{15}\text{N}$ ) and the X-axis represents the chemical shift positions of the amide protons ( $^1\text{H}$ ). The additional broad amide peaks marked by arrows indicate the oligomerization due to halothane in the presence of thiopental. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3. Results and discussions

A $\beta$  peptide adopts a random coil conformation in aqueous medium which leads to a time-dependent aggregation of A $\beta$  peptide in aqueous solution [15–20]. It is important to note that the structure of A $\beta$  peptides in membranes is of high interest, as it is believed that A $\beta$  damages the cellular membrane resulting in a cytotoxic effect [21]. Previous findings from our laboratory demonstrated brain membrane alterations in both AD autopsy samples and probable AD subjects [22–28]. The SDS micelle environment provides an appropriate membrane mimicking system for NMR studies. In the present work, we have studied the A $\beta$ 40 interactions with isoflurane, propofol, thiopental and thiopental combined with halothane in a membrane mimic SDS medium.

#### 3.1. A $\beta$ peptide interactions with single anesthetics

##### 3.1.1. Isoflurane interaction with A $\beta$ 40

Fig. 2 is an overlay of four HSQC spectra of uniform  $^{15}\text{N}$  labeled A $\beta$ 40 with and without 3  $\mu\text{l}$ , 6  $\mu\text{l}$  and 10  $\mu\text{l}$  isoflurane. These NMR

experiments were designed to investigate the concentration dependence of isoflurane interactions with A $\beta$ 40 peptide. The sequence specific assignments of amide proton of A $\beta$ 40 (without isoflurane) are shown in blue. The amide protons of A $\beta$ 40 are shown in different colors with the addition of 3  $\mu\text{l}$  (red), 6  $\mu\text{l}$  (green) and 10  $\mu\text{l}$  (magenta) isoflurane. Changes in chemical shift due to addition of isoflurane were observed for the critical amide proton of G29, A30 and I31 (Fig. 2). The isoflurane concentration after addition of 3  $\mu\text{l}$ , 6  $\mu\text{l}$  and 10  $\mu\text{l}$  in a 500  $\mu\text{l}$  SDS solution was found to be 26.57, 53.26 and 81.69 mM as experimentally determined by  $^{19}\text{F}$  NMR using two coaxial NMR tubes (5 mm and 3 mm) [9].

##### 3.1.2. Propofol interactions with A $\beta$ 40

Fig. 3 is an overlay of three HSQC spectra of uniform  $^{15}\text{N}$  labeled A $\beta$ 40 with and without 32 mM and 64 mM propofol. The amide protons of A $\beta$ 40 are shown in blue (without propofol) and in the presence of 32 mM (red) and 64 mM (green) propofol concentrations. Changes in chemical shift observed for amino acid residues, G29, A30 and I31 were similar to A $\beta$ 40–isoflurane as shown in Fig. 2.

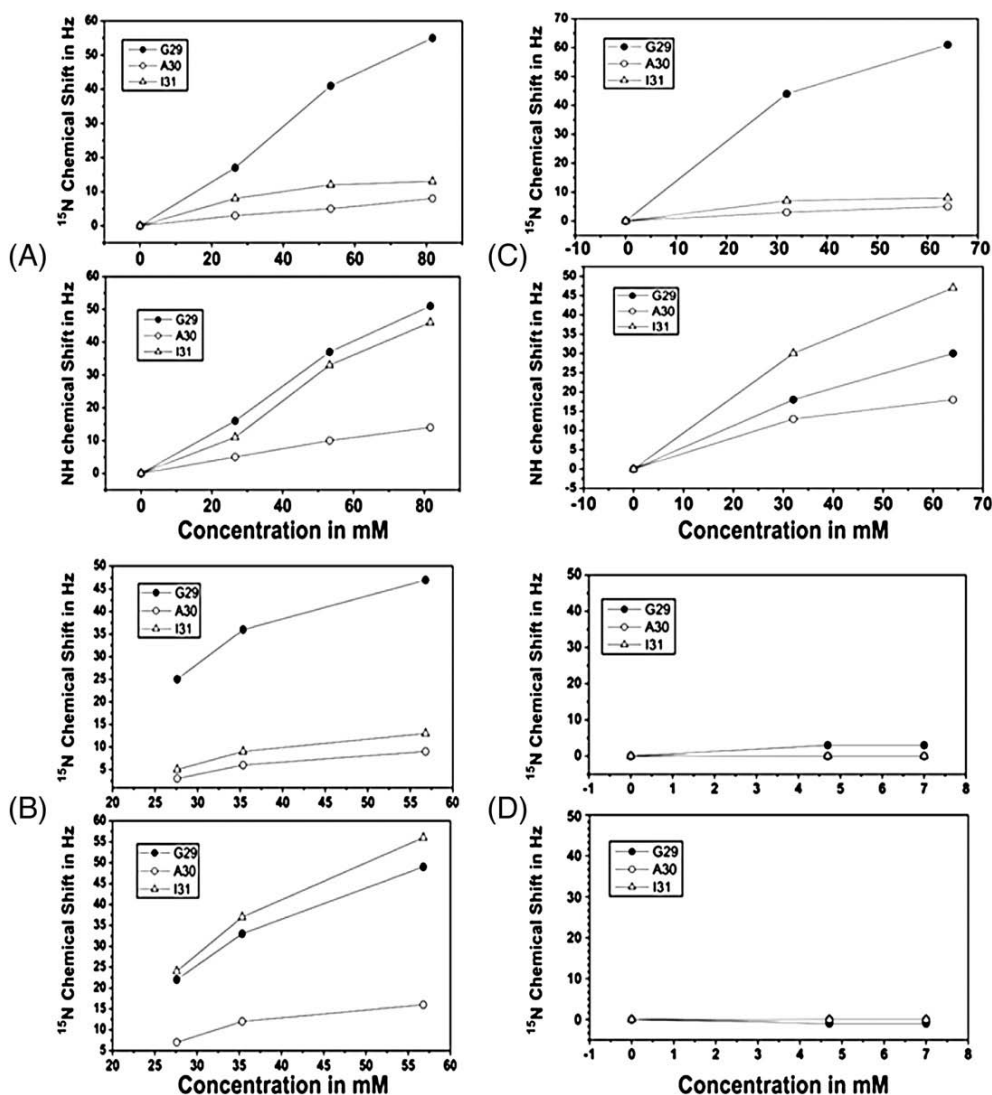


Fig. 7. Chemical shift changes (NH and  $^{15}\text{N}$ ) of three residues (G29, A30 and I31) due to addition of isoflurane (A), thiopental with halothane (B), propofol (C) and with thiopental (D) at various concentrations.

### 3.1.3. Thiopental interactions with A $\beta$ 40

Fig. 4 is an overlay of three HSQC spectra; one without thiopental (A $\beta$  only, blue), the others with 4.8 mM (green) and 7 mM (magenta) thiopental concentrations. No change in chemical shift was observed for critical amino acid residues, G29, A30, and I31 due to thiopental. A $\beta$  peptide remains in monomeric form in the presence of 7 mM thiopental. It is very important to note that other residues (i.e. F20, Q15 etc) show chemical shift in the presence of isoflurane, thiopental and propofol.

### 3.2. A $\beta$ peptide interactions with thiopental and halothane in combination

Fig. 5 is an overlay of four HSQC spectra of uniform  $^{15}\text{N}$  labeled A $\beta$ 40 without thiopental (blue), with 4 mM thiopental (red), 4 mM thiopental+4  $\mu\text{l}$  halothane (green), and 4 mM thiopental+6  $\mu\text{l}$  halothane (magenta), respectively. These NMR experiments were designed to investigate the effect halothane on A $\beta$  in the presence of thiopental with different physiochemical characteristics. Changes in chemical shift were observed for the amide protons of amino acid residues, G29 and I31 after the addition of halothane (Fig. 5). Fig. 6 is an overlay of two HSQC spectra of uniformly  $^{15}\text{N}$  labeled A $\beta$ 40 without thiopental (blue) and with 4 mM thiopental+56.8 mM halothane (red) after 28 h (Fig. 6). We have determined experimentally that 4  $\mu\text{l}$ , 6  $\mu\text{l}$  and 8  $\mu\text{l}$  halothane in 505  $\mu\text{l}$  SDS solution corresponds to 27.6, 35.35 and 56.8 mM using a recently developed technique using 3 mm and 5 mm coaxial NMR tubes to measure inhaled anesthetic concentration [9].

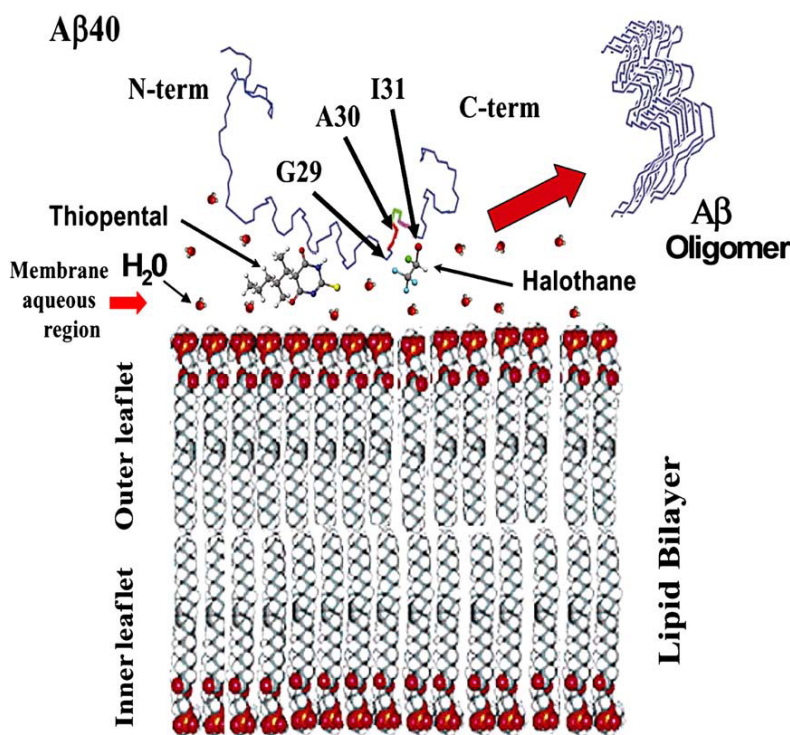
### 3.3. Topological evidence for A $\beta$ peptide and anesthetics interactions

It has been suggested that anesthesia might result from molecular interactions at the interface between aqueous and non-

polar media (e.g., membrane) [29]. This suggestion is supported by NMR and gas chromatography studies of clinical anesthetics with macromolecular surfaces [30]. Recent progress in computational studies of membrane aqueous interfaces has made it possible to estimate concentration profiles of anesthetics across these interfaces by computer simulations [31–33]. A $\beta$ 40 peptide is located in the membrane aqueous environment as determined by earlier studies using backbone dynamics studies [34]. Halothane, isoflurane and propofol are generally located in the membrane aqueous region [35–37]. Anesthetics and A $\beta$  peptide are localized in a similar domain/region and the interactions between A $\beta$  and anesthetics are supported due to topological coexistence.

### 3.4. Oligomerization of A $\beta$ peptide as observed by NMR spectroscopy

HSQC is an extremely powerful experiment to identify the oligomerization of uniformly labeled peptides or proteins. Under normal conditions, uniformly  $^{15}\text{N}$  labeled A $\beta$ 40 peptide (monomeric form) shows 40 unique amide peaks in the two-dimensional HSQC spectra (X-axis represents amide proton ( $^1\text{H}$ ) chemical shift and Y-axis represents  $^{15}\text{N}$  chemical shifts of A $\beta$  peptide). The chemical shift range of amide protons is generally 6.5 ppm to 10 ppm; however,  $^{15}\text{N}$  chemical shift range is generally 100 to 130 ppm. Due to wide dispersion of  $^{15}\text{N}$  chemical shift range, HSQC spectrum is less crowded for a small peptide like A $\beta$ 40. In the event of oligomerization of A $\beta$ 40, the HSQC spectra shows additional amide peaks [38]; the intensity of these additional peaks are generally lower because of oligomerization and slow molecular tumbling. The number of additional amide peaks indicates the extent of aggregation (e.g., dimeric, trimeric, tetrameric etc.) which exclusively depends on the system under study [38].



**Fig. 8.** A schematic diagram of both thiopental and halothane interactions with A $\beta$  on the extracellular side of the plasma membrane. The molecular volume of thiopental is greater than that of halothane. Hence, thiopental cannot access the critical amide protons in the loop regions of A $\beta$  and these residues are accessible by the smaller sized halothane. Halothane modulates these three critical residues and accelerates A $\beta$  oligomerization in the presence of thiopental.

### 3.5. Role of three amino acid residues in A $\beta$ oligomerization and a schematic model

Proteins are potential molecular targets of anesthetics [39]. It is reported by NMR spectroscopic studies that halothane binding results in chemical shift change in PDZ-95 PDZ2, a neuronal protein located at the synapses [40]. Fluorinated compounds are known to increase the helical content of peptides, most likely by desolvating the peptide [41,42]. Binding of the anesthetics molecules in the hydrophobic pocket of protein is also reported [39]. It has been shown that bound volatile general anesthetics alter both local protein dynamics and global protein stability [36,37,43]. A $\beta$ 40 peptide has two  $\alpha$ -helices [ $\alpha$ -helix-I (residues 15–23) and  $\alpha$ -helix-II (residues 32–36)] connected by a more flexible kink region [44]. The dynamics of the kink (hinge) region of A $\beta$  peptide is altered due to interaction with anesthetics as indicated by the profound chemical shift changes of G29 and I31. This could bring the two helices of A $\beta$  closer and it might initiate the cascade of events leading to A $\beta$  oligomerization.

NMR data indicate that isoflurane, propofol and halothane in the presence of thiopental interact with the critical residues G29, A30 and I31. On contrary, thiopental alone does not interact with these residues. In this study, four anesthetics (halothane, isoflurane, propofol and thiopental) consisting of two inhaled (halothane and isoflurane) and two intravenous anesthetics (propofol and thiopental). The physicochemical characteristics (identified by chemical structure as well as their physical properties) of these anesthetics may play an important role for interaction with A $\beta$  peptide. Halothane and isoflurane have similar physicochemical character and among these two anesthetics, halothane is much smaller than isoflurane. So molecular size is a factor comparing these two anesthetics. It is very important to note that halothane (in the presence of thiopental) induces more chemical changes of three critical residues than isoflurane with similar concentration range (Fig. 7). In the case of intravenous anesthetics, propofol and thiopental have similar physicochemical characteristics. Thiopental is larger than propofol and similarly the size factor between these anesthetics may be important for the differential pattern of interaction with A $\beta$  peptide. This explains why no changes in chemical shifts were observed for G29, A30 or I31 of A $\beta$ 40 peptide with the addition of thiopental (Fig. 7). Also, no A $\beta$ 40 oligomerization was observed in the presence of thiopental.

The peak intensity of three peaks (G29, A30 and I31) is also reduced significantly due to halothane (in the presence of thiopental), isoflurane and propofol (figure not shown) and G29, A30 and I31 peak intensity did not change due to thiopental (figure not shown). Thiopental did not interact with the three critical residues and did not influence the interaction the halothane–A $\beta$  interactions. A schematic diagram [8] of anesthetic-induced A $\beta$  oligomerization of A $\beta$  peptide is shown in Fig. 8. This schematic diagram is intended to illustrate our hypothesis that anesthetics interact with A $\beta$  peptide in the membrane aqueous domain and induce structural changes that lead to A $\beta$  oligomerization. Fig. 8 also shows that due to its bulkier size, thiopental could not fit the hydrophobic pocket. Hence the interaction of halothane with these three residues is not influenced in the presence of thiopental. The A $\beta$ 40 structure is taken from the PDB data bank (1BA4) [45]. To investigate the influence of anesthetic size on A $\beta$  oligomerization, future research with bulkier anesthetics (e.g. midazolam, etomidate and ketamine) are warranted.

## 4. Conclusions

This report reveals the molecular details of A $\beta$  peptide interactions with several anesthetics that differ in physicochemical properties and molecular volumes. Existing literature indicates that amyloid load is increased in transgenic mice with halothane exposure [6]. These animal model studies need to be extended to intravenous as well as

combination of different types of anesthetics. The NMR experimental approach utilized in this study can be extended to other amyloid peptides including the islet amyloid polypeptides [46–51].

The anesthetic concentrations used in our NMR studies were higher than anesthetic concentrations used in clinical settings. Clinically relevant concentrations of halothane and isoflurane are approximately 0.3 mM [52–54]. In our NMR studies the A $\beta$  peptide concentration was 0.45 mM, much higher than *in vivo* ( $21 \pm 7 \mu\text{M}$ ) concentrations [5]. However, A $\beta$  peptide concentrations used in *in vitro* studies are usually  $\sim 22$  fold higher than *in vivo* concentrations, in keeping with the higher anesthetic concentrations used in our biophysical studies. Our NMR studies of A $\beta$  peptide with different anesthetics are being extended at clinically relevant concentrations. Future studies with A $\beta$ -anesthetic interactions in lipid bilayers environment will be performed.

## Acknowledgement

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