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## **In vivo magnetic resonance imaging and spectroscopy of Alzheimer\_s disease in transgenic mice**

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# 5 Correlation between the severity of amyloid- $\beta$ deposition and altered neurochemical profile in a transgenic mouse model of Alzheimer's disease, observed by $\mu$ MRI and high resolution two-dimensional MRS\*

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## 5.1 Abstract

Localized two-dimensional magnetic resonance spectroscopy was applied in combination with high resolution  $\mu$ MRI to follow the exact sequential relationship between the severity of A $\beta$  deposition and altered neurochemical profile with age in the brain of a transgenic mouse model of Alzheimer's disease. Localized 2D MR spectra obtained from the cortex-hippocampus area of the 18 month old transgenic mice show a significant alteration in a number of neurometabolites. N-acetylaspartate, glutamate and glutathione show a clear decline, while the level of taurine shows an increase in AD mice as compared to controls. In addition, a significant increase (~75%) was detected in the level of glycerophosphocholine in AD mice. A correlation between age dependent changes in the level of GPC measured by 2D MRS and severity of A $\beta$  deposition visualized by  $\mu$ MRI has been followed in the same transgenic mice. Our results show that the increase in GPC with age in transgenic mice is directly correlated with the severity of plaque deposition up to 18 months of age. Interestingly the level of GPC drops drastically at the age of 20 months, which may signify gross membrane impairment at later stages of AD. This study provides the first direct *in vivo* evidence for the increase in GPC in plaque affected areas and suggests that altered GPC may be a valuable marker for early diagnosis and for testing therapeutics in the AD mouse model.

## 5.2 Introduction

Alzheimer's disease is the most common form of dementia, afflicting mainly the elderly. With the rapid aging of western societies, this disease is set to become a major problem in healthcare. Currently several methods are employed in attempts to diagnose AD; In

addition to psychological examination and examination of metabolite levels from collected blood samples, several neuroimaging techniques have been employed in an attempt to visualize AD pathological hallmarks (see Chapter 1). However, a definitive diagnosis of AD is only possible post-mortem, by detecting the two hallmarks of the disease- amyloid plaques and neurofibrillary tangles in the brain tissue (1). There is thus a great need for biomarkers to diagnose AD with high specificity and sensitivity in a clinical setting in humans, as well as in transgenic animal models for preclinical drug research.

Proton magnetic resonance spectroscopy provides a non-invasive way to investigate *in vivo* neurochemical abnormalities of many brain disorders. Several groups have applied MRS in the study of AD, both in transgenic mice (2-4) and in humans (5-10). In these studies the most commonly altered metabolites in AD were *myo*-inositol, taurine, glutamate, glutamine and N-acetylaspartate. Although a decrease in NAA, glutamate, glutamine and an increase in *myo*-inositol or taurine has been consistently observed in AD, these changes are not specific to only AD, since they have been shown to occur in other neurodegenerative diseases such as Huntington's disease (11,12), Parkinson's disease (13,14) as well as in other brain disorders (15-17). Therefore, specific *in vivo* MRS markers of AD are still missing. While transgenic mouse models of AD might be instrumental in discovering new *in vivo* biomarkers of AD, the use of localized *in vivo* 1D MRS in mice is often hampered by low sensitivity of local measurements due to both the small size of the brain resulting in limited signal-to-noise ratio and low concentrations of several brain metabolites. In addition a considerable overlap of peaks of metabolites with coupled spin systems restrict the number of molecules which can be uniquely assigned in 1D MRS (18).

To overcome the problem of spectral resolution and peak overlap, we have recently employed a 2D MRS at 9.4T and obtained the first highly resolved localized 2D MR spectra from the hippocampus-cortex regions of the living mouse brain (Chapter 4). Due to spreading of resonances in two dimensional surfaces, a large number of metabolites were unambiguously assigned based on their network of cross-peaks in localized region of the mouse brain *in vivo*. These results show that localized 2D MRS at high field strength can offer a better scope for identifying potential biomarkers of various brain diseases, including AD, using the various available transgenic mouse models.

In the present study we have mapped the neurochemical composition of transgenic AD mice and control mice, and followed the neurochemical changes with age in the same

animals, using high resolution localized 2D MRS. A noteworthy finding in this study was an increase in the level of GPC in Tg2576 mice compared to control mice. A correlation between age dependent changes in the level of GPC measured by 2D MRS and severity of A $\beta$  deposition visualized by  $\mu$ MRI has been followed in the same transgenic mice. Our results show that the age-dependent increase in GPC in transgenic mice correlates well with the severity of plaque deposition. Altered GPC levels may be a valuable marker to test therapeutics in the AD mouse model.

### **5.3 Materials & methods**

#### **5.3.1 Transgenic mice**

The transgenic mice used in this study contain as transgene the Swedish double mutation of the human amyloid precursor protein (APP<sub>695</sub>), as developed and described previously by Hsiao *et al.* (19). The transgene is expressed in C57B6 breeders. The N2 generation mice of both genders (n=30) were studied at ages between 8 and 24 months. Age-matched non-transgenic littermates served as controls. All animal experiments were approved by the Institutional Animal Care and Animal Use Committee in accordance with the NIH Guide for the care and Use of Laboratory Animals. For all *in vivo* MR measurements the mice were anesthetized using Isoflurane (Forene, Abbott, UK) inhalation anesthesia, together with air and oxygen (1:1) at 0.3 l/minute. The anesthetic gas was administered via a special face mask, which also served as a fixation device for the mouse head by coupling it with a specially designed toothbar to hold the head in place (Bruker Biospin). While inside the probe, the respiration rate of the mouse was constantly monitored by means of a pressure transducer placed on the abdomen. The transducer was connected to an SA Instruments MR compatible small animal monitoring and gating system, which was interfaced to an SA Instruments command module and laptop running SAM PC monitoring software (SA Instruments, Inc. Stony Brook, NY, USA).

#### **5.3.2 MR Measurements**

All  $\mu$ MRI and 1D & 2D MRS measurements were conducted at 25° on a vertical wide-bore 9.4T Bruker Avance 400WB spectrometer, with a 1000 mTm<sup>-1</sup> actively shielded imaging gradient insert (Bruker BioSpin). The RF coil used was a 25 mm volume coil, specifically, a birdcage transmit/receive coil (Bruker BioSpin). The system was interfaced to a Linux pc running Topspin 1.5 and Paravision 4.0 imaging software (Bruker BioSpin).

***μMRI***

All μMRI measurements were done using a RARE sequence (20) which employs a single excitation step followed by the collection of multiple phase encoded echoes. Basic measurement parameters used for the RARE sequence were: TE = 10.567 ms (22.45 ms effective), TR = 6 s, flip angle = 90°, NA = 4, RARE factor (echo train length) = 4. The field of view was  $2.00 \times 2.00 \text{ cm}^2$ , with image matrix of  $256 \times 256$ . This yields an effective in-plane resolution of approximately 78 μm. Coronal (transverse) images were obtained with a slice thickness of 0.5 mm in a total scan time of approximately 25 minutes.

***MR spectroscopy***

To select a volume of interest, localized  $T_2$ -weighted images were acquired using the RARE sequence as described above. The MRS voxels were localized either in: (a) the middle of the mouse brain, covering predominantly the thalamus region and some parts of the hippocampus ( $4 \times 4 \times 4 \text{ mm}^3$ ; 64 μl), or (b) in the cortex-hippocampus regions in the mouse brain ( $1.7 \times 4 \times 4 \text{ mm}^3$ ; 27 μl). The local field homogeneity was optimized by adjustment of first- and second-order shim coil currents using the FASTMAP sequence. The field homogeneity in a 27-64 μl voxel typically resulted in water line-widths of ~16-20 Hz in live mouse brain.

The PRESS sequence (21) was used for localized 1D MR spectroscopy. This sequence uses 3 hermite RF pulses (90°, 180°, 180°). The sequence details are described by Mandal et al. (22). The repetition time and echo time were 1500 ms and 15 ms respectively. The PRESS sequence used 2048 complex points, with a spectral width of 11 ppm. The 1D spectra were collected in 512 scans with a scan time of approximately 13 minutes.

The localized 2D MRS was measured using the 2D L-COSY sequence (18) as described in Chapter 4. The sequence consists of three RF pulses (90°, 180°, 90°), slice-selective along 3 orthogonal axes. The last slice-selective 90° RF pulse also served as a coherence transfer pulse for the L-COSY spectrum necessary for correlating the metabolites peak in the second dimension. Optimized hermite 90° and 180° RF pulses with 1 ms durations were used for localization. The bandwidth of 90° and 180° RF pulses were 5.4 KHz and 3.4 KHz, respectively. A total of 16 phase cycles for the three RF pulses were used for each localized  $\Delta t_1$  increment. To achieve a short echo time of 15 ms, the duration of the spoiler gradient necessary to dephase the unwanted magnetization from outside the voxel was kept to a minimum. Both the 1D PRESS sequence and 2D L-COSY sequence were

preceded by a VAPOR sequence (23) for global water suppression. The sequence consists of 7 variable power RF pulses with an optimized relaxation delay. The relaxation delays  $\tau_1$ - $\tau_7$  between the consecutive pulses were 150, 80, 160, 80, 100, 37.11 and 57.36 ms, respectively. The water suppression bandwidth was set at 350 Hz. Outer volume suppression was combined in an interleaved mode with the water suppression scheme, thus improving the localization performance and reducing the demands for spoiler gradients. The OVS scheme used a total of 18 hyperbolic secant RF pulses, each with 90° nominal flip angle and 1 ms pulse length. The OVS slice thickness was 4 mm with a 0 mm gap to the voxel.

Localized 2D MR spectra were recorded using a TE of 15 ms ( $TE_1=6$ ms and  $TE_2=9$ ms), and a TR of 1500 ms (Fig. 5.1). In order to obtain a feasible scan time, 2D MR spectra were recorded using 2048 complex points along  $F_2$  and 192 incremental steps along  $F_1$ , with a spectral width of 11 ppm and 20 averages per excitation step. This resulted in a total of 3840 scans ( $192 \Delta t_1$  increments and 20 NEX/ $\Delta t_1$ ), yielding a total scan time of approximately 1 hr 36 min. For the data in the  $F_2$  direction only the first 1024 data points were used. The data in the  $F_1$  direction was zero-filled to 1024 points, yielding a square matrix. Subsequently a squared sine windowing function was applied, with a sine bell shift of 8. For presentation spectra were symmetrized to eliminate noise and obtain clearly defined cross-peaks. Processed data is presented in magnitude mode. All 2D spectra were referenced to the diagonal peak of tCr at 3.02 ppm. Further assignments are based on the study by Govindaraju *et al.* (24)

### 5.3.3 Data Processing and Analysis

A $\beta$  plaque-load and numerical density in MR images were quantified by SCIL image software (25) as described in Chapter 3.

Localized 1D MR spectra were analyzed by using LCModel (26), which calculates the best fit of the experimental spectrum based on a linear combination of model spectra. The final analysis is performed in the frequency domain; however, raw data (FIDs) are used as standard data input. The following metabolites were included in the basis set for LCModel: alanine, aspartate, creatine,  $\gamma$ -aminobutyric acid, glucose, glutamate, glutamine, glycerophosphocholine, phosphocholine, *myo*-inositol, lactate, N-acetylaspartate, N-acetylaspartylglutamate, phosphocreatine, phosphoethanolamine, *scyllo*-inositol, and taurine. Quantification was obtained by using the tCr resonance as an internal standard. The LCModel fitting was performed over the spectral range from 1.0 to 4.4 ppm.

Metabolites in the localized 2D spectra were quantified by calculating the cross-peak volumes of properly scaled non-symmetrized 2D data sets using Sparky NMR assignment and integration software (T.D. Goddard & D.G. Kneller; Sparky 3, University of California, San Francisco). Volume calculation was achieved using the sum 'over ellipse' integration method, allowing linewidths to be adjusted and the baseline to be fit. Additionally, only data above the lowest contour was used for quantification. Manual selection and integration afforded better reproducibility than with the automatic peak selection and integration techniques in the Sparky analysis software.

## 5.4 Results & Discussion

Figures 5.1b and 5.1c show characteristic 1D MR spectra obtained from a 27  $\mu$ l voxel placed in the cortex-hippocampus area of the brain of an 18 month old control and a transgenic mouse of the same age, as illustrated in Fig. 5.1a. The most significant changes were increased taurine and decreased NAA and Glutamate in the cortex and hippocampus of transgenic mice, compared to the WT. The quantification of MR spectra revealed an increase in the taurine/Cr ratio of ~20% and a decrease in the NAA/Cr and Glu/Cr ratios of ~18 and ~24%, respectively, in the transgenic mice compared to control mice. Similar neurochemical changes have been observed in the cingulate cortex in previous MRS studies using the same mouse model of AD at 4.7T (2) and at 9.4T (3). In another transgenic mouse model of AD, APP/PS1 mice, Marjanska *et al.* have observed an increase in *myo*-inositol instead of taurine compared to control mice (3). They proposed that the ratio of NAA and *myo*-inositol might be a sensitive spectroscopic marker for following AD in human disease as well as in mouse models such as the APP/PS1.

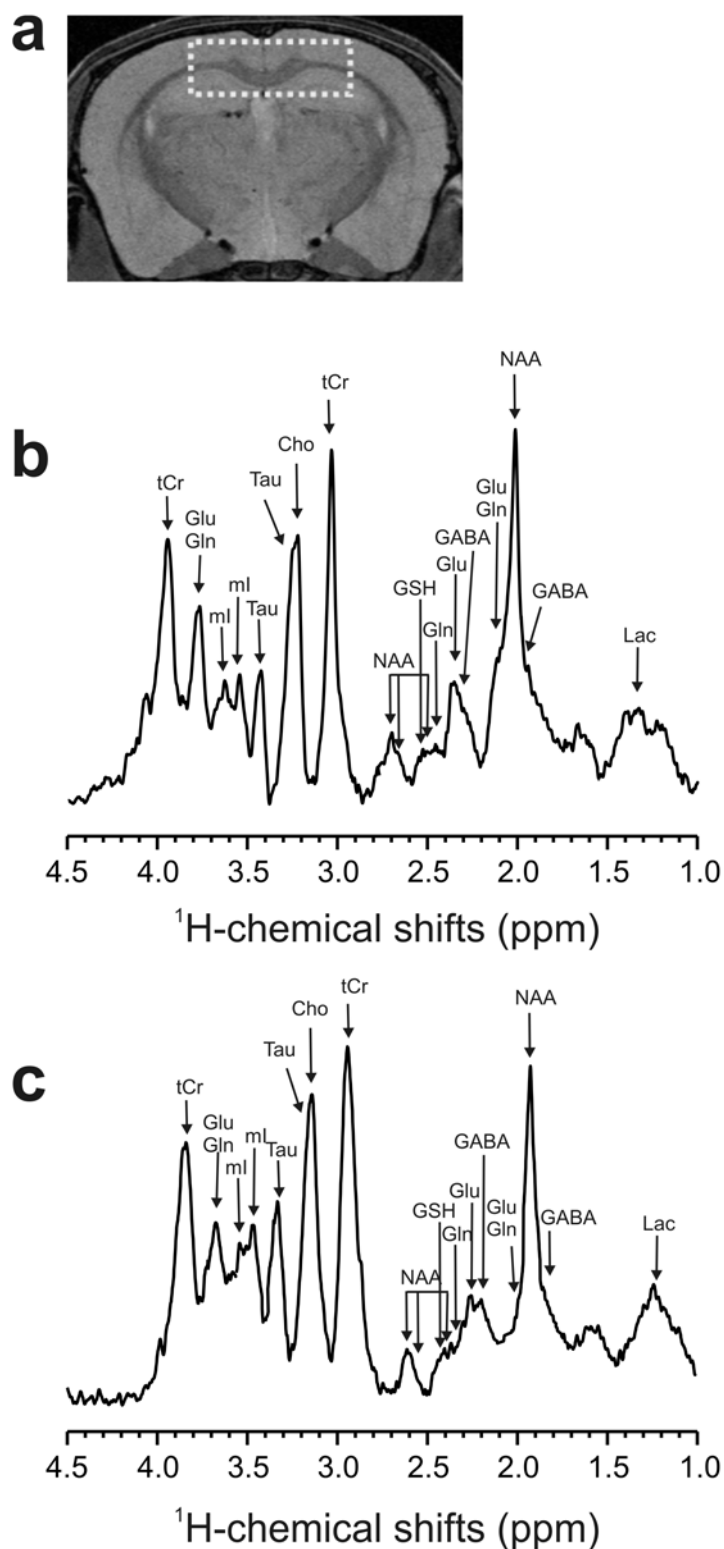


Figure 5.1: Representative MR image and spectra of the mouse brain. (a) A coronal MR image of the mouse brain, obtained using a RARE sequence, showing the position of the selected 27  $\mu$ l ( $1.7 \times 4 \times 4$  mm<sup>3</sup>) voxel covering the cortex-hippocampus region. One-dimensional localized <sup>1</sup>H-MR spectra obtained from the selected 27  $\mu$ l voxel placed in the brain of an 18 month old control (b) and AD (c) mouse. Spectra were obtained using a PRESS sequence with TR=1500 ms and TE=15 ms at 9.4T.



Although a decrease in NAA and glutamate and an increase in *myo*-inositol or taurine have been consistently observed in AD brain, these changes are not specific to only AD, since they have been shown to occur in other neurodegenerative diseases such as Huntington's disease (11,12), Parkinson's disease (13,14) as well as in other brain disorders (15-17). Since only a limited number of metabolites can be reliably detected with 1D MRS, several metabolites and neurotransmitters that are present in low concentrations cannot be unambiguously quantified with this approach. This limits the application of 1D MRS to identify potential biomarkers of AD. For unambiguous characterization of the neurochemical profile in transgenic AD mouse brains, we have applied *in vivo* localized 2D MRS. Figures 5.2a and 5.2b show characteristic 2D MR spectra obtained from a 27  $\mu$ l voxel placed in the cingulate cortex and covering some parts of the hippocampus (Fig. 5.1a) of an 18 month old control and a transgenic mouse, respectively. Both spectra are scaled and plotted with the same absolute contour levels. As has been shown in Chapter 4, 2D MRS in mouse brain at high magnetic field allows separation of most overlapping signals for the coupled spin systems that gave rise to off-diagonal peaks. As a result, a wider range of neurochemicals can be detected in a 2D MR spectrum compared to a 1D spectrum obtained from the same volume.

The 2D metabolite ratios and the raw volume integrals of the signals on the diagonal and of the cross peaks in data collected from the mouse brain were very well reproduced, and the variation of the 2D peak volumes between individuals was sufficiently small to allow for the reliable detection of differences between normal and AD mice. In line with the 1D spectra, an increase in the intensity of cross peaks of taurine, and reduced intensities of the cross peaks of glutamate and NAA were observed in the transgenic mice relative to controls. In addition, a decrease in the concentration of glutathione was observed. Interestingly, the cross-peaks labeled as U2 and U3 which could not be assigned to any of the 35 known brain metabolites in control mouse brain were absent in transgenic mouse brain (see also chapter 4). On the other hand, two unknown cross-peaks labeled as U4 and U5, were present only in transgenic mouse brain and not in controls. Although further studies would be needed to assign these resonances to new metabolites or macromolecules present in the cortex-hippocampus region of the control or transgenic mouse brain, their presence or absence in the transgenic brain may indicate a potential role as marker for AD.

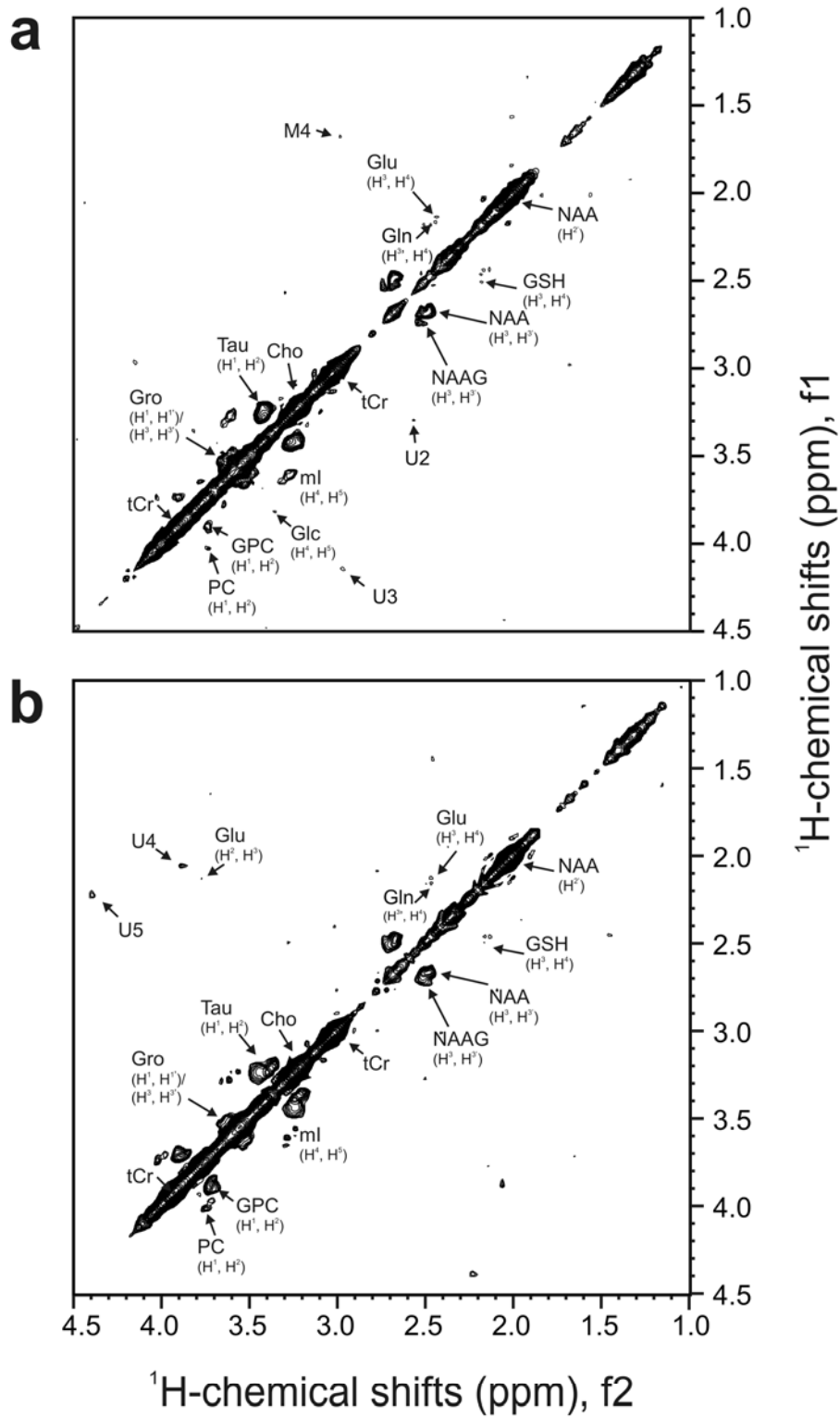


Figure 5.2: Two dimensional in vivo localized MR spectra obtained from a 27  $\mu\text{l}$  ( $1.7 \times 4 \times 4 \text{ mm}^3$ ) voxel covering the cortex-hippocampus region of the brain of an 18 month old control (a) and AD (b) mouse. Spectra were obtained using the PRESS based 2D L-COSY sequence at 9.4T with TR=1500 ms and TE=15 ms. The 2D data set was apodized with a QSINE function and zero filled to 1024 in both dimensions. U2, U3, U4 and U5 are unassigned cross-peaks (see also Chapter 4). The peak M4 arises from macromolecules and is labeled in accordance with Behar and Ogino (27).

A diagonal peak at 3.2 ppm arises from methyl protons of choline-containing compounds. This signal contains contribution from free choline, GPC and PC. Since the concentration of choline in brain is well below the NMR detection limit, the total choline response at 3.2 ppm arises mainly from GPC and PC. Due to the very small difference in chemical shifts, PC and GPC cannot be separated in 1D  $^1\text{H}$ -MRS. Separation could in principle be achieved through smaller resonances from the methylene proton in the region between 3.6 and 3.9 ppm. However, due to the strongly coupled spin system these resonances are difficult to separate from the resonances of other metabolites in the same region in 1D MRS. As shown in Fig. 5.2, the cross-peaks of GPC were clearly separated in the 2D MR spectrum. When the concentration of GPC in the transgenic mice was compared with control mice, GPC levels were strongly (~75%) and significantly ( $P < 0.01$ ) increased in AD mice. This observation is similar to what has been observed by HPLC in CSF samples of human AD patients and post-mortem  $^{31}\text{P}$  MRS studies of human AD brain extracts (10,28-30). It has been proposed that an increase in GPC during AD may be linked to the increased breakdown of membrane phospholipids, such as phosphatidylcholine, during progression of neurodegeneration due to the activation of calcium dependent phospholipase  $A_2$  (29). This inference is supported by a previous study, which revealed that phospholipase  $A_2$  is responsible for NMDA-induced formation of GPC in rat hippocampal slices (31). It is also supported by the detection of elevated cPLA $_2$  immunoreactivity in AD brain (32). In addition, amyloid peptide *in vitro* induces direct activation of cPLA $_2$  as well as direct release of cellular choline (33). Other studies have shown that GPC promotes amyloid aggregation (34,35). A putative chain of events to explain an increase in the level of GPC in AD brain and its relationship with soluble A $\beta$  and A $\beta$  plaques is shown in Fig. 5.3. In this cascade of events, soluble A $\beta$  protein produced during abnormal cleavage of APP is likely to be the initiating factor in the disease cascade. Increased levels of A $\beta$  can cause an influx of calcium in neuronal cells, leading to calcium overload. This subsequently activates calcium dependent phospholipase  $A_2$ , which breaks down PtdCho, which ultimately leads to an elevation in the level of GPC and other breakdown products (29). Under normal conditions GPC is unable to leave the cell. However, as the cell becomes leaky due to membrane breakdown, GPC can be released into the extracellular fluid and the CSF. This extracellular GPC can subsequently enhance A $\beta$  aggregation leading to plaque deposition (29,33-35). In addition, a defective membrane metabolism could expose APP to abnormal proteolytic cleavage (28) leading to an increase in toxic soluble A $\beta$  peptide which can disrupt normal membrane phospholipid turnover (Fig. 5.3).

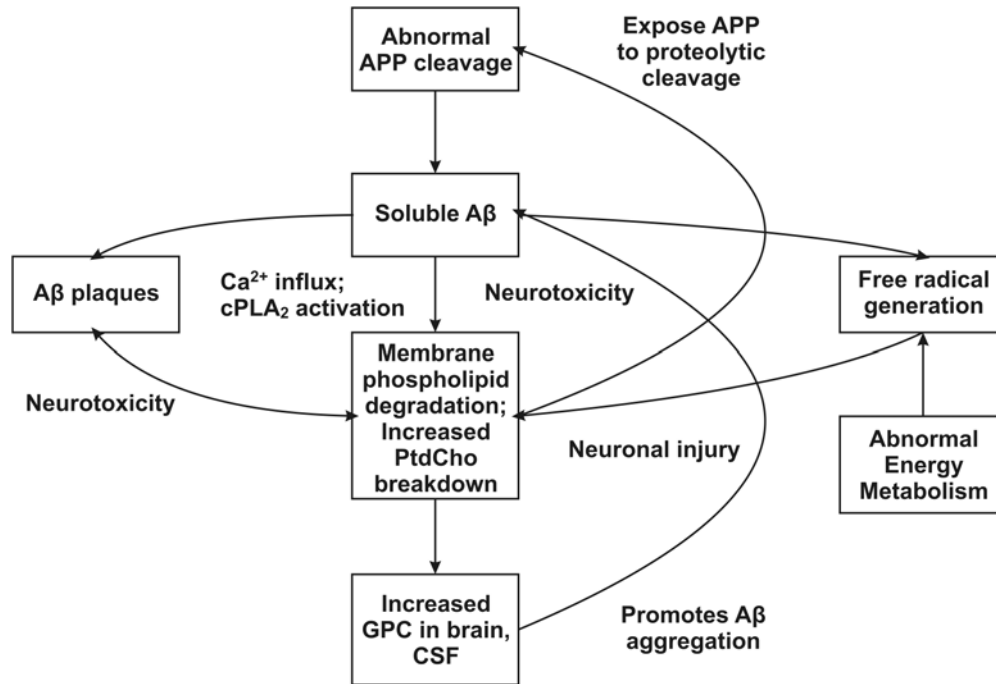


Figure 5.3: Simplified disease pathways of AD, illustrating the interactions of both soluble and aggregated A $\beta$  with membrane phospholipids. As a result of these interactions, GPC levels increase. Increasing GPC levels may account for the observed increase in plaque deposition, or vice versa.

The exact sequential relationship between amyloid aggregation and membrane breakdown during AD progression *in vivo* is not yet known. To investigate the temporal relation between plaque formation and changes in GPC during AD progression, we simultaneously tracked plaque deposition and the changes in GPC levels in the same transgenic AD mice with age using longitudinal *in vivo*  $\mu$ MRI and 2D MRS. Fig. 5.4 (upper panel), shows representative MR images of the same transgenic AD mouse which was imaged at 12, 16, 18, 20 and 22 months of age. A consistent increase in plaque-load was observed with age. This increase in plaque-load correlated well with an increase in GPC levels until 18 months of age (Fig. 5.4; lower panel). Interestingly, a very rapid decline in GPC was observed at the age of 20 months. Figure 5.5a shows the changes in GPC level in control and transgenic mice at ages between 8 and 22 months detected by 2D MRS. The level of GPC in the cortex/hippocampus region of control and AD mice was similar up to 12 months of age (Fig. 5.5a). Consequently an increase is observed in the transgenic mice up to 18 months of age, with levels in control mice being consistently lower (Fig. 5.5a). At the 20th month of age, GPC levels in transgenic mice apparently drop to resemble the levels found for control mice. Quantification of A $\beta$  plaque-load in AD mice with age is shown in Fig. 5.5b. A marked age-related increase in both plaque-load and numerical density of A $\beta$  plaques is evident in this figure.

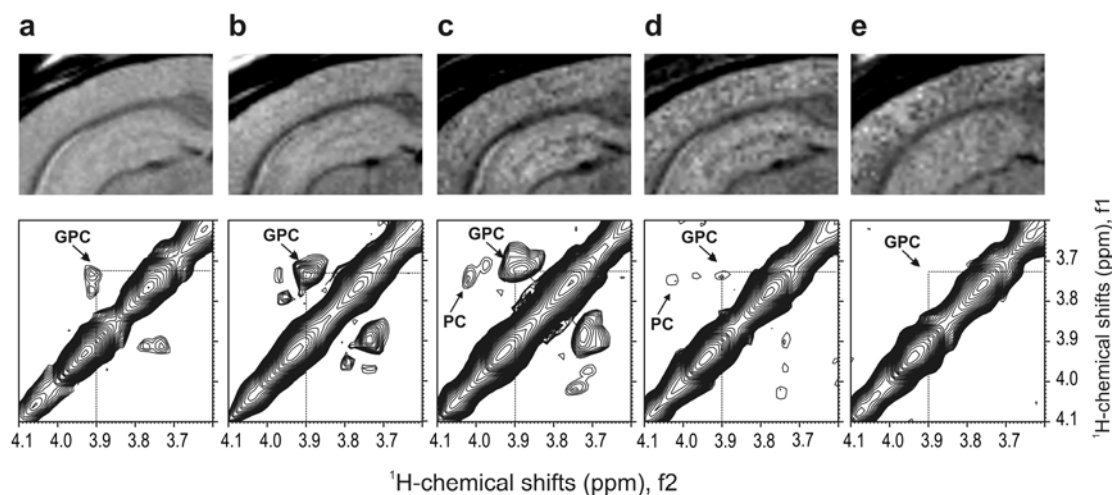


Figure 5.4: Correlation between the development of A $\beta$  plaques visualized by  $\mu$ MRI (upper panel) and the changes in the levels of glycerophosphocholine monitored by 2D MRS (lower panel) in the cortex-hippocampus region of the same AD mouse brain with age. A $\beta$  plaques were visualized in the same transgenic mouse (Tg2576) at the ages of 12 months (a), 16 months (b), 18 months (c), 20 months (d), and 22 months. The MR images were obtained using a RARE sequence, with an in-plane resolution of  $78 \mu\text{m} \times 78 \mu\text{m}$ , TE = 10.567 ms, TR = 6 s, echo train length = 4, averages = 4 and a total scan time of  $\sim 25$  min. Concurrently with the visualization of A $\beta$  development, 2D spectra were obtained using the L-COSY sequence in the same mice at the ages of 12 months (a), 16 months (b), 18 months (c), 20 months (d), and 22 months.

A comparison of the GPC levels with plaque-load at different time points shows that GPC levels remain unchanged until 12 months of age, when plaques were also not detected. Subsequently a marked age-related increase in both plaque-load and GPC level was observed between 14 and 18 months (Fig. 5.5). These results advocate a direct correlation between the severity of A $\beta$  deposition and the increase in GPC levels in AD brain. In contrast to the cortex-hippocampus region, the thalamus region does not show significant A $\beta$  deposition, while the GPC levels remain the same (data not shown). This provides additional support for a direct correlation between GPC levels and A $\beta$  deposition. The reason for the sudden decline in the GPC level beyond 20 months of age is not clear (Fig. 5.4 and 5.5). It is possible that significant neuronal membrane impairment up to this age results in GPC leaving the cells and being released into the extracellular fluid and CSF, which leads to a decline of GPC in the affected brain regions. This assumption is supported by the observation of an increase in GPC levels in CSF during AD in a previous study (29). Alternatively, down-regulation of the membrane phospholipid turnover may be responsible for the decline in GPC.

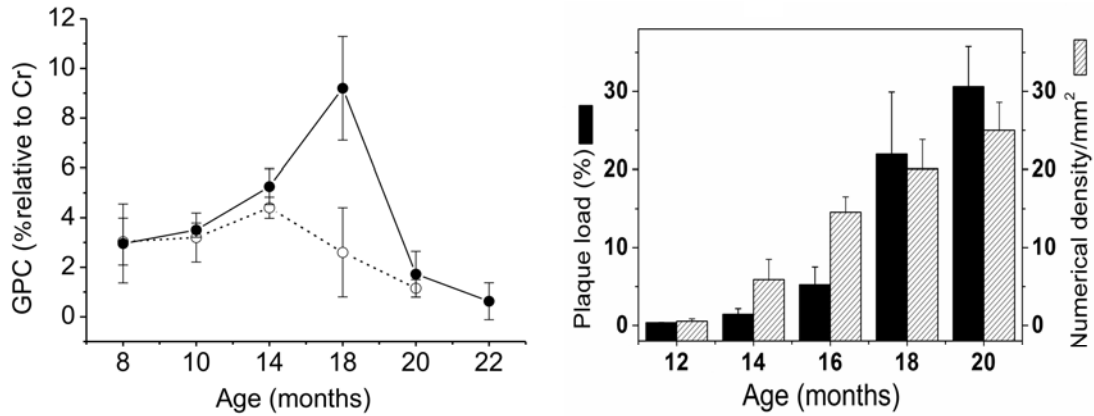


Figure 5.5: Age-dependent changes in A $\beta$  plaque-load and numerical density of A $\beta$  lesions in the cortex-hippocampus region of transgenic mice detected by  $\mu$ MRI are shown in the right panel, while GPC levels in the cortex-hippocampus region of AD mice (●) and control mice (○) detected by 2D MRS are shown in the left panel. Data represent the mean of  $n=5$  ( $\pm$ SD). The GPC levels were estimated from localized 2D datasets collected at the age of 8, 10, 14, 18, 20 and 22 months. Plaque-load and numerical density of plaques was calculated from the  $\mu$ MR images at the age of 12, 14, 16, 18 and 20 months.

In summary, the present study demonstrates that localized 2D MRS in combination with  $\mu$ MRI provides a powerful non-invasive method to follow the correlation between age-dependent neurochemical changes and plaque deposition in AD transgenic mice. Due to the non-invasive and repeatable nature, these methods can substantially accelerate drug discovery. Based on the results from this study, it can be concluded that elevated GPC levels correlate well with AD progression and GPC can be a useful *in vivo* biomarker of AD. Further experimental studies are needed to identify the large number of unassigned resonances detected on 2D MRS spectra, as these may be relevant to pathophysiological conditions during AD.

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