Association among Amyloid Plaque, Lipid, and Creatine in Hippocampus of TgCRND8 Mouse Model for Alzheimer Disease*

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Amyloid peptide (Aβ) aggregation in the brain is a characteristic feature of Alzheimer disease (AD). Previously, we reported the discovery of focally elevated creatine deposits in brain tissue from TgCRND8 mice, which express double mutant (K670N/M671L and V717F) amyloid protein precursor. In this study, frozen hippocampal tissue sections from 5-, 8-, 11-, 14-, and 17-month old TgCRND8 and littermate control mice were examined with Fourier transform infrared microspectroscopy to explore the distribution of lipid, creatine, and dense core plaque deposits. Lipid distribution throughout the hippocampus was similar in transgenic (Tg) and non-Tg littermates at all ages. Dense core plaques were always found to lie within a thin (30–50 μm) lipid envelope, confirmed by imaging through serial sections. Creatine deposits were found in all TgCRND8 mice; the extent of deposition increased with age. Minor creatine deposits appeared in the oldest littermate controls. Distribution in the serial sections showed moderate correlation between layers, slightly disturbed by the freeze/thaw process. Creatine deposits in Tg mice were not specifically co-localized with plaques or lipid halos. The dimension of the lipid envelope is comparable with that of the diffuse halo of nonaggregated amyloid, implying a dynamic association in vivo, postulated to have a significant role in the evolving neurotoxicity.

Alzheimer disease (AD) is a slowly progressing, heterogeneous neurodegenerative disorder characterized by memory impairment, emotional imbalances, and dementia (1). The pathological hallmarks include extracellular deposits of amyloid peptides (Aβ) derived from the amyloid precursor protein (APP), and neurofibrillary tangles formed within the neurons due to hyperphosphorylated tau protein (2). Spatial, temporal, and biochemical connections between Aβ deposits and hyperphosphorylated tau tangles are still debated (3, 4). A dynamic equilibrium between the aggregated Aβ and the possibly more toxic protofibrils, mediated by neuronal membrane lipids, has been demonstrated in vitro (5).

Several theories have been developed to explain AD pathogenesis, including amyloid cascade, neurofibrillar tangle formation, oxidative stress and inflammation, although the exact mechanism that causes neuronal dysfunction and death remains unclear (6–9). Reactive oxygen species and lipid peroxidation lead to oxidation of protein, DNA, and RNA (10). Although oxidative damage is believed to contribute to disease progression, an antioxidant diet that reduced oxidized end products did not reduce plaque load or slow learning impairment in APP mice (11).

We have reported Fourier transform infrared (FTIR) and Raman analyses (12, 13) of brain sections from the TgCRND8 mouse model (14, 15), which expresses a double mutant form of human APP695 (K670N/M671L and V717F), and nontransgenic littermate controls. The position and intensity of the bands in an infrared (IR) spectrum reflect the biomolecular composition of tissue (12, 13, 16–18). Our IR and Raman spectra reveal plaques in TgCRND8 brain (13). The clearly defined dense core plaques are surrounded by phospholipids; significantly, no such lipid structures surround diffuse plaques.

Pathological processes in neurodegenerative disorders such as AD are often accompanied by changes in brain metabolites (19, 20). The creatine/phosphocreatine system, regulated by creatine kinase, helps shuttle phosphate to regenerate ATP (21). We discovered numerous large creatine deposits in transgenic (Tg) mouse brain sections as well as in human AD, whereas only traces appeared in the oldest littermate controls (12). APP and the precursor of ubiquitous mitochondrial creatine kinase may interact directly within cells, supporting a possible relationship among AD, cellular energy homeostasis, and mitochondrial function (22).
Here, we report the results of further analysis of cryosectioned hippocampal tissue from TgCRND8 and littermate control mice. The goals were to evaluate the spatial relationship of phospholipid, creatine and dense core plaque in Tg and control mice brain sections at different ages and to depth profile selected plaques and associated creatine and lipid deposits through consecutive serial sections, to explore possible interactions among creatine, dense core plaque and lipids in the progression of AD.

**MATERIALS AND METHODS**

**Transgenic Mice**—TgCRND8 mice and non-Tg littermate pairs were killed at ages 5, 8, 11, 14, and 17 months. The set included four Tg(K670N/M671L + V717F) (C3H/C57) mice and their gender-matched, non-Tg littermates and one pair of Tg(K670N/M671L + V717F)19959 and the non-Tg littermate (8-month-old animals). The Tg19959 line derives from the same transgene construct as TgCRND8 but is maintained on a different genetic background (129SvEv/Tac). The animals were bred at the Centre for Research in Neurodegenerative Diseases, University of Toronto. All experimental protocols for animal studies were approved by appointed Protocol Management Review committees located at the University of Toronto and University of Manitoba, following guidelines established by the Canadian Council for Animal Care. Mice were killed by cervical dislocation; brains were removed and bisected at the midline. The left hemisphere of the brain was dipped in OCT compound, snap-frozen by immersion in liquid nitrogen for 30 s, and then stored at −80 °C until cryosectioning. The right half was placed in cold 3% paraformaldehyde in 100 mM PBS for fixation, then dehydrated and embedded in paraffin. For FTIR studies, the unfixed, snap-frozen tissue was warmed to −20 °C. For depth profile studies, 20 serial sections from each of the 14-month brains were cryosectioned at 8-μm thickness and mounted on gold-coated microscope slides fabricated in-house. Additional cryosections from these and all other mice were mounted on IR reflective slides (Low-e MirrIR™; Kevley Technologies, Chesterland, OH). All sections exhibited occasional small folds or imperfections. The best sections were chosen for spectral image analysis.

**IR Microspectroscopy**—All spectra were acquired in reflectance mode at 4-cm⁻¹ resolution, from 4000 to 800 cm⁻¹, using a Happ-Genzel apodization function, and saved in log (1/R) format. Typically, 64 interferograms were co-added and ratioed to a similar background scan recorded at a blank region of the slide.

Synchrotron FTIR (sFTIR) data were recorded on a Nicolet Magna 550 FTIR with Continuum IR microscope (SRC, University of Wisconsin, Madison). sFTIR maps were acquired in raster scan mode, using a step size of 10 μm and matching aperture. Maps were analyzed with Omnic/Atlas software (Version 7.3; Thermo-Nicolet), on unprocessed, original data, to avoid the introduction of artifacts.

Focal plane array maps (FTIR-FPA) were collected on a Varian 670-IR FTIR spectrometer with a Varian 620-IR Imaging Microscope (University of Manitoba). FTIR-FPA maps were acquired using a 64 × 64-pixel array with a pixel size of 5.5 μm. Maps are composed of up to 4 × 6 tiles of (64 × 64) pixels, giving 98,304 spectra/map. Maps were analyzed with Resolutions Pro software (Version 5.0.0.700; Varian).

**IR Spectral Analysis**—White and gray matter were distinguished by differences in the intensities of the symmetric CH₂ band envelope (area under curve between 2858 and 2844 cm⁻¹,
base line 3012–2750 cm−1) and the lipid carbonyl band envelope (area under curve between 1750 and 1720 cm−1, base line 1761–1704 cm−1). A dense core plaque was identified either through a ratio of the area of the β-sheet amide I band envelope (1662–1652 cm−1, base line 1806–900 cm−1) to that of α-helix amide I (1630–1620 cm−1, base line 1806–900 cm−1) or by processing for the area of the β-sheet amide I shoulder alone. The area of a sharp doublet (located between 1410 and 1384 cm−1, base line 1410–1384 cm−1) was found to be adequate for detection of all creatine deposits within the tissue. Spectral images processed for distribution of lipid, protein, creatine, and dense core plaque are either juxtaposed or superimposed to illustrate tissue composition, co-localization, and microenvironment.

RESULTS

A photomicroscopic image of a typical TgCRND8 mouse hippocampus, with densely packed neurons in the cornu ammonis and dentate gyrus is shown in Fig. 1A. The FTIR-FPA map, processed for lipid distribution using intensity of symmetric CH2 stretch band, reveals the tissue morphology (Fig. 1B). Characteristic spectra for major tissue components: neurons in gray matter, neurons in the CA1 region, lipid membrane (mainly myelin) in white matter, dense core plaques and creatine, were extracted from this map (Fig. 1C).

To explore the evolution of tissue composition with age and disease progression, comparable sections of hippocampus from littermate pairs of Tg and non-Tg mice, killed at ages of 5, 8, 11, 14, and 17 months, were mapped with FTIR-FPA. These maps, processed for tissue morphology and creatine distribution, are illustrated in Fig. 2.

Consistently, the neurons were revealed by lowest lipid intensity; neurophil showed higher lipid, whereas the entire hippocampus was surrounded by high lipid (white matter structures including the alveus). For Tg mice, in every section, creatine deposits were dispersed throughout the hippocampus and generally followed the curve of the cornu ammonis. Many deposits extended across several hundred micrometers. No creatine was detected in 5-, 8-, and 11-month control brain. Two small streaks of creatine and one punctuate deposit were detected in the 14-month brain; two larger, multistreak deposits were seen at 17 months.

As seen in Fig. 2 and noted earlier (12), creatine deposits often appear as streaks across the tissue. To assess the three-dimensional nature of the creatine deposits, a depth profile experiment was designed, in which the entire hippocampus from each of nine consecutive serial sections was imaged with FTIR-FPA. Three consecutive sections are shown in Fig. 3A. The maps were processed for the symmetric CH2 band to illustrate tissue morphology and lipid distribution (gray scale), with location of creatine deposits superimposed (color scale).

A map of the CA1 region of a 17-month Tg brain was obtained with the sFTIR microscope to examine location and distribution of creatine, dense core plaque, and lipids (Fig. 3B). Single, aperture pixel sFTIR can reveal slightly more detail than the bench source FPA (18) because of minor spread of spectral information into adjacent pixels of the FPA.

Finally, dense core plaques from the 14-month-old Tg mouse were explored in a second depth profile experiment, again using sFTIR, to study the three-dimensional nature and environment more closely. Tissue markers, including outline of hippocampus, shape of the dentate gyrus, and distortion of tissue due to plaque presence, were used to ensure that the same plaque was mapped in each section.
Amyloid Plaque, Lipid, Creatine in APP Mouse Brain

In the processed map of the TgCRND8 hippocampus (Fig. 1, A and B), the darkest regions correspond to neurons of the cornu ammonis and dentate gyrus; they have the least membrane content, and thus spectra have the lowest intensity of the symmetric CH\(_2\) stretch band envelope (maximum at 2851 cm\(^{-1}\)), ascribed to lipid membrane bilayers (Fig. 1C). In contrast, dense white matter tracts produce the highest intensity of this band; in the gray scale processed image, the alveus surrounding the hippocampus is white. Between these two extremes, the gray matter containing numerous cellular processes has a generally gray tone, indicative of roughly uniform, moderate membrane composition.

The position of the amide carbonyl absorption maximum can be used to identify secondary protein structure (13, 18, 26). Typical \(\alpha\)-helical conformation gives rise to a single-band envelope with a maximum around 1655–1665 cm\(^{-1}\). Tissue that includes dense core A\(\beta\) plaque is marked by a distinct amide I doublet at 1628 cm\(^{-1}\), due to the presence of \(\beta\)-sheet conformation (Fig. 1C).

No changes in general lipid distribution were evident in comparisons of the hippocampus between control and Tg littermates or between age groups (Fig. 2). As was reported earlier (13), closer examination shows that individual plaques are enveloped in a thin sheath of high lipid character (see below and Figs. 3 and 4).

The amount of creatine increased with age in both transgenic and non-Tg mice (Fig. 2), but relative amounts differed significantly. A few small deposits were detected in the hippocampus of the 14- and 17-month controls (Fig. 2, Control, second column, bottom two images). Moderate increase in creatine may be the result of normal aging; similar results have been noted in some human tissue (12, 27–29) and may accompany the same process in the non-Tg littermates.

In marked contrast, >30 creatine deposits were detected in the 5-month TgCRND8 brain; in older mice, extensive deposits appeared throughout the hippocampus, but never in the white matter (Fig. 2, TgCRND8, last column, ages 5–17 months, top to bottom). This observation strongly suggests that creatine elevation arises at an early stage of the disease and increases in parallel with the progression of neurodegeneration. Although a minor elevation may be a part of the normal aging process, this is enhanced or accelerated in the TgCRND8 mouse.

Three of nine serial sections (Fig. 3A) show that creatine deposits in one section do not seem to be physically connected with the creatine deposits in the adjacent sections. Thus, creatine could have been extracellular or intracellular and released when the tissue was frozen, sectioned, or both, then pooling and drying into crystalline rivulets. The proximity to the cornu

Results from a depth profile through nine consecutive sections on a plaque located just inside the dentate gyrus (box in Fig. 1B) are shown in Fig. 4. Visible images of tissue from sections 1–9, as seen under the sFTIR microscope, are shown in Fig. 4A, Row I. Maps processed for lipid, dense core plaque and creatine, are shown in Fig. 4A, Rows II, III, and IV, respectively. The global relationship among the lipid, plaque core, and creatine is illustrated in Row V, wherein pixels with the highest intensity for each component are superimposed on the tissue image, color-coded as lipid (red), dense core plaque (yellow), and creatine (green).

Changes in the amide I spectral region, through and across the dense core, provide a multidimensional spectral record of the plaque environment. Spectra selected to best represent a straight line directly through the sections (boxes 1–9 in Row I) are presented in Fig. 4B. Spectra from the column of pixels in section 7 identified by boxes a–g (seventh image in Row I) are presented in Fig. 4C.

**DISCUSSION**

The pathology of AD involves numerous interrelated processes, including A\(\beta\) aggregation, oxidative stress, inflammation, dysfunction in mitochondria and synaptic transmission, and neuronal death (3, 23). The hippocampus is affected significantly by neurodegeneration early in AD (24, 25). In this study, we analyze the distribution of several key tissue components in the hippocampus from the TgCRND8 mouse model for AD and nontransgenic littermate controls, aged 5–17 months. FTIR has been applied to identify and monitor changes in the distribution of lipid, dense core plaque, and creatine, with age and through serial sections.

FTIR–FPA maps can be processed for characteristic spectral features to display tissue morphology and spatial relationships. In the hippocampus of the TgCRND8 mouse (Fig. 1, A and B), the darkest regions correspond to neurons of the cornu ammonis and dentate gyrus; they have the least membrane content, and thus spectra have the lowest intensity of the symmetric CH\(_2\) stretch band envelope (maximum at 2851 cm\(^{-1}\)), ascribed to lipid membrane bilayers (Fig. 1C). In contrast, dense white matter tracts produce the highest intensity of this band; in the gray scale processed image, the alveus surrounding the hippocampus is white. Between these two extremes, the gray matter containing numerous cellular processes has a generally gray tone, indicative of roughly uniform, moderate membrane composition.

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**FIGURE 3.** A, depth profile of hippocampal tissue through three consecutive sections of 14-month-old TgCRND8 mouse brain. FTIR-FPA maps were processed for band area of CH\(_2\) symmetric stretch to show tissue morphology (gray scale below). Creatine locations from map processed on a creatine band area at 1400 cm\(^{-1}\) are overlaid (color gradient below). Scale bar, 100 \(\mu\)m. B, sFTIR analysis of dense core plaque from 17-month-old TgCRND8 mouse, a, light microscope image. b–d, sFTIR map was processed for: \(\beta\)-sheet in amide I region (b), creatine band at 1400 cm\(^{-1}\) (c), and lipid membrane from band area of CH\(_2\) symmetric stretch (d). Scale bar, 50 \(\mu\)m.

Changes in the amide I spectral region, through and across the dense core, provide a multidimensional spectral record of the plaque environment. Spectra selected to best represent a straight line directly through the sections (boxes 1–9 in Row I) are presented in Fig. 4B. Spectra from the column of pixels in section 7 identified by boxes a–g (seventh image in Row I) are presented in Fig. 4C.
Dense core plaques have been found in the transgenic TgCRND8 mouse tissue as early as 3 months (14) and are detectable by FTIR in our youngest, 5-month-old, mice (13). In 4G8-stained tissue (13), the dense core plaques were found in the transgenic TgCRND8 mouse tissue as early as 3 months (14) and are detectable by FTIR in our youngest, 5-month-old, mice (13). In 4G8-stained tissue (13), the dense core plaques were found to be surrounded by a halo, ∼20-μm depth, of diffuse, nonaggregated Aβ. This halo lies within the radius of the lipid enveloping the dense plaques that we can detect with FTIR.

Here, we find that the creatine deposits are found exclusively in hippocampal gray matter and not in white matter tracts such as the neighboring alveus (Fig. 3B). One of the creatine deposits is concentrated within the envelope of lipid surrounding the large dense core plaque. The other deposit surrounds a smaller plaque, penetrating the lipid envelope but also extending beyond it to the apparently normal gray matter. This image is typical of the distribution that we have observed in all samples.

The interaction between plaque and lipid is apparently dynamic. Aggregated Aβ peptides can bind with membrane lipids, phospholipids, and cholesterol due to their hydrophobic nature (5, 31, 32). Binding to lipid membranes is postulated to induce and/or facilitate its fibrillogenesis in AD. An in vitro study demonstrated that liposomes composed from a complex mixture of naturally occurring lipids can rapidly revert aggregated Aβ(1–42) into toxic protofibrils (5). Injection of such reverted protofibrils into mice caused the same types of learning and memory impairment as protofibrils formed directly from monomeric Aβ. The lipid envelope that we image with FTIR is comparable in magnitude to the halo of diffuse, nonaggregated amyloid detected through staining, suggesting that protofibrils are present here because of such dynamic reversion in vivo.

Disturbed tissue associated with the plaque core can be seen in the sFTIR microscope images of unstained tissue Fig. 4A, Row I. After spectral processing, the false color maps show the phospholipid halo (Row II) that surrounds the dense core revealed by the β-sheet shoulder at 1628 cm⁻¹ in the amide I band (Row III, sections 4–7). Although all dense core plaques are enveloped by elevated lipid, the lipid signature is low within the core. Creatine is present in the general vicinity of the plaque (Row IV, sections 1 and 5–9), but relative locations of the three components are not otherwise correlated from one layer to another (Row V).

There are many processes and hypotheses concerning the etiology of AD. In this study, we show the temporal development and spatial distribution of dense core plaque, lipid, and creatine in a transgenic mouse model for AD. Dense core Aβ plaques were detected by FTIR-FPA and sFTIR in hippocampi of all transgenic mice from 5 to 17 months. Elevated lipid surrounds dense core plaques from the earliest age and is postu-
related to play a role in the dynamics of amyloid aggregation. Although a few small deposits of creatine were found in the oldest littermate controls, significant creatine deposits were detected in all transgenic animals. Depth profile analyses showed no spatial correlation between the position of individual creatine deposits and either the plaque cores or their environs. The number and magnitude of these creatine deposits increased with age, in parallel to disease progression. Its origin and possible roles in the underlying pathophysiological processes require further investigation.

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