Short Communication

Potentially Pathogenic Calcium Oxalate Dihydrate and Titanium Dioxide Crystals in the Alzheimer's Disease Entorhinal Cortex

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Abstract. Knowing that Alzheimer's disease (AD) nucleates in the entorhinal cortex (EC), samples of 12 EC specimens were probed for crystals by a protocol detecting fewer than 1/5000th of those present. Of the 61 crystals found, 31 were expected and 30 were novel. Twenty-one crystals of iron oxides and 10 atherosclerosis-associated calcium pyrophosphate dihydrate crystals were expected and found. The 30 unexpected crystals were NLRP3-inflammasome activating calcium oxalate dihydrate (12) and titanium dioxide (18). Their unusual distribution raises the possibility that some were of AD origination sites.

Keywords: Alzheimer's disease pathogenesis, calcium oxalate dihydrate, inflammasome activating crystals, titanium dioxide

Alzheimer's disease (AD) nucleates in a small volume-element of the entorhinal cortex (EC) [1, 2], spreads in the EC, expands to the hippocampus then to other parts of the cortex. AD's hallmark aggregated amyloid-β (Aβ) peptide and hyperphosphorylated tau protein are established propagators of AD, but it is not known if they are its cause. Their presence at the EC initiation-point has not been established. The initiation-point could be microbial [3], cholesterol comprising [4, 5], or as will be discussed below, it could comprise pathogenic crystals.

Macrophage [6] and microglial NLRP3 [7–10] and NLRP1 [11, 12] inflammasomes assemble upon phagocytosis of species having certain molecular patterns. The patterns include those of aggregated

$A\beta_{42}$ [7, 8], of aggregated hyperphosphorylated tau [10, 13, 14], of pathogens or their fragments [13, 15], and of biogenic and exogenous crystals. The family of inflammasome-activating biogenic crystals includes sodium urate [16–18], cholesterol [19, 20], calcium oxalate dihydrate (COD) [17, 18], calcium pyrophosphate dihydrate (CPPD) [16, 17], and cystine [17]. The family of exogenous crystals includes quartz [21–23], asbestos [21], titanium dioxide (TiO_2) [18, 23], and sintered indium tin oxide [24].

Activation of the NLRP3 inflammasome initiates release of IL-1β and other inflammatory cytokines and induces, through a multistep process, pyroptosis, the death of proximal cells. Upon death of proximal neurons more aggregating Aβ and hyperphosphorylated tau are released and more neurons and synapses perish [10].

The commonality of microbial patterns [13, 15], cholesterol [19, 20], Aβ aggregates [7, 25], and hyperphosphorylated tau aggregates [10] is that all

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activate the NLRP3 inflammasome. In light of this commonality, we ask here if there are NLRP3 inflammasome-activating crystals in postmortem EC specimens of AD donors [17, 18] that could also be among the point-initiators of AD.

Duplicate 200 mg samples of 12 formalin-fixed entorhinal cortex specimens, provided by the NIH Harvard Neurobiobank, were probed. Six of the specimens were of Braak and Braak stages I or II [1] and 6 were of stage V [1]. The samples were homogenized with 1 mL of an aqueous solution containing 40 mg of Proteinase K from *Tritirachium album* (Sigma P2308). Instead of using the usual crystalline zirconia beads that would have introduced zirconia crystals, the samples were homogenized using 3 mm-diameter non-crystalline Pyrex glass beads. After overnight proteolysis at ambient temperature, the fluid was centrifuged at 13,000 G for 1 h, the supernatant was discarded, and the centrifugate was re-suspended in 0.5 mL de-ionized water and re-centrifuged for 1 h. To strip the water-soluble salts that would have crystallized upon evaporation of the water, the centrifugate was re-suspended in 0.5 mL water, centrifuged at 13,000 G for 10 min, and the supernatant liquid was discarded. The washing-centrifugation cycle was repeated four times, then the final centrifugate was suspended in 0.5 mL water. Five μL of the suspension was withdrawn from the bottom of the vial and applied to a 3 mm diameter, 314 squares, 200 mesh carbon type-B transmission electron microscopy grid support (Ted Pella, Redding CA). The crystals were observed on duplicate grids from duplicate EC samples, i.e., in $2 \times 5 = 10 \,\mu L$, applied to each grid-pair. The grid-pairs were TEM scanned for presence of crystals, then 5 squares of the grid-pair were probed. Because 10 μL of the 0.5 mL processed volume was applied to the $2 \times 314 = 628$ squares, and because only 5 squares were probed, fewer than 0.015% of the present crystals were detected; furthermore, because the 2×200 mg samples comprised less than 1/5th of the entorhinal cortices, each detected crystal signaled the presence of more than 25,000.

Each detected crystal was TEM-imaged; after confirmation of its crystallinity by its electron diffraction pattern, the crystal's approximate (± 5 atom %) elemental composition was determined by energy-dispersive X-ray spectroscopy. Oxygen-assays of those crystals that rapidly lost their water of hydration in the vacuum of the TEM chamber were less accurate; and with the TEM grids being of carbon, and with tissue-residue adhering to the crystals, their

Table 1
Anticipated and found crystals in the AD entorhinal cortex

EC Specimen	AD Stage	Ferrihydrite/ Goethite	Magnetite	CPPD
8191	I	0	2	0
9232	I	1	0	0
9078	I	1	0	3
6675	I	4	1	3
1202	II	0	1	0
17142	II	0	0	3
2818	V	1	0	0
0275	V	1	0	0
13857	V	2	1	1
9540	V	1	0	0
8818	V	0	0	0
3273	V	5	0	0
Total		16	5	10

Table 2
Novel inflammasome-activating crystals in the AD entorhinal cortex

Donor	Age	AD Stage	COD	TiO$_2$
8191	65	I	0	0
9232	58	I	1	0
9078	60	I	1	0
6675	66	I	1	3
1202	74	II	1	0
17142	83	II	0	1
2818	73	V	0	0
275	74	V	2	1
13857	62	V	0	3
9540	86	V	0	3
8818	85	V	6	0
3273	85	V	0	7
Total			12	18

carbon was not assayed. If cholesterol crystals were present, these would not have been observed because their $1.05 \,\mathrm{g\,cm^{-3}}$ density prevents their separation by centrifugation.

Tables 1 and 2 list the 61 detected crystals: Table 1 listing those that we expected and Table 2 listing those that surprised us. We expected the iron oxide crystals, including the 16 ferrihydrite crystals dehydrating in the TEM's vacuum chamber to crystalline goethite (Supplementary Figure 1, top left), and the 5 magnetite crystals, from earlier studies of crystals in the AD cortex [26–28]. The crystalline oxides of iron have not been reported to activate the NLRP3 inflammasome. The 10 CPPD crystals, dehydrating in the TEM's vacuum chamber to calcium pyrophosphate, did not surprise us, because the NIH Harvard Neurobiobank histopathology reports of their donor brains included atherosclerotic plaques [29]. We note, nevertheless, that CPPD is inflammasome-activating [16].

The 30 unanticipated crystals are listed in Table 2. Twelve were COD crystals of $0.8 \times 0.5\,\mu m$ average imaged area (Supplementary Figure 1, top right) and 18 were TiO_2 crystals of $0.3 \times 0.2\,\mu m$ averaged imaged area (Supplementary Figure 1, bottom). The 30 crystals were unevenly distributed between the early and the advanced stage AD specimens, with 22 in the 6 specimens of stage V, and 8 in the 6 specimens of stages I or II. Specimen 8818 of stage V contained half of all the 12 COD crystals and specimen 3273, also of stage V, contained 7 of the 18 TiO_2 crystals.

The COD crystals lost their water of hydration, fracturing, but remaining crystalline in the vacuum of the TEM chamber, then decomposing under the electron beam to crytalline calcium carbonate and finally to crystalline calcium oxide, $CaC_2O_4.2H_2O \longrightarrow CaC_2O_4 + 2H_2O\uparrow \longrightarrow CaCO_3 + CO\uparrow \longrightarrow CaO + CO_2\uparrow$. Correspondingly, the Ca/O atom ratio increased from $1:6$ to $1:4$, then from $1:4$ to $1:3$, then from $1:3$ to $1:1$.

Phagocytized COD crystals are residents of chronic sterile inflammations of the kidneys and of the cyst [30]. They are found also in the brain after xylitol administration [31], after ethylene glycol (antifreeze) poisoning [32], and in hyperoxaluria [33]. We also found these in the *substantia nigra* of Parkinson's disease donors [34].

COD precipitates when the $[Ca^{2+}][oxalate]$ product-defined solubility-limit is exceeded, i.e., when the Ca^{2+} concentration is high, when the oxalate concentration is high, or when both are higher than normal. Because exaggerated endothelial reticulum Ca^{2+} release, leading to elevation of neuronal cytosolic Ca^{2+} concentration, precedes in the mouse model aggregation of $A\beta_{42}$ [35], the precipitation of inflammasome-activating COD crystals is not likely to be a result of AD, and can be one of its causes. In the absence of xylitol or ethylene glycol poisoning the oxalate precursors in the CNS is ascorbate [36]. The neuronal concentration of ascorbate is as high as $10\,mM$ [37], twice that of glucose; and the concentration of ascorbate in stimulated macrophages is massive [38], making it likely that it is similarly massive in stimulated microglia. Excessive ascorbate concentration is a recognized cause of sterile inflammatory COD disease [39].

The TiO_2 particles of the observed size constitute the white pigment of walls. Smaller TiO_2 particles, constituting the white pigment of medications, foods, and cosmetics, were found in the pancreas of obese type 2 diabetic donors [40], their number density scaling with obesity [41].

In conclusion, our study suggests that entorhinal cortex COD and TiO_2 crystals should be added to the existing list of potential AD initiators, all known to activate the NLRP3 inflammasome.

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SUPPLEMENTARY MATERIAL

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