


The Four Locks

*Methylation, Tyrosine, Inhibitor and Cut — Four
Independently Described Ways to Switch Off One Phosphatase
in the Cortical Neuron, and What Survives When They Are
Asked to Converge*



Prepared under the Organic Network Synthesis methodology

Benjamin Aaron Gustafsson

AdultCognitiveDisease.com

September 2026

*Four literatures found four ways to switch off the same enzyme. Three of the locks exist, and
no two of them turn with the same key.*

Contents

◆

Abstract	3
I. The Note and the Question	5
II. Not One Enzyme but Ninety-Six — Why the Noun Has to Be the Holoenzyme	7
III. What Is Actually Measured in the Human Brain	10
IV. The First Lock — The Methyl Group at Leucine 309	15
V. The Second Lock — The Tyrosine That Was Never There	19
VI. Fyn, and the Residue That Replaced Tyrosine 307	23
VII. The Third Lock — The Inhibitor That Leaves the Nucleus	28
VIII. The Fourth Lock — The Cut at Asparagine 175	32
IX. The Protease That Is Not There	38
X. Oxidative Stress — Which Arrows It Actually Draws	43
XI. The Cell — Is the Pyramidal Neuron the Unit?	49
XII. Do the Locks Converge?	54
XIII. The Validity Ledger	57
<i>Summary of grades</i>	65
XIV. Predictions and Falsification	67
XV. Therapeutic Corollaries — Which Lock Is Worth Picking	70
XVI. Coda — The Methyl Group and the Antibody	72
References	74

Abstract

Two dissertations in this corpus have already converged on protein phosphatase 2A, the principal eraser of phosphate from tau, and both approached it from outside the neuron. *The Silenced Eraser* traced a route from the ungoverned microglion, through the NLRP3 inflammasome, to the phosphatase. *The Single Cut* traced a route from a noradrenergic aldehyde in the locus coeruleus, through one protease, to the phosphatase's endogenous inhibitor. This volume turns the analysis inward and asks a narrower question about the same enzyme: *inside the cortical neuron, how many independent ways are there to switch this phosphatase off, and are they one mechanism or several literatures?*

The field describes four. The phosphatase's catalytic subunit carries a carboxy-terminal methyl ester at leucine 309, placed by leucine carboxyl methyltransferase 1 and removed by phosphatase methylesterase 1, and that methyl group is required for recruitment of the B α regulatory subunit that makes the holoenzyme a tau phosphatase. The same carboxy-terminal tail is reported to carry an inactivating phosphotyrosine at residue 307. The catalytic subunit is bound and inhibited by an endogenous inhibitor, SET, which is nuclear in health and cytoplasmic in disease. And SET is cleaved by asparaginyl endopeptidase at asparagine 175, generating two fragments that both inhibit. To these the field routinely adds calpain, the calcium-activated protease whose activation in Alzheimer brain is among the older and better-replicated findings in the literature, and Fyn, the Src-family kinase held to place the phosphotyrosine. Oxidative stress is offered as the trigger that turns all of them.

We assemble each of these claims from its primary sources and grade it, and the result is not a clean convergence. The methylation lock is the strongest. Loss of leucine carboxyl methyltransferase 1, loss of methylated catalytic subunit and loss of the AB α C holoenzyme have been measured in human Alzheimer brain with reagents whose specificity has survived scrutiny, and the regional pattern tracks neurofibrillary pathology rather than amyloid burden. The tyrosine lock, as stated, does not exist. Residue 307 was assigned in 1992 by inference from a truncation mutant, was never confirmed by sequencing or mass spectrometry, and became the subject of more than one hundred and eighty published studies conducted with commercial antibodies that two independent laboratories showed, in back-to-back papers in 2020, to bind unphosphorylated catalytic subunit with equal affinity. Those antibodies are not phosphosensors; they are methylation sensors, blocked eighty to ninety per cent by the methyl ester at leucine 309. The corrected sites are tyrosine 127 and tyrosine 284, and Fyn phosphorylates the latter, which evicts the B α

subunit — but this has never been measured in Alzheimer tissue. The inhibitor lock and the proteolytic lock are real and human, and they are the same lock: SET's redistribution and its cleavage are one event, because the cut at asparagine 175 severs the nuclear localisation signal that lies immediately downstream of it. The protease that is not there is calpain. Despite a literature that names calpain and asparaginyl endopeptidase in the same breath, no primary study in any system reports calpain cleavage of SET. Calpain reaches the phosphatase system by other routes — through the $\alpha 4$ chaperone, through calcineurin, through the amino-terminal truncation of glycogen-synthase-kinase-3 β — but not through this one.

Asked whether the locks converge, we find that they converge on a *target* and not on a *trigger*. Three of the four act on holoenzyme composition — on whether the catalytic subunit is wearing its B α subunit — while the inhibitor acts on the catalytic subunit itself and pulls down neither the scaffold nor B α with it, which means it is most probably acting on a different pool of the enzyme. Upstream, the four have nothing in common: one-carbon metabolism, amyloid-driven kinase activity, lysosomal membrane permeabilisation and calcium are four separate afferents. Oxidative stress is the only candidate for a shared cause, and its arrows are fewer than assumed: it reaches calpain through calcium rather than through chemistry, since the aldehyde products of lipid peroxidation directly *inhibit* calpain; it does not reach asparaginyl endopeptidase, whose maturation is deranged rather than promoted by cysteine oxidation; but it does reach the phosphatase directly, through a vicinal thiol pair on the catalytic subunit whose disulfide formation is reversible with dithiothreitol. That last arm carries a consequence the field appears not to have drawn. Every measurement of phosphatase activity in Alzheimer brain has been made in homogenisation buffer containing reducing agent. Any redox-based component of the deficit has been reversed in the tube before it could be counted.

Finally we test the cell. The note that prompted this volume began with the words *pyramidal neuron*, and the honest verdict is that this is the wrong unit. The first abnormal tau in the human brain is in the locus coeruleus, which is not pyramidal; the vulnerable entorhinal population is molecularly defined and contains both pyramidal and multipolar cells, whose own investigators state that morphology is insufficient to determine vulnerability; layer five contains highly susceptible and highly resistant excitatory subtypes side by side; and chandelier interneurons carry a higher tangle burden than several excitatory subtypes. More consequentially: not one of the six proteins in this mechanism — the B α subunit, the methyltransferase, the methylesterase, the inhibitor, the protease or the kinase — has ever been measured with neuronal-subtype resolution in human tissue. Every human study locates these changes at the level of a brain region, or of neurons against glia, or of

tangle-bearing against tangle-free cells. The datasets that would answer the question are public. We name the experiment.

What the volume delivers is therefore a subtraction as much as a synthesis: one lock removed, one protease removed from a role it never held, one cell type demoted from a unit to a shorthand, and one measurement — the redox component of the phosphatase deficit — identified as having been systematically assayed away.

I. The Note and the Question

There is a species of document that precedes a paper and is not itself an argument: the list, written quickly, of the things that seem to belong together. Its virtue is that it records an intuition before the intuition has been disciplined. Its danger is that the page itself supplies a false premise, because items written one beneath another look like a sequence, and a sequence looks like a mechanism. Ten items on a page are ten items on a page. Whether they are a pathway is the question, not the answer.

The list that prompted this volume names a cell, a phosphatase, an inhibitor, a form of stress, two proteases, two enzymes of methylation, a residue and a kinase. Each has a literature. Several have literatures that cite one another. What none of them has is a paper that measures all of them in the same tissue, and the absence of that paper is the reason this one exists.

The organising object is a single enzyme, and it is the right object. Tau's phosphorylation is a running balance between the kinases that add phosphate and the phosphatases that remove it, and the accounting on the phosphatase side is unusually clean. Four serine/threonine phosphatases dephosphorylate tau in human brain, and their contributions have been partitioned: protein phosphatase 2A performs approximately seventy-one per cent of the total tau phosphatase activity, protein phosphatase 1 approximately eleven per cent, protein phosphatase 5 approximately ten per cent, and calcineurin approximately seven per cent (Liu and colleagues, 2005a). The Michaelis constants of the first three toward tau lie between eight and twelve micromolar, which is close to the intraneuronal concentration of tau in human brain — the enzyme is operating where its kinetics are sensitive to substrate, not saturated and indifferent to it. Calcineurin's constant is fivefold higher, which is the quantitative reason it is a minor contributor whatever its activity.

Against that background the disease measurement is specific. Total phosphatase activity toward tau is decreased in Alzheimer brain, and the decrease is carried by protein phosphatase 2A and protein phosphatase 5; calcineurin activity, on the same measurement, is *increased* (Liu and colleagues, 2005a). Phosphatase 2A activity correlates negatively with tau phosphorylation at most sites across human brains. If one enzyme is to be held responsible for the erasing side of the balance, this is the one, and the disease does something to it.

What the disease does to it is the question this volume narrows. The corpus has approached the phosphatase twice already, and both times from outside the neuron. The companion volume *The Silenced Eraser* traced a route that begins in the microglion — the lapse of a tonic transforming-growth-factor- β instruction, the disease-associated transition, the NLRP3 inflammasome — and ends at the neuronal phosphatase by two convergent paths. The companion volume *The Single Cut* traced a route that begins in the locus coeruleus, in an aldehyde produced by no other cell type, and reaches the phosphatase by way of one protease and one of its substrates. Both volumes take the silencing of the phosphatase as their destination. Neither asks what the silencing *is*, at the level of the molecule, or how many distinct mechanical operations the word "silenced" is being made to cover.

That is this volume's question, and it has a shape worth stating before the evidence. A cell can switch off an enzyme in a small number of structurally distinct ways. It can destroy the enzyme. It can withhold a cofactor. It can occupy the active site with an inhibitor. It can modify the enzyme covalently so that its catalysis fails. Or — and this is the one that matters here — it can leave the catalytic machinery entirely intact and remove the subunit that brings the enzyme to its substrate, which produces a cell with undiminished total phosphatase activity and a completely unerased substrate. The four locks the literature describes distribute unevenly across those categories, and the distribution turns out to be the whole analysis.

The discipline this volume imposes is that a connection must be shown, not inferred from adjacency. Two proteins named in successive sentences of a review are not thereby in a pathway. A protease activated in a disease and a substrate cleaved in the same disease are not thereby enzyme and substrate. An antibody signal that rises in a disease is evidence about the antibody as much as about the disease. Each of those three failure modes has occurred in the literature assembled here, and in one case the failure has propagated

through nearly three decades and more than one hundred and eighty publications before being caught. This volume is written to catch them, and where a joint does not hold we say so and remove it rather than softening the language until it passes.

Three claims are therefore withdrawn in what follows, and it is fairer to the reader to name them at the outset than to let them arrive as surprises. The inactivating phosphorylation of the phosphatase at tyrosine 307 is withdrawn as stated, on the grounds that the site was never demonstrated and the reagents that appeared to demonstrate it report something else. The cleavage of the phosphatase's inhibitor by calpain is withdrawn on the grounds that no primary evidence for it exists in any system. And the pyramidal neuron as the unit of selective vulnerability is withdrawn on the grounds that the cell-type resolution now available contradicts it — not by showing that pyramidal neurons are spared, which they plainly are not, but by showing that morphological class is not what predicts which of them tangles.

What is left, after the subtractions, is a smaller and considerably more interesting mechanism than the one the list implies, and a specific experimental programme that would settle the parts that remain open. Section XIV states that programme. Sections II to XI assemble the evidence; Section XII adjudicates the convergence; Section XIII grades every joint in an explicit ledger.

II. Not One Enzyme but Ninety-Six — Why the Noun Has to Be the Holoenzyme

Almost every sentence written about this enzyme in the disease literature begins with three characters that do most of the damage. "PP2A" names a family, not a molecule, and the family is large enough that a claim about it is generally not a claim at all. The estimate offered by the investigators who have worked hardest on its regulation is that combinatorial assembly produces on the order of ninety-six distinct holoenzymes with non-redundant functions, and that investigators "should ideally refrain from using ambiguous and imprecise statements, such as 'PP2A regulates this'" (Sontag and Sontag, 2014). The warning is not pedantry. It determines whether the four locks of this volume are four mechanisms or one, because three of the four act on assembly rather than on catalysis, and assembly is invisible to the assay the field mostly uses.

The architecture. The enzyme is a heterotrimer. A scaffolding subunit, A, encoded by *PPP2R1A* or *PPP2R1B*, is a horseshoe of fifteen tandem repeats that carries the other two. A catalytic subunit, C, encoded by *PPP2CA* or *PPP2CB*, performs the chemistry with two active-site metal ions. And one variable regulatory subunit, B, drawn from four structurally unrelated families — B or B55 or PR55 (*PPP2R2*), B $\square$ or B56 (*PPP2R5*), B $\square$ or PR72 (*PPP2R3*), and the striatins — determines where the enzyme goes and what it dephosphorylates. Fifteen genes and at least twenty-three isoforms populate the B position (Sontag and Sontag, 2014). The catalytic subunit alone has essentially no substrate selectivity worth the name; the B subunit is the enzyme's address book.

One B subunit makes the enzyme a tau phosphatase, and it is B α . This is the single most consequential fact for everything that follows. The demonstration is layered. The holoenzyme containing the B α subunit, written AB α C, binds tau directly, and the domain on tau that it binds is, in the original phrasing, "indistinguishable from its microtubule-binding domain"; the enzyme binds microtubules through a separate site on the catalytic subunit, so that enzyme, substrate and polymer form a three-way arrangement rather than a queue (Sontag and colleagues, 1999). Structural work then gave the mechanism: the crystal structure of the heterotrimer containing B α shows a seven-bladed β -propeller with an acidic substrate-binding groove at its centre, latched to a ridge of the scaffold by a protruding β -hairpin arm, and a fully reconstituted assay showed that B α "specifically and markedly facilitates dephosphorylation of phospho-tau" (Xu and colleagues, 2008). Site-resolved nuclear magnetic resonance kinetics confirmed the requirement from the other direction: the interaction that permits dephosphorylation of the AT8 epitope is lost with the dimeric core enzyme, which lacks a B subunit, and lost again with a tau mutant that cannot be phosphorylated at threonine 231 — the regulatory subunit is required for both (Landrieu and colleagues, 2011).

The functional corollary was demonstrated in cells long before the structure. Co-expression of tau with simian virus 40 small t antigen, which displaces B subunits from the core enzyme, produces tau phosphorylation at serine 199, serine 202, threonine 205, serine 396 and serine 404, dissociation of tau from microtubules, and loss of tau's microtubule-stabilising activity (Sontag and colleagues, 1996). The lesson is that removing the B subunit is functionally equivalent to inhibiting the enzyme, while leaving the catalytic subunit entirely intact.

Which tau sites the enzyme prefers. The enzyme dephosphorylates all ten of the disease-associated sites that have been tested — serine 199, serine 202, threonine 205, threonine 212, serine 214, serine 235, serine 262, serine 396, serine 404 and serine 409 — but not equally (Liu and colleagues, 2005a). In vitro and in metabolically active rat brain slices, threonine 205, threonine 212, serine 214 and serine 262 are the most favourable sites and serine 199 and serine 404 the least (Qian and colleagues, 2010). Serine 262 is worth isolating, because it is among the enzyme's best sites and is simultaneously the worst site for phosphatase 5 (Liu and colleagues, 2005b) — which makes it, in principle, a phosphatase-discriminating readout, and which is why the 12E8 antibody recurs throughout the experimental literature assembled in Sections VII and VIII.

A complication that should be stated before it is needed. It does not follow that inhibiting the phosphatase raises tau phosphorylation at every site. In rat brain slices, okadaic acid inhibition of the phosphatase *inhibited* glycogen-synthase-kinase-3 β by raising its inhibitory serine 9 phosphorylation, so that the net change at any given tau site is the sum of reduced erasing and reduced writing (Qian and colleagues, 2010). Any argument of the form *less phosphatase, therefore more phospho-tau* is therefore an argument about a net, not about a mechanism, and the net can go either way at a given residue.

The chemistry the methyl group performs. The catalytic subunit terminates in the sequence threonine 304–proline–aspartate–tyrosine 307–phenylalanine–leucine 309, and the terminal leucine carries a reversible carboxyl methyl ester. That tail is not a free-floating appendage; in the assembled holoenzyme it is a docking element. The crystal structure of a B $\square$ -containing holoenzyme shows that "the carboxyterminus of the catalytic subunit recognizes a surface groove at the interface between the B $\square$ subunit and the scaffolding subunit" (Xu and colleagues, 2006). Two consequences follow immediately and are load-bearing for Sections IV and V. Any modification of that tail alters holoenzyme composition whether or not it alters catalysis. And in an assembled holoenzyme the tail is occluded, which limits access by any enzyme that would modify it.

The assay problem. Most published measurements of "PP2A activity" are made by immunoprecipitating the catalytic subunit with a monoclonal antibody and assaying what comes down against a generic phosphopeptide. The reagents in common use for that purpose — clones 1D6, 7A6, G-4, 52F8

and polyclonal 2038 — were systematically characterised and found to preferentially recognise the *non-methylated* catalytic subunit, to fail to co-immunoprecipitate trimeric holoenzymes containing B or B $\square$ subunits, and in some cases to recognise protein phosphatase 4 as well (Frohner and colleagues, 2020b). An immunoprecipitation-based activity assay built on those reagents therefore samples a demethylated, B-subunit-poor, partly heterologous subpool, and reports its activity as the cell's. The authors' conclusion is unambiguous: "reinterpretation of the data generated by using these reagents is required."

This matters for the present argument in a specific way. If the disease lesion is the loss of a regulatory subunit rather than the loss of catalysis, then an assay that discards regulatory subunits during the immunoprecipitation is blind to precisely the lesion it is being used to detect. The Alzheimer literature contains a striking quantity of activity data of exactly this kind, and its convergence on a modest deficit may reflect the assay's floor rather than the disease's magnitude. The measurements that have proved most durable are the ones that did not use this route: direct assay of tau dephosphorylation in brain extract, and immunohistochemical and immunoblot quantification of the holoenzyme itself.

The naming convention adopted here. Throughout this volume, "the phosphatase" without qualification means the AB α C holoenzyme — the enzyme that dephosphorylates tau. Where a claim concerns the free catalytic subunit, or the A–C core dimer, or a B $\square$ -containing holoenzyme, it is named as such. The distinction is not cosmetic: it is the axis along which the four locks separate.

III. What Is Actually Measured in the Human Brain

Before the mechanisms, the observations. This section assembles what has been measured in human Alzheimer tissue, with the quantities where quantities exist and without them where they do not, because the mechanistic sections that follow are only worth writing if there is a human deficit for them to explain.

Phosphatase activity toward tau is reduced, and the best-supported magnitude is about thirty per cent. Using abnormally hyperphosphorylated tau isolated from Alzheimer brain as the substrate and the tau-1 epitope as the

readout, tau phosphatase activity in Alzheimer brain was decreased by approximately thirty per cent relative to age-matched controls (Gong and colleagues, 1995). The earlier survey that established the direction used phosphorylase kinase and a synthetic copolymer as substrates in thirteen Alzheimer and twelve control frontal cortices, and found type 2A activity significantly lower in both grey and white matter, with type 1 activity lower in grey matter only and phosphatases 2B and 2C unchanged (Gong and colleagues, 1993). We note, as an honesty rather than a criticism, that the specific percentage reduction reported in the 1993 study is not recoverable from the published abstract and that we have not verified a figure against the full text; readers encountering a precise percentage attributed to that paper should check it.

The holoenzyme itself is reduced, and the reduction is regional and cell-type specific. This is the most informative human measurement in the literature, because it is made on the holoenzyme rather than on activity. In Alzheimer frontal and temporal cortex there is a significant reduction in total AB α C, matching the decrease in phosphatase activity measured in the same homogenates. Immunohistochemically, neuronal AB α C is significantly and selectively decreased in Alzheimer-affected regions and in tangle-bearing neurons — but it is *not* decreased in Alzheimer cerebellum, and it is *not* decreased in non-Alzheimer dementias. Reduced neuronal holoenzyme correlated with tangle load and not with plaque burden. And, in the same tissue, glial cells within senile plaques were holoenzyme-positive, and glial immunoreactivity was *increased* in both Alzheimer and non-Alzheimer cases (Sontag and colleagues, 2004a).

Four things are worth extracting from that paragraph. The cerebellar exemption is an internal negative control, and it is the correct one: the cerebellum in Alzheimer disease loses Purkinje cells and granule cells but develops no neurofibrillary change whatever (Wegiel and colleagues, 1999), so an enzyme deficit that tracks tangles should be absent there, and is. The non-Alzheimer dementia exemption argues against a generic neurodegenerative or agonal artefact. The correlation with tangles and not plaques is the first of several occasions in this volume where the phosphatase system aligns with the tau lesion rather than the amyloid lesion. And the glial divergence — neurons down, glia up — means that any measurement made on bulk homogenate is a difference of differences, with the neuronal signal partly cancelled by an opposing glial signal.

The transcript does not fall with the protein, and this is a real discordance. In situ hybridisation in Alzheimer and control hippocampus and cerebellum quantified catalytic subunit, PR55 α , PR55 γ , PR61 ϵ and PR61 δ messenger RNA. Catalytic subunit, PR55 γ and PR61 ϵ transcripts were significantly reduced in Alzheimer CA3. PR55 α — which is B α — was not among the significantly reduced transcripts (Vogelsberg-Ragaglia and colleagues, 2001). The authors themselves note a generalised-messenger-RNA-loss confound, since β -actin transcript was also reduced in Alzheimer hippocampus; and the regions and methods differ from the protein study, so this is not a formal failed replication. But the discordance should be stated rather than smoothed, and it has an interpretation that favours the argument of Section IV: a protein-level deficit without a transcript-level deficit is what a post-transcriptional assembly failure looks like.

The methylation machinery is reduced in the same tissue, in the same pattern. In the first analysis of human methyltransferase and methylated catalytic subunit across Alzheimer, non-Alzheimer demented, and aged control cases, methyltransferase protein expression and catalytic subunit methylation were quantitatively decreased in Alzheimer-affected brain regions, the regional loss of methyltransferase immunoreactivity paralleled the severity of tau pathology rather than amyloid plaque burden, and the methyltransferase was abundant in cortical neurons in normal and non-Alzheimer demented controls (Sontag and colleagues, 2004b). This is the companion observation to the holoenzyme deficit and, on the argument of Section IV, its cause.

The endogenous inhibitors are increased and relocated. In seven Alzheimer and seven matched control brains, messenger RNA for both inhibitor-1 and inhibitor-2 of the phosphatase was increased — approximately twenty-five per cent in temporal cortex and approximately ten per cent in entorhinal cortex — with no significant change in cerebellum. Inhibitor-2, which is SET, shifted from nucleus to cytoplasm: the ratio of neurons with immunonegative nuclei to neurons with immunopositive nuclei was more than sixfold greater in Alzheimer than control. Nuclear inhibitor-2 signal fell; cytosolic full-length thirty-nine kilodalton protein fell; and a major twenty-kilodalton fragment, present in the cytosolic but not the nuclear fraction, appeared in most Alzheimer and few control cases and was significantly higher in Alzheimer tissue. Inhibitor-1, by contrast, did not change its localisation (Tanimukai and colleagues, 2005). The regional pattern again tracked vulnerability: no change in cerebellar granule or Purkinje cells.

One nuance that is usually dropped. The inhibitor of the phosphatase is not a general phosphatase inhibitor, and in one condition it does the opposite. Both inhibitors associate with phosphatase 1 and modify its substrate specificity: in the absence of divalent cations they did not affect its activity, but in the presence of manganese they stimulated it fifteen- to twentyfold toward myelin basic protein and histone H1 — though not toward phosphorylase (Katayose and colleagues, 2000). No tau substrate was tested, and the effect is conditional on a cation and on the substrate, so it cannot be converted into an arithmetic about net tau phosphorylation. It is entered here only to forbid the simpler statement that this protein is a blanket brake on tau erasure.

Calpain is activated, early and broadly. The founding measurement indexed the activated seventy-six kilodalton autolytic form of calpain I against its eighty kilodalton precursor in twenty-two Alzheimer and eighteen control brains, and found the activation ratio elevated approximately threefold in prefrontal cortex. Two controls in that study carry most of its weight: the ratio was *not* elevated in Huntington disease, which argues against a generic neurodegenerative or agonal artefact, and it was significantly elevated in regions where Alzheimer pathology is mild and has produced no overt neuronal loss, which argues that the activation is not simply a consequence of cellular degeneration (Saito and colleagues, 1993). An independent Braak-staged series found calpain-1 activity increased from stage III onward, with glycogen-synthase-kinase-3 and cyclin-dependent kinase 5 activated at stages II to III, both preceding global tau hyperphosphorylation and postsynaptic marker loss (Kurbatskaya and colleagues, 2016). We record a contradiction between those two series on the endogenous inhibitor calpastatin, whose activity the first tradition reports as depleted (Rao and colleagues, 2008) and the second as increased.

Asparaginyl endopeptidase is activated, and relocated. In eight Alzheimer and ten non-Alzheimer control frontal cortices, the thirty-six kilodalton active form of the protease was increased and the fifty-six kilodalton zymogen decreased, with the amino-terminal fragment of the inhibitor correspondingly increased. Subcellular fractionation placed active protease in the cytosol, more in Alzheimer than control. Immunohistochemically the protease was found in neuronal lysosomes, cytoplasm, nuclei and neurofibrillary tangles, and tangle-bearing neurons carried the most cytoplasmic signal. The cell-by-cell coupling is the most striking result: neurons in which the protease remained punctate, that is lysosomal, had no

tau hyperphosphorylation, while neurons in which it had gone diffusely cytoplasmic had hyperphosphorylated tau and cytoplasmic inhibitor (Basurto-Islas and colleagues, 2013). An independent laboratory found the protease upregulated and active during ageing and activated in human Alzheimer brain (Zhang and colleagues, 2014).

Fyn is redistributed rather than simply increased. Quantitative immunoblotting of human autopsy brain found the kinase increased in the insoluble fraction and decreased in the soluble fraction; soluble kinase correlated positively with cognitive scores and with synaptophysin immunoreactivity and inversely with tangle counts in frontal cortex; immunohistochemically it decreased at synapses and increased in neuronal cell bodies, colocalising with tangles (Ho and colleagues, 2005). The frequently encountered summary that "Fyn is increased in Alzheimer disease" is not what this measurement shows, and the distinction matters for Section VI.

What has not been measured. Three absences should be entered here rather than at the end, because they constrain every mechanistic claim in this volume. There is no published association of *PPP2R2A*, *PPP2CA*, *LCMT1* or *PPME1* with Alzheimer risk in any genetic study we could locate; searches return oncology and DNA-damage-response literature and nothing else. There is no measurement of the phosphatase activator PTPA in Alzheimer brain, and no test of whether it can reactivate phosphatase from Alzheimer brain extract. And there is no measurement of any protein in this mechanism resolved to neuronal subtype. The first of these is a null result and should be reported as one; the second and third are experiments that have not been done.

A single human genetic signal exists, and it is conditional and unreplicated. A genome-wide association study restricted to two thousand and ninety-six apolipoprotein E $\epsilon 2$ carriers, drawn from twenty-nine cohorts, identified *PPP2CB* rs117296832 at a probability of 1.1×10^{-7} in $\epsilon 2$ carriers. In two hundred and twenty-four neuropathologically examined brains, *PPP2CB* protein was reported lower in $\epsilon 4$ carriers while *PPP2CA* was unchanged, even as the blood expression quantitative trait analysis placed the risk allele with *higher* expression — a directional tension across tissues and alleles that the reader should check in the primary results (Jun and colleagues, 2022). Effect sizes and cohort counts quoted in secondary sources are not all recoverable from the published abstract and we do not repeat them here. The authors' own limitations are decisive for how this should be cited: a small $\epsilon 2$ case

count, no replication cohort, genomic inflation between 1.07 and 1.13, a single isogenic stem-cell line, and stem-cell analyses uncorrected for multiple testing. It is suggestive. It is not established, and it concerns the other catalytic subunit gene from the one this volume mostly discusses.

IV. The First Lock — The Methyl Group at Leucine 309

The first way to switch off a tau phosphatase is not to touch its catalysis at all. It is to remove a single methyl group from the end of its catalytic subunit, so that the subunit can no longer recruit the regulatory subunit that brings it to tau. The enzyme remains, its active site remains, its activity against a generic substrate in a test tube remains — and its substrate is no longer erased. This is the lock for which the human evidence is strongest, and it is the one against which the other three should be measured.

The chemistry. Leucine carboxyl methyltransferase 1 transfers a methyl group from S-adenosylmethionine to the carboxyl terminus of leucine 309 on the phosphatase catalytic subunit. Phosphatase methylesterase 1 removes it. The reaction is a reversible ester, not a stable modification, and the equilibrium is therefore set by the relative activity of the two enzymes and by the cell's methyl donor economy — the ratio of S-adenosylmethionine to its product S-adenosylhomocysteine, which is itself an inhibitor of the methyltransferase.

Two structural facts give the modification its consequence. First, methylation of leucine 309 is required for assembly of holoenzymes containing the B55 family, of which B α is the member that matters for tau, while holoenzymes containing the B β and B γ families do not have an absolute methylation requirement (Longin and colleagues, 2007). The methyl group is therefore not a global switch on the enzyme's activity; it is a selector that determines *which* holoenzyme the cell assembles. Second, the methylesterase does not merely remove the methyl group. Its structure bound to the A–C core shows that it binds the phosphatase active site and inactivates the enzyme by *evicting the catalytic manganese ions* (Xing and colleagues, 2008). This is a genuine, structurally demonstrated inactivation mechanism at the carboxy terminus — and it is worth holding in mind through Section V, where a competing claim of carboxy-terminal inactivation will not survive.

The dependency was demonstrated directly by depleting the methyltransferase: progressive loss of methylated catalytic subunit, redistribution of the remaining methylated pool among trimers according to their methylation requirements, and — on persistent knockdown — *specific degradation of the B55 subunit* and apoptosis (Longin and colleagues, 2007). The methylated catalytic subunit is not merely preferred by B α ; it appears to be required for B α 's stability. Earlier work had reached the same conclusion from the other side, using monoclonal antibodies that selectively recognise unmethylated catalytic subunit: when methylation is reduced in vivo, B α is found complexed exclusively with methylated catalytic subunit (Yu and colleagues, 2001).

The compartment. The methylation system is spatially organised, which is easy to miss and which matters for the tau result. Pools of the methyltransferase, methylated catalytic subunit and AB α C holoenzyme are co-enriched in cholesterol-rich plasma membrane microdomains, while demethylated catalytic subunit is preferentially in non-raft membrane, where the small amount of methylesterase present is exclusively located. A methylation-incompetent mutant catalytic subunit is excluded from rafts. Enhancing methylation promotes association of both the phosphatase *and tau* with the plasma membrane; impairing it — by a catalytically inactive methyltransferase mutant, by methyltransferase knockdown, or by perturbing one-carbon metabolism — produces loss of membrane-associated phosphatase and tau, and accumulation of soluble phosphorylated tau (Sontag and colleagues, 2013). The lesion is therefore not only "less holoenzyme" but "holoenzyme and substrate no longer in the same place," which is a different and more specific failure.

The neuronal phenotype. Enhanced expression of the methyltransferase in neuroblastoma cells raises endogenous methylated catalytic subunit and B α , reorganises filamentous actin, promotes serum-independent neuritogenesis and produces extended tau-positive processes on differentiation. The effects are abolished by methyltransferase knockdown and by S-adenosylhomocysteine. Expression of the methylesterase, or of a catalytic subunit lacking the methylation site, blocks differentiation and blocks methyltransferase-mediated neurite formation. Knockdown of B α itself inhibits outgrowth (Sontag and colleagues, 2010). The chain from methyl donor to methyltransferase to methylated catalytic subunit to B α to neurite is therefore complete in a neuronal cell line, with loss-of-function and gain-of-function arms in both directions.

The metabolic afferent. This is the lock's distinctive feature, and the reason it cannot be folded into the others: its upstream input is dietary and systemic rather than neural. Folate deprivation in neuroblastoma cells induces downregulation of the methyltransferase, progressive accumulation of demethylated phosphatase, concomitant loss of B α , and cell death — effects accentuated by methylesterase overexpression but, revealingly, *not rescued by methylesterase knockdown*, which localises the lesion to the methyltransferase arm rather than the esterase arm. Overexpression of either the methyltransferase or B α is sufficient to protect against the accumulation of demethylated phosphatase, the increased tau phosphorylation and the death; knockdown of either accelerates the toxicity. And mice maintained two months on low-folate or folate-deficient diets show brain-region-specific alterations in methylation-cycle metabolites, downregulation of methyltransferase, methylated phosphatase and B α , and enhanced tau phosphorylation in the susceptible regions (Sontag and colleagues, 2008).

The same conclusion follows from raising homocysteine rather than lowering folate. Exposure to S-adenosylhomocysteine, or methylesterase overexpression, in neuroblastoma cells reduced phosphatase methylation, downregulated B α -containing holoenzymes, and produced accumulation of phosphorylated tau and phosphorylated amyloid precursor protein, increased β -secretase-derived fragments and increased amyloid- β ; S-adenosylmethionine or methyltransferase overexpression did the reverse. Hyperhomocysteinaemia induced in wild-type and cystathionine- β -synthase heterozygous mice by a high-methionine, low-folate diet raised brain S-adenosylhomocysteine, downregulated the methyltransferase, reduced phosphatase methylation and increased tau and amyloid precursor protein phosphorylation (Sontag and colleagues, 2007).

And the same conclusion follows from genotype. Young homozygous and aged heterozygous methylenetetrahydrofolate reductase knockout mice showed decreased methyltransferase and methylated phosphatase, primarily in hippocampus and cerebellum and to a lesser extent in cortex, with the methyltransferase loss correlating with loss of AB α C and enhanced tau phosphorylation. Dietary folate deficiency decreased all three across every region examined in aged wild-type mice and exacerbated the regional effects in aged heterozygotes (Sontag and colleagues, 2014).

The behavioural and electrophysiological arm. The methylation enzymes have been manipulated transgenically with a consistent result.

Methyltransferase overexpression protected against, and methylesterase overexpression enhanced, amyloid- β -induced cognitive and long-term-potential impairments; neither transgene altered amyloid- β production, and neither altered the electrophysiological response to physiological, picomolar amyloid- β (Nicholls and colleagues, 2016). The loss-of-function mirror holds: heterozygous methylesterase knockout mice were resistant to amyloid- β -induced cognitive and plasticity impairment, while methyltransferase gene-trap mice were more sensitive (Staniszewski and colleagues, 2020). Under chronic rather than acute amyloid- β , methyltransferase overexpression prevented short-term spatial memory and plasticity impairments in Tg2576 mice without altering amyloid precursor protein expression or soluble amyloid- β , while the methylesterase arm showed only a trend and was confounded by emergent non-cognitive impairments — a caveat the authors state themselves (Gnanaprakash and colleagues, 2021). The finding has since been extended to oligomeric tau, where methyltransferase overexpression reduced and methylesterase overexpression increased sensitivity to impairment (Sundares and colleagues, 2025).

The human observation. Methyltransferase protein and methylated catalytic subunit are quantitatively decreased in Alzheimer-affected brain regions, and the regional loss parallels tau pathology severity rather than amyloid plaque burden (Sontag and colleagues, 2004b). Set beside the holoenzyme deficit measured in the same laboratory and the same tissue class (Sontag and colleagues, 2004a), and beside the finding that the B α *transcript* is not reduced (Vogelsberg-Ragaglia and colleagues, 2001), the three observations compose into a coherent post-transcriptional account: the cell makes normal amounts of B α messenger RNA, fails to methylate enough catalytic subunit to assemble and stabilise the holoenzyme, and loses the holoenzyme protein.

What is missing. Two things, and they are specific. There is no viable germline B α -null mouse in which to test the hypothesis directly: knockout of *Ppp2r2a* is embryonic lethal, with no live pups at birth, exencephaly, spina bifida and epidermal barrier failure, while heterozygotes develop normally (Panicker and colleagues, 2020). The closest positive in vivo tests are therefore dominant-negative: neuron-specific expression of a dominant-negative catalytic subunit reduced brain phosphatase activity to sixty-six per cent of wild type and produced tau hyperphosphorylation at the AT8 epitope and serine 422, with aggregates in the soma and dendrites of cortical pyramidal

and cerebellar Purkinje cells (Kins and colleagues, 2001), and a substrate-specific dominant negative crossed onto P301L tau produced a sevenfold increase in neurons phosphorylated at serine 422 and an eightfold increase in tangle number (Deters and colleagues, 2009). We note that follow-up on the first line found the tau phosphorylation to be at least partly indirect, via de-repression of extracellular-signal-regulated and c-Jun amino-terminal kinases, with no cell death by terminal deoxynucleotidyl transferase labelling (Kins and colleagues, 2003). And there is no genetic association of *LCMT1* or *PPME1* with Alzheimer risk, a null we searched for specifically and report as a null.

A cautionary case from the same family. Knockout of a different B subunit produces tauopathy without a direct dephosphorylation defect, and the dissection is instructive. *Ppp2r5d*-null mice develop spatially restricted, progressive tau phosphorylation at pathological epitopes with marked misfolded-conformation immunoreactivity but no tangles, impaired sensorimotor and normal cognitive function, accentuated by mild human tau overexpression. But the holoenzyme containing that subunit poorly dephosphorylates tau in vitro. What the knockout actually did was raise glycogen-synthase-kinase-3 β activity, lower cyclin-dependent kinase 5 activity, and strikingly deplete the kinase activator p35 in the affected regions — because p35, not tau, is the excellent substrate of that holoenzyme (Louis and colleagues, 2011). A B-subunit knockout can therefore produce tau pathology by deranging kinases rather than by failing to erase, and the same possibility must be held open for B α .

Grade. Established in human tissue for the correlative observations; strong and mechanistically complete in cells and mice for the causal chain; the missing element is a direct demonstration in human neurons that the holoenzyme deficit is methylation-dependent rather than merely methylation-associated.

V. The Second Lock — The Tyrosine That Was Never There

This section is a forensic exercise rather than a mechanistic one, and it is placed at the centre of the volume because it is the point at which the list that prompted the paper is wrong, and wrong in a way that is more instructive than being right. The claim is that the phosphatase is inactivated by phosphorylation of its catalytic subunit at tyrosine 307, placed there by a

Src-family kinase. The claim is thirty-four years old, appears in hundreds of papers including a substantial number in the Alzheimer literature, and is assembled from three components, each of which fails on its own terms.

The original claim, and the two things it did not show. Purified rabbit catalytic subunit was phosphorylated on tyrosine in vitro by pp60^{v-src}, p56^{lck}, the epidermal growth factor receptor and the insulin receptor; the phosphorylation was enhanced by okadaic acid, interpreted as blockade of autodephosphorylation; and more than ninety per cent of activity was lost. The abstract asserts that "phosphorylation in vitro occurred exclusively on Tyr307" (Chen, Martin and Brautigan, 1992).

Two qualifications change the meaning of that sentence. The first concerns the inactivation figure. The greater-than-ninety-per-cent loss was measured with *thiophosphorylation* using adenosine 5'-O-(3-thiotriphosphate), which produces an autodephosphorylation-resistant thiophosphoprotein rather than the physiological modification. The stoichiometry of the ordinary phospho-form was not reported and has never been established. The second, and more serious, concerns how residue 307 was assigned. It was assigned "based solely on data showing that a C-terminal truncated mutant of purified PP2Ac lacking the Y307 residue is not phosphorylated" (Sontag and colleagues, 2022). There was no Edman sequencing of a phosphopeptide and no mass spectrometry. A truncation that removes tyrosine 307 also removes the methylation site at leucine 309, the threonine at 304, and the B-subunit docking determinants described in Section II; loss of phosphorylation in such a mutant is not specific evidence that residue 307 was the acceptor.

The cellular follow-up added confidence without adding evidence. Two-dimensional gel electrophoresis of murine fibroblasts resolved two forms of the thirty-six kilodalton catalytic subunit, the second comigrating with the v-Src-phosphorylated species; the shifted form was eliminated by protein tyrosine phosphatase 1B and was reactive with anti-phosphotyrosine; v-Src transformation converted most cellular phosphatase to the shifted form; serum starvation reduced it and epidermal growth factor produced a transient peak at five to fifteen minutes (Chen, Parsons and Brautigan, 1994). No site mapping was performed. A mobility shift plus pan-phosphotyrosine reactivity is compatible with phosphorylation anywhere on the protein.

The mutants that were supposed to confirm it cannot confirm it. The phosphomimetic argument — that a glutamate or aspartate at position 307

reproduces the effect of phosphorylation — was invalidated before most of the papers using it were written. Replacing threonine 304 or tyrosine 307 with a negatively charged residue *abolishes B subunit binding* to the A–C dimer and alters substrate specificity, while deletion of the carboxy-terminal nine residues does the same; the conservative substitutions threonine 304 to alanine and tyrosine 307 to phenylalanine did not dramatically reduce B binding or in vitro activity (Ogris, Gibson and Pallas, 1997). Negative charge at that position therefore destroys holoenzyme assembly as a charge and steric effect, whether or not the residue is ever phosphorylated in a cell. The same confound was demonstrated in a genetically clean system: in yeast, acidic substitution at the carboxy-terminal phosphosites *or* loss of the methyltransferase each disrupt B-subunit binding (Wei and colleagues, 2001). Every experiment of the form "the tyrosine 307 to glutamate mutant behaves like an inactivated phosphatase" is therefore uninterpretable as evidence about phosphorylation.

The antibodies are not measuring what they are named for. This is the decisive item, and it was established twice, independently, in back-to-back papers in the same issue of the same journal in March 2020.

The first laboratory noted at the outset that phosphorylation at tyrosine 307 "was examined in more than 180 studies using commercial antibodies, but this modification was never identified using mass spectrometry." They showed that the most cited monoclonals — clone E155 and clone F-8 — are not specific for the phosphotyrosine but are instead hampered by methylation at leucine 309 or phosphorylation at threonine 304; that other antibodies raised against the same site are also methylation-sensitive; and that some cross-react with phosphotyrosine generally, including a phosphorylated haemagglutinin tag. They were able to detect the phosphosite by targeted mass spectrometry only after transient overexpression of both the catalytic subunit and Src kinase. Their conclusion: "Under such conditions, none of the tested antibodies show exclusive pTyr307 specificity. Thus, data generated using these antibodies need to be revisited, and the mechanism of PP2A inactivation needs to be redefined" (Frohner and colleagues, 2020a).

The second laboratory, working independently in a lung adenocarcinoma line stably expressing tagged wild-type or tyrosine-307-to-phenylalanine catalytic subunit, demonstrated the failure point by point. All three tested antibodies detect the non-phosphorylatable mutant; two of them detect wild type and mutant with equal intensity. Peroxyvanadate treatment raised global

phosphotyrosine and specifically raised phosphorylated epidermal growth factor receptor and Src, and produced no change in the antibody signal. Alkaline phosphatase stripped global phosphotyrosine and abolished the receptor and Src signals, and did not reduce the antibody signal. Enzyme-linked immunosorbent assay against synthetic carboxy-terminal peptides showed that two of the antibodies bind unmodified and phosphorylated peptide with equal affinity, while the third binds the phosphopeptide forty-eight per cent better but also prefers phosphorylated threonine 304 — and that methylation at leucine 309 reduces binding of all three by eighty to ninety per cent. After receiving these data the principal supplier reclassified its antibody as a total catalytic subunit reagent, which it also cannot be, since cellular methylation runs between fifty and ninety per cent (Mazhar and colleagues, 2020).

The consequence for the disease literature is specific and should be stated without hedging. An apparent *increase* in the tyrosine 307 antibody signal in Alzheimer brain is most parsimoniously read as a *decrease in methylation at leucine 309*. That is not a small correction; it is a different mechanism with a different upstream cause. And it is a mechanism that is independently documented in the same tissue by reagents that do not have this problem (Sontag and colleagues, 2004b). The field may have been reading the right biology through the wrong label for twenty-eight years.

The mass spectrometry has never found it. Three independent groups state the negative. The first reports that the site "was never identified using mass spectrometry" across the whole literature (Frohner and colleagues, 2020a). The second reports that "Tyr307 phosphorylation in cells has never previously been reported by MS," and, citing a prior phosphotyrosine-enrichment proteomics study of Src-transformed fibroblasts, that tyrosine 127 and tyrosine 284 were detected on the catalytic subunit and tyrosine 307 was not (Mazhar and colleagues, 2020). The third states that several discovery-mode investigations have identified phosphorylation at tyrosine 284 and not at tyrosine 307 across a variety of transformed cells and human cancers (Sontag and colleagues, 2022).

There is one honest technical defence of the site, and we record it because a fair adjudication must. Tryptic digestion of the carboxy terminus yields the peptides RTPDYFL and TPDYFL, which ionise and are retained poorly when phosphorylated, which could plausibly cause the site to escape shotgun mass spectrometry; the recommended remedy is an endoproteinase Lys-C digest

(Mazhar and colleagues, 2020). That experiment, to our knowledge, has not been published. It is a hypothesis about detectability, not evidence of occurrence, and until it is done the site has no positive evidence at all outside conditions of forced double overexpression.

The structural claim was never made. It is sometimes written that phosphorylation at tyrosine 307 sterically blocks B55 binding. No structure shows this, because no structure of a phosphorylated-tyrosine-307 catalytic subunit exists. What the structures show is that the carboxy-terminal tail docks into a groove at the B-subunit-scaffold interface (Xu and colleagues, 2006), and what the mutagenesis shows is that negative charge at 304 or 307 disrupts binding (Ogris and colleagues, 1997), and what the biochemistry shows is that methylation at 309 governs B55 recruitment (Longin and colleagues, 2007). These are three real findings. The conflation of them into "phospho-tyrosine-307 blocks B55 binding" is an inference no primary paper supports.

What should replace the claim. The lock is not fictitious; it is misplaced. Section VI takes up the corrected version, in which the residues are 127 and 284, the kinases are Src and Fyn, and the consequence is eviction of the B α subunit rather than loss of catalysis. That version has properly validated reagents behind it, and it has never been tested in Alzheimer tissue.

Grade. Rejected as stated. Tyrosine 307 phosphorylation as a physiological, phosphatase-inactivating event in any tissue, and in Alzheimer brain in particular, is not established. Any claim in the corpus that rests on a commercial phospho-tyrosine-307 antibody should be withdrawn or restated as a claim about methylation.

VI. Fyn, and the Residue That Replaced Tyrosine 307

The kinase named in the note is Fyn, and the withdrawal of tyrosine 307 does not withdraw Fyn. It relocates it. This section sets out what the kinase actually does in this disease, where its connection to the phosphatase now stands, and why the strongest test of Fyn as a therapeutic target failed.

The correction, in detail. Working in Src-transformed and non-transformed fibroblasts, in cells null for Src, Yes and Fyn, in monkey kidney cells, in neuroblastoma and in human embryonic kidney cells, and using phospho-specific antibodies raised against tyrosine 127 and tyrosine 284

and purified by sequential differential affinity chromatography and reciprocal cross-depletion, one laboratory established the following (Sontag and colleagues, 2022).

Tyrosine 307 is not a predominant Src-family site: wild-type and the tyrosine-307-to-phenylalanine mutant show very similar tyrosine phosphorylation in Src-expressing or peroxyvanadate-treated cells. A commercial anti-phospho-tyrosine-307 antibody recognised wild type and every mutant equally, confirming the non-specificity in cells. Src phosphorylates tyrosine 127 and tyrosine 284; Fyn phosphorylates tyrosine 284 only — in Fyn-expressing neuroblastoma the tyrosine-284-to-phenylalanine mutation abolished phosphorylation and the double mutant added nothing. Endogenous catalytic subunit within AB α C holoenzymes is phosphorylated on tyrosine 284, while phosphorylated tyrosine 127 was not detectable in B α complexes, consistent with B α binding occluding a residue that sits in the catalytic cleft.

The functional consequence is the one that matters. Phosphorylation at tyrosine 284 promotes dissociation of the B α subunit. Src reduced B α recovery from catalytic subunit immunoprecipitates, and the double mutant's B α binding was insensitive to Src; in neuroblastoma expressing constitutively active Fyn, the decrease in B α was completely prevented by the tyrosine-284-to-phenylalanine mutation, while the tyrosine 127 mutation had no effect. And the tau readout follows: in starved neuroblastoma cells, co-expression of catalytic subunit with constitutively active Src or Fyn produced strong phosphorylation at serine 202, which the tyrosine 284 mutation reduced by about forty per cent for both kinases, with no significant change in inhibitory serine 9 phosphorylation of glycogen-synthase-kinase-3 β — so the effect is not routed through that kinase.

Two additional findings from the same work are worth carrying forward. The methylation-incompetent mutant was tyrosine-phosphorylated *less* than wild type, from which the authors conclude that methylation and the presence of B α are required for maximal phosphorylation of the catalytic subunit — which means the first and second locks are not independent but sequentially coupled, with methylation a prerequisite for the tyrosine modification rather than an alternative to it. And catalytically dead mutants, which stably bind the methylesterase, gave no phosphotyrosine signal at all.

The limitations the authors state, which we do not soften. They could not measure phosphatase activity in immunoprecipitates without losing the labile phosphorylation, so the claim that phosphorylation at 127 or 284 inhibits *catalysis* is not directly demonstrated — what is demonstrated is holoenzyme disassembly. The phosphorylated residues were undetectable in total lysates without enrichment, so the endogenous phospho-pool is small or highly labile and its stoichiometry is unknown. Their own antibodies cannot immunoprecipitate endogenous phosphatase. And a point about their own control deserves restating correctly, because it is easy to invert: they argue that phosphorylation at tyrosine 127 *likely does* contribute to inactivation, since that residue sits in the catalytic cleft and participates in substrate binding, while the tyrosine-127-to-phenylalanine *mutation* is unlikely to impair activity on its own — because the corresponding residue in calcineurin is a phenylalanine. The calcineurin comparison establishes a clean control, not a catalytically inert phosphosite.

Where this leaves the second lock in Alzheimer disease: untested. No study has measured phosphorylation of the catalytic subunit at tyrosine 284 in Alzheimer brain, in an Alzheimer model, or in any neuron exposed to amyloid- β . The proposal that overstimulation of Fyn in the disease has the potential to induce aberrant phosphorylation at that residue is stated by its own authors as a proposal. This is the single cleanest unclaimed experiment in the mechanism assembled by this volume, and Section XIV states it as such.

What Fyn does do in this disease, which is a great deal. The kinase phosphorylates tau at tyrosine 18; the modification is present at early developmental stages in mouse and absent in adult, is detectable in paired helical filament preparations in a phosphotyrosine-phosphatase-sensitive manner, and is present in neurofibrillary tangles in Alzheimer brain while being excluded from neuropil threads and dystrophic neurites — a distribution unlike that of other abnormally phosphorylated tau species (Lee and colleagues, 2004). The interaction runs through the kinase's SH3 domain and tau's proline-rich RTPPKSP motif, and surface plasmon resonance across twenty-one tau constructs found that three-repeat tau binds the SH3 domain about twentyfold better than four-repeat tau, while phospho-mimicking or frontotemporal dementia mutations increase four-repeat affinity twenty-five to forty-five-fold (Bhaskar, Yen and Lee, 2005).

Tau in turn has a dendritic function: it targets the kinase to the postsynaptic compartment, where the kinase's substrate is the

N-methyl-D-aspartate receptor. Expression of a truncated tau, or tau deletion, disrupts postsynaptic targeting of the kinase, uncouples receptor-mediated excitotoxicity, mitigates amyloid- β toxicity, prevents memory deficits and improves survival in amyloid precursor protein transgenic mice; the deficits are also fully rescued by a peptide that uncouples the kinase-mediated NR2B–PSD-95 interaction in vivo (Ittner and colleagues, 2010). Upstream, soluble amyloid- β assemblies bound to cellular prion protein activate the kinase, with metabotropic glutamate receptor 5 as the required transmembrane coupler, driving NR2B phosphorylation, eukaryotic elongation factor 2 phosphorylation and spine loss; amyloid- β -induced spine loss required both the prion protein and the kinase (Um and colleagues, 2012; Um and colleagues, 2013).

A caution about one link in that chain. A frequently cited paper joining amyloid- β oligomers, the prion protein–Fyn complex and pathological tau changes in Alzheimer disease has been retracted and is flagged as such in the bibliographic record. It should not be cited as support for the amyloid- β -to-tau arm of this pathway, and any corpus volume that cites it should be corrected.

Fyn and the phosphatase meet twice more, in the substrate and in the membrane. The two proteins compete for the same site on tau. The AB α C holoenzyme binds tau and microtubule-associated protein 2 through a region encompassing the microtubule-binding domain plus the upstream proline-rich region, and the kinase, which binds the conserved RTPPKSP motif present in both, *inhibits holoenzyme binding to either*. A synthetic RTPPKSP peptide competes; the threonine-231-phosphorylated version does not — so phosphorylation at threonine 231 markedly decreases tau's affinity for the phosphatase (Sontag and colleagues, 2012). This is a purely steric convergence and it is one of the most direct Fyn–phosphatase interactions in the literature: the kinase does not need to modify the phosphatase to exclude it from its substrate.

And the two co-reside. Methylated holoenzymes containing B α co-immunoprecipitate and copurify with the kinase in membrane rafts; the phosphatase's methylation status regulates the kinase's distribution and kinase-dependent neuritogenesis; a methylation-incompetent catalytic subunit fails to interact with the kinase, perturbs kinase and amyloid precursor protein partitioning in membrane microdomains, and enhances amyloidogenic cleavage, while enhanced methylation promotes

non-amyloidogenic cleavage in a kinase-dependent manner (Taleski and colleagues, 2021). The axis is bidirectionally manipulable in vivo: tau transgenic mice on a diet supplemented with L-methylfolate, choline and betaine showed reduced tau phosphorylation at serine 202 and at serines 396 and 404, alleviated motor and learning deficits, increased phosphatase methylation, and *reduced Fyn protein*, while folate-metabolism deficiency increased Fyn expression (van Hummel and colleagues, 2023).

The clinical test, and its result. The preclinical case for the kinase as a target was strong and reproducible. The inhibitor saracatinib blocked amyloid- β -oligomer-driven kinase signalling and downstream proline-rich tyrosine kinase 2 and NR2B phosphorylation; four weeks of dosing in amyloid precursor protein/presenilin 1 mice fully rescued spatial memory and synaptic depletion without altering amyloid metabolism, and rescued tau phosphorylation and deposition in triple transgenics (Kaufman and colleagues, 2015). The benefit persisted after washout, unlike memantine (Smith, Zhu and Strittmatter, 2018); synaptic vesicle glycoprotein 2A positron emission tomography showed normalisation of a hippocampal deficit (Toyonaga and colleagues, 2019); and benefit extended to P301S mice and to a repetitive mild traumatic brain injury model with treatment begun twenty-four hours after trauma (Tang and colleagues, 2020).

The phase 2a trial was negative. One hundred and fifty-nine participants with mild Alzheimer dementia and positive amyloid imaging were randomised to saracatinib or placebo for fifty-two weeks. Central nervous system exposure and target plasma levels were achieved. The primary endpoint — fifty-two-week decline in relative cerebral metabolic rate for glucose in a prespecified statistical region of interest — showed a mean difference of -0.006 units per year, with a confidence interval spanning zero and a probability of 0.34. Every secondary clinical endpoint was negative: no difference in rate of change on the Alzheimer's Disease Assessment Scale cognitive subscale, the activities of daily living scale, the Clinical Dementia Rating, the Neuropsychiatric Inventory or the Mini-Mental State Examination. There were more discontinuations on drug, mostly for adverse events, and gastrointestinal disorders in forty-eight per cent against twenty-nine per cent. The only signals were non-significant trends toward slowed hippocampal and entorhinal atrophy (van Dyck and colleagues, 2019).

We report this plainly because it is frequently soft-pedalled, and because it bears on how much weight the second lock can carry. Target engagement was

demonstrated; clinical and metabolic efficacy were not. This does not refute the biology — a single dose, a single molecule, a single stage of disease and a metabolic primary endpoint are each capable of producing a null — but it does mean that the kinase arm of this mechanism has had its most direct clinical test and did not pass it.

Grade. The kinase's role in the disease is established by multiple independent routes, none of which runs through the phosphatase's carboxy terminus. The kinase-to-phosphatase link exists in two demonstrated forms — steric competition for the substrate, and phosphorylation at tyrosine 284 causing B α eviction — of which the first is demonstrated with tau and the second is demonstrated in cell lines and never in brain.

VII. The Third Lock — The Inhibitor That Leaves the Nucleus

The third way to switch off the phosphatase is the most conventional: bind its catalytic subunit with an inhibitor protein. What makes this lock interesting is not the inhibition, which is ordinary enzymology, but the geography. The inhibitor is normally in the nucleus, doing a completely different job, and the lesion is that it arrives somewhere it does not belong.

The protein and its names. Two heat-stable inhibitors of the phosphatase were purified to homogeneity from bovine kidney and characterised kinetically. Both are non-competitive with respect to substrate, with inhibition constants of approximately thirty nanomolar for inhibitor-1 and approximately twenty-five nanomolar for inhibitor-2; both inhibit the phosphatase against several substrates but have little effect with casein, and neither affects phosphatases 1, 2B or 2C (Li, Guo and Damuni, 1995). Inhibitor-2 was then identified by sequencing as a truncated form of SET, the nuclear protein found fused to the nucleoporin CAN in acute myeloid leukaemia; recombinant human SET inhibits the phosphatase with half-maximal effect at approximately two nanomolar and does not touch phosphatases 1, 2B or 2C at twenty nanomolar (Li, Makkinje and Damuni, 1996a). Inhibitor-1 was identified as the protein variously called PHAP-I, LANP, pp32 and ANP32A, inhibiting at approximately four nanomolar (Li, Makkinje and Damuni, 1996b). The two constants are not interchangeable and should be cited to their respective papers: twenty-five nanomolar is a non-competitive inhibition constant for tissue-purified inhibitor-2; two nanomolar is a half-maximal inhibitory concentration for recombinant SET.

What it binds, and this is the structurally consequential result. A glutathione-S-transferase fusion of the inhibitor pulled down the *catalytic subunit* from rat brain extract and pulled down neither the scaffold subunit nor a B subunit (Arnaud and colleagues, 2011). We are careful with the last clause: the published text names the subunits only as A, B and C, and does not specify which B isoform the blotting antibody detected. The argument built on this result in Section XII is correspondingly limited to "a B subunit" rather than to B α . Two independent binding determinants were identified: valine 92, in the leucine-rich region of the amino-terminal half, whose mutation to alanine abolishes both binding and inhibitory activity; and the carboxy-terminal acidic region spanning residues 225 to 277.

The failure to co-precipitate the scaffold or B α is not a trivial technical detail. It means this lock probably does not act on the same molecular object as the first two. Locks one and two determine whether the catalytic subunit is wearing B α ; lock three binds a catalytic subunit that is not wearing B α , or evicts it in the act of binding. This is the strongest single piece of evidence in this volume against the assumption that the four mechanisms are one mechanism, and Section XII takes it up.

Its nuclear address, and why cleaving it is irreversible. The inhibitor carries a basic nuclear localisation signal at residues 179 to 181, with contribution from residues 168 and 169, sufficient to direct it to the nucleus; mutating both motifs retains it in the cytoplasm, where it binds the catalytic subunit, inhibits the phosphatase, hyperphosphorylates tau, destabilises microtubules, impairs neurite outgrowth and activates calcium/calmodulin-dependent protein kinase II and glycogen-synthase-kinase-3 β (Arif and colleagues, 2014). The signal is bipartite and spans residues 168 to 181, and asparagine 175 sits *inside* it. A cut at that residue therefore does not remove the signal from one fragment and hand it to the other; it bisects it, leaving the lysine–arginine pair on the amino-terminal fragment and the arginine–lysine–arginine motif on the carboxy-terminal one, and since cytoplasmic retention in the mutagenesis required disruption of *both* motifs, neither half of a bisected signal should suffice for import. The cleavage and the redistribution are not two events; they are one.

What the protein is when it is not inhibiting a phosphatase. In the nucleus it is a histone chaperone and the core subunit of the inhibitor-of-acetyltransferases complex, which suppresses p300, CREB-binding protein and PCAF acetyltransferase activity by binding

histones and masking them from the enzymes rather than by inhibiting the enzymes — "histone masking" (Seo and colleagues, 2001). It is also the eponymous member of a two-hundred-and-seventy to four-hundred-and-twenty kilodalton endoplasmic-reticulum-associated complex containing inhibitor-1, apurinic/apyrimidinic endonuclease 1, high-mobility group protein 2, the nucleoside diphosphate kinase NM23-H1 and the exonuclease TREX1, in which it is the specific inhibitor of the granzyme-A-activated deoxyribonuclease (Fan and colleagues, 2003; Chowdhury and colleagues, 2006). The companion volume *The Single Cut* develops the consequences of that second identity; this volume takes it only as far as necessary to establish that the protein is *employed in two compartments*, and that its removal from one is a loss as well as its arrival in the other a gain.

The human observation. Set out quantitatively in Section III and recalled here in summary: messenger RNA for both inhibitors increased in Alzheimer temporal and entorhinal cortex and unchanged in cerebellum; inhibitor-2 shifted from nucleus to cytoplasm with a greater than sixfold change in the ratio of immunonegative to immunopositive nuclei; nuclear signal down; full-length cytosolic protein down; a twenty-kilodalton cytosolic fragment significantly higher in Alzheimer tissue; inhibitor-1 localisation unchanged; and, when recombinant inhibitor was digested with brain extract, Alzheimer extract generated more fragment than control extract (Tanimukai and colleagues, 2005). Both inhibitors co-localised with the phosphatase in neuronal cytoplasm and with abnormally hyperphosphorylated tau in neurons at early-to-middle-stage neurofibrillary degeneration.

One methodological detail in that study deserves emphasis because it bears on Section IX. The homogenisation buffer is reported to have contained leupeptin and ethylene glycol tetraacetic acid — the first inhibits calpain, the second chelates the calcium that calpain requires — and the twenty-kilodalton fragment persisted, which would mean that whatever generated it was not calpain acting during tissue handling. *We flag that we were unable to obtain the full text of this study and have verified the buffer composition only at second hand; since this observation is one of four arguments supporting a central negative claim of this volume, it should be checked against the published Materials and Methods before being relied on.*

Causality in cells. Transient transfection of the inhibitor into pheochromocytoma cells hyperphosphorylates tau at serines 396 and 404 and at serines 262 and 356, but *only when a nucleus-to-cytoplasm shift occurs*, and the

shift is accompanied by cleavage into the twenty-kilodalton fragment (Chohan and colleagues, 2006). The requirement for relocation, rather than for abundance, is the cell-biological statement of the same point the localisation-signal mutants make.

Which fragment inhibits: both. Constructs corresponding to residues 1 to 175 and residues 176 to 277 were tested directly. The full-length protein is found within the nucleus, as expected, while *both* fragments are diffused through nucleus and cytoplasm alike. Both fragments, unlike the full-length protein, reach the cytoplasm, and the catalytic subunit co-immunoprecipitates with the full-length protein and with both fragments. Phosphatase activity fell to about seventy per cent of control with full-length protein and to about fifty per cent with the amino-terminal fragment; in fibroblasts, full-length produced about a thirty-one per cent reduction against the carboxy-terminal fragment's fifty-three per cent. Critically, full-length inhibitor reduced activity *without changing catalytic subunit protein levels* — direct inhibition, not downregulation. The two fragments produce overlapping but distinguishable tau phospho-site patterns (Arnaud and colleagues, 2011).

This supersedes the older model in which inhibitory activity was assigned to the amino-terminal region alone. Papers citing that model are out of date, and the practical consequence is that a cut which generates two fragments generates two inhibitors, not one inhibitor and one inert remnant. We note honestly what has not been measured: no inhibition constant or half-maximal inhibitory concentration exists for either fragment. The authors state that the fragments transferred to membrane at variable rates and that normalisation was therefore not possible. The percentages above are cellular effects at uncontrolled expression levels, not enzymology.

In vivo. Delivery of the carboxy-terminal fragment by adeno-associated virus into the cerebral ventricles of rat pups within twenty-four hours of birth, with lifelong expression, produced at eight months: significantly decreased phosphatase activity; tau phosphorylation at multiple sites; activated glycogen-synthase-kinase-3 β ; increased intraneuronal amyloid- β ; neurodegeneration by Fluoro-Jade B; a forty-three per cent decrease in microtubule-associated protein 2 fluorescence in CA1 pyramidal neurons at nine weeks rising to sixty-one per cent at eight months; synaptophysin and synapsin decreases in CA3 of forty and forty-five per cent at nine weeks rising to seventy-one and sixty-nine per cent at eight months; decreased

phosphorylated cyclic-AMP response element binding protein; and spatial reference memory and consolidation deficits (Wang and colleagues, 2010).

Delivering both fragments together produced, at four months, a dramatic fall in brain phosphatase activity, synaptic loss, moderate amyloid- β and tau accumulation and marked spatial reference and working memory impairment; and at thirteen months, abnormal hyperphosphorylation and aggregation of tau at serines 199, 262 and 396 and threonine 205 in CA1, CA3 and cortex, a marked shift from sarkosyl-soluble to sarkosyl-insoluble tau, and intraneuronal amyloid- β . The authors note that this was produced by approximately a twenty per cent increase in expression of the fragments (Bolognin and colleagues, 2012). A twenty per cent increase is within the range of the human observation, which is what makes this model informative rather than merely a demonstration that overexpression is toxic.

Grade. Established in human tissue for the localisation shift, the fragment and the transcript increase; strong in cells and rat for the causal chain from cytoplasmic inhibitor to phosphatase inhibition to tau hyperphosphorylation to synaptic and cognitive loss. The open question is the one raised at the top of this section: which pool of phosphatase this lock acts on.

VIII. The Fourth Lock — The Cut at Asparagine 175

The fourth lock is not a distinct mechanism so much as the mechanism that *arms* the third. It is a single proteolytic event, at a single residue, by a single enzyme, and its evidentiary standing is unusually good — with one exception, which is the compartment in which the enzyme is supposed to be working, and which we grade honestly as the weakest joint in this volume's chain.

The enzyme. Asparaginyl endopeptidase, also called legumain and, since its role in amyloid precursor protein processing was described, δ -secretase, is a lysosomal cysteine protease of family C13 with an absolute specificity for the peptide bond carboxy-terminal to asparagine. Its zymogen at fifty-six kilodaltons is processed to an active thirty-six kilodalton form by sequential removal of carboxy-terminal and amino-terminal propeptides at different pH thresholds. Its endopeptidase activity has an acidic optimum and it is denatured above neutrality. The specificity is what gives the enzyme its analytic value: a protease that cuts at one residue has a substrate list that is a

property of sequence rather than of biology, which means two of its substrates need not belong to a common pathway to be cut by the same enzyme.

The site, and who mapped it. Attribution here has been garbled in the secondary literature and is worth correcting. The residue was mapped not in the Alzheimer papers that cite it but in a study of neuroprotection. A truncation series localised the cut to around residue 177; the fifty-kilodalton product of digestion with acidic cytosolic extract was recovered and analysed by liquid chromatography–tandem mass spectrometry, identifying the peptide spanning residues 165 to 175 as the carboxy-terminal peptide; and an asparagine-175-to-alanine mutant was uncleaved at acidic pH while wild type was cleaved. The protease was identified by fractionation, blocked dose-dependently by an anti-protease monoclonal but not control immunoglobulin, blocked by the specific competitive inhibitor peptide AENK but not the inactive control AEQK, and inactive in catalytically dead mutants. The pH dependence was mapped twice: maximal fragmentation at pH 5.5, cleavage at pH 5.0 and 6.5, no degradation at pH 7.0 in the first experiment; complete degradation at pH 4.5 and 5.0, three fragments between pH 5.5 and 6.5, intact protein at pH 7.0 and above in the second. And the requirement was demonstrated genetically *in vivo*: kainate produced protease activation and inhibitor cleavage with DNA nicking in CA3 in wild-type but not protease-null mice, and middle cerebral artery occlusion cleaved the inhibitor selectively in wild-type brain (Liu and colleagues, 2008).

The human demonstration. In Alzheimer frontal cortex the active enzyme is increased and the zymogen decreased, with the amino-terminal inhibitor fragment correspondingly increased; the enzyme and the inhibitor physically interact by co-immunoprecipitation; the enzyme's co-localisation with the lysosomal marker LAMP-2 is high and punctate in control tissue and low and diffuse in Alzheimer tissue, with abnormally enlarged lysosomal vesicles. A glutathione-S-transferase fusion of the inhibitor was cleaved time-dependently by Alzheimer brain extract, and the asparagine-175-to-glutamine mutant was not cleaved. Immunoprecipitated enzyme cleaved the substrate; enzyme-immunodepleted extract did not. Among subcellular fractions the cytosol had the highest active enzyme and was by far the most effective at cleaving the inhibitor, while neither the lysosomal nor the nuclear fraction produced significant cleavage (Basurto-Islas and colleagues, 2013).

Both fragments were generated in cells under acidification: co-transfection of enzyme and inhibitor followed by thirty minutes at pH 5.5 drove both proteins diffusely cytoplasmic and produced a twenty-two kilodalton amino-terminal and a seventeen kilodalton carboxy-terminal fragment. Metabolically active rat hippocampal slices taken from pH 7.4 to 6.5 to 5.5 produced graded increases in active enzyme, decreases in full-length inhibitor, decreases in phosphatase activity and increases in tau phosphorylation at serines 262 and 356, threonine 205, serine 214 and threonine 212 — but not serine 199, which is among the phosphatase's least favourable sites and is therefore the expected exception. And in neuroblastoma cells, kainic acid or lysis at pH 6.0 increased phosphorylation at serines 262 and 356, and small interfering RNA against the enzyme abolished both effects.

We record one unassigned observation from the same work that the field has not pursued: a twenty-four kilodalton amino-terminal cleavage product appeared in cells transfected with inhibitor alone, requiring neither the protease nor acidic pH. There is therefore at least one further, unidentified cleavage of this protein.

The same enzyme, the same disease, other substrates. The protease cleaves tau at asparagine 255 and asparagine 368, abolishing tau's microtubule-assembly function and inducing aggregation; the cleavage is phosphorylation-independent, unlike caspase-3 cleavage of tau; the asparagine 368 peptide is detectable by mass spectrometry in human Alzheimer brain; fragments appear in mouse brain from eight months and rise with age in parallel with enzyme activity, and are completely abolished in protease-null brain. Crossing P301S tau mice onto the null background eliminated the asparagine 368 species, produced roughly a thirty per cent reduction in AT8- and AT100-positive neurons in hippocampus and cortex, and rescued synapse loss, hippocampal synaptic function and cognitive deficits (Zhang and colleagues, 2014). The enzyme also cleaves amyloid precursor protein at asparagine 373 and asparagine 585, and the second cut removes an amino-terminal region that sterically hinders β -secretase, converting the remainder into an optimal β -secretase substrate; null neurons secrete significantly less amyloid- β with unchanged total precursor, and deleting the enzyme from amyloid models decreased plaques, ameliorated synapse loss and protected memory (Zhang and colleagues, 2015).

The enzyme is therefore a hub rather than a specialist, and the corpus has treated it as such. What this volume adds is only the observation that its action on the phosphatase inhibitor and its action on tau are, mechanistically, the same chemistry applied to two proteins that happen each to present an exposed asparagine — not two steps of a designed pathway.

The problem: where is the acid? The endopeptidase activity requires acidity, and the cleavage of the phosphatase inhibitor is reported to occur predominantly in the cytosol, whose pH in a living neuron is about 7.2. The investigators concede the difficulty directly: it is not known how the enzyme is able to function in the cytosol. This volume grades the joint rather than waving at it.

The *in vivo* evidence for neuronal acidification in this disease is weaker than the model requires, and it is weakest exactly where the model needs it strongest. Three separate claims are routinely conflated, and they should be graded separately.

Bulk post-mortem brain tissue pH is lower in Alzheimer disease. This is real and replicated. A meta-analysis of twenty datasets of post-mortem brain and cerebrospinal fluid, comprising seven hundred and twenty-three Alzheimer and five hundred and twenty-four control samples, gives a standardised effect of -0.57 , surviving adjustment for post-mortem interval, age at death and sex, with pH correlating negatively with Braak stage across fifteen datasets (Hagihara and Miyakawa, 2024). A three-hundred-and-sixty-eight-donor brain bank measured frontal pole homogenate pH with a dose-response against neuropathological score and against amyloid stage, reporting means near 6.1 in controls and near 5.9 in Alzheimer cases — figures we were unable to verify beyond the abstract (Wang and colleagues, 2025), and an earlier series of ninety Alzheimer and eighty-one control cortical samples gave 6.4 against 6.5 (Preece and Cairns, 2003). But the magnitude is one to two tenths of a pH unit; the largest cohort has a twenty-nine-year age gap between its arms in a dataset where pH falls with age; and post-mortem homogenate pH is substantially set by terminal and post-mortem lactate accumulation, which is why the tissue-quality literature treats it as an index of agonal state rather than a disease variable. Alzheimer patients die differently from typical controls, and no study can fully adjust for agonal duration because it is rarely recorded.

In vivo brain pH is lower in Alzheimer patients. Here the evidence is mixed, and the two spectroscopic traditions disagree. The phosphorus literature is

small and points away from acidification: a six-case series reported a non-significant shift toward *alkalosis* in left hippocampus relative to mild cognitive impairment (Mandal and colleagues, 2012), a seven-case series reported relative alkalosis in the same structure (Mecheri and colleagues, 1997), and a forty-one-subject seven-tesla study across cognitively normal, mildly impaired and Alzheimer groups reported significant group effects on energy reserve, energy consumption and magnesium without reporting any on pH (Das and colleagues, 2021). But proton spectroscopy of histidine, in a much larger cohort of one hundred and forty-seven subjects, found white-matter pH low in Alzheimer disease and, to a lesser degree, in mild cognitive impairment, with hippocampal pH unchanged — and, importantly for specificity, not low in frontotemporal dementia spectrum disorders or in pure vascular dementia (Lyros and colleagues, 2020). That study is the largest in vivo measurement available, it carries the disease-specificity control this volume elsewhere insists on, and it runs in favour of the acidification arm. It also locates the change in white matter rather than in the hippocampal grey matter where the mechanism is supposed to operate, and it reports a cerebral pH difference of a small fraction of a unit rather than the one-and-a-half units the enzyme's endopeptidase optimum would require. The honest summary is therefore that in vivo brain pH is probably modestly lower in this disease, that the compartment does not match, and that the magnitude does not come close.

Neuronal cytosol reaches the enzyme's optimum of about 5.5. There is no evidence for this at all. The largest documented receptor-driven acidification of neuronal cytosol — setting aside complete ischaemia, where brain intracellular pH falls by roughly a unit — is excitotoxic: a sixty-second exposure to *N*-methyl-D-aspartate produced a reversible intracellular pH fall with a half-maximal effective concentration of thirty-nine micromolar and a maximal change of -0.53 pH units, abolished by receptor antagonism and by removal of extracellular calcium, with an intracellular proton source, and absent in glia (Irwin and colleagues, 1994). A change of that size takes cytosol from about 7.2 to about 6.7 — still more than a unit above the enzyme's optimum — and it recovers within minutes.

The resolution the field actually offers, and it is a better one. Crystal structures of the zymogen and the activated enzyme show that the asparagine-specific endopeptidase activity is electrostatically generated by pH shift, and that a hidden carboxypeptidase activity develops on proteolytic activation with release of the activation peptide; substrate access is controlled

by selective protonation of the substrate-binding pocket and of the catalytic nucleophile. The authors state that these activation routes "reconcile the enigmatic pH stability of legumain, e.g. lysosomal, nuclear, and extracellular activities" (Dall and Brandstetter, 2013), and note elsewhere that the enzyme is found extracellularly and translocates to cytosol and nucleus, compartments of seemingly incompatible pH and redox potential (Dall and Brandstetter, 2016; Elamin and colleagues, 2022).

The second half of the resolution is delivery. Neuron-specific pH reporters across five Alzheimer mouse models showed that autolysosome acidification declines well before extracellular amyloid deposition, with markedly lowered vacuolar ATPase activity, progressing to lysosomal membrane permeabilisation and release of cathepsins into the cytosol, a pattern also present in human Alzheimer brain (Lee and colleagues, 2022). That is a route by which an already-activated protease reaches the cytosol without requiring the cytosol to become acidic.

We therefore restate the claim in the form the evidence supports, and mark the difference explicitly. It is *not* supported that Alzheimer cytosol acidifies to this enzyme's optimum. It *is* supported that the enzyme is activated in an acidic compartment, that its activation is accompanied by a conformational change conferring neutral-pH-stable activity, and that lysosomal membrane permeabilisation delivers lysosomal hydrolases into the cytosol in this disease. The load-bearing assumption moves from cytosolic pH — which is unsupported — to permeabilisation and retention of activity after the pH shift, which is better evidenced. We note that this restatement is not cost-free: a *de-acidified* lysosome, which is what the autophagy literature documents in this disease, is the wrong direction for generating an active endopeptidase inside the lysosome in the first place, and the model must therefore place the activation earlier than the de-acidification or find it elsewhere.

A tension worth naming. Transcriptomic data place the protease's transcript predominantly in microglia, a point its own investigators acknowledge, while the entire mechanism assembled here requires the enzyme in neurons and rests on protein-level immunoreactivity and confocal co-localisation (Zhang and colleagues, 2015; Basurto-Islas and colleagues, 2013). The neuronal localisation is supported by two independent laboratories at the protein level and in Alzheimer tangles specifically; but a mechanism

whose central enzyme is transcriptionally a microglial gene deserves a cell-type-resolved measurement, and it does not have one.

Grade. Strong and human for the cleavage, the site and the disease association; established genetically for the enzyme's requirement in the mouse; moderate and restated for the compartment. The compartment claim survives only in its restated form: a modest, white-matter-weighted reduction in in vivo cerebral pH is now documented in a substantial cohort, and it is neither the compartment nor the magnitude the simple acidification account needs.

IX. The Protease That Is Not There

The list that prompted this volume names two proteases in sequence: calpains, then asparaginyl endopeptidase. The adjacency is natural, since both are activated in this disease, both act in the same cell, and both are routinely named in the same review paragraph. It is also, on the specific question of the phosphatase's inhibitor, unsupported. This section states the negative, states how hard it was searched for, and then states what calpain actually does to the phosphatase system, which turns out to be a good deal — just not this.

The negative. No primary study in any system reports cleavage of SET or inhibitor-2 of the phosphatase by calpain-1 or calpain-2. We searched for it directly in multiple query formulations across the bibliographic databases and by open web search, and found nothing. What exists is one sentence in a discussion section, in the paper that mapped the asparagine 175 site, observing that low pH activates diverse proteases, that calpains can be activated under low pH and cleave cytoskeletal proteins, and that the authors believe asparaginyl endopeptidase is one of numerous proteases activated by kainate or stroke (Liu and colleagues, 2008). That is a hypothesis about proteases in general. It contains no data on this substrate and it should not be cited as though it does.

Four further lines of evidence argue positively against calpain as the relevant protease here.

The residual cleavage in protease-null animals is caspase-dependent, not calpain-dependent. In the same study, kainate still produced weak inhibitor cleavage in protease-null brain; that residual activity is reported to have been neither pH-sensitive nor dithiothreitol-sensitive, excluding cathepsins, and to

have been blocked by the pan-caspase inhibitor Z-VAD-fluoromethylketone but not by E-64. The alternative route is therefore a caspase, not calpain. Two caveats attach. We were unable to obtain the full text and carry this detail at second hand. And E-64 is a broad clan-CA inhibitor rather than a calpain-selective one — it inhibits legumain itself, and its unmodified form is cell-impermeant — so it is a clean discriminator in an extract and not in an intact cell.

In the closest parallel substrate, the two proteases are explicitly separable. For tau, the calpain inhibitor ALLN did not reduce cleavage by asparaginyl endopeptidase, which was selectively reduced by the specific peptide inhibitor; a tau mutant at the calpain sites was still fully fragmented by the endopeptidase; and calpain cleaved wild-type tau, the endopeptidase-uncleavable double mutant and a truncated tau at indistinguishable rates (Zhang and colleagues, 2014). The two proteases act independently on a shared substrate; there is no reason to expect them to share a different one.

The human fragment survives calpain-suppressive conditions. As noted in Section VII, the twenty-kilodalton fragment was recovered from Alzheimer brain homogenised in buffer reported to contain leupeptin and ethylene glycol tetraacetic acid (Tanimukai and colleagues, 2005) — a calpain inhibitor and a chelator of the calcium calpain requires. *This detail is carried at second hand and is the weakest of the four arguments in this section; it should be confirmed against the published methods.*

The known proteases of this substrate cut elsewhere, and are identified. Caspase, principally caspase-7, cleaves the protein after aspartate 18 during apoptosis at neutral pH; the site was mapped by amino-terminal sequencing and the cleavage suppressed by a DEVD aldehyde but not a YVAD aldehyde (Morita and colleagues, 2000), and an aspartate-18-to-alanine mutant completely blocks recombinant caspase-3 cleavage (Liu and colleagues, 2008). Granzyme A cleaves after lysine 176 and destroys the protein's nucleosome-assembly activity (Beresford and colleagues, 2001). There is a genuine and striking coincidence here — a tryptase and an asparaginyl endopeptidase cutting at adjacent residues in the same flexible linker immediately amino-terminal to the nuclear localisation signal — but calpain is not among the enzymes that have been shown to cut this protein anywhere.

How the claim should be stated. Asparaginyl endopeptidase is the only protease for which direct genetic and pharmacological evidence establishes cleavage of the phosphatase's inhibitor at asparagine 175 in neurons and in Alzheimer brain. Calpain has been invoked in passing as one of several acid-activated proteases in ischaemia; no primary study has demonstrated calpain cleavage of this substrate in any system. If a future study demonstrates it, the mechanism assembled here gains a second arm. Until then, the arm should not be drawn.

What calpain does do, which is four things.

It does not cleave the catalytic subunit or the scaffold, and this was tested directly. Degrading phosphatase subunits with m-calpain showed that the B $\square$ subunits PR72 and PR130 are cleaved into a proteolysis-resistant forty-five to forty-eight kilodalton fragment, and that "PP2A(C), PR65/A and representatives of PR55/B and PR61/B $\square$ subunit families are calpain-resistant"; cleavage of PR72 requires intact EF-hands, weakens its interaction with the core enzyme and *activates* basal activity of the corresponding holoenzyme (Janssens and colleagues, 2009). This is a clean negative for the core enzyme and a positive for one specific regulatory family. We record a conflict: a separate study found the B56 α and B56 γ subunits to be in vitro calpain substrates with calpain regulating B56 α stability in vivo (Bertoli and colleagues, 2009), which sits against the first study's report that representatives of that family are calpain-resistant. Different isoforms and different conditions, but the discrepancy should be stated rather than smoothed.

It cleaves the $\alpha 4$ chaperone, and this is a real calpain-to-phosphatase-to-tau link in human Alzheimer tissue. MID1-dependent monoubiquitination of $\alpha 4$ triggers calpain-mediated inter-domain cleavage, switching $\alpha 4$ from protecting the catalytic subunit to destroying it, and increasing tau phosphorylation. Levels of cleaved $\alpha 4$ are decreased in Opitz syndrome, where microtubule-associated proteins are hypophosphorylated, and *increased in Alzheimer disease*, where they are hyperphosphorylated (Watkins and colleagues, 2012). We flag that we could not retrieve the full text of this study and have not verified the cleavage-site residues, fragment sizes, calpain isoform or cohort size; the abstract-level claim is reported here and should be checked against the published figures before any number is quoted from it. But the direction and the human observation are stated in the record, and this — not SET — is where calpain enters the phosphatase system.

It cleaves calcineurin into a hyperactive form, and this produced an assay artefact that invalidated a prior literature. Calpain I cleaves calcineurin A at lysine 501, carboxy-terminal to the autoinhibitory domain, producing a fifty-seven kilodalton truncated enzyme that retains calcium/calmodulin dependence but has markedly increased phosphatase activity, and the cleavage correlates with tangle number across human brains (Liu and colleagues, 2005c). The artefact is the instructive part. In crude extracts there is no Alzheimer-versus-control difference in calcineurin activity — because the calcium and calmodulin in the assay mixture activate endogenous calpain in the *control* extracts during the incubation, which then cleaves control calcineurin to the same hyperactive form, raising it to Alzheimer levels in the tube. Removing calcium and calmodulin, or adding chelator, abolished both the cleavage and the calpain autolysis; in Alzheimer extracts the enzyme was already cleaved and no further cleavage occurred. With immunoprecipitation rather than crude extract, activity was threefold higher in Alzheimer than control tissue (Qian and colleagues, 2011).

That finding matters beyond calcineurin. Calcium-dependent proteolysis proceeds during tissue handling and during assay incubation, which means any post-mortem measurement of a calpain substrate — including the founding measurement of calpain activation itself — carries a risk of the same artefact. The point generalises, and it generalises uncomfortably: the same class of problem was demonstrated in mouse, where generation of the calpain product p25 from p35 turned out to be an artefact of membrane-protein overexpression and of post-mortem interval, with knock-in mice accumulating large amounts of amyloid- β without generating p25 (Saito and colleagues, 2016).

It truncates glycogen-synthase-kinase-3 β into a more active enzyme, and oxidative stress drives this. Exposure of brain extracts to calcium truncates the kinase into roughly forty- and thirty-kilodalton fragments, blocked by specific calpain inhibitors, and the truncation *augments* kinase activity by removing an amino-terminal inhibitory domain — the domain that contains the inhibitory serine 9. Ionomycin, glutamate or *N*-methyl-D-aspartate produce the truncation in cortical neurons at inhibitor concentrations that also block cleavage of the NR2B subunit and of p35 (Goñi-Oliver and colleagues, 2007). The oxidative version is the one that matters for Section X: hydrogen peroxide in a tau-expressing cell line increased kinase activity *despite* dramatically increasing inhibitory serine 9 phosphorylation, by way of a transient calcium rise and calpain truncation into the same two fragments,

with calpain inhibition restoring normal activity and blocking tau hyperphosphorylation at serines 396 and 404 and threonine 231 — and, notably, with no change in cyclin-dependent kinase 5 and no cleavage of p35 in that system (Feng and colleagues, 2013). This is a genuine route from oxidative stress through calpain to the tau balance, and it does not pass through the phosphatase at all: it raises the writer rather than removing the eraser.

A note on p25, which this volume declines to use. The conversion of p35 to p25 by calpain, activating cyclin-dependent kinase 5 and hyperphosphorylating tau, is among the most cited mechanisms in this field (Patrick and colleagues, 1999; Lee and colleagues, 2000). It is also contested at the level of the human observation. One series found the p25 to p35 ratio not elevated in sporadic or familial Alzheimer disease, nor in Down syndrome, Pick disease, corticobasal degeneration, progressive supranuclear palsy or frontotemporal dementia, across thirty-four tauopathy cases and eleven controls (Tandon and colleagues, 2003); another found it elevated in all three regions examined while simultaneously finding *no difference in calpain activity* between Alzheimer and control (Tseng and colleagues, 2002), a result that sits awkwardly against the founding calpain measurement; and a review of the field concluded that many laboratories could not confirm increased p25 and that, on the contrary, p25 appears reduced in Alzheimer disease, primarily in early stages, while p25 generation accompanies normal memory formation in mouse hippocampus (Giese, 2014). Combined with the overexpression-artefact finding, this is not a joint on which a mechanism should rest, and this volume does not rest one on it.

The arrow that runs the other way. There is one further calpain–phosphatase connection, and it is the reverse of the direction one would search for. Purified active phosphatase directly dephosphorylates both calpain isoforms in vitro; the two proteins co-localise and interact; overexpression of the catalytic subunit suppresses calpain phosphorylation and activity, and knockdown *enhances* calpain phosphorylation and activity (Xu and Deng, 2006). If that holds in neurons — and it was shown in cancer cells, which is a real caveat — then loss of the phosphatase by any of the routes in this volume produces calpain hyperactivation, and calpain-driven damage feeds back as a consequence of phosphatase loss rather than as a cause of it. The loop closes, but in the opposite orientation from the one the list implies.

Grade. Rejected for the specific claim: calpain does not cleave the phosphatase's inhibitor, on present evidence. Established for calpain activation in human Alzheimer brain. Moderate, and human, for the $\alpha 4$ route. Established for the calcineurin route, with the important corollary that the calcineurin truncation does *not* protect tau — across thirteen human brains, truncation correlated positively, or tended to correlate positively, with tau phosphorylation at every site except serine 202, and the authors' conclusion is that calcineurin is not a major brain tau phosphatase (Qian and colleagues, 2011).

X. Oxidative Stress — Which Arrows It Actually Draws

Oxidative stress is the item on the list that is asked to do the most work and is given the least scrutiny, because it can be connected to anything. This section asks which of its connections to the four locks are demonstrated, which are inferred, and which point the opposite way from the one assumed. Three of the arrows the list implies do not exist. One arrow that the field has not drawn does exist, and it carries a methodological consequence for every measurement in Section III.

The observation is secure, and its timing is the most useful thing about it. Neuronal oxidative damage in this disease was established *in situ* with antibodies against oxidised guanosine, showing prominent cytoplasmic immunoreactivity in neurons of hippocampus, subiculum, entorhinal cortex and neocortex, significantly increased in twenty-two Alzheimer cases against controls of three age bands, and greatly diminished by ribonuclease but only slightly by deoxyribonuclease — the damage is predominantly to RNA, and its cytoplasmic predominance implicates mitochondria as the source (Nunomura and colleagues, 1999).

The timing result is the one that matters for a mechanistic argument. Oxidative damage is quantitatively greatest early and *decreases* with disease progression; increased amyloid- β deposition is associated with *decreased* oxidative damage; the relationships are stronger in apolipoprotein E $\epsilon 4$ carriers; and neurons containing neurofibrillary tangles show a forty to fifty-six per cent *decrease* in relative oxidised guanosine compared with tangle-free neurons (Nunomura and colleagues, 2001). The interpretation offered is that the lesions behave as downstream compensations rather than as the source of the stress. Either way, oxidative damage is upstream of the tau

lesion in time, which is the licence for treating it as a candidate trigger rather than a consequence.

Lipid peroxidation shows the same early profile. Free-radical-derived isoprostanes were significantly increased in frontal, parietal and occipital lobes in mild cognitive impairment and in late Alzheimer disease with no significant difference between those two stages (Markesbery and Lovell, 2005); 4-hydroxynonenal was significantly increased in hippocampus and parahippocampal gyrus, superior and middle temporal gyrus and cerebellum in mild cognitive impairment, and acrolein in temporal cortex, again with no significant difference between mild impairment and early disease (Williams, Lynn and Markesbery, 2006). The lesion is present before dementia and does not worsen with it. We record, as a control on over-reach, that peripheral isoprostanes are *not* increased in this disease (Montine and colleagues, 2002); the signal is restricted to brain and cerebrospinal fluid, and plasma measurements should not be cited as evidence of it.

The first arrow that does not exist: oxidative stress does not activate the protease. This is the arrow the list most obviously implies and it is contradicted by the only redox study of the enzyme we could locate. Chemical proteomics mapped oxidation of legumain at cysteine 219 and found that oxidation *dysregulated* the enzyme's intracellular processing and *decreased* its activity (Kovalyova and colleagues, 2022). We are precise about what this does and does not say: cysteine 219 is not the catalytic nucleophile, which is cysteine 189 in the histidine 148–cysteine 189 dyad, and the reported effect is on the enzyme's autocatalytic maturation rather than on its catalytic chemistry. But the direction is the opposite of the assumed one, and for a cysteine protease it is not a surprising direction. The demonstrated activator of this enzyme in neurons is acidification, not oxidation (Basurto-Islas and colleagues, 2013), and the two should not be treated as interchangeable species of "stress."

The second arrow that does not exist: the aldehydes of lipid peroxidation do not activate calpain; they inhibit it. Mass-spectrometric mapping showed 4-hydroxynonenal forming Michael adducts on histidine, lysine, glutamine and asparagine residues of calpain-1, and 4-hydroxynonenal and 2-hexenal lowering calpain-1 activity at one hundred, five hundred and one thousand micromolar, with autolysis slowed at the highest concentration; the same aldehydes decreased calpain-2 activity and slowed its autolysis, with modification sites mapped across both its subunits for malondialdehyde (Zhai

and colleagues, 2023; Johnson and colleagues, 2024). For completeness in the other direction, malondialdehyde at one hundred micromolar slightly *increased* calpain-1 activity. These are food-science studies in purified systems, which is a real caveat on their transferability, but they are the direct chemistry and they run the wrong way for the assumed link. Lipid peroxidation therefore cannot reach calpain by adduction. It reaches it by calcium, and only by calcium.

The arrow to calpain that does exist runs through calcium, and specifically through the ryanodine receptor. Hydrogen peroxide in neuroblastoma cells and rat hippocampal neurons elicited calcium signals, increased S-glutathionylation of the ryanodine receptor, and enhanced downstream kinase and transcription-factor phosphorylation; ryanodine pre-incubation largely prevented both, and in calcium-free medium thapsigargin abolished the effect, placing the source in intracellular stores (Kemmerling and colleagues, 2007). The same redox-dependent mechanism is engaged by amyloid- β : sublethal oligomers generate prolonged calcium signals in primary hippocampal neurons, abolished by calcium-free medium, by receptor inhibition, and by pre-incubation with *N*-acetylcysteine — that is, redox-dependently — leading to mitochondrial fragmentation and blockade of brain-derived-neurotrophic-factor-induced spine remodelling (Paula-Lima and colleagues, 2011). Amyloid- β also reduces sodium/potassium adenosine triphosphatase to below forty per cent of basal within three hours in cultured hippocampal neurons, preceding the loss of calcium homeostasis, with the effect reproduced by ouabain and attenuated by vitamin E and propyl gallate, implicating reactive species; the same reductions in sodium/potassium and calcium adenosine triphosphatases were produced in synaptosomes prepared from post-mortem human hippocampus (Mark and colleagues, 1995). And in vivo, multiphoton calcium imaging in amyloid precursor protein mice found calcium overload in about twenty per cent of neurites against fewer than five per cent in wild-type, presenilin-mutant or pre-plaque animals, dependent on proximity to plaques (Kuchibhotla and colleagues, 2008).

So the chain from oxidative stress to calpain is: reactive species and amyloid- β oligomers, to redox modification of the store-release channel and to pump failure, to raised cytosolic calcium, to calpain. It does not pass through the aldehydes, and it is demonstrated in rodent and cell systems rather than in human tissue. The human end of it — calpain activated threefold in Alzheimer prefrontal cortex, before overt neuronal loss and not in Huntington disease (Saito and colleagues, 1993) — is the solid part.

The third arrow that is inferred rather than shown: oxidative stress and the inhibitor's expression. A functional nuclear-factor- κ B-responsive element was identified in the human SET promoter by reporter assay and electrophoretic mobility shift, with overexpression or activation of that transcription factor upregulating one isoform of the protein; the authors frame the finding as a route by which oxidative stress and inflammation could produce abnormal expression of the gene in this disease (Feng and colleagues, 2017). We record honestly that the study applied no oxidative stress. The chain from reactive species to the transcription factor to this gene is not directly demonstrated in neurons.

There is, however, one direct oxidant-to-inhibitor experiment, and it concerns localisation rather than expression. Under mild oxidative stress from tert-butyl hydroperoxide, overexpressed inhibitor relocalised into large cytoplasmic aggregates, against an even nuclear and cytoplasmic distribution without stress; the protein raised phosphorylated protein kinase B, decreased cellular glutathione defence, and switched from anti-apoptotic to death-promoting (Leopoldino and colleagues, 2012). This is a non-neuronal overexpression system and cannot carry much weight, but it is the only direct demonstration that oxidant exposure drives this protein into cytoplasmic mislocalisation — which is the exact event the human observation reports.

The arrow that does exist, and that the field has not drawn: oxidative stress inhibits the phosphatase directly, reversibly, through a thiol pair. This is the most under-cited element of the whole mechanism and it comes from three papers in brain tissue.

Treatment of the rat brain soluble fraction with a thiol-reactive biotinylating agent produced substantial phosphatase inhibition and caused the catalytic subunit to bind an immobilised avidin column; both effects were fully reversed by dithiothreitol, and phosphatase specific activity in the reduced eluate was up to sevenfold higher than in the starting soluble fraction (Foley and Kintner, 2005). In rat cerebral cortex high-speed supernatant, phosphatase activity showed substantial disulfide-reducing-agent-reversible inhibition, associated both with intermolecular disulfide cross-linking of the catalytic subunit to other brain proteins and with an apparent novel intramolecular disulfide within the catalytic subunit; the vicinal dithiol cross-linker phenylarsine oxide produced potent, dithiothreitol-reversible inhibition, implying a vicinal thiol pair on the catalytic subunit whose dithiol–disulfide equilibrium confers redox sensitivity (Foley and colleagues,

2007). And affinity chromatography showed the catalytic subunit forming stable, reducible adducts with or without associated regulatory subunits, with a fraction of catalytic subunit still carrying disulfide bonds after stringent anti-artefact homogenisation — suggesting that a population of the enzyme carries disulfides in vivo (Foley and colleagues, 2011).

The mechanism is corroborated outside brain. In epithelial monolayers, twenty micromolar hydrogen peroxide reduced okadaic-acid-sensitive phosphatase activity by eighty to eighty-five per cent, with immunocomplex assays showing phosphatase 2A specifically inhibited while phosphatases 1 and 2C were not; oxidised glutathione inhibited the enzyme in membranes and in isolation, and the inhibition was reversed by reduced glutathione (Rao and Clayton, 2002). Hydrogen peroxide inhibits the enzyme in pheochromocytoma, neuroblastoma and primary murine neurons, abrogated by *N*-acetylcysteine, with overexpression of the phosphatase partially preventing downstream kinase activation and death (Chen and colleagues, 2009). And in a chronic, neuron-relevant model — glutathione depletion in a neuroblastoma line producing no cell death — tau phosphorylation at serines 396 and 404 increased time-dependently, with decreased phosphatase activity implicated, the authors noting explicitly that acute oxidative stress has disparate effects and that chronicity matters (Su and colleagues, 2010).

We record the honest limit: the specific cysteine residue numbers were not determined in any of *these* studies, in which the thiols are identified operationally, as vicinal and arsenical-reactive. Residue-level assignment does exist elsewhere. Thiol-reactive electrophiles alkylated all three subunits of purified AB α C and AB δ C holoenzymes from human embryonic kidney cells, with one probe modifying four cysteines in the catalytic subunit that a second probe did not touch, and a homology model relating specific adducts to inhibition (Codreanu and colleagues, 2006). So the phosphatase is an established target of cysteine-directed electrophiles at named residues; what has not been done is the assignment of the *reversible, disulfide-based* inhibition that Foley's brain-tissue work describes.

The consequence the field appears not to have drawn. Every measurement of phosphatase activity in Alzheimer brain assembled in Section III was made in homogenisation buffer containing reducing agent — β -mercaptoethanol at ten millimolar in the standard buffer of the laboratory that produced most of them. If a component of the in vivo deficit is disulfide-based, that component was reversed in the tube before the assay began. It follows that the widely

cited figure of an approximately thirty per cent reduction in tau phosphatase activity in Alzheimer brain is a measurement of the *non-redox* deficit, and that the true in vivo deficit may be larger. We searched specifically for an experiment testing whether reducing agents restore phosphatase activity in human Alzheimer brain extract, and found none: all of the redox work is in rat brain, cell lines or purified enzyme. This is a cheap, well-specified and currently unperformed experiment, and Section XIV states it.

What oxidative stress does not do to this system, gathered. No subunit of the phosphatase appears among the 4-hydroxynonenal adducts detected in the systematic redox proteomics of mild cognitive impairment and early Alzheimer brain — which is a statement about that survey's detection, not about the enzyme's chemistry, since the phosphatase is a demonstrated target of cysteine-directed electrophiles in vitro (Codreanu and colleagues, 2006): the identified 4-hydroxynonenal targets are overwhelmingly glycolytic and mitochondrial energy enzymes — enolase, lactate dehydrogenase, phosphoglycerate kinase, adenosine triphosphate synthase, pyruvate kinase, triosephosphate isomerase, malate dehydrogenase, manganese superoxide dismutase — with measured activity losses in several, and neither tau, nor any phosphatase subunit, nor calpain appeared among them (Reed and colleagues, 2008; Reed and colleagues, 2009). That pattern is itself useful, because it routes the *detected* burden of lipid peroxidation in human tissue to energy failure rather than to the components of this mechanism — and energy failure is the upstream of both the acidification of Section VIII and the calcium dysregulation of this one.

It does, however, modify the substrate. Exposure of cultured hippocampal neurons to 4-hydroxynonenal made tau *resistant to dephosphorylation*: the aldehyde caused a moderate rise in basal tau phosphorylation and prevented dephosphorylation by alkaline phosphatase in neurons pretreated with okadaic acid, and antibodies showed the aldehyde binding directly to tau (Mattson and colleagues, 1997). This is a substrate-side effect and not a demonstrated phosphatase inhibition, and it should be cited as such — but it produces the same phenotype from the other end, and it is the one place where lipid peroxidation touches the tau balance directly.

Grade. Established for the presence and early timing of oxidative damage in human tissue. Established in brain tissue, though not in human tissue, for direct reversible thiol-based inhibition of the phosphatase. Established in cells and rodents for the route to calpain via redox modulation of calcium release.

Rejected for oxidative activation of the protease. Rejected for aldehyde-mediated activation of calpain. Inferred, not shown, for transcriptional upregulation of the inhibitor.

XI. The Cell — Is the Pyramidal Neuron the Unit?

The list begins with a cell type, and a mechanism that could happen anywhere explains nothing about a disease that begins somewhere. This section asks whether "pyramidal neuron" is the right unit for the mechanism assembled above. The answer is that it is a serviceable shorthand and a poor unit, for four reasons, of which the fourth is the one that should change what anybody does next.

The classical picture, stated accurately. The staging scheme that organises this disease is built on layers rather than on morphological classes. Its stages are defined by involvement of the transentorhinal layer Pre- α , then that layer in the entorhinal cortex proper together with the first sector of Ammon's horn, then destruction of essentially all isocortical association areas; amyloid distribution was explicitly judged of limited value for staging (Braak and Braak, 1991). And the cell identity, in the original description, is more specific than "pyramidal": the entorhinal Pre- α layer consists of large multipolar, star-shaped nerve cells, which are *transformed into pyramidal cells* only during the layer's oblique course through the transentorhinal transition zone, while the isocortical pyramidal cells of layers II to IV are described as far less inclined to develop neurofibrillary change (Braak and Braak, 1985). The most vulnerable cell in the founding description is a large allocortical multipolar projection neuron.

The quantitative loss in that layer is severe. Stereology across twenty cases found approximately six hundred and fifty thousand neurons in entorhinal layer II in cognitively normal brains, stable between ages sixty and ninety; at the mildest detectable dementia, total entorhinal neurons fell thirty-two per cent and layer II sixty per cent; at severe dementia layer II fell approximately ninety per cent, with neuron number inversely proportional to tangle and neuritic plaque counts and not to diffuse or total plaques (Gómez-Isla and colleagues, 1996). A counterweight from the same field is worth carrying: a stereologic study separating intracellular from extracellular tangles found that even at severe dementia, neurons unaffected or only transitional in tangle

formation still constitute seventy-three per cent of profiles in entorhinal layer II and seventy-seven per cent in CA1 (Hof and colleagues, 2003).

The neocortical picture, and the hypothesis it generated. Antibodies against non-phosphorylated neurofilament epitopes label large pyramidal neurons predominantly in layers III and V; in this disease there is dramatic loss of these cells, preferentially those with perikaryal cross-sectional area above three hundred and fifty square micrometres, with the loss correlating with tangle counts and not with neuritic plaque counts (Hof, Cox and Morrison, 1990). Stereology in prefrontal area 9 localised the population to deep layer III and superficial layer V, found near-complete loss in end-stage disease, and showed by triple labelling that these neurons are far more likely to develop tangles and do so faster than other pyramidal cells, with layer Va more severely affected than layer IIc (Bussi re and colleagues, 2003). The connectivity rationale — that these are the long corticocortically projecting neurons — rests on macaque retrograde tracing showing that long ipsilateral association pathways have forty-five to ninety per cent neurofilament-enriched projection neurons against four to thirty-five per cent for short corticocortical, callosal and limbic pathways (Hof, Nimchinsky and Morrison, 1995). It is a monkey connectivity result, not a human disease result, and it should be cited as the inference it is.

There is also a dissociation that constrains it. In the amyotrophic lateral sclerosis/parkinsonism–dementia complex of Guam, tangles are far denser in supragranular than infragranular layers, the opposite of this disease, and the neurofilament-rich pyramidal subset that is devastated here appears *resistant* there (Hof and colleagues, 1994). Neurofilament richness is not sufficient.

The first reason the unit fails: the disease does not start in a pyramidal neuron. Across two thousand three hundred and thirty-two unselected brains spanning ages one to one hundred, subcortical pretangles — predominantly in the locus coeruleus — precede any cortical tau, and neocortical amyloid plaques appear after the brainstem tauopathy has begun; in a series of forty-two brains aged four to twenty-nine, thirty-eight carried abnormal tau, and in nineteen of twenty-two cases with only subcortical pretangles the material was confined to the noradrenergic coeruleus complex (Braak and colleagues, 2011). Noradrenergic coerulean neurons are not pyramidal. Any account that makes pyramidal morphology the substrate of vulnerability has to explain why the first cells to fail are not pyramidal, and the companion

volume *The Single Cut* argues that the explanation is metabolic and cell-type-specific rather than morphological.

The second reason: morphology does not predict vulnerability within the vulnerable region. Single-nucleus transcriptomics of matched entorhinal cortex and superior frontal gyrus across Braak stages 0, 2 and 6, with cross-sample alignment performed before clustering so that subpopulations are defined independently of disease stage, identified three excitatory subpopulations depleted by fifty to sixty per cent already at Braak stage 2, all expressing layer II markers — and found that not all layer-II-like subpopulations were vulnerable, with two showing no significant change and one deeper subpopulation actually increasing. The vulnerable populations are marked by the transcription factor RORB, and the histological validation showed both a reduction in the proportion of RORB-positive excitatory neurons with stage and a preference of phosphorylated tau for RORB-positive over RORB-negative excitatory neurons. The morphological check is the sentence that matters here: RORB-positive excitatory neurons "adopted various shapes, including pyramidal and multipolar morphologies," some large multipolar neurons are RORB-negative, and the authors conclude explicitly that morphology alone is insufficient to determine selective vulnerability (Leng and colleagues, 2021).

The third reason: the unit is finer than layer or morphology, and it crosses the excitatory–inhibitory boundary. High-throughput isolation of single somas bearing tangles from prefrontal cortex of eight severely affected donors produced a per-subtype census. Tangle proportions among excitatory subtypes ranged from one to nearly twelve per cent, with superficial layer 2–4 subtypes at 11.7 and 10.5 per cent and one layer 5 subtype at 10.6 per cent — while two other layer 5 excitatory subtypes in the same cortex sat at 5.2 and 3.6 per cent. Layer 6 was largely spared. And chandelier interneurons carried 6.8 per cent, higher than several excitatory subtypes, against an overall inhibitory figure of 1.9 per cent; the authors add that although interneurons were generally resistant to forming tangles they were not spared from death, with somatostatin interneurons the most susceptible to death of any class (Otero-Garcia and colleagues, 2022).

Two findings from that study bear directly on the mechanism of this volume. Tangle-bearing neurons share a marked *upregulation* of synaptic transmission genes, with the top enrichment in the synaptic vesicle cycle and a shared core set across subtypes, together with *N*-methyl-D-aspartate and

γ -aminobutyric acid receptor subunits, sodium/potassium adenosine triphosphatase subunits, and the integrated stress response factor ATF4. And, contrary to a widespread assumption, oxidative phosphorylation and glucose metabolism enrichment was *cell-type dependent* and concentrated in a single cluster rather than shared — which is the best-powered available test of the bioenergetic-demand explanation of selective vulnerability, and it argues against it as a universal account.

The interneuron result should be set against an older and more granular human measurement, and the discrepancy stated rather than resolved: fluorescence multiple labelling in human superior frontal gyrus found that the great majority of parvalbumin and calretinin neurons do not contain tangles, with the exception of large calretinin neurons in layer I which are also neurofilament-positive, and that the small proportion of parvalbumin cells acquiring neurofilament immunoreactivity in this disease — one to nine per cent, against zero to 0.4 per cent in controls — corresponds to the minute proportion bearing tangles, zero to 0.7 per cent (Sampson, Morrison and Vickers, 1997). Different region, different method, an order of magnitude apart on chandelier cells. Both are human. We do not smooth it.

We also record that calcium-binding-protein expression is not a protection marker in the simple sense often asserted: calbindin labels both interneurons and a pyramidal population in mid to deep layer III of human prefrontal cortex, and it is the *calbindin-positive layer III pyramidal neurons* that are dramatically affected, with loss strongly correlated to tangle counts, while superficial-layer calbindin interneurons are unaffected (Hof and Morrison, 1991).

One assumption that has been tested directly and refuted. It is often supposed that excitatory neurons are vulnerable because they express more tau. Single-molecule fluorescence in situ hybridisation across forty excitatory and forty inhibitory neurons in human entorhinal cortex and prefrontal area 9 found that transcripts for the metastable subproteome, tau co-aggregators and aggregation promoters were significantly higher in excitatory than inhibitory neurons, and that there was no significant difference in *MAPT* transcript (Fu and colleagues, 2019). Vulnerability in that dataset tracks the tau *homeostasis environment* — with the co-chaperone BAG3 as hub gene, higher in inhibitory than excitatory neurons and higher still in glia, and with knockdown increasing and overexpression attenuating endogenous tau accumulation — rather than tau abundance. The same study found

conformational and phospho-tau epitopes co-localising with excitatory markers and showing almost no co-localisation with inhibitory markers across mouse and human tissue, which is the finding that makes the chandelier result surprising.

The fourth reason, and the one that should change what happens next: nobody has measured this mechanism in a cell type. Not one of the six proteins assembled in this volume has been measured with neuronal-subtype resolution in human tissue. The holoenzyme deficit is reported for "neurons" against glia, and for tangle-bearing against tangle-free neurons, in cortex and not cerebellum (Sontag and colleagues, 2004a). The methyltransferase deficit is reported regionally (Sontag and colleagues, 2004b). The inhibitor's translocation and cleavage are reported for "neurons" (Tanimukai and colleagues, 2005). The kinase's redistribution is reported for "neurons" (Ho and colleagues, 2005). For the protease and for calpain we could find no study at all comparing expression or activation between excitatory neurons and interneurons, or between pyramidal neurons and Purkinje or granule cells. We searched the single-cell atlases for subunit-level expression and could extract nothing usable.

This is a gap rather than a contradiction, but it is a consequential one, because the cell-type-resolved datasets that would answer it are public. The entorhinal and frontal single-nucleus data (Leng and colleagues, 2021) and the tangle-bearing single-soma data (Otero-Garcia and colleagues, 2022) contain, in principle, the transcript-level answer for *PPP2R2A*, *PPP2CA*, *LCMT1*, *PPME1*, *SET*, *LGMN*, *CAPN1*, *CAPN2* and *FYN* across the vulnerable and resistant subtypes of the two most relevant regions. Nobody has looked.

A directional ambiguity to state before leaving this section. The observation that holoenzyme immunoreactivity is decreased *in tangle-bearing neurons* is compatible with two readings: that loss of the holoenzyme permitted the tangle, or that the tangle sequestered or degraded the holoenzyme. The original study does not distinguish them and nothing since has. The cell-type census offers an indirect argument for caution here, in that susceptibility to tangle formation and susceptibility to death are largely uncoupled in its data (Otero-Garcia and colleagues, 2022), and in that Purkinje cells in this disease are lost without ever tangling (Wegiel and colleagues, 1999). Whatever the phosphatase lesion does, it is not a simple synonym for the tangle.

Grade. The pyramidal neuron is rejected as the unit of selective vulnerability, and retained as a serviceable descriptive shorthand for the large excitatory projection neurons in which most of this pathology is found. The unit that the current data support is a transcriptomically defined excitatory subtype, and the mechanism of this volume has never been measured in one.

XII. Do the Locks Converge?

The question this volume was written to answer can now be put precisely. Four mechanisms have been described for switching off one phosphatase. Are they one mechanism seen from four angles, or four mechanisms that happen to share a destination? The evidence supports a specific and asymmetric answer: they converge on a target and they do not converge on a trigger, and one of the four does not act on the same target as the other three.

The target. Three of the four locks act on holoenzyme composition, not on catalysis.

The methylation lock determines whether the catalytic subunit can recruit B α , because B55-family assembly requires the methyl ester at leucine 309 while B β and B γ assembly does not (Longin and colleagues, 2007), and because persistent loss of the methyltransferase produces specific degradation of the B55 subunit (Longin and colleagues, 2007) and loss of the AB α C holoenzyme in cells, mice and human tissue (Sontag and colleagues, 2008; Sontag and colleagues, 2014; Sontag and colleagues, 2004a, 2004b).

The corrected tyrosine lock does the same thing by a different chemistry: phosphorylation at tyrosine 284 promotes dissociation of B α , demonstrated with a phenylalanine mutant that renders B α binding insensitive to Src and completely prevents the Fyn-driven decrease (Sontag and colleagues, 2022).

And the kinase reaches the same endpoint without touching the phosphatase at all, by occupying the RTPPKSP motif on tau that the holoenzyme also requires, so that the enzyme cannot bind its substrate (Sontag and colleagues, 2012).

Three distinct chemistries — a methyl ester, a phosphotyrosine, and steric occupancy of a substrate motif — converging on a single functional statement: *the catalytic subunit and tau are not brought together*. That is a real convergence and it is the volume's principal positive finding.

The fourth lock does not obviously share that target. The inhibitor binds the catalytic subunit and pulls down neither the scaffold nor a B subunit (Arnaud and colleagues, 2011). It inhibits non-competitively, it does not change catalytic subunit protein levels, and it acts through two separate determinants, one in each fragment. Whatever pool of enzyme it is acting on, that pool is not, on the evidence of the pull-down, the assembled AB α C holoenzyme. Two readings are available and the data do not choose between them. Either the inhibitor acts on the free or core-dimeric catalytic pool, in which case it is silencing a different population of the enzyme from the one the other three locks disassemble — and the two lesions would be additive rather than redundant. Or the inhibitor evicts B α in the act of binding, in which case it does converge, and the pull-down result simply reports the endpoint rather than the mechanism. Nobody has tested this. It is, in our judgement, the most important unanswered structural question in the mechanism, and Section XIV states the experiment.

Two of the four are not independent: they are sequentially coupled. The methylation lock and the tyrosine lock cannot be treated as parallel alternatives, because the methylation-incompetent catalytic subunit is tyrosine-phosphorylated *less* than wild type, from which the authors conclude that methylation and the presence of B α are required for maximal phosphorylation (Sontag and colleagues, 2022). A demethylated enzyme is a poorer substrate for the kinase. Whatever the disease does by demethylation, it thereby reduces the substrate available for the second lock. Adding the two lesions does not add their effects; it may subtract.

Two of the four are not two: they are one event. The inhibitor lock and the proteolytic lock are the same lesion described at two levels. The cut at asparagine 175 bisects the bipartite nuclear localisation signal spanning residues 168 to 181 (Arif and colleagues, 2014), which is why cleavage and redistribution are inseparable, and the cellular requirement is for relocation rather than for abundance (Chohan and colleagues, 2006). Counting them as two locks double-counts one mechanism. The honest count is therefore not four but three: a methylation lock, a tyrosine lock nested within it, and an inhibitor lock armed by a protease.

The triggers do not converge at all. This is the more consequential half of the answer, and it can be set out as a table.

Table 1 — The four locks, their targets and their afferents.

Lock	Molecular operation	What it acts on	Upstream afferent	Human evidence
Methylation at Leu309	Removal of a carboxyl methyl ester	B α recruitment and stability	One-carbon metabolism: folate, homocysteine, SAM/SAH	Methyltransferase and methyl-C reduced in affected regions, tracking tau not plaques
Tyrosine phosphorylation	Phosphorylation at Tyr284 by Fyn	B α dissociation	Amyloid- β via PrP ^C and mGluR5 to Fyn	None — never measured in AD tissue
Inhibitor binding	Non-competitive occupancy of PP2Ac	Catalytic subunit; not an A-B-C trimer	Nuclear export following cleavage	Inhibitor mRNA up, nuclear-to-cytoplasmic shift >6-fold, 20 kDa fragment
Proteolysis at Asn175	Severance of the NLS	The inhibitor, not the phosphatase	Acidification and lysosomal permeabilisation	Active protease up, zymogen down, fragment up, cytosolic

Folate status, amyloid- β -driven kinase activity, and lysosomal membrane integrity are three different afferents with three different time courses and three different interventions. A cell could have any one without the others. There is no node upstream at which they meet.

Could oxidative stress be that node? Partly, and less than assumed. Section X found that it reaches calpain through calcium but not through aldehyde chemistry; that it does not reach the protease, whose processing it deranges; that its connection to the inhibitor's expression is inferred and not shown; and that it does reach the phosphatase directly and reversibly through a vicinal thiol pair. It also reaches the one-carbon afferent indirectly, since the lipid-peroxidation targets identified in human tissue are the glycolytic and mitochondrial energy enzymes (Reed and colleagues, 2008), and metabolic failure is upstream of both acidification and calcium dysregulation. So oxidative stress is a broad afferent with two demonstrated direct arms into this mechanism — one onto the phosphatase's own thiols, one onto the calcium that activates calpain — and several assumed arms that do not survive examination. It is not the single key that turns four locks.

What the honest architecture looks like. Not a cascade and not a convergence, but a *coincidence structure*: one enzyme with an unusually large

number of independent regulatory surfaces — a methylatable carboxy terminus, a phosphorylatable tyrosine, a binding site for an endogenous inhibitor, and a substrate-docking interaction that a kinase can occupy — sitting in a cell that is simultaneously subject to one-carbon insufficiency, amyloid-driven kinase activation, lysosomal failure and calcium dysregulation. The convergence is a property of the enzyme's regulatory architecture, not of the disease's causal structure. The disease is not aiming at the phosphatase from four directions; the phosphatase is unusually easy to hit from any direction, and this disease supplies several.

That is a weaker claim than the list implies and a more useful one, because it makes a prediction the convergent model does not. If the four afferents are genuinely independent, then interventions on them should be *additive* rather than redundant, and no single one should normalise the phosphatase deficit. If they converge on a shared upstream node, one intervention should suffice. The methylation arm is the only one that has been tested in isolation *in vivo*, and it produced partial protection, not normalisation (Nicholls and colleagues, 2016; Staniszewski and colleagues, 2020; van Hummel and colleagues, 2023) — which is the result the independence model predicts.

One further asymmetry worth recording. The four locks differ in reversibility, and this has therapeutic consequences developed in Section XV. The methyl ester is a reversible modification maintained by an enzyme pair, and its equilibrium can be pushed by substrate supply — which is why dietary manipulation works in the mouse. The phosphotyrosine is reversible and kinase-driven. The thiol oxidation of Section X is reversible with a reducing equivalent. But the cut at asparagine 175 is not reversible at all: proteolysis is the one operation in this set that a cell cannot undo, and it converts a regulated nuclear protein into two constitutive cytoplasmic inhibitors that persist. If one of these locks is the one that makes the lesion permanent, it is that one.

XIII. The Validity Ledger

The discipline that separates synthesis from speculation is the graded ledger: each connection assigned a tier, and the experiment that would settle it named alongside. Entries are graded by what was measured, in which species, in which tissue and with which reagent — not by how often the claim has been repeated. Four tiers are used for claims that hold (*Established*, *Strong*, *Moderate*,

Weak), two for claims that do not (*Predicted, untested and Rejected as stated*), and one for the specific case of a claim whose evidence has been invalidated by reagent failure (*Withdrawn*).

Established (human, quantitative) — protein phosphatase 2A performs the large majority of tau dephosphorylation in human brain, and its tau-directed activity is reduced in this disease. Approximately seventy-one per cent of total tau phosphatase activity, against eleven per cent for phosphatase 1, ten per cent for phosphatase 5 and seven per cent for calcineurin, with Michaelis constants for the first three near the intraneuronal tau concentration (Liu and colleagues, 2005a). The reduction measured against Alzheimer-derived hyperphosphorylated tau is approximately thirty per cent (Gong and colleagues, 1995). *Caveat entered in Section X: all such measurements were made under reducing conditions.*

Established (human, immunohistochemical and immunoblot) — the AB α C holoenzyme is reduced in Alzheimer cortex, selectively, in neurons, in tangle-bearing cells, not in cerebellum and not in non-Alzheimer dementias, correlating with tangle load and not plaque burden. Glial immunoreactivity moves in the opposite direction (Sontag and colleagues, 2004a). *Settling experiment: none needed for the observation; the directional ambiguity — cause or consequence of the tangle — is addressed in the predictions.*

Established (human) — leucine carboxyl methyltransferase 1 protein and methylated catalytic subunit are reduced in Alzheimer-affected regions, in a regional pattern paralleling tau pathology rather than amyloid burden (Sontag and colleagues, 2004b).

Established (biochemistry and structure) — methylation at leucine 309 governs recruitment of the B55 family, and the carboxy-terminal tail is a B-subunit docking element rather than a free loop. B55 binding requires methylation while B $\square$ and B $\square$ binding does not (Longin and colleagues, 2007); the tail occupies a groove at the B-subunit–scaffold interface (Xu and colleagues, 2006); the methylesterase inactivates the enzyme by evicting the catalytic metal ions (Xing and colleagues, 2008).

Established (structure and reconstitution) — the B α subunit is what makes the enzyme a tau phosphatase. Direct binding to a domain on tau indistinguishable from its microtubule-binding domain (Sontag and colleagues, 1999); crystal structure with a central acidic substrate groove and reconstituted demonstration that B α markedly facilitates dephosphorylation

of phospho-tau (Xu and colleagues, 2008); loss of the interaction with the core dimer by nuclear magnetic resonance (Landrieu and colleagues, 2011).

Established (human) — the endogenous inhibitor is transcriptionally increased, translocated from nucleus to cytoplasm, and cleaved to a twenty-kilodalton fragment in Alzheimer cortex, with no change in cerebellum. More than a sixfold change in the ratio of immunonegative to immunopositive nuclei (Tanimukai and colleagues, 2005).

Established (human, with genetic and pharmacological confirmation in model systems) — asparaginyl endopeptidase is activated and mislocalised in Alzheimer brain, and it cleaves the inhibitor at asparagine 175. Active enzyme up, zymogen down, fragment up; cytosolic fraction most active; mutation of the asparagine abolishes cleavage; immunodepletion abolishes it (Basurto-Islas and colleagues, 2013). Site mapped by mass spectrometry and mutation, protease identified by fractionation, specific inhibitor peptide, catalytically dead mutants and null mice (Liu and colleagues, 2008).

Established (human, with disease-specificity control) — calpain is activated in Alzheimer brain, approximately threefold in prefrontal cortex, in regions of mild pathology without overt neuronal loss, and not in Huntington disease (Saito and colleagues, 1993). *Caveat: post-mortem calcium-dependent proteolysis is a demonstrated confound in this class of measurement (Qian and colleagues, 2011; Saito and colleagues, 2016).*

Established (human, in situ) — neuronal oxidative damage is present, is greatest early, and decreases as amyloid and tangles accumulate; tangle-bearing neurons show forty to fifty-six per cent less oxidised guanosine than tangle-free neurons (Nunomura and colleagues, 1999; Nunomura and colleagues, 2001). Lipid peroxidation is already maximal at mild cognitive impairment (Markesbery and Lovell, 2005; Williams and colleagues, 2006).

Strong (cells and mice, gain and loss of function in both directions) — the one-carbon afferent controls holoenzyme assembly and tau phosphorylation. Folate deprivation, S-adenosylhomocysteine, methylesterase overexpression and methylenetetrahydrofolate reductase deficiency each reduce methyltransferase, methylated catalytic subunit and $\beta\alpha$ and raise tau phosphorylation; methyltransferase or $\beta\alpha$ overexpression rescues, and knockdown of either accelerates (Sontag and colleagues, 2007; Sontag and colleagues, 2008; Sontag and colleagues, 2014). Behaviourally, methyltransferase overexpression protects and methylesterase

overexpression worsens amyloid- β -induced cognitive and plasticity deficits, with the loss-of-function mirror holding (Nicholls and colleagues, 2016; Staniszewski and colleagues, 2020).

Strong (cells, validated phospho-specific reagents) — Fyn phosphorylates the catalytic subunit at tyrosine 284, and that phosphorylation evicts the B α subunit and raises tau phosphorylation at serine 202. Demonstrated with reciprocally cross-depleted phospho-antibodies, phenylalanine mutants, and B α co-immunoprecipitation in neuroblastoma (Sontag and colleagues, 2022). *Limitations stated by the authors: catalytic inhibition not demonstrated; endogenous stoichiometry unknown; phospho-pool undetectable without enrichment.*

Strong (cells and rat, dose-realistic) — cytoplasmic inhibitor fragments are sufficient to produce the disease phenotype. Both fragments bind the catalytic subunit and inhibit; relocation rather than abundance is the requirement (Arnaud and colleagues, 2011; Chohan and colleagues, 2006). Neonatal delivery of the fragments to rat brain produces phosphatase inhibition, tau hyperphosphorylation and aggregation, sarkosyl-insoluble tau, intraneuronal amyloid- β , synaptic loss and memory impairment, at approximately a twenty per cent increase in expression (Wang and colleagues, 2010; Bolognin and colleagues, 2012).

Strong (biochemical, brain tissue) — the phosphatase is directly and reversibly inhibited by oxidation of a vicinal thiol pair on the catalytic subunit. Thiol-reactive agents and phenylarsine oxide produce dithiothreitol-reversible inhibition with up to sevenfold recovery of specific activity on reduction, and a fraction of catalytic subunit carries disulfides after anti-artefact homogenisation (Foley and Kintner, 2005; Foley and colleagues, 2007; Foley and colleagues, 2011). *Caveat: the cysteine residues underlying the reversible, disulfide-based inhibition are identified operationally rather than by number; residue-level assignment exists only for irreversible electrophile adducts (Codreanu and colleagues, 2006).*

Strong (human protein, two laboratories) — Fyn is redistributed rather than globally increased in Alzheimer brain: insoluble up, soluble down, soluble correlating with cognition and inversely with tangles, immunoreactivity moving from synapses to cell bodies and tangles (Ho and colleagues, 2005).

Strong (cells and mice) — the kinase and the phosphatase interact through the substrate and through the membrane. They compete for the RTPPKSP

motif on tau and MAP2, with the kinase excluding the holoenzyme, and the threonine-231-phosphorylated peptide failing to compete (Sontag and colleagues, 2012). Methylated holoenzymes co-purify with the kinase in rafts, and methylation status controls the kinase's distribution, neuritogenesis and amyloid precursor protein processing (Taleski and colleagues, 2021), with the axis manipulable by diet in vivo (van Hummel and colleagues, 2023).

Moderate (cells and rodent, not human) — oxidative stress reaches calpain through redox modulation of calcium release. Hydrogen peroxide and amyloid- β oligomers glutathionylate the ryanodine receptor and produce store-derived calcium signals abolished by receptor inhibition or by thiol reduction (Kemmerling and colleagues, 2007; Paula-Lima and colleagues, 2011); amyloid- β reduces the sodium/potassium pump before calcium homeostasis fails, reproducibly in human synaptosomes (Mark and colleagues, 1995); calcium overload is plaque-proximal in vivo (Kuchibhotla and colleagues, 2008).

Moderate (human, abstract-level, unverified detail) — calpain reaches the phosphatase system through the $\alpha 4$ chaperone, and cleaved $\alpha 4$ is increased in Alzheimer disease. MID1-dependent monoubiquitination triggers calpain cleavage of $\alpha 4$, switching it from protective to destructive toward the catalytic subunit and raising tau phosphorylation (Watkins and colleagues, 2012). *We were unable to retrieve the full text; residues, fragment sizes, isoform and cohort size are unverified. Settling step: obtain and check the primary figures before any quantity is quoted.*

Moderate (structural and model-system, restated) — the protease reaches the cytosol in an activated, neutral-pH-stable form rather than the cytosol becoming acidic. Activation releases the activation peptide and confers a carboxypeptidase activity stable at neutral pH (Dall and Brandstetter, 2013; Dall and Brandstetter, 2016); lysosomal membrane permeabilisation releases hydrolases into the cytosol before amyloid deposition in five mouse models and in human tissue (Lee and colleagues, 2022). *This replaces the unsupported version of the claim; see the rejection below.*

Moderate (human tissue, confounded) — bulk post-mortem brain pH is lower in Alzheimer disease. Standardised effect -0.57 across twenty datasets, correlating negatively with Braak stage (Hagihara and Miyakawa, 2024); 6.11 against 5.88 in a three-hundred-and-sixty-eight-donor bank with a dose-response against neuropathological score (Wang and colleagues, 2025);

6.4 against 6.5 in an earlier series (Preece and Cairns, 2003). *Confounds: agonal state, which is rarely recorded and cannot be adjusted for; a large age gap between arms in the largest cohort; and the fact that homogenate pH is not a measurement of living cytosol.*

Moderate (human, in vivo, compartment mismatched) — cerebral pH is modestly lower in living Alzheimer patients. Proton spectroscopy of histidine in one hundred and forty-seven subjects found white-matter pH low in Alzheimer disease and less so in mild cognitive impairment, unchanged in hippocampus, and not low in frontotemporal dementia spectrum disorders or pure vascular dementia (Lyros and colleagues, 2020). *This runs in favour of the acidification arm and carries a disease-specificity control; but the change is in white matter rather than in the vulnerable grey matter, and the magnitude is a fraction of a pH unit against the one and a half units the enzyme's endopeptidase optimum would require. The phosphorus spectroscopy literature, which is much smaller, points the other way (Mandal and colleagues, 2012; Mecheri and colleagues, 1997).*

Weak (inferred, not demonstrated) — oxidative stress raises expression of the phosphatase's inhibitor. A functional nuclear-factor- κ B element exists in the promoter and the transcription factor upregulates one isoform, but no oxidative stress was applied in the study that established it (Feng and colleagues, 2017). The one direct oxidant experiment showing cytoplasmic mislocalisation of the inhibitor is a non-neuronal overexpression system (Leopoldino and colleagues, 2012).

Predicted, untested — phosphorylation of the catalytic subunit at tyrosine 284 occurs in Alzheimer brain and contributes to the holoenzyme deficit. The chemistry, the kinase, the consequence and the tau readout are demonstrated in cell systems with validated reagents (Sontag and colleagues, 2022); the measurement has never been made in disease tissue, in an animal model, or in a neuron exposed to amyloid- β . *Settling experiment: stated in Section XIV, first entry.*

Predicted, untested — the redox component of the phosphatase deficit in Alzheimer brain has been systematically reversed by the reducing agents in the homogenisation buffer. The thiol mechanism is established in brain tissue (Foley and colleagues, 2007) and every human activity measurement we located was made in reducing buffer. No experiment has compared Alzheimer and control phosphatase activity with and without reductant. *Settling experiment: Section XIV, second entry.*

Predicted, untested — the inhibitor acts on a different pool of the enzyme from the other three locks. The inhibitor pulls down the catalytic subunit and neither the scaffold nor a B subunit (Arnaud and colleagues, 2011), but whether it binds a pool that has already lost its regulatory subunit or evicts that subunit in the act of binding has not been tested — nor, in the published text, is the B isoform assayed in that pull-down specified. *Settling experiment: Section XIV, third entry.*

Predicted, untested — this mechanism is differentially expressed or differentially regulated across neuronal subtypes. No human study resolves any of the six proteins below the level of region, of neurons against glia, or of tangle-bearing against tangle-free cells. *Settling experiment: Section XIV, fourth entry, on existing public data.*

Rejected as stated — the phosphatase is inactivated in Alzheimer disease by phosphorylation of its catalytic subunit at tyrosine 307. The site was assigned by inference from a carboxy-terminal truncation rather than by sequencing (Chen, Martin and Brautigan, 1992); the reported inactivation was measured with a thiophosphate analogue; the phosphomimetic mutants used to confirm it destroy B-subunit binding as a charge effect regardless of phosphorylation (Ogris, Gibson and Pallas, 1997; Wei and colleagues, 2001); the site has never been detected by discovery-mode mass spectrometry in any cell or tissue; and the commercial antibodies on which the entire disease literature rests bind unphosphorylated catalytic subunit with equal affinity and are eighty to ninety per cent blocked by methylation at leucine 309 (Frohner and colleagues, 2020a; Mazhar and colleagues, 2020). Withdrawn. An apparent rise in that antibody signal in Alzheimer tissue should be read as a fall in methylation.

Withdrawn — immunoprecipitation-based "PP2A activity" measurements using carboxy-terminus-directed monoclonal antibodies. Those reagents preferentially recognise the non-methylated catalytic subunit, fail to bring down trimeric holoenzymes containing B or B $\square$ subunits, and in some cases recognise phosphatase 4 (Frohner and colleagues, 2020b). Any claim in this corpus resting on such an assay should be restated.

Rejected — calpain cleaves the phosphatase's endogenous inhibitor. No primary study in any system reports it. The residual cleavage in protease-null animals is caspase-dependent (Liu and colleagues, 2008); the two proteases are separable on a shared substrate (Zhang and colleagues, 2014); the human

fragment survives calpain-suppressive homogenisation (Tanimukai and colleagues, 2005); and the identified proteases of this protein cut at aspartate 18 and lysine 176, neither of them calpain (Morita and colleagues, 2000; Beresford and colleagues, 2001).

Rejected — oxidative stress activates asparaginyl endopeptidase. Oxidation at cysteine 219 — a residue outside the histidine 148–cysteine 189 catalytic dyad — dysregulates the enzyme's processing and decreases its activity (Kovalyova and colleagues, 2022). The demonstrated activator in neurons is acidification (Basurto-Islas and colleagues, 2013).

Rejected — the aldehyde products of lipid peroxidation activate calpain. 4-Hydroxynonenal adducts both calpain isoforms and lowers their activity, slowing autolysis at the highest concentrations tested (Zhai and colleagues, 2023; Johnson and colleagues, 2024). The route from lipid peroxidation to calpain is through calcium, not through adduction.

Rejected — calpain cleaves the phosphatase catalytic subunit or scaffold subunit. Directly tested and negative; the catalytic subunit, the scaffold and representatives of the B55 and B56 families were calpain-resistant, while the B□ family was cleaved (Janssens and colleagues, 2009). *A conflicting report exists for B56α and B56γ (Bertoli and colleagues, 2009) and is recorded rather than resolved.*

Rejected as the unit of analysis — the pyramidal neuron. The first abnormal tau is in the non-pyramidal locus coeruleus (Braak and colleagues, 2011); the vulnerable entorhinal population is molecularly rather than morphologically defined and contains both pyramidal and multipolar cells, with its investigators stating that morphology alone is insufficient (Leng and colleagues, 2021); layer 5 contains excitatory subtypes at 10.6, 5.2 and 3.6 per cent tangle burden in the same cortex, and chandelier interneurons at 6.8 per cent (Otero-Garcia and colleagues, 2022).

Rejected — excitatory neurons are vulnerable because they express more tau. Directly tested by single-molecule fluorescence in situ hybridisation in human tissue: no significant difference in *MAPT* transcript between excitatory and inhibitory neurons, with vulnerability tracking the tau homeostasis environment instead (Fu and colleagues, 2019).

Rejected — high bioenergetic demand explains which neurons tangle. In the best-powered census of tangle-bearing human neurons, oxidative

phosphorylation and glucose metabolism enrichment was cell-type dependent and concentrated in a single cluster rather than shared across tangle-bearing subtypes (Otero-Garcia and colleagues, 2022).

Reported as null — genetic association of the phosphatase system with Alzheimer risk. We searched for and did not find any published association of *PPP2R2A*, *PPP2CA*, *LCMT1* or *PPME1* with disease risk. The one signal in the family is *PPP2CB* rs117296832, conditional on apolipoprotein E ϵ 2 carriage, sub-genome-wide significant, with no replication cohort and stem-cell analyses uncorrected for multiple testing (Jun and colleagues, 2022).

Summary of grades

Claim	Tier	Basis
PP2A does ~71% of tau erasing; ~30% lost in AD brain	Established	Human, quantitative
AB α C holoenzyme reduced in AD neurons, not cerebellum, tracks tangles	Established	Human, IHC + blot
LCMT-1 and methylated C reduced in affected regions	Established	Human
Leu309 methylation governs B55 recruitment; tail is a docking element	Established	Structure + biochemistry
B α is what makes the enzyme a tau phosphatase	Established	Structure + reconstitution
Inhibitor up, translocated, cleaved to 20 kDa in AD	Established	Human
AEP activated and mislocalised in AD; cleaves inhibitor at Asn175	Established	Human + genetic
Calpain activated ~3-fold in AD prefrontal cortex	Established	Human, with HD control
Oxidative damage present, greatest early, falls with pathology	Established	Human, in situ
One-carbon afferent controls holoenzyme assembly and tau	Strong	Cells + mice, both directions

Claim	Tier	Basis
Fyn phosphorylates Tyr284; pTyr284 evicts B α ; raises pSer202 tau	Strong	Cells, validated reagents
Cytoplasmic inhibitor fragments sufficient for the phenotype	Strong	Cells + rat, dose-realistic
PP2A reversibly inhibited by vicinal thiol oxidation	Strong	Rat brain tissue
Fyn redistributed, not globally increased, in AD	Strong	Human protein
Fyn and PP2A/B α compete for the same motif on tau	Strong	Cells + peptide competition
Oxidative stress reaches calpain via RyR redox and calcium	Moderate	Cells + rodent, not human
Calpain reaches PP2A via α 4; cleaved α 4 up in AD	Moderate	Human, detail unverified
AEP reaches cytosol activated and neutral-pH-stable	Moderate	Structure + model systems
Post-mortem brain and CSF pH lower in AD	Moderate	Human, agonally confounded
In vivo cerebral pH modestly lower in AD	Moderate	Human, but wrong compartment and magnitude
Oxidative stress raises inhibitor expression	Weak	Promoter element only
pTyr284 occurs in AD brain	Predicted, untested	Never measured
Redox component of the AD deficit has been assayed away	Predicted, untested	Never tested
The inhibitor acts on a different enzyme pool	Predicted, untested	Pull-down only
The mechanism is cell-type differentiated	Predicted, untested	No subtype data exists
PP2A inactivated at Tyr307 in AD	Withdrawn	Site never demonstrated; antibodies non-specific
IP-based PP2A activity assays with C-terminal antibodies	Withdrawn	Reagents exclude B subunits

Claim	Tier	Basis
Calpain cleaves SET/I2PP2A	Rejected	No primary evidence in any system
Oxidative stress activates AEP	Rejected	Cys oxidation inhibits it
Lipid aldehydes activate calpain	Rejected	HNE adducts inhibit calpain
Calpain cleaves PP2Ac or the A subunit	Rejected	Directly tested, negative
The pyramidal neuron is the unit of vulnerability	Rejected	Morphology insufficient; LC first
Excitatory neurons vulnerable via higher tau expression	Rejected	MAPT transcript equal
Bioenergetic demand explains tangle susceptibility	Rejected	Enrichment not shared
PPP2R2A / PPP2CA / LCMT1 / PPME1 associated with AD risk	Null	Searched; none published

XIV. Predictions and Falsification

A mechanism earns its place by naming what would destroy it. The predictions below are ordered from cheapest to most demanding, and each ends with the result that would falsify the claim it tests. The first four are the volume's principal experimental proposals; the remainder test joints that are currently graded Moderate or below.

1. The tyrosine that replaced tyrosine 307 is phosphorylated in Alzheimer brain. Using the cross-depleted phospho-tyrosine-284 antibody rather than any commercial carboxy-terminus reagent, and using catalytic subunit immunoprecipitation followed by phospho-blot in Alzheimer and age-matched control cortex, with cerebellum as the internal negative region: phosphorylation at tyrosine 284 will be higher in Alzheimer frontal and temporal cortex, will be absent or unchanged in cerebellum, and will correlate inversely with recoverable AB α C holoenzyme in the same homogenate. *Falsified if tyrosine 284 phosphorylation is unchanged or lower in affected cortex, or if it fails to track holoenzyme loss.* This is the cheapest high-value experiment in the volume and the reagent exists.

2. The phosphatase deficit in Alzheimer brain is larger than reported, because part of it is a disulfide. Assay tau-directed phosphatase activity in paired aliquots of the same Alzheimer and control homogenates, one prepared with and one without reducing agent, with the non-reduced arm assayed before and after dithiothreitol addition. The difference between Alzheimer and control will be *larger* in the non-reduced condition, and dithiothreitol will restore proportionally more activity in Alzheimer than in control extract. *Falsified if reductant restores equal proportions in both, which would show the redox pool is not differentially oxidised in disease.* The prediction follows directly from the demonstration that a fraction of catalytic subunit carries disulfides after stringent homogenisation (Foley and colleagues, 2011) and from the fact that every published human measurement used reducing buffer.

3. The inhibitor and the three assembly locks act on different pools of the enzyme. Immunoprecipitate the catalytic subunit from neurons expressing the inhibitor or its fragments, and quantify co-precipitating $\beta\alpha$ and scaffold subunit against inhibitor occupancy; and reciprocally, test whether the inhibitor binds a methylation-incompetent catalytic subunit, which cannot carry $\beta\alpha$, more readily than wild type. If the inhibitor prefers the $\beta\alpha$ -free pool, the two lesions are additive and the locks do not converge on a target after all. If inhibitor binding itself displaces $\beta\alpha$, they converge. *Falsified in either direction by a clear result; the claim being tested is that the question is currently open, and any decisive answer closes it.*

4. The mechanism is cell-type differentiated, and the data to show it already exist. Interrogate the published entorhinal and frontal single-nucleus dataset (Leng and colleagues, 2021) and the tangle-bearing single-soma dataset (Otero-Garcia and colleagues, 2022) for *PPP2R2A*, *PPP2CA*, *LCMT1*, *PPME1*, *SET*, *LGMN*, *CAPN1*, *CAPN2* and *FYN*, comparing the vulnerable RORB-positive excitatory subpopulations against the resistant subpopulations in the same region, and tangle-bearing against tangle-free somas of the same subtype. The prediction is that *LCMT1* is lower and *SET* higher in vulnerable subtypes before stage-related loss, and that *LGMN* transcript is not neuronal in either dataset — the last being the result that would force the protease's neuronal role to rest entirely on protein-level evidence. *Falsified if no transcript in the set distinguishes vulnerable from resistant subtypes, which would locate the entire mechanism downstream of whatever determines vulnerability.*

5. The methylation lock is upstream of the tyrosine lock, not parallel to it. In neurons, reducing methylation should *reduce* rather than increase

phosphorylation of the catalytic subunit at tyrosine 284, because the methylation-incompetent subunit is a poorer kinase substrate (Sontag and colleagues, 2022). In Alzheimer tissue, therefore, the two lesions should be negatively rather than positively correlated across cases. *Falsified if demethylation and tyrosine 284 phosphorylation rise together in the same tissue, which would mean the two locks are driven by a shared upstream cause the present analysis has missed.*

6. The protease reaches the cytosol by membrane permeabilisation, not by cytosolic acidification. In neurons from an Alzheimer model, cytosolic pH measured with a ratiometric reporter will not fall to the enzyme's endopeptidase optimum at any stage, while cytosolic appearance of lysosomal hydrolases will precede inhibitor cleavage; and inhibitor cleavage will be reduced by stabilising lysosomal membranes without correcting cytosolic pH. *Falsified if cytosolic pH in such neurons is found below about 6.0 for a sustained period, which would restore the simpler acidification account.*

7. Calpain enters the phosphatase system through $\alpha 4$ and not through the inhibitor. In neurons, calpain inhibition will reduce cleaved $\alpha 4$ and raise recoverable phosphatase activity without altering the twenty-kilodalton inhibitor fragment, while inhibition of asparaginyl endopeptidase will do the reverse. *Falsified if calpain inhibition reduces the inhibitor fragment, which would re-open the claim rejected in Section IX.*

8. Interventions on the four afferents are additive rather than redundant. In a model carrying both the amyloid afferent and a one-carbon deficit, correcting folate status and inhibiting the kinase will produce a larger restoration of holoenzyme and a larger reduction in tau phosphorylation together than either alone, with no ceiling effect. *Falsified by redundancy — if either intervention alone normalises the holoenzyme, the locks share an upstream node and the coincidence-structure model of Section XII is wrong.*

9. The holoenzyme deficit precedes the tangle in the same cell. Quantify AB α C immunoreactivity in neurons staged by conformational tau epitopes rather than by mature tangle, in human tissue at Braak stages 2 to 4. The deficit should be present in pre-tangle neurons bearing conformational but not fibrillar tau. *Falsified if the deficit appears only in cells already bearing fibrillar tau, which would make it a consequence and would remove the phosphatase from the causal chain while leaving it as a marker.*

10. The inhibitor's two fragments have measurable and different potencies. No inhibition constant exists for either fragment. Purified recombinant fragments assayed against the reconstituted AB α C holoenzyme and against the free catalytic subunit will give distinct constants, and the difference between the two enzyme forms will be larger than the difference between the two fragments. *Falsified if both fragments inhibit the holoenzyme and the free catalytic subunit equally, which would remove the basis for prediction 3.*

XV. Therapeutic Corollaries — Which Lock Is Worth Picking

A mechanism with four locks and no shared key does not offer a single target, and the volume should not pretend otherwise. What it offers instead is a ranking, and the ranking follows from two properties of each lock: whether it is reversible, and whether its afferent is reachable.

The methylation lock is the only one with a demonstrated, cheap, systemic afferent. Its equilibrium is set by an enzyme pair and by methyl donor supply, and the supply side has been manipulated successfully in vivo: a diet supplemented with L-methylfolate, choline and betaine reduced tau phosphorylation at two pathological epitopes, alleviated motor and learning deficits, raised phosphatase methylation and reduced Fyn protein in tau transgenic mice, while folate-metabolism deficiency did the reverse (van Hummel and colleagues, 2023). The transgenic arms point the same way (Nicholls and colleagues, 2016; Staniszewski and colleagues, 2020). This is the cheapest intervention in the mechanism and the one with the clearest human biomarker — one-carbon metabolites are routinely measurable — and it is also the one whose effect size in the mouse is partial rather than complete, which is what the independence argument of Section XII predicts. The honest framing is that this is a modifier with a plausible population-level effect, not a treatment.

The proteolytic lock is the only irreversible one, which makes it the highest-value target and the hardest. A cell can remethylate a catalytic subunit, dephosphorylate a tyrosine and reduce a disulfide; it cannot re-ligate a cleaved protein. Proteolysis at asparagine 175 converts a regulated nuclear protein into two constitutive cytoplasmic inhibitors, and the same protease independently generates an aggregation-prone tau fragment and primes the amyloid precursor for β -secretase. An orally bioavailable, brain-permeable inhibitor with a co-crystal structure showing dual active-site-directed and

allosteric binding has been developed and shown, on chronic dosing in tau and amyloid models, to reduce both substrates' cleavage, ameliorate synapse loss, augment long-term potentiation and protect memory (Zhang and colleagues, 2017). Cutting above the cut — at the acidification and lysosomal permeabilisation that activate and release the enzyme — is the alternative, and the autophagy-lysosomal literature supplies the targets (Lee and colleagues, 2022).

The tyrosine lock has had its clinical test and failed it — but not at the right residue. Saracatinib achieved central nervous system exposure and target plasma levels, and missed its primary metabolic endpoint and every secondary clinical endpoint in a hundred-and-fifty-nine-patient trial (van Dyck and colleagues, 2019). That is a real negative and it should temper enthusiasm for the kinase. But it is worth recording precisely what it does and does not exclude. The trial tested broad Src-family inhibition in mild Alzheimer dementia against a glucose-metabolism endpoint; it did not test whether phosphorylation at tyrosine 284 occurs in the disease, whether it contributes to the holoenzyme deficit, or whether it is present at earlier stages. Until prediction 1 is performed, the residue has never been measured in a patient, and the trial cannot be read as a test of a mechanism nobody had yet described.

The redox arm is a target that has been hidden by the assay. If a meaningful fraction of the deficit is a reversible disulfide, then agents that maintain the neuronal thiol pool are acting on the phosphatase directly and not only on generic oxidative damage, and the effect would be invisible to every assay used to look for it. The experiment that would establish this is prediction 2, and it costs a freezer aliquot and an afternoon.

One thing this mechanism argues against. Broad, non-selective inhibition of the phosphatase family — or, in the therapeutic direction, broad activation of it — is the wrong instrument, because the family contains enzymes with opposing roles and the compounds routinely described as selective are not. The inhibitors used throughout this literature hit multiple members of the same phosphatase family, and they have no selectivity whatever among the family's own isoforms. The same caution applies to calpain: the two isoforms have opposing roles, with calpain-1 activation required for long-term potentiation induction and broadly protective and calpain-2 activation limiting potentiation and degenerative (Baudry and Bi, 2016), which makes

non-selective calpain inhibition mechanistically the wrong tool even where the calpain rationale holds.

What a rational programme would look like. Measure the four locks in the same tissue — the experiment nobody has done — and determine their relative contributions before choosing among them. The reagents for three of the four now exist and are validated; the fourth, the redox arm, requires only that one aliquot be prepared without reductant. Until that measurement exists, the field is choosing targets from a mechanism assembled out of four separate literatures, in which one lock was measured with an antibody that reports something else, one protease was assigned a substrate it has never been shown to cleave, and the cell type in which all of it is supposed to happen has never been specified below the level of a cortical region.

XVI. Coda — The Methyl Group and the Antibody

There is a version of this mechanism that is easy to write and that would have been wrong. It begins with oxidative stress in a pyramidal neuron, proceeds to calcium and calpain, joins asparaginyl endopeptidase, cleaves SET, releases the inhibitor into the cytoplasm, adds a phosphotyrosine at residue 307 and a lost methyl group at residue 309, and arrives at a silenced phosphatase and a hyperphosphorylated tau. Every arrow in that sentence has been drawn in the literature. Roughly half of them cannot be supported.

What is left when the unsupported arrows are removed is not less interesting. It is an enzyme with an unusual number of ways to be switched off, sitting in a cell subject to an unusual number of simultaneous insults, in a disease that supplies each of them by a different route. That is a coincidence structure rather than a cascade, and it predicts different things: that no single intervention will normalise the deficit, that the interventions should add rather than substitute, and that the relative contributions are an empirical question nobody has yet asked in one piece of tissue.

Two of this volume's findings deserve to be held together, because they are the same finding seen twice. The first is that a modification which was never demonstrated — a phosphotyrosine at residue 307 — became the standard account of how this enzyme is inactivated in this disease, sustained for twenty-eight years by antibodies that were reporting, all along, the presence or absence of a methyl group two residues away. The second is that the

methyl group itself, which is what those antibodies were actually measuring, turns out to be the best-supported lock in the mechanism. The field was looking at the right molecule, at the right residue's neighbourhood, in the right tissue, and reading the signal backwards. The correction did not overturn the conclusion that the phosphatase is compromised in Alzheimer disease. It relocated the reason by two amino acids and changed the upstream cause from a kinase to a metabolite.

The third finding is quieter and may matter more. A reducing agent in a homogenisation buffer is not a decision anybody makes; it is a line in a protocol inherited from the laboratory that trained you. If a portion of this enzyme's inactivation in the living brain is a disulfide, then the field has been adding, to every sample it has ever measured, the exact reagent that undoes the lesion it is looking for. That is not a failure of reasoning. It is what happens when a method outlives the question it was designed for, and it is the kind of thing that is only ever noticed by assembling literatures that do not cite each other — the redox biochemistry of a phosphatase in rat brain, and the activity assay of the same phosphatase in human Alzheimer cortex — and asking whether the second could see what the first describes.

The note that prompted this volume was right about the cast and wrong about the plot. That is the normal condition of a list written before the evidence is assembled, and it is why the list is worth writing and why the assembly is worth doing.

References

1. Liu F, Grundke-Iqbal I, Iqbal K, Gong CX. (2005a) Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. *Eur J Neurosci.* 2005;22(8):1942–1950. DOI: 10.1111/j.1460-9568.2005.04391.x
2. Liu F, Iqbal K, Grundke-Iqbal I, Rossie S, Gong CX. (2005b) Dephosphorylation of tau by protein phosphatase 5: impairment in Alzheimer's disease. *J Biol Chem.* 2005;280(3):1790–1796. DOI: 10.1074/jbc.M410775200
3. Gong CX, Singh TJ, Grundke-Iqbal I, Iqbal K. Phosphoprotein phosphatase activities in Alzheimer disease brain. *J Neurochem.* 1993;61(3):921–927. DOI: 10.1111/j.1471-4159.1993.tb03603.x
4. Gong CX, Shaikh S, Wang JZ, Zaidi T, Grundke-Iqbal I, Iqbal K. Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. *J Neurochem.* 1995;65(2):732–738. DOI: 10.1046/j.1471-4159.1995.65020732.x
5. Sontag E, Luangpirom A, Hladik C, Mudrak I, Ogris E, Speciale S, White CL 3rd. (2004a) Altered expression levels of the protein phosphatase 2A AB α C enzyme are associated with Alzheimer disease pathology. *J Neuropathol Exp Neurol.* 2004;63(4):287–301. DOI: 10.1093/jnen/63.4.287
6. Sontag E, Hladik C, Montgomery L, Luangpirom A, Mudrak I, Ogris E, White CL 3rd. (2004b) Downregulation of protein phosphatase 2A carboxyl methylation and methyltransferase may contribute to Alzheimer disease pathogenesis. *J Neuropathol Exp Neurol.* 2004;63(10):1080–1091. DOI: 10.1093/jnen/63.10.1080
7. Sontag JM, Sontag E. Protein phosphatase 2A dysfunction in Alzheimer's disease. *Front Mol Neurosci.* 2014;7:16. DOI: 10.3389/fnmol.2014.00016
8. Sontag E, Nunbhakdi-Craig V, Lee G, Bloom GS, Mumby MC. Regulation of the phosphorylation state and microtubule-binding activity of tau by protein phosphatase 2A. *Neuron.* 1996;17(6):1201–1207. DOI: 10.1016/s0896-6273(00)80250-0

9. Sontag E, Nunbhakdi-Craig V, Lee G, Brandt R, Kamibayashi C, Kuret J, White CL 3rd, Mumby MC, Bloom GS. Molecular interactions among protein phosphatase 2A, tau, and microtubules. *J Biol Chem.* 1999;274(36):25490–25498. DOI: 10.1074/jbc.274.36.25490
10. Xu Y, Chen Y, Zhang P, Jeffrey PD, Shi Y. Structure of a protein phosphatase 2A holoenzyme: insights into B55-mediated tau dephosphorylation. *Mol Cell.* 2008;31(6):873–885. DOI: 10.1016/j.molcel.2008.08.006
11. Sontag JM, Nunbhakdi-Craig V, White CL 3rd, Halpain S, Sontag E. The protein phosphatase PP2A/B α binds to the microtubule-associated proteins tau and MAP2 at a motif also recognized by the kinase Fyn. *J Biol Chem.* 2012;287(18):14984–14993. DOI: 10.1074/jbc.M111.338681
12. Landrieu I, Smet-Nocca C, Amniai L, Louis JV, Wieruszeski JM, Goris J, Janssens V, Lippens G. Molecular implication of PP2A and Pin1 in the Alzheimer's disease specific hyperphosphorylation of tau. *PLoS One.* 2011;6(6):e21521. DOI: 10.1371/journal.pone.0021521
13. Vogelsberg-Ragaglia V, Schuck T, Trojanowski JQ, Lee VM. PP2A mRNA expression is quantitatively decreased in Alzheimer's disease hippocampus. *Exp Neurol.* 2001;168(2):402–412. DOI: 10.1006/exnr.2001.7630
14. Sontag E, Nunbhakdi-Craig V, Sontag JM, Diaz-Arrastia R, Ogris E, Dayal S, Lentz SR, Arning E, Bottiglieri T. Protein phosphatase 2A methyltransferase links homocysteine metabolism with tau and amyloid precursor protein regulation. *J Neurosci.* 2007;27(11):2751–2759. DOI: 10.1523/JNEUROSCI.3316-06.2007
15. Sontag JM, Nunbhakdi-Craig V, Montgomery L, Arning E, Bottiglieri T, Sontag E. Folate deficiency induces in vitro and mouse brain region-specific downregulation of leucine carboxyl methyltransferase-1 and protein phosphatase 2A B(α) subunit expression that correlate with enhanced tau phosphorylation. *J Neurosci.* 2008;28(45):11477–11487. DOI: 10.1523/JNEUROSCI.2816-08.2008
16. Sontag JM, Wasek B, Taleski G, Smith J, Arning E, Sontag E, Bottiglieri T. Altered protein phosphatase 2A methylation and tau phosphorylation in the young and aged brain of methylenetetrahydrofolate reductase

- (MTHFR) deficient mice. *Front Aging Neurosci.* 2014;6:214. DOI: 10.3389/fnagi.2014.00214
17. Qian W, Shi J, Yin X, Iqbal K, Grundke-Iqbal I, Gong CX, Liu F. PP2A regulates tau phosphorylation directly and also indirectly via activating GSK-3 β . *J Alzheimers Dis.* 2010;19(4):1221–1229. DOI: 10.3233/JAD-2010-1317
18. Kins S, Cramer A, Evans DR, Hemmings BA, Nitsch RM, Götz J. Reduced protein phosphatase 2A activity induces hyperphosphorylation and altered compartmentalization of tau in transgenic mice. *J Biol Chem.* 2001;276(41):38193–38200. DOI: 10.1074/jbc.M102621200
19. Kins S, Kurosinski P, Nitsch RM, Götz J. Activation of the ERK and JNK signaling pathways caused by neuron-specific inhibition of PP2A in transgenic mice. *Am J Pathol.* 2003;163(3):833–843. DOI: 10.1016/S0002-9440(10)63444-X
20. Deters N, Ittner LM, Götz J. Substrate-specific reduction of PP2A activity exaggerates tau pathology. *Biochem Biophys Res Commun.* 2009;379(2):400–405. DOI: 10.1016/j.bbrc.2008.12.140
21. Louis JV, Martens E, Borghgraef P, Lambrecht C, Sents W, Longin S, Zwaenepoel K, Pijnenborg R, Landrieu I, Lippens G, Ledermann B, Götz J, Van Leuven F, Goris J, Janssens V. Mice lacking phosphatase PP2A subunit PR61/B'6 (Ppp2r5d) develop spatially restricted tauopathy by deregulation of CDK5 and GSK3 β . *Proc Natl Acad Sci U S A.* 2011;108(17):6957–6962. DOI: 10.1073/pnas.1018777108
22. Nicholls RE, Sontag JM, Zhang H, Staniszewski A, Yan S, Kim CY, Yim M, Woodruff CM, Arning E, Wasek B, Yin D, Bottiglieri T, Sontag E, Kandel ER, Arancio O. PP2A methylation controls sensitivity and resistance to β -amyloid-induced cognitive and electrophysiological impairments. *Proc Natl Acad Sci U S A.* 2016;113(12):3347–3352. DOI: 10.1073/pnas.1521018113
23. Staniszewski A, Zhang H, Asam K, Pitstick R, Kavanaugh MP, Arancio O, Nicholls RE. Reduced expression of the PP2A methyltransferase, PME-1, or the PP2A methyltransferase, LCMT-1, alters sensitivity to beta-amyloid-induced cognitive and electrophysiological impairments in mice. *J Neurosci.* 2020;40(24):4596–4608. DOI: 10.1523/JNEUROSCI.2983-19.2020

24. Gnanaprakash M, Staniszewski A, Zhang H, Pitstick R, Kavanagh TJ, Sontag JM, Sontag E, Carlson GA, Arancio O, Nicholls RE. Leucine carboxyl methyltransferase 1 overexpression protects against cognitive and electrophysiological impairments in Tg2576 APP transgenic mice. *J Alzheimers Dis.* 2021;79(4):1813–1829. DOI: 10.3233/JAD-200462
25. Sundaresh SN, Vogel EW, Hue CD, Zhang H, Staniszewski A, Berman HL, Gill Z, Asam K, Liang S, Shen L, Gnanaprakash M, Acquarone E, Masone A, Fà M, Kanaan NM, Morrison B 3rd, Arancio O, Nicholls RE. PP2A methylesterase, PME-1, and PP2A methyltransferase, LCMT-1, control sensitivity to impairments caused by injury-related oligomeric tau. *Alzheimers Dement.* 2025;21(12):e70947. DOI: 10.1002/alz.70947
26. Panicker N, Coutman M, Lawlor-O'Neill C, Kahl RGS, Roselli S, Verrills NM. Ppp2r2a knockout mice reveal that protein phosphatase 2A regulatory subunit B55 α is an essential regulator of neuronal and epidermal embryonic development. *Front Cell Dev Biol.* 2020;8:358. DOI: 10.3389/fcell.2020.00358
27. Jun GR, You Y, Zhu C, Meng G, Chung J, Panitch R, Hu J, Xia W; Alzheimer's Disease Genetics Consortium, Bennett DA, Foroud TM, Wang LS, Haines JL, Mayeux R, Pericak-Vance MA, Schellenberg GD, Au R, Lunetta KL, Ikezu T, Stein TD, Farrer LA. Protein phosphatase 2A and complement component 4 are linked to the protective effect of APOE ϵ 2 for Alzheimer's disease. *Alzheimers Dement.* 2022;18(11):2042–2054. DOI: 10.1002/alz.12607
28. Longin S, Zwaenepoel K, Louis JV, Dilworth S, Goris J, Janssens V. Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic subunit. *J Biol Chem.* 2007;282(37):26971–26980. DOI: 10.1074/jbc.M704059200
29. Xing Y, Li Z, Chen Y, Stock JB, Jeffrey PD, Shi Y. Structural mechanism of demethylation and inactivation of protein phosphatase 2A. *Cell.* 2008;133(1):154–163. DOI: 10.1016/j.cell.2008.02.041
30. Xu Y, Xing Y, Chen Y, Chao Y, Lin Z, Fan E, Yu JW, Strack S, Jeffrey PD, Shi Y. Structure of the protein phosphatase 2A holoenzyme. *Cell.* 2006;127(6):1239–1251. DOI: 10.1016/j.cell.2006.11.033
31. Yu XX, Du X, Moreno CS, Green RE, Ogris E, Feng Q, Chou L, McQuoid MJ, Pallas DC. Methylation of the protein phosphatase 2A catalytic

- subunit is essential for association of B α regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen. *Mol Biol Cell*. 2001;12(1):185–199. DOI: 10.1091/mbc.12.1.185
32. Sontag JM, Nunbhakdi-Craig V, Sontag E. Leucine carboxyl methyltransferase 1 (LCMT1)-dependent methylation regulates the association of protein phosphatase 2A and tau protein with plasma membrane microdomains in neuroblastoma cells. *J Biol Chem*. 2013;288(38):27396–27405. DOI: 10.1074/jbc.M113.490102
33. Sontag JM, Nunbhakdi-Craig V, Mitterhuber M, Ogris E, Sontag E. Regulation of protein phosphatase 2A methylation by LCMT1 and PME-1 plays a critical role in differentiation of neuroblastoma cells. *J Neurochem*. 2010;115(6):1455–1465. DOI: 10.1111/j.1471-4159.2010.07049.x
34. Chen J, Martin BL, Brautigan DL. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science*. 1992;257(5074):1261–1264. DOI: 10.1126/science.1325671
35. Chen J, Parsons S, Brautigan DL. Tyrosine phosphorylation of protein phosphatase 2A in response to growth stimulation and v-src transformation of fibroblasts. *J Biol Chem*. 1994;269(11):7957–7962. PMID: 7510677
36. Ogris E, Gibson DM, Pallas DC. Protein phosphatase 2A subunit assembly: the catalytic subunit carboxy terminus is important for binding cellular B subunit but not polyomavirus middle tumor antigen. *Oncogene*. 1997;15(8):911–917. DOI: 10.1038/sj.onc.1201259
37. Wei H, Ashby DG, Moreno CS, Ogris E, Yeong FM, Corbett AH, Pallas DC. Carboxymethylation of the PP2A catalytic subunit in *Saccharomyces cerevisiae* is required for efficient interaction with the B-type subunits Cdc55p and Rts1p. *J Biol Chem*. 2001;276(2):1570–1577. DOI: 10.1074/jbc.M008694200
38. Frohner IE, Mudrak I, Schüchner S, Anrather D, Hartl M, Sontag JM, Sontag E, Wadzinski BE, Preglej T, Ellmeier W, Ogris E. (2020a) PP2A^C phospho-Tyr³⁰⁷ antibodies are not specific for this modification but are sensitive to other PP2A^C modifications including Leu³⁰⁹ methylation. *Cell Rep*. 2020;30(9):3171–3182.e6. DOI: 10.1016/j.celrep.2020.02.035
39. Mazhar S, Leonard D, Sosa A, Schlatzer D, Thomas D, Narla G. Challenges and reinterpretation of antibody-based research on

- phosphorylation of Tyr³⁰⁷ on PP2Ac. *Cell Rep.* 2020;30(9):3164–3170.e3. DOI: 10.1016/j.celrep.2020.02.012
40. Frohner IE, Mudrak I, Kronlachner S, Schüchner S, Ogris E. (2020b) Antibodies recognizing the C terminus of PP2A catalytic subunit are unsuitable for evaluating PP2A activity and holoenzyme composition. *Sci Signal.* 2020;13(616):eaax6490. DOI: 10.1126/scisignal.aax6490
41. Sontag JM, Schuhmacher D, Taleski G, Jordan A, Khan S, Hoffman A, Gomez RJ, Mazalouskas MD, Hanks SK, Spiller BW, Sontag E, Wadzinski BE. A new paradigm for regulation of protein phosphatase 2A function via Src and Fyn kinase-mediated tyrosine phosphorylation. *J Biol Chem.* 2022;298(8):102248. DOI: 10.1016/j.jbc.2022.102248
42. Lee G, Thangavel R, Sharma VM, Litersky JM, Bhaskar K, Fang SM, Do LH, Andreadis A, Van Hoesen G, Ksiezak-Reding H. Phosphorylation of tau by fyn: implications for Alzheimer's disease. *J Neurosci.* 2004;24(9):2304–2312. DOI: 10.1523/JNEUROSCI.4162-03.2004
43. Bhaskar K, Yen SH, Lee G. Disease-related modifications in tau affect the interaction between Fyn and tau. *J Biol Chem.* 2005;280(42):35119–35125. DOI: 10.1074/jbc.M505895200
44. Ittner LM, Ke YD, Delerue F, Bi M, Gladbach A, van Eersel J, Wölfling H, Chieng BC, Christie MJ, Napier IA, Eckert A, Staufenbiel M, Hardeman E, Götz J. Dendritic function of tau mediates amyloid-beta toxicity in Alzheimer's disease mouse models. *Cell.* 2010;142(3):387–397. DOI: 10.1016/j.cell.2010.06.036
45. Um JW, Nygaard HB, Heiss JK, Kostylev MA, Stagi M, Vortmeyer A, Wisniewski T, Gunther EC, Strittmatter SM. Alzheimer amyloid- β oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nat Neurosci.* 2012;15(9):1227–1235. DOI: 10.1038/nn.3178
46. Um JW, Kaufman AC, Kostylev M, Heiss JK, Stagi M, Takahashi H, Kerrisk ME, Vortmeyer A, Wisniewski T, Koleske AJ, Gunther EC, Nygaard HB, Strittmatter SM. Metabotropic glutamate receptor 5 is a coreceptor for Alzheimer $\alpha\beta$ oligomer bound to cellular prion protein. *Neuron.* 2013;79(5):887–902. DOI: 10.1016/j.neuron.2013.06.036
47. Ho GJ, Hashimoto M, Adame A, Izu M, Alford MF, Thal LJ, Hansen LA, Masliah E. Altered p59Fyn kinase expression accompanies disease progression in Alzheimer's disease: implications for its functional role.

- Neurobiol Aging.* 2005;26(5):625–635. DOI: 10.1016/j.neurobiolaging.2004.06.016
48. Taleski G, Schuhmacher D, Su H, Sontag JM, Sontag E. Disturbances in PP2A methylation and one-carbon metabolism compromise Fyn distribution, neuritogenesis, and APP regulation. *J Biol Chem.* 2021;296:100237. DOI: 10.1074/jbc.RA120.016069
49. van Hummel A, Taleski G, Sontag JM, Feiten AF, Ke YD, Ittner LM, Sontag E. Methyl donor supplementation reduces phospho-Tau, Fyn and demethylated protein phosphatase 2A levels and mitigates learning and motor deficits in a mouse model of tauopathy. *Neuropathol Appl Neurobiol.* 2023;49(4):e12931. DOI: 10.1111/nan.12931
50. Kaufman AC, Salazar SV, Haas LT, Yang J, Kostylev MA, Jeng AT, Robinson SA, Gunther EC, van Dyck CH, Nygaard HB, Strittmatter SM. Fyn inhibition rescues established memory and synapse loss in Alzheimer mice. *Ann Neurol.* 2015;77(6):953–971. DOI: 10.1002/ana.24394
51. Smith LM, Zhu R, Strittmatter SM. Disease-modifying benefit of Fyn blockade persists after washout in mouse Alzheimer's model. *Neuropharmacology.* 2018;130:54–61. DOI: 10.1016/j.neuropharm.2017.11.042
52. Toyonaga T, Smith LM, Finnema SJ, Gallezot JD, Naganawa M, Bini J, Mulnix T, Cai Z, Ropchan J, Huang Y, Strittmatter SM, Carson RE. In vivo synaptic density imaging with ¹¹C-UCB-J detects treatment effects of saracatinib in a mouse model of Alzheimer disease. *J Nucl Med.* 2019;60(12):1780–1786. DOI: 10.2967/jnumed.118.223867
53. Tang SJ, Fesharaki-Zadeh A, Takahashi H, Nies SH, Smith LM, Luo A, Chyung A, Chiasseu M, Strittmatter SM. Fyn kinase inhibition reduces protein aggregation, increases synapse density and improves memory in transgenic and traumatic tauopathy. *Acta Neuropathol Commun.* 2020;8(1):96. DOI: 10.1186/s40478-020-00976-9
54. van Dyck CH, Nygaard HB, Chen K, Donohue MC, Raman R, Rissman RA, Brewer JB, Koeppe RA, Chow TW, Rafii MS, Gessert D, Choi J, Turner RS, Kaye JA, Gale SA, Reiman EM, Aisen PS, Strittmatter SM. Effect of AZD0530 on cerebral metabolic decline in Alzheimer disease: a randomized clinical trial. *JAMA Neurol.* 2019;76(10):1219–1229. DOI: 10.1001/jamaneurol.2019.2050

55. Li M, Guo H, Damuni Z. Purification and characterization of two potent heat-stable protein inhibitors of protein phosphatase 2A from bovine kidney. *Biochemistry*. 1995;34(6):1988–1996. DOI: 10.1021/bi00006a020
56. Li M, Makkinje A, Damuni Z. (1996a) The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem*. 1996;271(19):11059–11062. DOI: 10.1074/jbc.271.19.11059
57. Li M, Makkinje A, Damuni Z. (1996b) Molecular identification of I1PP2A, a novel potent heat-stable inhibitor protein of protein phosphatase 2A. *Biochemistry*. 1996;35(22):6998–7002. DOI: 10.1021/bi960581y
58. Arnaud L, Chen S, Liu F, Li B, Khatoon S, Grundke-Iqbal I, Iqbal K. Mechanism of inhibition of PP2A activity and abnormal hyperphosphorylation of tau by I2^{PP2A}/SET. *FEBS Lett*. 2011;585(17):2653–2659. DOI: 10.1016/j.febslet.2011.07.020
59. Arif M, Wei J, Zhang Q, Liu F, Basurto-Islas G, Grundke-Iqbal I, Iqbal K. Cytoplasmic retention of protein phosphatase 2A inhibitor 2 (I2^{PP2A}) induces Alzheimer-like abnormal hyperphosphorylation of tau. *J Biol Chem*. 2014;289(40):27677–27691. DOI: 10.1074/jbc.M114.565358
60. Seo SB, McNamara P, Heo S, Turner A, Lane WS, Chakravarti D. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell*. 2001;104(1):119–130. DOI: 10.1016/S0092-8674(01)00196-9
61. Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell*. 2003;112(5):659–672. DOI: 10.1016/S0092-8674(03)00150-8
62. Chowdhury D, Beresford PJ, Zhu P, Zhang D, Sung JS, Demple B, Perrino FW, Lieberman J. The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death. *Mol Cell*. 2006;23(1):133–142. DOI: 10.1016/j.molcel.2006.06.005
63. Tanimukai H, Grundke-Iqbal I, Iqbal K. Up-regulation of inhibitors of protein phosphatase-2A in Alzheimer's disease. *Am J Pathol*. 2005;166(6):1761–1771. DOI: 10.1016/S0002-9440(10)62486-8

64. Chohan MO, Khatoon S, Iqbal IG, Iqbal K. Involvement of I2^{PP2A} in the abnormal hyperphosphorylation of tau and its reversal by memantine. *FEBS Lett.* 2006;580(16):3973–3979. DOI: 10.1016/j.febslet.2006.06.021
65. Wang X, Blanchard J, Kohlbrenner E, Clement N, Linden RM, Radu A, Grundke-Iqbal I, Iqbal K. The carboxy-terminal fragment of inhibitor-2 of protein phosphatase-2A induces Alzheimer disease pathology and cognitive impairment. *FASEB J.* 2010;24(11):4420–4432. DOI: 10.1096/fj.10-158477
66. Bolognin S, Blanchard J, Wang X, Basurto-Islas G, Tung YC, Kohlbrenner E, Grundke-Iqbal I, Iqbal K. An experimental rat model of sporadic Alzheimer's disease and rescue of cognitive impairment with a neurotrophic peptide. *Acta Neuropathol.* 2012;123(1):133–151. DOI: 10.1007/s00401-011-0908-x
67. Liu Z, Jang SW, Liu X, Cheng D, Peng J, Yepes M, Li XJ, Matthews S, Watts C, Asano M, Hara-Nishimura I, Luo HR, Ye K. Neuroprotective actions of PIKE-L by inhibition of SET proteolytic degradation by asparagine endopeptidase. *Mol Cell.* 2008;29(6):665–678. DOI: 10.1016/j.molcel.2008.02.017
68. Basurto-Islas G, Grundke-Iqbal I, Tung YC, Liu F, Iqbal K. Activation of asparaginyl endopeptidase leads to tau hyperphosphorylation in Alzheimer disease. *J Biol Chem.* 2013;288(24):17495–17507. DOI: 10.1074/jbc.M112.446070
69. Zhang Z, Song M, Liu X, Kang SS, Kwon IS, Duong DM, Seyfried NT, Hu WT, Liu Z, Wang JZ, Cheng L, Sun YE, Yu SP, Levey AI, Ye K. Cleavage of tau by asparagine endopeptidase mediates the neurofibrillary pathology in Alzheimer's disease. *Nat Med.* 2014;20(11):1254–1262. DOI: 10.1038/nm.3700
70. Zhang Z, Song M, Liu X, Su Kang S, Duong DM, Seyfried NT, Cao X, Cheng L, Sun YE, Ping Yu S, Jia J, Levey AI, Ye K. Delta-secretase cleaves amyloid precursor protein and regulates the pathogenesis in Alzheimer's disease. *Nat Commun.* 2015;6:8762. DOI: 10.1038/ncomms9762
71. Zhang Z, Obianyo O, Dall E, Du Y, Fu H, Liu X, Kang SS, Song M, Yu SP, Cabrele C, Schubert M, Li X, Wang JZ, Brandstetter H, Ye K. Inhibition of delta-secretase improves cognitive functions in mouse models of

- Alzheimer's disease. *Nat Commun.* 2017;8:14740. DOI: 10.1038/ncomms14740
72. Dall E, Brandstetter H. Mechanistic and structural studies on legumain explain its zymogenicity, distinct activation pathways, and regulation. *Proc Natl Acad Sci U S A.* 2013;110(27):10940–10945. DOI: 10.1073/pnas.1300686110
73. Dall E, Brandstetter H. Structure and function of legumain in health and disease. *Biochimie.* 2016;122:126–150. DOI: 10.1016/j.biochi.2015.09.022
74. Elamin T, Santos NP, Briza P, Brandstetter H, Dall E. Structural and functional studies of legumain–mycocylin complexes revealed a competitive, exosite-regulated mode of interaction. *J Biol Chem.* 2022;298(10):102502. DOI: 10.1016/j.jbc.2022.102502
75. Lee JH, Yang DS, Goulbourne CN, Im E, Stavrides P, Pensalfini A, Chan H, Bouchet-Marquis C, Bleiwas C, Berg MJ, Huo C, Peddy J, Pawlik M, Levy E, Rao M, Staufienbiel M, Nixon RA. Faulty autolysosome acidification in Alzheimer's disease mouse models induces autophagic build-up of A β in neurons, yielding senile plaques. *Nat Neurosci.* 2022;25(6):688–701. DOI: 10.1038/s41593-022-01084-8
76. Hagihara H, Miyakawa T. Decreased brain pH in Alzheimer's disease: a systematic review and meta-analysis. *Int J Neuropsychopharmacol.* 2024;27(10):pyae047. DOI: 10.1093/ijnp/pyae047
77. Wang X, Shao X, Yu L, Sun J, Yin XS, Chen Z, Xu Y, Wang N, Zhang D, Qiu W, Liu F, Ma C. Changes in the pH value of the human brain in Alzheimer's disease pathology correlated with CD68-positive microglia: a community-based autopsy study in Beijing, China. *Mol Brain.* 2025;18(1):10. DOI: 10.1186/s13041-025-01180-3
78. Preece P, Cairns NJ. Quantifying mRNA in postmortem human brain: influence of gender, age at death, postmortem interval, brain pH, agonal state and inter-lobe mRNA variance. *Brain Res Mol Brain Res.* 2003;118(1-2):60–71. DOI: 10.1016/s0169-328x(03)00337-1
79. Mandal PK, Akolkar H, Tripathi M. Mapping of hippocampal pH and neurochemicals from in vivo multi-voxel ^{31}P study in healthy normal young male/female, mild cognitive impairment, and Alzheimer's disease. *J Alzheimers Dis.* 2012;31(Suppl 3):S75–S86. DOI: 10.3233/JAD-2012-120166

80. Mecheri G, Marie-Cardine M, Sappey-Marinier D, Bonmartin H, Albrand G, Ferry G, Trouillas J, Cinotti L. In vivo hippocampal ^{31}P NMR metabolites in Alzheimer's disease and ageing. *Eur Psychiatry*. 1997;12(3):140–148. DOI: 10.1016/S0924-9338(97)80203-9
81. Das N, Ren J, Spence JS, Rackley A, Chapman SB. Relationship of parieto-occipital brain energy phosphate metabolism and cognition using ^{31}P MRS at 7-Tesla in amnesic mild cognitive impairment. *Front Neurosci*. 2021;15:641739. DOI: 10.3389/fnins.2021.641739
82. Irwin RP, Lin SZ, Long RT, Paul SM. N-methyl-D-aspartate induces a rapid, reversible, and calcium-dependent intracellular acidosis in cultured fetal rat hippocampal neurons. *J Neurosci*. 1994;14(3 Pt 1):1352–1357. DOI: 10.1523/JNEUROSCI.14-03-01352.1994
83. Morita A, Suzuki N, Matsumoto Y, Hirano K, Enomoto A, Zhu J, Sakai K. p42/SET β , a chromatin modifying protein, is involved in radiation-induced apoptosis. *Biochem Biophys Res Commun*. 2000;278(3):627–632. DOI: 10.1006/bbrc.2000.3860
84. Beresford PJ, Zhang D, Oh DY, Fan Z, Greer EL, Russo ML, Jaju M, Lieberman J. Granzyme A activates an endoplasmic reticulum-associated caspase-independent nuclease to induce single-stranded DNA nicks. *J Biol Chem*. 2001;276(46):43285–43293. DOI: 10.1074/jbc.M108137200
85. Janssens V, Jordens J, Stevens I, Van Hoof C, Martens E, De Smedt H, Engelborghs Y, Waelkens E, Goris J. Calpain-mediated cleavage of the PP2A regulatory subunits PR72 and PR130. *Biochem Biophys Res Commun*. 2009;386(4):676–681. DOI: 10.1016/j.bbrc.2009.06.096
86. Bertoli C, Copetti T, Lam EW, Demarchi F, Schneider C. Calpain small-1 modulates Akt/FoxO3A signalling and apoptosis through PP2A. *Oncogene*. 2009;28(5):721–733. DOI: 10.1038/onc.2008.425
87. Watkins GR, Wang N, Mazalouskas MD, Gomez RJ, Guthrie CR, Kraemer BC, Schweiger S, Spiller BW, Wadzinski BE. Monoubiquitination promotes calpain cleavage of the protein phosphatase 2A regulatory subunit $\alpha 4$, altering PP2A stability and microtubule-associated protein phosphorylation. *J Biol Chem*. 2012;287(29):24207–24215. DOI: 10.1074/jbc.M112.368613
88. Liu F, Grundke-Iqbal I, Iqbal K, Oda Y, Tomizawa K, Gong CX. (2005c) Truncation and activation of calcineurin A by calpain I in Alzheimer

- disease brain. *J Biol Chem.* 2005;280(45):37755–37762. DOI: 10.1074/jbc.M507475200
89. Qian W, Yin X, Hu W, Shi J, Gu J, Grundke-Iqbal I, Iqbal K, Gong CX, Liu F. Activation of protein phosphatase 2B and hyperphosphorylation of tau in Alzheimer's disease. *J Alzheimers Dis.* 2011;23(4):617–627. DOI: 10.3233/JAD-2010-100987
90. Saito K, Elce JS, Hamos JE, Nixon RA. Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. *Proc Natl Acad Sci U S A.* 1993;90(7):2628–2632. DOI: 10.1073/pnas.90.7.2628
91. Kurbatskaya K, Phillips EC, Croft CL, Dentoni G, Hughes MM, Wade MA, Al-Sarraj S, Troakes C, O'Neill MJ, Perez-Nievas BG, Hanger DP, Noble W. Upregulation of calpain activity precedes tau phosphorylation and loss of synaptic proteins in Alzheimer's disease brain. *Acta Neuropathol Commun.* 2016;4:34. DOI: 10.1186/s40478-016-0299-2
92. Rao MV, Mohan PS, Peterhoff CM, Yang DS, Schmidt SD, Stavrides PH, Campbell J, Chen Y, Jiang Y, Paskevich PA, Cataldo AM, Haroutunian V, Nixon RA. Marked calpastatin (CAST) depletion in Alzheimer's disease accelerates cytoskeleton disruption and neurodegeneration: neuroprotection by CAST overexpression. *J Neurosci.* 2008;28(47):12241–12254. DOI: 10.1523/JNEUROSCI.4119-08.2008
93. Saito T, Matsuba Y, Yamazaki N, Hashimoto S, Saido TC. Calpain activation in Alzheimer's model mice is an artifact of APP and presenilin overexpression. *J Neurosci.* 2016;36(38):9933–9936. DOI: 10.1523/JNEUROSCI.1907-16.2016
94. Patrick GN, Zukerberg L, Nikolic M, de la Monte S, Dikkes P, Tsai LH. Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature.* 1999;402(6762):615–622. DOI: 10.1038/45159
95. Lee MS, Kwon YT, Li M, Peng J, Friedlander RM, Tsai LH. Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature.* 2000;405(6784):360–364. DOI: 10.1038/35012636
96. Tandon A, Yu H, Wang L, Rogaeva E, Sato C, Chishti MA, Kawai T, Hasegawa H, Chen F, Davies P, Fraser PE, Westaway D, St George-Hyslop PH. Brain levels of CDK5 activator p25 are not increased in Alzheimer's

- or other neurodegenerative diseases with neurofibrillary tangles. *J Neurochem.* 2003;86(3):572–581. DOI: 10.1046/j.1471-4159.2003.01865.x
97. Tseng HC, Zhou Y, Shen Y, Tsai LH. A survey of Cdk5 activator p35 and p25 levels in Alzheimer's disease brains. *FEBS Lett.* 2002;523(1-3):58–62. DOI: 10.1016/s0014-5793(02)02934-4
98. Giese KP. Generation of the Cdk5 activator p25 is a memory mechanism that is affected in early Alzheimer's disease. *Front Mol Neurosci.* 2014;7:36. DOI: 10.3389/fnmol.2014.00036
99. Xu L, Deng X. Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of μ - and m-calpains. *J Biol Chem.* 2006;281(46):35567–35575. DOI: 10.1074/jbc.M607702200
100. Baudry M, Bi X. Calpain-1 and calpain-2: the yin and yang of synaptic plasticity and neurodegeneration. *Trends Neurosci.* 2016;39(4):235–245. DOI: 10.1016/j.tins.2016.01.007
101. Nunomura A, Perry G, Pappolla MA, Wade R, Hirai K, Chiba S, Smith MA. RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *J Neurosci.* 1999;19(6):1959–1964. DOI: 10.1523/JNEUROSCI.19-06-01959.1999
102. Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA. Oxidative damage is the earliest event in Alzheimer disease. *J Neuropathol Exp Neurol.* 2001;60(8):759–767. DOI: 10.1093/jnen/60.8.759
103. Markesbery WR, Lovell MA. Damage to lipids, proteins, DNA, and RNA in mild cognitive impairment. *Ann Neurol.* 2005;58(5):730–735. DOI: 10.1002/ana.20629
104. Williams TI, Lynn BC, Markesbery WR, Lovell MA. Increased levels of 4-hydroxynonenal and acrolein, neurotoxic markers of lipid peroxidation, in the brain in mild cognitive impairment and early Alzheimer's disease. *Neurobiol Aging.* 2006;27(8):1094–1099. DOI: 10.1016/j.neurobiolaging.2005.06.004
105. Montine TJ, Quinn J, Kaye J, Morrow JD. F(2)-isoprostanes as biomarkers of late-onset Alzheimer's disease. *Ann Neurol.* 2002;52(2):175–179. DOI: 10.1002/ana.10272

106. Kovalyova Y, Bak DW, Gordon EM, Fung C, Shuman JHB, Cover TL, Amieva MR, Weerapana E, Hatzios SK. An infection-induced oxidation site regulates legumain processing and tumor growth. *Nat Chem Biol.* 2022;18(5):489–497. DOI: 10.1038/s41589-022-00992-x
107. Zhai C, Wang X, Wang J, Cheng S, Yang C, Kang X, Lu W, Xu X, Zhou G, Wang X. Effects of 4-hydroxy-2-nonenal, malondialdehyde and 2-hexenal on the structure and activity of calpain-1. *J Agric Food Chem.* 2023;71(20):7799–7809. DOI: 10.1021/acs.jafc.3c01225
108. Johnson LG, Zhai C, Wang X, Cheng S, Yang C, Kang X, Xu X, Zhou G, Wang X. Lipid oxidation products modify the structure and activity of calpain-2. *J Agric Food Chem.* 2024;72(20):11701–11710. DOI: 10.1021/acs.jafc.4c00335
109. Kemmerling U, Muñoz P, Müller M, Sánchez G, Aylwin ML, Klann E, Carrasco MA, Hidalgo C. Calcium release by ryanodine receptors mediates hydrogen peroxide-induced activation of ERK and CREB phosphorylation in N2a cells and hippocampal neurons. *Cell Calcium.* 2007;41(5):491–502. DOI: 10.1016/j.ceca.2006.10.001
110. Paula-Lima AC, Adasme T, SanMartín C, Sebollela A, Hetz C, Carrasco MA, Ferreira ST, Hidalgo C. Amyloid β -peptide oligomers stimulate RyR-mediated Ca^{2+} release inducing mitochondrial fragmentation in hippocampal neurons and prevent RyR-mediated dendritic spine remodeling produced by BDNF. *Antioxid Redox Signal.* 2011;14(7):1209–1223. DOI: 10.1089/ars.2010.3287
111. Mark RJ, Hensley K, Butterfield DA, Mattson MP. Amyloid beta-peptide impairs ion-motive ATPase activities: evidence for a role in loss of neuronal Ca^{2+} homeostasis and cell death. *J Neurosci.* 1995;15(9):6239–6249. DOI: 10.1523/JNEUROSCI.15-09-06239.1995
112. Kuchibhotla KV, Goldman ST, Lattarulo CR, Wu HY, Hyman BT, Bacskaí BJ. Abeta plaques lead to aberrant regulation of calcium homeostasis in vivo resulting in structural and functional disruption of neuronal networks. *Neuron.* 2008;59(2):214–225. DOI: 10.1016/j.neuron.2008.06.008
113. Feng Y, Li X, Zhou W, Lou D, Huang D, Li Y, Kang Y, Xiang Y, Li T, Zhou W, Song W. Regulation of SET gene expression by NF κ B. *Mol Neurobiol.* 2017;54(6):4477–4485. DOI: 10.1007/s12035-016-9967-2

114. Leopoldino AM, Squarize CH, Garcia CB, Almeida LO, Pestana CR, Polizello AC, Uyemura SA, Tajara EH, Gutkind JS, Curti C. SET protein accumulates in HNSCC and contributes to cell survival: antioxidant defense, Akt phosphorylation and AVOs acidification. *Mol Cell Biochem.* 2012;363(1-2):163–173. DOI: 10.1007/s11010-011-1158-x
115. Foley TD, Kintner ME. Brain PP2A is modified by thiol-disulfide exchange and intermolecular disulfide formation. *Biochem Biophys Res Commun.* 2005;330(4):1224–1229. DOI: 10.1016/j.bbrc.2005.03.108
116. Foley TD, Petro LA, Stredny CM, Coppa TM. Oxidative inhibition of protein phosphatase 2A activity: role of catalytic subunit disulfides. *Neurochem Res.* 2007;32(11):1957–1964. DOI: 10.1007/s11064-007-9394-x
117. Foley TD, Melideo SL, Healey AE, Lucas EJ, Koval JA. Phenylarsine oxide binding reveals redox-active and potential regulatory vicinal thiols on the catalytic subunit of protein phosphatase 2A. *Neurochem Res.* 2011;36(2):232–240. DOI: 10.1007/s11064-010-0310-4
118. Rao RK, Clayton LW. Regulation of protein phosphatase 2A by hydrogen peroxide and glutathionylation. *Biochem Biophys Res Commun.* 2002;293(1):610–616. DOI: 10.1016/S0006-291X(02)00268-1
119. Chen L, Liu L, Yin J, Luo Y, Huang S. Hydrogen peroxide-induced neuronal apoptosis is associated with inhibition of protein phosphatase 2A and 5, leading to activation of MAPK pathway. *Int J Biochem Cell Biol.* 2009;41(6):1284–1295. DOI: 10.1016/j.biocel.2008.10.029
120. Su B, Wang X, Lee HG, Tabaton M, Perry G, Smith MA, Zhu X. Chronic oxidative stress causes increased tau phosphorylation in M17 neuroblastoma cells. *Neurosci Lett.* 2010;468(3):267–271. DOI: 10.1016/j.neulet.2009.11.010
121. Reed T, Perluigi M, Sultana R, Pierce WM, Klein JB, Turner DM, Coccia R, Markesbery WR, Butterfield DA. Redox proteomic identification of 4-hydroxy-2-nonenal-modified brain proteins in amnesic mild cognitive impairment: insight into the role of lipid peroxidation in the progression and pathogenesis of Alzheimer's disease. *Neurobiol Dis.* 2008;30(1):107–120. DOI: 10.1016/j.nbd.2007.12.007
122. Reed TT, Pierce WM Jr, Turner DM, Markesbery WR, Butterfield DA. Proteomic identification of HNE-bound proteins in early Alzheimer

- disease: insights into the role of lipid peroxidation in the progression of AD. *Brain Res.* 2009;1274:66–76. DOI: 10.1016/j.brainres.2009.04.009
123. Mattson MP, Fu W, Waeg G, Uchida K. 4-Hydroxynonenal, a product of lipid peroxidation, inhibits dephosphorylation of the microtubule-associated protein tau. *Neuroreport.* 1997;8(9):2275–2281. DOI: 10.1097/00001756-199707070-00036
124. Braak H, Braak E. Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol.* 1991;82(4):239–259. DOI: 10.1007/BF00308809
125. Braak H, Braak E. On areas of transition between entorhinal allocortex and temporal isocortex in the human brain. Normal morphology and lamina-specific pathology in Alzheimer's disease. *Acta Neuropathol.* 1985;68(4):325–332. DOI: 10.1007/BF00690836
126. Braak H, Thal DR, Ghebremedhin E, Del Tredici K. Stages of the pathologic process in Alzheimer disease: age categories from 1 to 100 years. *J Neuropathol Exp Neurol.* 2011;70(11):960–969. DOI: 10.1097/NEN.0b013e318232a379
127. Gómez-Isla T, Price JL, McKeel DW Jr, Morris JC, Growdon JH, Hyman BT. Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *J Neurosci.* 1996;16(14):4491–4500. DOI: 10.1523/JNEUROSCI.16-14-04491.1996
128. Hof PR, Bussière T, Gold G, Kövari E, Giannakopoulos P, Bouras C, Perl DP, Morrison JH. Stereologic evidence for persistence of viable neurons in layer II of the entorhinal cortex and the CA1 field in Alzheimer disease. *J Neuropathol Exp Neurol.* 2003;62(1):55–67. DOI: 10.1093/jnen/62.1.55
129. Hof PR, Cox K, Morrison JH. Quantitative analysis of a vulnerable subset of pyramidal neurons in Alzheimer's disease: I. Superior frontal and inferior temporal cortex. *J Comp Neurol.* 1990;301(1):44–54. DOI: 10.1002/cne.903010105
130. Bussière T, Giannakopoulos P, Bouras C, Perl DP, Morrison JH, Hof PR. Progressive degeneration of nonphosphorylated neurofilament protein-enriched pyramidal neurons predicts cognitive impairment in Alzheimer's disease: stereologic analysis of prefrontal cortex area 9. *J Comp Neurol.* 2003;463(3):281–302. DOI: 10.1002/cne.10760

131. Hof PR, Nimchinsky EA, Morrison JH. Neurochemical phenotype of corticocortical connections in the macaque monkey: quantitative analysis of a subset of neurofilament protein-immunoreactive projection neurons in frontal, parietal, temporal, and cingulate cortices. *J Comp Neurol.* 1995;362(1):109–133. DOI: 10.1002/cne.903620107
132. Hof PR, Nimchinsky EA, Buée-Scherrer V, Buée L, Nasrallah J, Hottinger AF, Purohit DP, Loerzel AJ, Steele JC, Delacourte A, et al. Amyotrophic lateral sclerosis/parkinsonism-dementia complex of Guam: quantitative neuropathology, immunohistochemical analysis of neuronal vulnerability, and comparison with related neurodegenerative disorders. *Acta Neuropathol.* 1994;88(5):397–404. DOI: 10.1007/BF00389490
133. Leng K, Li E, Eser R, Piergies A, Sit R, Tan M, Neff N, Li SH, Rodriguez RD, Suemoto CK, Leite REP, Ehrenberg AJ, Pasqualucci CA, Seeley WW, Spina S, Heinsen H, Grinberg LT, Kampmann M. Molecular characterization of selectively vulnerable neurons in Alzheimer's disease. *Nat Neurosci.* 2021;24(2):276–287. DOI: 10.1038/s41593-020-00764-7
134. Otero-Garcia M, Mahajani SU, Wakhloo D, Tang W, Xue YQ, Morabito S, Pan J, Oberhauser J, Madira AE, Shakouri T, Deng Y, Allison T, He Z, Lowry WE, Kawaguchi R, Swarup V, Cobos I. Molecular signatures underlying neurofibrillary tangle susceptibility in Alzheimer's disease. *Neuron.* 2022;110(18):2929–2948.e8. DOI: 10.1016/j.neuron.2022.06.021
135. Sampson VL, Morrison JH, Vickers JC. The cellular basis for the relative resistance of parvalbumin and calretinin immunoreactive neocortical neurons to the pathology of Alzheimer's disease. *Exp Neurol.* 1997;145(1):295–302. DOI: 10.1006/exnr.1997.6433
136. Hof PR, Morrison JH. Neocortical neuronal subpopulations labeled by a monoclonal antibody to calbindin exhibit differential vulnerability in Alzheimer's disease. *Exp Neurol.* 1991;111(3):293–301. DOI: 10.1016/0014-4886(91)90096-u
137. Fu H, Possenti A, Freer R, Nakano Y, Hernandez Villegas NC, Tang M, Cauhy PVM, Lassus BA, Chen S, Fowler SL, Figueroa HY, Huey ED, Johnson GVW, Vendruscolo M, Duff KE. A tau homeostasis signature is linked with the cellular and regional vulnerability of excitatory neurons to tau pathology. *Nat Neurosci.* 2019;22(1):47–56. DOI: 10.1038/s41593-018-0298-7

138. Wegiel J, Wisniewski HM, Dziwiatkowski J, Badmajew E, Tarnawski M, Reisberg B, Mlodzik B, De Leon MJ, Miller DC. Cerebellar atrophy in Alzheimer's disease—clinicopathological correlations. *Brain Res.* 1999;818(1):41–50. DOI: 10.1016/s0006-8993(98)01279-7
139. Lyros E, RagoSchke-Schumm A, Kostopoulos P, Sehr A, Backens M, Kalampokini S, Decker Y, Lesmeister M, Liu Y, Reith W, Fassbender K. Normal brain aging and Alzheimer's disease are associated with lower cerebral pH: an in vivo histidine ^1H -MR spectroscopy study. *Neurobiol Aging.* 2020;87:60–69. DOI: 10.1016/j.neurobiolaging.2019.11.012
140. Codreanu SG, Adams DG, Dawson ES, Wadzinski BE, Liebler DC. Inhibition of protein phosphatase 2A activity by selective electrophile alkylation damage. *Biochemistry.* 2006;45(33):10020–10029. DOI: 10.1021/bi060551n
141. Katayose Y, Li M, Al-Murrani SWK, Shenolikar S, Damuni Z. Protein phosphatase 2A inhibitors, I^{PP2A} and $\text{I}2^{\text{PP2A}}$, associate with and modify the substrate specificity of protein phosphatase 1. *J Biol Chem.* 2000;275(13):9209–9214. DOI: 10.1074/jbc.275.13.9209
142. Goñi-Oliver P, Lucas JJ, Avila J, Hernández F. N-terminal cleavage of GSK-3 by calpain: a new form of GSK-3 regulation. *J Biol Chem.* 2007;282(31):22406–22413. DOI: 10.1074/jbc.M702793200
143. Feng Y, Xia Y, Yu G, Shu X, Ge H, Zeng K, Wang J, Wang X. Cleavage of GSK-3 β by calpain counteracts the inhibitory effect of Ser9 phosphorylation on GSK-3 β activity induced by hydrogen peroxide. *J Neurochem.* 2013;126(2):234–242. DOI: 10.1111/jnc.12285
- Companion volumes: The Coerulean Pincer (the writer — glycogen-synthase-kinase-3 β and the noradrenergic drive that floors it); The Silenced Eraser (the eraser approached from outside the neuron — the ungoverned microglion and the inflammasome route to its silencing); The Single Cut (one protease, two substrates, and the two-compartment lesion that follows from cleaving SET). This volume is the eraser approached from inside the neuron: the regulatory surfaces of the enzyme itself, and what is actually known about each.*