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Does Traumatic Brain Injury Hold the Key to the Alzheimer's Puzzle?

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Abstract

Introduction—Neurodegenerative disorders have been a graveyard for hundreds of well-intentioned efforts at drug discovery and development. Concussion and other traumatic brain injury (TBI) and Alzheimer's disease (AD) share many overlapping pathologies and possible clinical links.

Methods—We searched the literature since 1995 using Medline and Google Scholar for the terms concussion, AD, and shared neuropathologies. We also studied a TBI animal model as a supplement to transgenic (tg) mouse AD models for evaluating AD drug efficacy by preventing neuronal losses. To evaluate TBI/ AD pathologies and neuronal self-induced cell death (apoptosis) we are studying brain Extracellular Vesicles (EVs) in plasma and (–)-phenserine pharmacology to probe, in animal models of AD and humans, apoptosis and pathways common to concussion and AD.

Results—Neuronal cell death and a diverse and significant pathological cascade follow TBI. Many of the developing pathologies are present in early AD. The use of an animal model of concussion as a supplement to tg mice provides an indication of an AD drug candidate's potential for preventing apoptosis and resulting progression towards dementia in AD. This weight drop supplementation to tg mouse models, the experimental drug (–)-phenserine, and plasma derived EVs enriched for neuronal origin to follow biomarkers of neurodegenerative processes, each and in combination show promise as tools useful for probing the progression of disease in AD, TBI/AD pathologies, apoptosis, and drug effects on rates of apoptosis both preclinically and in humans. (–)-Phenserine both countered many subacute post-TBI pathologies that could initiate

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clinical AD and, in the concussion and other animal models, showed evidence consistent with direct inhibition of neuronal preprogrammed cell death in the presence of TBI/AD pathologies.

Conclusions—These findings may provide support for expanding preclinical tg mouse studies in AD with a TBI weight drop model, insights into the progression of pathological targets, their relations to apoptosis, and timing of interventions against these targets and apoptosis. Such studies may demonstrate the potential for drugs to effectively and safely inhibit preprogrammed cell death as a new drug development strategy for use in the fight to defeat AD.

Keywords

Concussion; Alzheimer's disease; neuropathology; anecrotic cell death; preprogrammed cell death; timing of therapeutic interventions; drug targets

1. INTRODUCTION

Since the identification of a Nucleus Basalis of Meynert lesion in Alzheimer's disease (AD) brains in 1983, over three decades of intensive preclinical investigation and drug candidate evaluations have failed to mitigate the onset of dementia for affected patients [1]. AD investigators are not alone. Neurodegenerative disorders have been a graveyard for hundreds of well-intentioned efforts at drug discovery and development [2,3]. In spite of promising effects in animal models, all attempts at drug arrests of degenerative brain pathologies have failed in clinical trials. No drug has successfully altered the disease course in AD, arrested the pathologies in Parkinson's disease (PD), mitigated the pathological effects from Traumatic Brain Injuries including Concussions (TBI), and course of other neurodegenerative disorders.

After a review of the literature we concluded earlier that, under current conditions, for scientific [3] and regulatory [4] reasons, development of a clinically effective anti-A β ₄₂ amyloid targeted therapy was unlikely. As a consequence, we considered how AD investigators might turn current research emphases in different directions that potentially offer opportunities to outflank, rather than frontally assault, AD. In general neurodegenerations, while distinguished by specific pathologies and clinical presentations, share general features; specifically, chronic, slowly unfolding cascades of poorly understood associated pathologies. One potentially promising approach to understanding these shared and probably important contributory pathological sources for disease progression in neurodegenerations may arise from the controversial but frequently reported association between AD and concussion/TBI [5–9]. Chronic degenerative processes may be difficult to understand in part due to the slow and unpredictable unfolding of events. For example, A β deposits have been detected during the fourth decade of life in brains of persons regarded at increased risk of later dementia. Yet, extensive A β amyloid deposits and neurofibrillary tangles comprised of hyperphosphorylated tau, both pathognomonic neuropathological features of AD, have been reported in cognitively unimpaired persons in their eighth and ninth decades of life. Many of the pathologies found in this five decades of unfolding AD pathologies have also been reported as present in and unfolding within weeks following TBI: inflammatory, glutaminergic, oxidative, A β , cholinergic, and other environmental stressors of neurons [10,11]. We undertook the opportunity to study in more depth TBI as a possible

model contributory to better understanding the puzzle of AD. We were motivated to turn in this direction as a result of the overlapping AD/ TBI pathologies and the potential advantages from being able to study the interrelations among pathologies during their accelerated unfolding following their provocation by the stressor TBI.

As a result of this turn we have found a range of potential opportunities deserving further consideration.

- ◆ As background we provide a comparison of known TBI and AD pathologies and claimed clinical associations where patients report a history of TBI preceding the onset of AD.
- ◆ These overlaps reveal not only the possibility of identifying, in further studies of TBI, causal interrelations among AD neuropathologies, but also a pressing need for plasma based measures reflecting activities in brain of these pathologies.
- ◆ As a consequence, under Methods and Results/ Discussion, we pursue and describe the current potential for evaluating biomarkers derived from plasma extracellular vesicles (EVs) (sometimes termed exosomes) expressing neuronal markers for brain origin to better inform AD clinical research about the state of pathologies in patients.
- ◆ A third implication from our initial studies of TBI pathologies may be the utilities from using a weight drop animal model for concussive injury as a supplemental assessment of candidate AD drugs.
- ◆ The ultimate aim of AD drug intervention is to prevent the onset of dementia due to losses of neurons. We have found the TBI animal weight drop model provides the opportunity to study AD drug effects on neuronal death occurring secondary to induced pathologies.
- ◆ There is a pressing need for plasma biomarkers of brain specific neurochemical processes. Neuronally and astrocyte marked EVs show promise as plasma sourced indicators of brain neurochemical activities.
- ◆ As we discuss with EVs, used as an intervention, a drug, in both *in vitro* and *in vivo* animal model studies, serves potentially as both a pharmacological probe and a therapeutic candidate.
- ◆ As a result from probing anoxia, weight drop, and AD related models and *in vitro* mechanisms with (–)-phenserine, we are able to report that the indirect neuroprotective activities of drugs from mitigation of disease pathologies may be capable of countering AD and related neurodegenerations with a direct anti-programmed cell death drug activity.
- ◆ Finally, this preliminary evidence from (–)-phenserine probing of various animal models suggests to us the utility of introducing into AD drug development increased uses of small intensively assessed clinical trials.
- ◆ Hence, in synopsis, to aid development of AD drug interventions that prevent the onset of dementia due to dysfunction and losses of neurons, the application

of a TBI animal model (such as the weight drop model) provides an opportunity to study AD drug effects on neuronal death occurring secondary to induced pathologies. Up till now, most candidate AD drugs gain traction from successes in AD Tg mouse models that represent critical pathologies regarded as responsible for clinical AD. However, interventions against these pathologies have failed when translated into humans in clinical trials; with hundreds of promising preclinical drug candidates failing to arrest the progression of AD over the past decade. The screening of drugs in animal models requires a change, by being more rigorous and including models for alternative pathologies. Inclusion of a TBI model provides accelerated development of neurodegenerative brain pathologies and neuronal compromise and death, and can supplement or possibly replace existing models for drug interventions against AD and perhaps other neurodegenerative diseases whose time-dependent process can be evaluated by the use EV technology that provides a window to the brain.

- ◆ These findings support the utility of combining studies of AD patients with studies of concussion/ TBI patients using candidate AD drugs to characterize the time-dependent biochemistry of apoptosis in these disorders in humans and the effects of a drug intervention.

EVs are small membranous particles (30–150 nm in size) that support intercellular communication by conveying proteins, DNA, RNA and bioactive lipids [12]. The contents of EVs reflect the cellular and metabolic processes of their cells of origin, which may be modified by pathological changes in their microenvironment as well as by pharmacological and behavioral interventions, and serve as personalized biomarkers for following such responses [12]. By the use of a two-step method (first precipitating total EVs, then, immunoprecipitating EVs that express select membrane proteins defining their cellular origin, such as the neuronal cell adhesion molecule L1CAM to generate a subpopulation enriched for neuronal origin), EVs can be time-dependently isolated from plasma or serum as probes of AD pathological mechanistic activities in humans and efficacy sensitive small clinical trial capabilities. Using such techniques AD research may be empowered to better take advantage of integrated preclinical-clinical mechanistic translational studies for drug discoveries and their methods of assessment.

2. BACKGROUND

2.1 Why look to TBI: The quest for disease modification of AD

During the 1980's the National Institute on Aging, NIH, identified the advantage that AD potentially offers investigators. Because of the late age of onset of sporadic AD, a delay of onset by only five years will reduce the cost and prevalence of the disease by half [13]. Unfortunately, this clear and modest aim has presented an elusive target for researchers. Two neuropathologies still prominently interrelate in medicine's current theory of AD [14]. Following failures to benefit clinical AD with anti-A β ₄₂ amyloid drugs after clinical disease becomes apparent, the research community developed a general agreement that earlier interventions are needed to slow the accumulation of pathologies and neuronal losses [3,4].

Currently, multiple primary or secondary prevention studies are underway mainly focused on individuals at imminent risk of clinical AD [3,4]. Unsettled issues haunt these approaches. First, A β ₄₂ amyloid accumulates as early as age 45 in some persons and, second, hyperphosphorylated tau (p-Tau) can appear 15 years later, but still some 15 years prior to the onset of clinical AD [15–19]. The long latency before clinical symptoms appear from these most prominent of AD pathologies undermines the feasibility of effective applications of early drug interventions [3]. Additional impediments include a lack of knowledge of possibly critical stages in the progression of involved pathologies and ignorance of the causes for neuropathologies to sometimes manifest as clinical disease and other times remain asymptomatic during aging. What is known is not heartening. Studies in laboratories have shown that the presence of A β ₄₂ amyloid can provoke the emergence of p-Tau. P-Tau, once present in brain, appears capable of spreading to synaptically connected unaffected neurons where it provokes the abnormal phosphorylation of neuronal tau into neurotoxic p-Tau [16].

Recent investigations have shown that an unnatural form of p-Tau, such as cis p-Tau or a rare high molecular weight form, that is not readily cleared by cells, accumulates and polymerizes to form AD's classical neurofibrillary tangles [17–19]. To prevent the emergence of self-sustaining p-Tau pathologies and cumulative A β ₄₂ amyloid toxicity, anti-A β ₄₂ amyloid drug interventions may have to take place 15 or more years before the onset of clinical AD [2,3]. This may, likewise, be the case for familial AD. Under current regulatory standards, because of the long latency between initial pathologies and clinical symptoms, unless disease arresting and precise stage-targeted drug interventions can be developed for AD, proper evaluation and commercialization of anti-A β ₄₂ amyloid drugs may simply be impossible [3,4].

Other potential problems with the current drugs, animal models, and the amyloid theory of AD also hinder progress at disease modification. Current AD transgenic (Tg) mouse models do not display extensive synaptic de-arborization and neuronal losses at levels comparable to those found in humans with cognitive decline and dementia. Seen from this clinical AD perspective, current Tg mouse models express some pathological features but not the profound synaptic pruning, dendritic de-arborization, and neuronal death and progressive cognitive behavioral deficits typical of dementia in AD [20]. These findings in AD Tg mice are unexpected because A β ₄₂ has been shown toxic for neurons in tissue culture [20,21]. Inconsistent with their effects on cultured neurons, anti-A β ₄₂ or β -amyloid drugs reduce concentrations of A β ₄₂ and β -amyloid plaques in both mouse and human AD subjects, but without evidence for synaptic benefits or increased neuronal survival and cognitive and functional benefits. Neither fibrillar A β ₄₂, or A β ₄₂ amyloid, or p-Tau, or animal models for these pathologies may serve as a target capable of identifying drugs effective against AD.

2.2 Turning to TBI to understand AD pathologies

Patients report a history of TBI preceding an onset of AD, epidemiological data support some overlapping distributions of clinical cases, and neuropathological cascades following concussion/ TBI involve the same pathologies—inflammatory, glutamergic, oxidative, A β ₄₂, cholinergic, and others—present in AD and other neurodegenerations. In each of these

disorders the final common pathway into clinical symptomatology involves the malfunctioning and death of neurons. One not well-understood or entirely documented outcome from each of these pathologies, premature self-induced necrotic, apoptotic, or other types of preprogrammed neuronal cell death have been hypothesized as causes for neuronal losses in neurodegenerative disorders [22,23]. These associations seem to invite further study.

The elderly are at increased risk for both falls and AD. Epidemiologic studies have suggested that a history of head trauma is associated with an increased risk of AD [8]. In earlier clinical anecdotal observations we found that some patients presenting with AD reported falling and hitting their heads approximately two years prior to their symptoms [8]. A single head injury with loss of consciousness has been associated with a 50% increased risk of AD dementia [24]. Time to onset of AD has been found to be significantly reduced in those who sustained a TBI, compared to a control population [24]. Head trauma has been associated with an earlier age at onset of AD, particularly among APOE $\epsilon 4$ carriers [25]. Recent gene expression studies across animal models of TBI have revealed the triggering of pathways leading to AD and Parkinson's disease (PD) induced by mild and more severe injury forms [26,27]. Axonal injury as a consequence of TBI results in the release and accumulation of amyloid precursor protein (APP), whose enzymatic cleavage can generate A β peptides [28, 29]. The risk of PD was found substantially increased in subjects with mild or more severe TBIs [8] and α -Synuclein is a potential pathological link between TBI and PD [30,31]. A β plaques and intra-axonal A β deposits have been found in approximately one-third of TBI subjects dying shortly after injury [32,33]. On the other hand, imaging studies of subjects diagnosed with Mild Cognitive Impairment found increased levels of amyloid in brain even though the frequency of self-reported head trauma did not differ between cognitively normal and MCI groups [34,35]. p-Tau immunoreactive NFTs have also been observed in young individuals within weeks to months following their last concussion [36]. Mice deficient in amyloid- β -converting enzymes [37] as well as mice administered an antibody to APP [38] have significantly improved pathologic and behavioral outcomes following a head injury. This finding is consistent with A β_{42} accumulations leading to progressive brain atrophy [37,38]. Overlaps in the neuropathologies of these different neurodegenerations are so frequent to suggest a possible single or basically similar pathological cascade provoking or accompanying the pathologies specific to the dementias or other clinical outcomes from these neurodegenerations.

A review of TBI-associated brain damage reveals that pathologies can be segregated into two key phases (Table 1). First, an initial primary damage phase occurs at the moment of insult, and potentially includes contusion and laceration, diffuse axonal injury, intracranial hemorrhage, and immediate (necrotic) cell death [28,32,36]. These initial consequences are followed by an extended second phase that involves cascades of biological processes initiated at the time of injury: neuroinflammation, glutamate toxicity, astrocyte reactivity, apoptosis, and local or more general ischemia [28,32,36]. While primary brain injury events may require surgical interventions, secondary brain responses to injuries may be pharmacologically reversible, in whole or in part, and provide potential targets for drug interventions.

Elements comprising the delayed secondary phase can be both a cause of significant neuronal losses following TBI and introduce pathological activations that may function as initiators of pathologies that can develop into clinical AD. These pathologies offer targets that can be modified with interventions. For example, within 6 h after TBI, a robust increase in p53-labeled cells is evident within the site of maximal injury (contusion) in the brain [39]. Apoptosis or other programmed cell death are active in AD [23,40]. In animal studies, inhibition of p53 transcriptional activity (a key gatekeeper of neuronal apoptosis [22], with a therapeutic window of 5 to 7 h after injury, minimized apoptotic cell death and ensuing neuronal losses and cognitive deficits [39,41,42]. Likewise, inhibition of neuroinflammation in preclinical animal models of TBI with a therapeutic window of 12 hours can fully mitigate secondary injury [43], indicating that such processes underpinning it are potentially reversible and that neurodegenerative pathways triggered by TBI can also be pharmacologically switched off.

Autopsy studies have reported significant amyloid- β deposition in up to 30% of persons who die acutely following a brain injury, including children [44–46]. CSF levels of A β_{42} are lower after TBI, consistent with A β_{42} amyloid deposition in brain and its lower clearance into CSF. As expected from axonal ruptures and neuronal death, tau levels are raised consistent with the severity of TBI [46,47]. Consistent with these findings, in animal models following acute injury, a release of neurotransmitters and ionic fluxes occurs which, in turn, leads to changes in cell membrane functions, microglial activation, and a glucose metabolic depression phase of 1–10 or more days duration [32,36,48]. During this period the brain becomes more vulnerable to repeated injury [50]. With the exception of anatomical trauma, each of these pathologies is present early in the development of AD. In sum, both epidemiological and molecular mechanistic evidence point towards a possible role of concussion and other traumatic brain injury in the activation of AD in some elderly persons.

2.3 Potential advantages from using TBI as a supplemental model for AD

One major impediment to characterization of the cascade of pathologies found in AD has been the five decade long lag between the first reported brain pathologies appearing in the fourth decade of life and the persistence of pathologies into the ninth decade, sometimes without detectable evidence of clinical functional impairments or disabilities in neuropathologically affected persons [2,3]. TBI offers investigators the opportunity to induce many of the shared neuropathologies using animal models for concussion/ TBI or study them in patients who have suffered a head injury. Concussion/ TBI offers basic and clinical investigators a temporally condensed microenvironment potentially reflecting within days and weeks neuropathological progressions that can be studied in AD only by cross-sectional sampling of at risk subjects chosen from a population spread across decades. The broad range of pathologies of interest to AD investigators—inflammatory, blood-brain barrier changes, extraneuronal matrix cell support changes, glutamergic toxicity, oxidative stress, increased A β_{42} synthesis and accumulation, cholinergic deficiencies from Nucleus Basalis of Meynert injury [1,14,29,31–33,51–55], and others—have each been reported following head injury offering a rich field for neuropathological investigation. Epidemiological studies, anecdotal reports, and autopsy records support a relationship that may include the potential for TBI to activate AD neuropathologies in persons at risk.

To take full advantage of these promising TBI neuropathological insights into AD available from coordinated studies of animal models and human injuries, investigators will require new tools for measuring in humans the mechanistic interactions of brain pathologies. In response, one of our efforts has been focused on deriving brain EVs from plasma or serum samples for measuring biomarkers of the various pathologies common between TBI and AD, another on the characterization of (–)-phenserine pharmacology for potential use with EVs as a probe of TBI and AD disease mechanisms. We find (–)-phenserine of interest as it has been administered safely in 645 people for up to one year duration.

2.4 Do Extracellular Vesicles have a role in TBI/AD research?

EVs are generated and released by most viable cells and accumulate in biological fluids, including the blood, where they can be sampled in a time-dependent manner. The isolation of a EV subpopulation enriched for a particular cellular origin is possible using cell-specific surface markers [12]. The presence of neural cell adhesion molecule L1 (L1CAM) on a subpopulation of plasma EVs allows the selective immunoprecipitation of EVs that are enriched for neuronal origin. Analysis of the contents of such EVs provides a window to neurons within the brain and can be used to assess their response to physiological challenges, disease and its progression and drugs [12]. Hence, in recent years probing the contents of plasma EVs is providing data to aid bridge the diagnostic gap to identify neurodegenerative disorders, their progression and response to therapy in both preclinical and clinical settings [56]. Evaluation of the classical AD markers A β ₄₂ and p-tau (phosphorylated at S396 or T181) found them significantly elevated in L1CAM+ EVs of AD patients with a high sensitivity of distinguishing AD patients from age matched controls; these markers may be attaining a plateau as early as 10 years before AD clinical diagnosis [57]. Recent analysis of other proteins within the cargo of in L1CAM+ EVs is providing insight into mechanisms involved in AD development, with the occurrence of brain insulin resistance [58] and reductions in the levels of cellular survival factors [59] and synaptic proteins [60]; processes likely shared with TBI in which elevations in p-Tau within neural EVs have likewise recently been reported [61]. These processes, together with measures of neuroinflammation by evaluating astrocyte derived [62] and/or microglial-derived EVs [63] may provide insight into time-dependent mechanisms occurring in TBI, factors involved in progression to later AD and responses to interventions.

3. METHODS

To proceed with coordinated study of TBI and AD pathologies, using TBI as a temporally accelerated model of the mechanistic cascade found in AD and potentially other neurodegenerations, we pursued a number of interrelated studies. Already reported methods and results include *in vitro* studies of (–)-phenserine activities against inflammation, glutamate toxicity, oxidative stress, amyloid precursor protein synthesis, acetylcholinesterase (AChE) [55]. Methods used to study (–)-Phenserine activities in anoxia-stroke, weight drop-TBI, and tg-AD animal models have also been reported [11,64–66].

To identify plasma EV-based biomarkers of brain pathological activities common to TBI and AD, we searched the literature since 1995 using Medline and Google Scholar for the terms

“Extracellular Vesicles” and “exosomes” in association with “concussion”, “TBI”, “AD”, “apoptosis” or “programmed neuronal cell death” (of neurons), and each of the pathologies listed in Table 1. In addition, we searched the bibliographies in papers identified for these terms of interest. Because of the breadth of this review, we concluded each search when further references contributed no additional information relevant to the specific potential pathway or other hypotheses under study.

To validate biomarkers based on plasma/serum EVs enriched for neuronal origin we have been assaying brain pathological markers in preparations of L1CAM+ plasma/serum EVs obtained from mouse in a time-dependent manner after TBI and in human studies by following published procedures [12,57–60,62]. Quantitative evaluation of proteins present in EVs has been undertaken using the platforms of Meso Scale Diagnostics (Rockville, MD) and SomaLogic (Boulder, CO), in line with the manufacturers’ procedures.

Following initial animal model studies, we sought to confirm possible (–)-phenserine prevention of neurons proceeding into programmed cell death by assessing immunohistochemical neuronal cell losses and neuroinflammation markers 72 hours after weight drop injury, following the methodology of Tweedie et al., [11] (unpublished data and [39,41,42,64,65]). Cellular loss and neuroinflammation were evaluated by Fluoro Jade C and by IBA1 and TNF- α , respectively, following published immunohistochemical procedures [42,67].

4. RESULTS (Extracellular Vesicles (EVs) and (–)-Phenserine)

4.1 Studies of EVs enriched for neuronal origin

EVs enriched for neuronal origin can be time-dependently sampled from the plasma/serum of animal models and humans following TBI or AD, and their analysis allows quantitative evaluation of not only classical markers of AD but also of TBI [12,68], many of which are detailed in Table 1. These provide insight into time-dependent mechanisms underpinning TBI, and factors that may trigger the cascade eventually leading to AD. Our group is evaluating the use of EVs enriched for neuronal origin obtained from blood to follow brain changes occurring after a TBI challenge to mice (a 30 g close head weight drop to a 30 g mouse [11], in line with two humans of equal weight clashing heads). Ongoing analysis of these EVs has demonstrated early changes (within 8 hr) in proteins associated with pathways related to inflammatory cytokines and acute phase responses, as well as to apoptosis, cell adhesion and glucogenesis. These led to later changes (24 hr to 30 days) in EV markers of cytokine/chemokine activity as well as signaling cascades and proteins associated with neural development, differentiation, proliferation and migration, together with fatty acid biosynthesis and insulin resistance (unpublished data). Many of these same processes are also known to be involved in AD. In parallel, we have been conducting human case-control studies, in veterans that have suffered one or several TBIs during deployment, finding elevations in the levels of the classical AD biomarkers A β ₄₂ and tau compared to controls (unpublished data); these elevations are strikingly similar to those described in AD [57]. Moreover, higher elevations in A β ₄₂ are seen in veterans that suffered TBI and have residual non-specific neurological impairment (headaches, difficulty concentrating, etc.) compared to those without such impairment (unpublished data). Currently, we are focused on identifying

a practical array of proteins with the ability to interrelate findings between preclinical animal and human studies of TBI and AD, which will additionally allow insight into mechanisms underpinning drugs and their potential impact on both diseases.

4.2 (–)-Phenserine as a pharmacological probe

(–)-Phenserine, ((–)-N-phenylcarbamoyl eseroline) is a carbamate analog of physostigmine. Following its initial pharmacological characterization as an experimental drug for AD at NIA, NIH, it was unsuccessfully developed by industry to exploit AChE and APP synthesis inhibitor activities [69–75]. As a result of continued pharmacological and kinetic studies, we found (–)-phenserine active against an unexpectedly wide range of brain pathologies prominent in TBI and AD: IL-1 β suppression without IL-10 effects; mitigation of glutamate induced excitotoxicity in rat primary hippocampal cultures; protection against H₂O₂-induced oxidative toxicity in the human immortal neuronal cell line SH-SY5Y; reduced A β levels in the cortex of AD Tg (APP Swedish + PS1) mice and in human plasma; enhanced neural precursor cell viability in cell culture with differentiation of neural precursor cells towards a glial phenotype reversed and elevation of neurotrophic BDNF, inhibition of APP and α -synuclein synthesis and AChE inhibition [11,64–66,76–79].

To test for possible *in vivo* effectiveness, we administered (–)-phenserine in studies of animal models of anoxia-stroke, weight drop-TBI, and tg-AD/TBI. Although representing an acute injury to the brain itself, anoxia/hypoxia is of relevance to and occurs in TBI [80,81]. In the anoxia-stroke rat model [65], (–)-phenserine reduced areas of infarction and apoptotic cell death (evaluated by TUNEL staining and NeuN immunohistochemistry, and by triphenyltetrazolium chloride (TTC) staining), increased brain-derived neurotrophic factor (BDNF) and B-cell lymphoma 2 (Bcl-2; an antiapoptotic protein associated with cell survival), but decreased, in brain and SH-SY5Y cells, activated-caspase 3 levels (a pro-apoptotic protein associated with cell death), amyloid precursor protein (APP) and glial fibrillary acidic protein (GFAP) (a marker of activated astrocytes) [65]. (–)-Phenserine also reduced matrix metalloproteinase 9 (MMP-9) in areas of anoxic brain [65]. MMP-9 is involved in the degradation of the extracellular matrix that supports neuronal viability and acts on pro-inflammatory cytokines, chemokines and other proteins to regulate inflammation. Additionally, there is increasing evidence for a role of MMP-9 in both establishing synaptic connections during development and in the restructuring of synaptic networks in the adult brain. Hence MMP-9 levels in brain are highly regulated, with elevations potentially leading to apoptosis and reductions as a therapeutic strategy in TBI and neurodegeneration [82]. After weight drop injury [11], (–)-phenserine preserved Y-Maze and Novel Object Recognition (two well characterized evaluations of spatial and visual memory, which are impaired following TBI in rodents and humans). (–)-Phenserine augmented homeostatic endogenous mechanisms within brain to mitigate oxidative stress, reversed injury-induced gene pathways associated with AD [11], and, at 72 hours post-weight drop injury, reduced neuronal cell losses and neuroinflammation (unpublished data). We interpret these data as demonstrating *in vitro* activities that may *in vivo* mitigate shared AD and TBI pathologies sufficiently to exert clinical benefits through direct effects on pathologies, possible direct inhibition of neuronal cell death responses to hostile environmental pathologies, or a combination of both. Our data also indicate that (–)-

phenserine is properly administered as a sustained release tablet to provide carefully monitored steady-state concentrations in brain between accumulated 25 and 200 nanomolar levels of (–)-phenserine and its metabolites (IC₅₀ ranges 25–100nM) [72]. Both (–)-phenserine and its metabolites inhibit acetylcholinesterase (AChE); therefore, AChE inhibition in red blood cell membranes can be monitored to predict cumulative (–)-phenserine / metabolite brain concentration activities.

5. DISCUSSION

The ultimate aim of AD drug intervention is to prevent the onset of dementia due to losses of neurons. We have found that the TBI animal weight drop model provides an opportunity to study AD drug effects on neuronal death occurring secondary to induced pathologies. Globally over recent decades, hundreds of promising preclinical drug candidates have failed to arrest the progression of AD. Most candidate AD drugs gain traction from successes in AD Tg mouse models. These Tg models represent critical pathologies regarded as responsible for clinical AD. Yet interventions against these pathologies have failed when translated in humans in clinical trials. This suggests that the screening of drugs in animal models should be more rigorous by including models for alternative pathologies. For instance, our TBI model provides accelerated development of neurodegenerative brain pathologies and neuronal compromise and death and can supplement or possibly replace existing models for drug interventions against AD and perhaps other neurodegenerative diseases, as supported by others [83,84,33].

Another way for more rigorous screening of drugs at the preclinical or early clinical trial phases is the insistence on demonstrating target engagement and biomarker response to treatment. Plasma/serum EVs enriched for neuronal origin can serve potentially as a new source of information about the more rapid progression of pathologies following TBI [12,56,68]. These patterns may prove useful in detecting and timing the more slowly evolving pathologies in AD and may also be used to monitor effects from drug interventions.

As a result from probing anoxia, weight drop, and AD related models and *in vitro* mechanisms with (–)-phenserine, we propose that the indirect neuroprotective activities of drugs from mitigation of disease pathologies may be able to be supplemented or even replaced with direct anti-programmed cell death drug activity. *In vitro* (–)-phenserine is active against many of the prominent pathologies present in the shared neurodegenerative cascade [64–66]. Yet, in AD multiple clinical trial interventions against these pathologies, anti-inflammatory, anti- Aβ₄₂ amyloid, cholinergic, antioxidative, and many more, have failed to arrest the disease course in clinical AD. In mice subjected to weight drop head injury, but not in AD tg mice, (–)-phenserine preserves cognitive and functional behaviors lost in the presence of shared neuropathologies [11]. (–)-Phenserine protects neurons against anoxia in a rat model of stroke [65], a finding recently replicated in an independent laboratory (Y Wang, Center for Neuropsychiatric Research, National Health Research Institutes, Taiwan, personal communication). We interpret these data as supporting the alternative explanatory hypothesis that direct neuronal protection against anecrotic cell death may account for (–)-phenserine behavioral effects in concussion/ TBI models and may be a necessary activity for drug efficacy against AD. In effect, the shared TBI-AD

neurodegenerative cascade may be a necessary condition for initiating anecrotic cell death in neurons, but the mitigation of these pathologies may not be a sufficient condition for preventing this anecrotic cell death. We suggest as a topic for further investigation whether neuroprotection and not cascade modification provides the most promising approach to an AD disease delaying therapy.

To succeed in the pursuit of effective treatments for AD and TBI will require a modification to the currently linear pursuit of human drug studies. Under this latter conception of clinical pharmacology, preclinical studies are completed as a preliminary step towards phased human studies leading to drug approval. This commercially compliant model neglects the obvious fact that humans are simply another species with potential for interspecies differences. Rather than reasoning from cumulative preclinical evidence to human proof of concept as a predictor of efficacy, we suggest the earlier use of “small-N” clinical trials involving small well-selected cohorts, with intensively monitored subjects in terms of safety, but also intensive research data acquisition, such that human pharmacodynamic data can become part of further drug development.

Under the current paradigm for drug development, pharmacological probing of the mechanisms in humans and proof of concept (POC) clinical trials to confirm (–)-phenserine efficacy, would likely involve hundreds of subjects and years to complete [85]. Drug synthesis and formulation under Current Good Manufacturing Processes, required for an FDA Investigational New Drug (IND) approval and, in turn, required for NIH clinical trial funding, extract high costs, which increase further when experience evidences the need for reformulations of drugs. Costs for POC studies themselves will easily exceed normal funding ceilings at the National Institutes for Health. In response to this limitation, we have turned our interests towards further development of small-N clinical trial POC methods, which we have already demonstrated as capable of yielding and replicating statistically significant outcome differences in AD [86–89]. Using these small-N clinical trial POC methods, investigators could avoid premature dependence on commercial funding (often accompanied by pressure for rapid advancement into clinical trials), avoid problems encountered when recruiting patients for large-N clinical trial POC studies, and flexibly test critical milestones (i.e. biomarker changes) in small groups of patients. Small-N trials also reduce the exposure of patients to drug candidates lacking any clinical evidence to support potential efficacy. This proposed strategy requires more stringent preclinical and initial clinical testing, such as evidence of drug activities in different but relevant animal models, pharmacodynamic studies probing mechanisms in humans, and finally replication of any successful POC trials to insure validity. Well over 100 AD drug candidates with large-N POC trials have failed in FDA Phase III [2,3,70,72,73,75,90]. As experience has shown, large-N Phase II trials too readily provide investigators with post-hoc analyses used to develop justifications that then fail in Phase III [73–75,91].

6. CONCLUSIONS

Prior studies of anti-inflammatory, cholinergic and other drugs, each one working via a single mechanism, have failed to address the broad range of pathologies following concussion and TBI. To our surprise, after literature review, we found that TBI potentially

provides AD investigators with an accelerated model of the AD's decades long slow progression. As a result, we have explored the use of plasma EVs enriched for neuronal origin to investigate pathological mechanistic activities in the brain and study the effects from pharmacological probing in AD and TBI. As part of these studies, (–)-phenserine emerged as a leading candidate with potential activities against the pathologies shared by TBI and AD. A diverse reversal of persisting or evoked disease specific pathologies may prove someday to decrease the risks for subsequent neurodegenerative disorders. The intriguing evidence we acquired supports possible (–)-phenserine anti-apoptotic and anti-preprogrammed cell death effects, and opens new options for drug developments in AD and other neurodegenerations. Given the widespread failures of drug interventions directly targeting the major AD pathologies, direct neuroprotection by halting apoptosis may prove to be an essential feature for an AD drug to be successful.

The soundest scientific position at present may be to accept that we do not know the critical targets for interventions against AD and that research should focus on probing for and qualifying targets rather than jumping into large clinical trials in a quest for a possible statistical signal rather than significant effect size. Premature large trials draw resources away from mechanistically oriented research into the neurodegenerative processes.

It may be past time for drug development in psychiatry and neurology to update their biology, eliminate errors plaguing research and patient care [74,89], insist upon sounder scientific preclinical and clinical stepwise grounding for all phases of drug development [75], and recognize medicine's primary responsibility to patients by controlling inadequately justified patient exposures to drug candidates and the associated costs [92,93]. Only in these ways can medicine bring Richard Feynman's scientific integrity fully into modern AD drug development and patient care [94]. At this moment, it remains unsettled whether these efforts, further studies of TBI, and the possible development of a drug with anti-programmed cell death activities can delay the onset of clinical AD by the long sought for five or more years. Our hope is that our further research with (–)-phenserine can identify the properties required by such a drug and the optimal conditions for its clinical development. These findings support the utility of combining studies of AD patients with studies of concussion/TBI patients using candidate AD drugs to characterize the time-dependent biochemistry of apoptosis in AD and the effects of a drug intervention.

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Research in Context

- I.** Systematic review: The authors reviewed the literature using PubMed and Google searches for relevant TBI/ AD pathologies. Searches were continued until further searching did not contribute to the data relevant to this study. Critically relevant papers are cited.
- II.** Interpretation: Our literature search findings suggested as plausible the hypothesis that TBIs share important pathologies with AD, which can potentially be probed with both plasma exosomes and (–)-phenserine tartrate. Further studies showed that (–)-phenserine tartrate may offer a possible flanking maneuver capable of informing the nature and timing of targets relevant to the progression of AD pathology and a candidate therapeutic for TBI and AD with the unusual mechanism of action of direct inhibition of self-induced programmed neuronal cell death. An animal model confirmed the potential use of (–)-phenserine to treat concussion/TBI and probe AD neuropathologies. The interpretation of these data suggests the hypothesis that protection against anecrotic or programmed cell death may be critical in the presence of the neurodegenerative cascade shared by concussion/ TBI and AD.
- III.** Future directions: The manuscript identifies the need for preclinical studies of the neurodegenerative cascade shared by concussion/ TBI and AD and for coordinated clinical trials to document and characterize for neurodegenerations' possible drug efficacies associated with neuroprotection from preprogrammed cell death.

Figure 1. Plasma Extracellular Vesicles (EVs) Enriched for Neuronal Origin

Schematic showing the assembly and release of EVs from cells within the brain. EVs are generated by the inward budding of endosomal membranes, with the associated recruitment and internalization of protein and RNA cargo. Following their cellular release EVs travel in the interstitial fluid to neighboring cells or, via the circulation, to distant targets to provide modulatory actions. EVs are found in and can be isolated from plasma/serum samples from animal models and humans. Enrichment for their cellular origin can be achieved by immunoprecipitation with biotinylated antibodies against surface markers to isolate sub-populations from different CNS cell types. The evaluation of their protein and RNA content provides a platform for their use as biomarkers to changes in neurological function, disease states and treatment conditions.

Table 1

Neuropathology in Concussion and Alzheimer's Disease.

Neuropathology	Concussion (TBI)	Alzheimer's disease
Aβ₄₂ Amyloid	<p>Aβ₄₂ amyloid deposits at injury sites [28].</p> <p>Amyloid precursor protein (APP) accumulates in some white matter injury sites [28].</p> <p>IL-1 activation in TBI known to provoke APP synthesis as</p> <ol style="list-style-type: none"> 1. a source for observed APP accumulations and 2. a source for increased Aβ₄₂ concentrations. <p>Aβ₄₂ decreased in the CSF reflects a reduction in Aβ₄₂ clearance in the brain and presumed increased Aβ₄₂ amyloid pathology [15].</p> <p>Plaques 11 months to 17 years post-TBI in areas shared with AD (posterior cingulate cortex) increase with time since injury.</p>	<p>IL-1 up-regulates APP synthesis and Aβ₄₂ in AD [51].</p> <p>Inflammation occurs early in AD [51].</p> <p>Aβ₄₂ amyloid progressively accumulates at affected (temporal and parietal regions) brain sites over 30 or more years. Aβ₄₂ amyloid concentrations are strong predictors of progression [14,15,51].</p> <p>In AD, the earliest pathological changes are low fibrillar Aβ₄₂ deposits with microglial activation only detectable later [14,15,51].</p> <p>A vicious circle occurs whereby Aβ₄₂ amyloid deposits stimulate further cytokine production by activated microglia. Cytokines, IL-1, increases synthesis rates of APP and its Aβ₄₂ metabolites [15,51].</p>
P-Tau	<p>When present the cis form of p-Tau is expected to be not metabolized, accumulate, spread to unaffected cells, and to be neurotoxic [19].</p> <p>P-Tau is found in mice following blast injury [27].</p> <p>In mice TBI activated caspase and calpain were found. These activated enzymes are capable of cleaving tau at various sites [28].</p> <p>Human focal perivascular accumulations of hyperphosphorylated tau (p-tau) as neurofibrillary tangles (NFTs) and neurites and TDP-43 immunopositive neurites are found in the white matter [36].</p>	<p>The cis form of p-Tau is not metabolized, accumulates, spreads to unaffected cells, forms neurofibrillary tangles, and is neurotoxic [17].</p> <p>Hyperphosphorylation of Tau is induced by Aβ₄₂ (See Aβ₄₂ Amyloid).</p>
Neuronal Injury	<p>Contusion and Laceration [28,36].</p> <p>Glial Fibrillary Acidic Protein increased.</p> <p>Neuron Specific Enolase irregularly reported increases. [28,36].</p>	<p>Physical injury to neurons not present as initiating events.</p>
Axonal Injury Markers	<p>Traumatic axonal injury is sometimes focal, multifocal or diffuse [28,29,36].</p> <p>Delayed secondary axotomy occurs [28,29,36].</p> <p>In concussion axons may be injured even in the absence of the death of the neuron of origin [28,36].</p> <p>Persistent axonal swelling and disconnection has been observed to continue for years with possible progressive disability in some individuals [28,36].</p> <p>Massive increases in intra-cellular and -axonal calcium [28].</p>	<p>Physical injury to axons not present as initiating events.</p>

Neuropathology	Concussion (TBI)	Alzheimer's disease
Astroglial Injury Markers	<p>Glial fibrillary acidic protein and S-100 detected in peripheral blood [36].</p> <p>Like microglia, astrocytes release cytokines, interleukins, nitric oxide, and other potentially cytotoxic molecules after exposure to Aβ₄₂, thereby exacerbating the neuroinflammatory response [28,36].</p>	<p>Like microglia, astrocytes release cytokines, interleukins, nitric oxide, and other potentially cytotoxic molecules after exposure to Aβ, thereby exacerbating the neuroinflammatory response [50,51].</p>
Activated microglia	<p>Perivascular clustering of activated microglia [28,36].</p> <p>Produce pro-inflammatory and anti-inflammatory cytokines and other neurotoxic products that generate free radicals [28,36].</p> <p>In mouse TBI Galectin 3 (LGALS3) increased, activated M 1 microglia release reactive oxygen species (ROS) and tumor necrosis factor-α (TNF-α) [28,36].</p> <p>In TBI activated microglia can remain activated for a long period time, sometimes from months to years [28,36].</p> <p>Chronic microglia activation has been linked to microglial aging and various neurodegenerative diseases like AD, amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD). [44].</p>	<p>Activated microglial cells produce and release potentially toxic products, including reactive oxygen species, pro-inflammatory cytokines, excitotoxins, and proteases, which could damage the neighboring neurons [50,51].</p> <p>Microglial activation is induced by aggregated Aβ₄₂ [50,51].</p> <p>Miliary foci with clusters of early (before neuronal loss) activated microglia [51].</p> <p>Activated M 1 microglia release reactive oxygen species (ROS) and tumor necrosis factor-α (TNF-α) [51].</p> <p>Presence of hallmarks of microglial aging: senescence, dystrophy, impaired movement, altered signaling, impaired phagocytosis, and impaired proteostasis [52].</p>
Neuroinflammation	<p>Neuroinflammation present [28,36].</p> <p>Fibrillar Aβ₄₂-amyloid deposits are known to induce, non-immune-mediated, chronic inflammatory-type responses [51].</p> <p>IL-1b, IL-6, IL-10 increased [36].</p> <p>C Reactive Protein in plasma over time raises the question as to whether systemic inflammation complicates clinical course.</p>	<p>Some plaque-associated pro-inflammatory cytokines (i.e., interleukin-1 [IL-1], IL-6, tumor necrosis factor-β [TNF-β], and acute-phase proteins [α 1-antichymotrypsin]) are risks factors for AD [51].</p> <p>Classical cellular immune-mediated responses are not involved in cerebral inflammation [51].</p> <p>Aβ₄₂-amyloid plaque formation [51,52].</p>
Glutamate Toxicity	Abrupt release of glutamate [11,36].	Glutamate-mediated excitotoxicity and neurodegeneration [53].
Oxidative Stress Toxicity	<p>Activated M-1 microglia release reactive oxygen species (ROS) [28,36,54].</p> <p>Aβ₄₂ induces oxidative stress in Tg-AD models, modifies metabolic enzymes with mitochondrial damage. Found in AD models similar activities can occur in TBI [54].</p>	<p>Aβ₄₂ stimulates the production of reactive oxygen species (ROS) and reactive nitrogen intermediates such as nitric oxide (NO). Oxidative stress caused by these species is a well-recognized contributor to neuronal loss and is observed early in AD, and activation of microglial NADPH oxidase is believed to be the primary source of ROS. Oxidative stress is also increased in aging. This is supported by gene-expression profiling of aged brains and age-related increases in the NADPH oxidase enzyme NOX2. NOX2 contributed to microglial ROS production in an LPS-induced model of systemic inflammation. [51,53,55].</p>

Neuropathology	Concussion (TBI)	Alzheimer's disease
Impaired Neurogenesis	In vitro and in vivo non human animal studies APP induces glial cells at the cost of neuronal genesis [95].	In vitro and in vivo non human animal studies APP induces glial cells at the cost of neuronal genesis [95].
Neurovascular Function and Anoxic Stress	<p>Hemorrhage, hematoma, vascular congestion, and diffuse petechial haemorrhages lead to increased intracranial pressure and anoxic stress [36,49].</p> <p>In children, diffuse swelling of the brain can occur after trivial injury due to reactive, rapidly progressive hyperemia [36].</p> <p>Diminished cerebral blood flow can occur and stress brain [36].</p>	<p>Neurovascular system in brain can be degraded by Aβ₄₂ collections and neuroinflammation leading to hypoxic brain stress and damage [51].</p> <p>Hypoxia directly induces amyloidogenic APP processing through several pathways involving β-secretase, γ-secretase, neprilysin, and reduces Aβ₄₂ clearance. [51].</p>
Nucleus Basalis of Meynert Injury	Cholinergic neuron loss within both the basal forebrain and hippocampus following TBI have been described in rodents and humans [36].	Cholinergic neuron loss within Nucleus Basalis of Meynert develops early in Alzheimer's disease. [1,72].
Disruption of Blood-Brain Barrier	<p>Disrupted in mouse TBI [19,36–38,50].</p> <p>Thrombin can enter brain parenchyma through the TBI-disrupted blood-brain barrier (BBB) inducing the proteolysis of tau proteins in the extracellular space after injury to axons.</p>	Blood-brain barrier (BBB) dysfunction may occur at AD onset [52].
Metabolism	"Neurometabolic cascade of concussion" with cellular energy crisis [36].	Impaired glucose metabolism is important to the progression of AD [15].
Mitochondria	Aberrant changes in mitochondrial function: suppression of mitochondrial oxidative phosphorylation, elevation in reactive oxygen species, loss of mitochondrial membrane potential and impaired calcium buffering, associated with post-injury neuronal dysfunction and death [11,19,36,54].	Mitochondrial dysfunction, abnormal dynamics, reduced calcium buffering, oxidative stress damage, lost mtDNA integrity occur causing neuronal and synaptic dysfunction [55].
Necrotic/Anecrotic Cell Death	Induced in animal models of TBI Due to injury induced metabolic cascade [28,36].	Both likely present [51].

TABLE 2

(–)-Phenserine Putative Effects on TBI and AD Shared Neuropathologies

(–)-Phenserine's Six Activities Against Shared TBI-AD Neurodegenerative Pathologies [64]

- Anti-inflammatory action: IL-1 β suppression with preserved IL-10 concentrations
- Suppression of glutamate induced excitotoxicity
- Protection against oxidative stress
- Inhibition of Amyloid Precursor Protein (APP) synthesis and Ab generation
- Neurogenesis/neurotrophic actions
- Counters cholinergic losses from Nucleus Basalis of Meynert (NBM) Injuries

(–)-Phenserine's Activities Against Shared TBI-AD Neuronal Preprogrammed Cell Death [11,64–66]

- Preserved Novel Object Recognition in wild type (wt) mice¹
- Preserved Y-Maze Function in wt mice¹
- Prevention of neuronal cell death² in wt and AD Tg mice¹
- Inhibited TNF- α in microglia in wt and AD Tg mice¹
- Decreases infarct size and neuronal cell death² in rats³
- Mimics anti-p53 outcomes in wt and AD Tg mice¹
- Regulated by the ERK-1/2 signaling pathway³
- Increases anti-apoptotic Bcl-2 and BDNF expression and attenuates pro-apoptotic Bax, activated caspase-3, and active MMP-9³
- Regulates the protein expression levels of APP, Bcl-2 and activated caspase-3, as well as phosphorylation levels of ERK-1/2 in SH-SY5Y cells
- Activities against apoptosis are independent of six activities against shared TBI-AD pathologies

¹Post weight drop concussive TBI challenge [11].

²Fluoro-Jade C and/or TUNEL assay detection of neuronal degeneration.

³Middle cerebral artery occlusion model of stroke [56].