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Insufficiency of Cellular Energy (ICE) May Precede Neurodegeneration in Alzheimer's Disease and Be Treatable via the Alternative Cellular Energy (ACE) Pathway

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Abstract

The term neurodegeneration emphasizes the destruction of neuronal cells as the primary explanation of many major neurological illnesses, including Alzheimer's disease. Specialized functioning of cells requires more cellular energy than is needed for basic cell survival. Cells can acquire energy both from the metabolism of food and from the alternative cellular energy (ACE) pathway. The ACE pathway is an added dynamic (kinetic) quality of the body's fluids occurring from the absorption of an external force termed KELEA (Kinetic Energy Limiting Electrostatic Attraction). KELEA is attracted to separated electrical charges and is seemingly partially released as the charges become more closely linked. As suggested elsewhere, the fluctuating electrical activity in the brain may attract KELEA from the environment and, thereby, contribute to the body's ACE pathway. Certain illnesses affecting the brain may impede this proposed antenna function of the brain, leading to a systemic insufficiency of cellular energy (ICE). Furthermore, individual neurons may derive some of the energy for their own activities from the repetitive depolarization of the cell. This may explain why hyper-excitability of neurons can occur in response to cell damage. This adaptive mechanism is unlikely to be sustainable, however, especially if there is a continuing need to synthesize neurotransmitters and membrane ion channels. The energy deficient neurons would then become quiescent and, although remaining viable, would not perform their intended specialized functions. Actual cell death would not necessarily occur till much later in the disease process. The distinction between quiescent and degenerated cells is important since the ACE pathway can be enhanced by several means, including the regular consumption of KELEA activated water. This, in turn, may improve the proposed antenna function of individual neurons, leading to a sustained restoration of specialized function via the ACE pathway. This paper

explores this novel concept and provides a rationale for clinical testing of KELEA activated water in patients with neurological and psychiatric illnesses, including Alzheimer's disease.

Keywords

Alzheimer's Disease, Alternative Cellular Energy, ACE, Insufficiency of Cellular Energy, ICE, Kinetic Energy Limiting Electrostatic Attraction, KELEA, Homeopathy, Enercel, Enerceutical, Calorie, Metabolism, Electrical Charge, Membrane Potential, Neurodegeneration, Psychiatry

1. Introduction

The ACE pathway was initially identified as providing a non-immunological defense mechanism against stealth adapted viruses [1]. These are derivative viruses, which have either lost or mutated the relatively few virus components normally targeted by the cellular immune system [2] [3] [4]. A cellular repair process occurs in the culturing of these viruses [1] [5]. It results from the production of chemical compounds, which typically self-assemble into particles and longer threads. These particulate materials are commonly pigmented, fluorescent, electrostatic, occasionally ferromagnetic and have electron donating, lipid synthesizing and water activating properties [1] [5]. The latter can be seen in the formation of vapor bubbles when the particles are placed into water [1] [5]. A striking feature of both *in vivo* and *in vitro* stealth adapted virus infected cells is the marked disruption of the cells' mitochondria (the main source of energy from the metabolism of food) [5]. Cellular survival in these cells is attributed to the energy transducing particulate materials, which are accordingly termed ACE pigments [1].

Refeeding of repaired stealth adapted virus infected cultures with fresh tissue culture medium leads to the rapid reactivation of the cytopathic effect (CPE). This can be prevented by adding ACE pigment particles to the refeeding medium [1] [5]. Inhibition of reactivation was also achieved using small amounts of a purportedly homeopathic remedy termed HANSI (Homeopathic Activator of the Natural System Immune). The demonstration of activity of HANSI in tissue cultures clearly excluded a direct role involving the immune system. Based on the ACE pathway concept, the United States manufacturer of HANSI renamed the product to Enercel.

The formulation of HANSI and the early productions of Enercel contained detectable levels of Lidocaine, a dipolar compound. Further studies on virus culture-derived ACE pigments and ACE pigments directly obtained from stealth adapted virus infected patients, led to studies showing that many dipolar chemicals can alter the physical and biophysical properties of water and other fluids [6] [7] [8]. The physical changes in water include the reduction in surface tension, increased volatility and more marked internal dynamic (kinetic) activity.

These changes are attributed to a reduction in the hydrogen bonding between water molecules [9]. A more general principle has emerged that KELEA is a fundamental force required to prevent the fusion and possible annihilation of electrostatically attracted opposite electrical charges. It is seemingly attracted to the separated electrical charges on dipolar molecules. Certain dipolar molecules can release KELEA to nearby water, possibly in an oscillatory manner. Various electrical devices with rapid on-off switching or which repetitively propel opposite electrical charges towards one another can similarly lead to the activation of nearby water [10] [11] [12] [13].

2. Separating and Rejoining Electrical Charges as a Source of Cellular Energy

The membrane partitioning of hydrogen ions and its subsequent channeling back through the membrane provides the driving force allowing ATP synthase to add a third phosphate onto adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) in both chlorophyll-mediated photosynthesis [14] and mitochondria-based food metabolism [15]. A reasonable question is where Nature initially derived the energy to form ATP synthase, chlorophyll, and the complex electron transferring molecules required for mitochondrial oxidative phosphorylation.

An intriguing possibility is that the membrane separation of electrical charges also allows for the attraction of KELEA, which could be partially released as the charges become more closely linked. KELEA could, thereby, provide a primary source of energy from which life has evolved. This process would likely be retained such that depolarization of the membrane potential of cells can act as an antenna to attract KELEA for transfer to both intracellular and extracellular water. This property would, therefore, be a basic energy-generating function of electrically excitable cells including neurons. The spontaneous electrical activity of the brain as reflected in the electroencephalogram (EEG) and in the oscillatory activities of various neurons may reflect the proposed antenna function of the brain in attracting KELEA into the body [16] [17] [18] [19]. The fluctuating electrical activities of muscles, including the heart, may similarly reflect KELEA attracting phenomena.

Support for the possibility that the body has an added energy-generating system is the realization that food metabolism is unlikely to totally provide the daily expenditure of energy by living organisms, including humans. Thus, for a 75 kilogram (Kg) individual to simply maintain body temperature at 20˚C above the average environmental temperature requires 1500 Calories (75 × 20). Since the body heat dissipates in less than 24 hours after death, these 1500 Calories are required daily. A typical diet of approximately 2000 Calories per day would leave insufficient Calories to reasonably account for skeletal muscle, cardiovascular, brain, liver and other physiological functions [20].

Additional support for the concept that the brain may have direct water activating capacity has come from observations on water samples placed within a

room of individuals participating in a laughing yoga class. These samples became more volatile, which is a measure of water activation, than did control samples not placed within the room [21]. The author has also encountered individuals with the ability to directly energize nearby water. They do so by adopting mental states, which they have individually found to be effective. This suggests that if indeed the brain is a major antenna for KELEA, then it is a variable property that can potentially be learned [20].

The ACE pathway provides more than just an addition to the energy derived from food metabolism. Specifically, it enhances resistance to infectious illnesses, having several advantages when compared to the immune system [22]. The ACE pathway may be able to bypass the metabolic blockades presumably preventing apoptosis in some tumors [23]. Ongoing clinical studies are highly suggestive of the ACE pathway contributing to functional activities of the brain that are not directly supported by food metabolism.

3. Insufficiency of Cellular Energy (ICE)

Neuronal cells can become deficient in cellular energy if their capacity to generate ATP from food metabolism is limiting. This can occur from reduced blood supply of nutrients and/or oxygen, along with an inability to effectively remove carbon dioxide, urea, and other metabolic waste products. Cellular energy deficiency can also arise from intrinsic defects in various metabolic pathways. These defects can be primarily genetic or secondary to external factors, including toxins and microbes. The normal functioning of neuronal cells is presumably also dependent upon the ACE pathway as it exists throughout the body. Individual neurons may also depend upon locally generated KELEA resulting from their own repetitive depolarization.

Establishing the membrane potential in most cell types, including neurons, requires the active transport of sodium (Na^+) ions from within the cell to the extracellular space. The same transporter imports one-third less potassium (K^+) ions into the cell [24]. Depolarization with the formation of an action potential occurs by an induced major influx of Na^+ ions into the cell. The inflowing Na^+ ions must then be secreted from the cell to repolarize the cell membrane. The Na/K transporter utilizes ATP as an energy source. Depolarization/repolarization, therefore, requires ongoing chemical energy [25]. It is proposed that some of this energy usage may be offset by, or at least essentially exchanged for the delivery of KELEA into the cell.

This energy gathering process is likely to be far more efficient for depolarizations occurring in unicellular organisms than for multicellular organisms with a networking nervous system. This is because the synthesis, secretion and reuptake of neurotransmitters at synaptic junctions add to the energy output of neuronal activity. Indeed, synaptic impulse transmissions utilize more ATP than does the generation of action potentials [26] [27] [28] [29] [30]. Actual studies on brain metabolism indicate that neuronal activities impose a significant drain on cellular metabolism. Still, if depolarization of unicellular organisms is an

evolutionarily net source of cellular energy, then more frequent depolarization may have persisted as a cellular adaptation of electrically excitable cells, including neurons, to ICE.

The full opening of the Na^+ channel is triggered at a threshold level that requires sufficient reduction in the differential electrical charge across the membrane. The electrical charge on the inner side of the resting cell membrane is approximately −70 millivolts (mV) with respect to the outside of the cell membrane. The Na^+ input channel is triggered at approximately −50 mV. Thus, a lessening of the membrane potential, for example from −70 mV to −60 mV, will lead to depolarization in response to minor stimuli that are unable to trigger cells with a normal membrane potential. Indeed, a lowered differential electrical charge is an early characteristic of neuronal cell damage [31] and by inference neuronal hyperactivity may be expected as an early manifestation of ICE.

As suggested above, repeated depolarization of a neuro-networking brain is a drain on the brain's cellular energy. In addition to the chemical energy demands of synaptic transmission, continuing hyperactivity appears to increase the turnover of ion channels. These channels are heavily glycosylated molecules and, therefore, cannot be readily brought back into the cell for recycling. If activation increases their turnover, then cellular energy will be required to maintain adequate numbers of ion channels within the external cell membrane [32]. The loss of ion channels would reduce ACE pathway input. The energy deficient neuronal cells would then enter a quiescent, survival mode of existence.

The progressive loss of cellular energy will eventually lead to cellular death and true neurodegeneration. This may, however, be a much latter phase of many neurological illnesses than is commonly envisioned. This reasoning applies to Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease and even aging.

4. Alzheimer's Disease

The underlying cause of Alzheimer's disease is still unknown. Genetic factors play a major role as does advancing age [33]. Overproduction of phosphorylated tau protein and both the overproduction and aberrant enzymatic cleavage of amyloid precursor protein (APP) occur in Alzheimer's disease patients [34]. Direct cellular toxicity has been attributed to both tau and amyloid-derived compounds, although efforts to reduce their levels have not shown major clinical benefits [35] [36]. As discussed elsewhere, it is feasible that neuronal cell damage leading to the overproduction of these materials may also occur from underlying stealth adapted virus infections. The notable accumulation of these materials in the brains of Alzheimer's disease patients, but not in younger stealth adapted virus infected patients, may be related to age-related inefficiency of a basic clearance mechanism [37].

Relevant to this paper are indications of possible hyperactivity within regions of the brain, including the hippocampus and motor cortex, as an early feature in Alzheimer's disease [38] [39] [40] [41] [42]. Neuronal hyperactivity in cognitive

pathways may contribute to dementia by distracting from the comprehension and interpretation of specific thought processes. Patients with Alzheimer's disease commonly display positive psychiatric symptoms, such as delusions, hallucinations, and agitation [43] [44]. Although potentially attributed to a loss of inhibitory neurons, these symptoms are consistent with intrinsic neuronal hyperactivity. The occurrence of new-onset epilepsy in conjunction with Alzheimer's disease [45] [46] [47] is also consistent with an initial hyperactivity phase of the illness.

Alzheimer's disease patients progress to illnesses in which there is clear hypofunctioning of multiple regions of the brain. The neurological deficits extend beyond impaired cognition in patients with advanced Alzheimer's disease. Most patients exhibit emotional apathy, social withdrawal, depression, blurred speech, impaired hearing, loss of smell, autonomic dysfunction, delayed reflexes, and poorly coordinated muscle activity [48] [49] [50] [51] [52]. Although the loss of neuronal cells can be demonstrated histologically, the signs and symptoms are not necessarily entirely due to neuronal degeneration. Rather, there may also be a major component of neuronal cells simply failing to engage in their intended specialized functions.

5. Clinical Improvements in Alzheimer's Disease Patients

Improvements resulting from lifestyle interventions have been observed in several Alzheimer's disease patients. The interventions primarily involve changes in diet, reduction of stress levels and/or increased aerobic exercise. This is still a controversial topic with some neurologists suggesting that significant reductions in symptoms preclude the earlier clinical diagnosis of Alzheimer's disease. Nevertheless, clinical improvements are consistent with neuronal cell dysfunction, as opposed to irreversible cellular degeneration. The dietary changes include switching to either a ketogenic [53] or a Mediterranean diet [54] usually in addition to consuming various dietary supplements. The common dietary supplements include medium chain triglycerides; phosphatidylcholine and other membrane lipids; *Moringa oleifera*; turmeric; cocoa; niacin; and others [55]-[62]. A trusted colleague has told me that she has achieved consistent cognitive improvements in well over fifty elderly Alzheimer's patients during the last several years. Her therapies include the regular consumption of water containing sodium chloride-depleted minerals from the Great Salt Lake, other dietary supplements and having her patients adopt an optimistic, mindfulness, mental attitude. Most alternative medical practitioners are likely to attribute any apparent clinical benefits of dietary supplements either to an assumed anti-oxidant activity or to the correcting of supposed underlying nutrient or mineral deficiencies [62]. KELEA is absorbed by dipolar chemicals and it can be argued, that the reported beneficial dietary compounds act by increasing the supply of KELEA to the body [7]. The term Enerceutical has been suggested for compounds with KELEA attracting and water activating properties [7]. It is further possible that even the willingness to make dietary changes reflects a basic change in brain activity, which may co-

incidentally enhance its KELEA antenna function. Similarly, the decision to minimize stress or to engage in more vigorous exercising may be shown in future clinical trials to increase the brain's KELEA absorbing capacity.

6. Controlled Studies on Enhancing the ACE Pathway

There are multiple ways to activate the ACE pathway and some are particularly well suited to double-blinded clinical trials. Among the more informative trials are the direct comparisons between matched groups of patients consuming either KELEA activated or regular water. Sufficient water activation for initial clinical studies can be provided by simply placing water into KELEA concentrating energy fields, as can be achieved by opposing fluctuating lights [12] and by other methods. Another approach is to use dipolar herbal components with subsequent, repeated dilutions to essentially reduce the residual concentrations to below detectable levels [63]. One study showed remarkable benefits of injecting and inhaling Enercel in tuberculosis-infected AIDS patients [64]. A striking feature of the study was the improved mood and cognition that occurred in addition to the clearance of the mycobacteria and the reduction in HIV levels. Current test protocols in this and other medical conditions now involve the drinking of approximately 500 ml per day of KELEA activated versus control water. Employing yet another protocol to enhance the ACE pathway, the healing of herpes virus infections has been expedited [65]. ACE pathway activation has clinically helped children with autism, including leading to the permanent suppression of epilepsy in a child [66].

7. Conclusion

This paper provides the rationale for clinical studies on the possible therapeutic value of activated water in patients with Alzheimer's disease. A major premise of the paper is that cells can acquire cellular energy via the alternative cellular energy (ACE) pathway. It is expressed as an added kinetic (dynamic) activity of the intracellular and extracellular fluids within the body. The energy for the ACE pathway comes from the absorption of a natural environmental force termed KELEA (Kinetic Energy Limiting Electrostatic Attraction). It is proposed that unlike most other cell types, neuronal cells may be able to directly attract KELEA from the external environment during their electrical depolarization. Indeed, repetitive depolarization may be an initial adaptive response of neuronal cells to an insufficiency of cellular energy (ICE). The damaged neuronal cells may progress to become hypo-responsive, quiescent cells [67]. As such, although still viable, the neurons would be unable to perform their intended more specialized functions. KELEA can be transferred into water for drinking and consuming KELEA activated water can be compared with consuming regular water for possible therapeutic benefits in patients with various neurological illnesses, including Alzheimer's disease. Clinical efficacy in such studies will naturally lead to major efforts at disease prevention through the support of the ACE pathway.

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Abbreviations

ACE	alternative cellular energy
ICE	insufficiency of cellular energy
KELEA	kinetic energy limiting electrostatic attraction
ATP	adenosine triphosphate
ADP	adenosine diphosphate
Na^+	sodium ion
K^+	potassium ion
mV	millivolt
Kg	kilogram

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Role of GSK3β and PP2A on Regulation of Tau Phosphorylation in Hippocampus and Memory Impairment in ICV-STZ Animal Model of Alzheimer's Disease

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Abstract

Intracerebroventricular administration (ICV) of streptozotocin (STZ) in rats has been associated to desensitization of the insulin receptor (IR) and biochemical changes similar to those occurring in Alzheimer's disease (AD) or older brains, so it has been proposed as a suitable model for studying some of the pathological features of AD sporadic type (SAD). In this study, we investigated the role of glycogen synthase kinase 3β (GSK3β) and protein phosphatase 2A (PP2A) in the regulation of the phosphorylation of tau (p-tau). Results showed that ICV-STZ treated rats had deficits in short- (1.5-h) and long-term (24- and 48-h) memory after one month of ICV-STZ treatment and six months relative to control rats. The memory deficit was associated to increasing [$F(3, 12) = 31.48$, $p < 0.0001$] p-tau in the hippocampus but not in prefrontal cortex (PFC). Likewise, STZ reduced phosphorylation of GSK3β (p-GSK3β) and PP2A in hippocampus and PFC, indicating that GSK3β and PP2A contributed to regulation of p-tau. These data supporting the model with ICV-STZ in rat are adequate to study the progressive memory impairment associated to hyperphosphorylation of tau and the cascade of insulin receptor signaling; confirm that phosphatidyl-inositol-3 kinase-protein kinase B (PI3K-PKB/Akt-GSK3β) and PP2A are involved in the modulation of proteins responsible for the regulation of neurodegeneration in AD.

Keywords

Memory Deficit, Tau Hyperphosphorylation, GSK3β, PP2A, Streptozotocin, Hippocampus

1. Introduction

Alzheimer's disease (AD) is the leading cause of dementia and is characterized by progressive memory loss and a gradual decline in cognitive function, eventually leading to premature death of the individual, which occurs typically 3 - 9 years after diagnosis [1]. The neuropathological hallmarks of AD are extracellular senile plaques containing aggregates of the β-amyloid peptide (Aβ), and intraneuronal neurofibrillary tangles (NFTs) [2] [3] [4] that consist mainly of intracellular and abnormally phosphorylated tau protein [3] [5]. These pathologic features are accompanied by decreased synaptic density, which eventually leads to widespread neurodegeneration, loss of synapses and failure of neurotransmitter pathways, particularly those of the basal forebrain cholinergic system, especially in the hippocampus and cortex [3] [6]. Tau phosphorylation (p-tau) is regulated by numerous Ser/Thr and phosphatases, including glycogen synthase kinase 3β (GSK3β) and protein phosphatase 2A (PP2A); both are considered to be the major kinase and phosphatase *in vivo* [7] [8] [9].

Several evidences have been associated between insulin dysfunction and AD [10]-[16]. For instance, hyperphosphorylation of tau protein has been associated with brain insulin deficiency or disorder of insulin signal transduction [17]. A *post-mortem* study showed that the hippocampal formation in AD cases without diabetes, exhibited markedly reduced receptor/insulin receptor substrate1/ phosphatidyli-inositol-3 kinase (IR-IRS1-PI3K) signaling pathway [18]. The other study showed that peripheral hyperinsulinemia correlated with an abnormal removal of Aβ and an increase in tau hyperphosphorylation, consequently of increased GSK3β activity [19]. In fact, hyperinsulinemia and Type II diabetes mellitus are considered risk factors for SAD [16] [20] [21] [22] [23]. Importantly, diabetes animal models suggest that peripheral insulin signaling dysfunction plays a key role in modulating AD pathology. Indeed, streptozotocin (STZ) administration (200 mg/kg i.p.) produced increase of tau phosphorylation in the brain of non-transgenic mice [7]. Likewise, the ICV administration of low STZ doses (1 - 3 mg/kg) reproduces aspects of SAD abnormalities, including decreased glucose utilization in rat cortical regions and hippocampus [24] [25] [26] [27], cholinergic deficits [25], increase in oxidative stress [28] [29] [30], decrease of IR expression and hyperphosphorylated tau protein in the hippocampus [31], and amyloid formation in leptomeningeal vessels [30]. All these changes are associated to memory impairment, and tau pathology, resulting in central insulin dysfunction [30] [31] [32] [33]. Therefore, herein we are testing that ICV-STZ animal model provides key information about the role of brain insulin disruption in AD pathology, hence, studying the effect at six months following icv STZ administration on memory function, phosphorylated levels of tau and GSK3β, and PP2A levels.

2. Methods

2.1. Animals

Male Wistar 4-month-old rats weighing 320 - 340 g were used. The animals were

placed on an individual cage after STZ administration during recovery time after surgery (one week). During the experimental phase they were maintained at 22˚C ± 2˚C and 12 h light/dark cycle, with free access to food and water previous to experimental manipulation. The experimental protocol was revised and approved by the Institutional Review Committee (CICUAL; Project No. 047/02) for the use of animal subjects in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication No. 85 - 23, revised 1985).

2.2. Surgical Procedure and Icv STZ Administration

Adult male Wistar rats were anesthetized with ketamine (75 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). The animal body was placed in position in the stereotaxic apparatus and in its head a midline sagittal incision was made in the scalp. Then, holes were drilled in the skull on both sides over the lateral ventricles. The following coordinates were used for ICV injection: 0.8 mm posterior to Bregma, 1.5 mm lateral to sagittal suture, 3.6 mm ventral from the surface of the brain [28]. Coordinates for placement of cannulas were determined by using the atlas of-Paxinos & Watson [34]. STZ was dissolved in citrate buffer (CB; pH 4.5; 28, 32) just prior to injection. The STZ group was injected bilaterally with STZ (3 mg/kg) in two divided doses on Days 1 and 3 asreported previously [28]. The concentration of STZ was adjusted so as to deliver 2 μL/ventricule of the solution as described previously [32]. In control group were given icv injection of the same volume of CB on 1 and 3 day as in STZ injected rats. Post-operatively, as above mentioned rats were maintained in an individual cage and received free access to food and water. One week previous to the behavioral task body weights were reduced to 85% by gradually reducing food intake until the last experimental day in order to perform the behavioral task.

2.3. Behavioral Protocol

2.3.1. Autoshaping Learning Task

In an autoshaping or sign-tracking setting, a hungry animal is placed in a conditioning chamber to find food pellets (unconditioned stimulus [US]) in the food-magazine and is then given a Pavlovian sequential pairing (stimulus-stimulus [S-S]) of a lighted key or a retractable-illuminated lever (conditioned stimulus [CS]) and food (US). After a number of such presentations, the animal approaches the CS and presents instrumental responses (conditioned response [CR]), such as peck, nose-poke, and lever-press responses. Then, CR or autoshaped responses result from the S-S association and are sustained by response-stimulus (R-S) association [35]. Importantly, within the continued progress of behavioral memory tasks development, a Pavlovian/Instrumental autoshaping (P/I-A) task combines both Pavlovian and instrumental conditioning; which offers the opportunity to study hippocampus-mediated declarative memory and striatum-mediated R-S "habit formation" [36]. Furthermore, except for magazine training, P/I-A is almost completely automatized, considerably reducing human intervention.

It is sensitive to small increases or decreases in various behavioral parameters (*i.e.*, not measuring the same event twice), including sign tracking (*i.e.*, conditioned behavior directed toward the localized retractable and illuminated lever; CS), and goal tracking (*i.e.*, the place where the US is delivered). The latest is quite important, as it allows the study of bidirectional expression of an enhanced or impaired memory formation. P/I-A clearly separates training for testing sessions, and it has been useful to detect changes in memory formation elicited by drugs or aging [35].

2.3.2. Food Magazine and Autoshaping Training

Following one month of the icv STZ administration, individually, each rat was placed in an experimental chamber for a habituation period (≈15 min) with access to 50 food-pellets (45 mg each) previously placed into the food magazine. The criterion was that once the animal ate all 50 food-pellets and presented 150 nose-pokes (as measured by a photocell) into the food-magazine, the autoshaping training program was initiated [35] [37]. The autoshaping program had been reported previously [37] [38], and this consisted of discrete trials. A trial began with the presentation of a retractable and illuminated lever for 8 s (conditioned stimulus; CS) followed by a food-pellet (unconditioned stimulus; US) delivery. There was an inter-trial interval time (ITI) of 60 s. When the animal pressed the CS, it was considered a conditioned response (CR), which shortened the trial, retracted the lever, turned off the light, and a US was delivered. The CR increment or decrement was considered an index for enhancement or impairment in memory consolidation, respectively. There was an autoshaping training session (10 trials) lasting nearly 12 min, and three training/testing (20 trails each) sessions, lasting nearly 24 min. All sessions were conducted over three consecutive days. The autoshaping training session is followed by consecutive training/testing sessions at 1.5 h for short-term memory (STM), and 24 and 48 h for long-term memory (LTM). Subsequently, a 20 minute testing session was conducted each month for 6 consecutive months (**Figure 1**).

2.4. Measured of Brain Proteins

2.4.1. Tissue Preparation

As previously reported [38], the rats treated with CB or STZ were sacrificed by decapitation after the autoshaping testing session at 6 months (**Figure 1**), and their brains were quickly removed, placed on ice in order to dissect the prefrontal cortex and hippocampus (from CA1 area to dentate gyrus) for each group according to Paxinos and Watson, 2005. Hippocampal and prefrontal cortex were homogenized with lysis buffer containing 150 mM NaCl, 50 mMTris-HCl, 5 mM EDTA and protease inhibitors (PMSF, aprotinin, leupeptin and pepstatin). The lysates were centrifuged at 12,000 g per 20 min at 4˚C. The supernatants were removed and kept in new Eppendorf tubes. Samples were frozen and stored at −70˚C until further analysis. Total protein concentration was determined. The amount of protein was assayed according to the method of [39] (Sigma; Cat. No. B6916). For calibration curve bovine serum albumin (BSA) standard was used (Sigma).

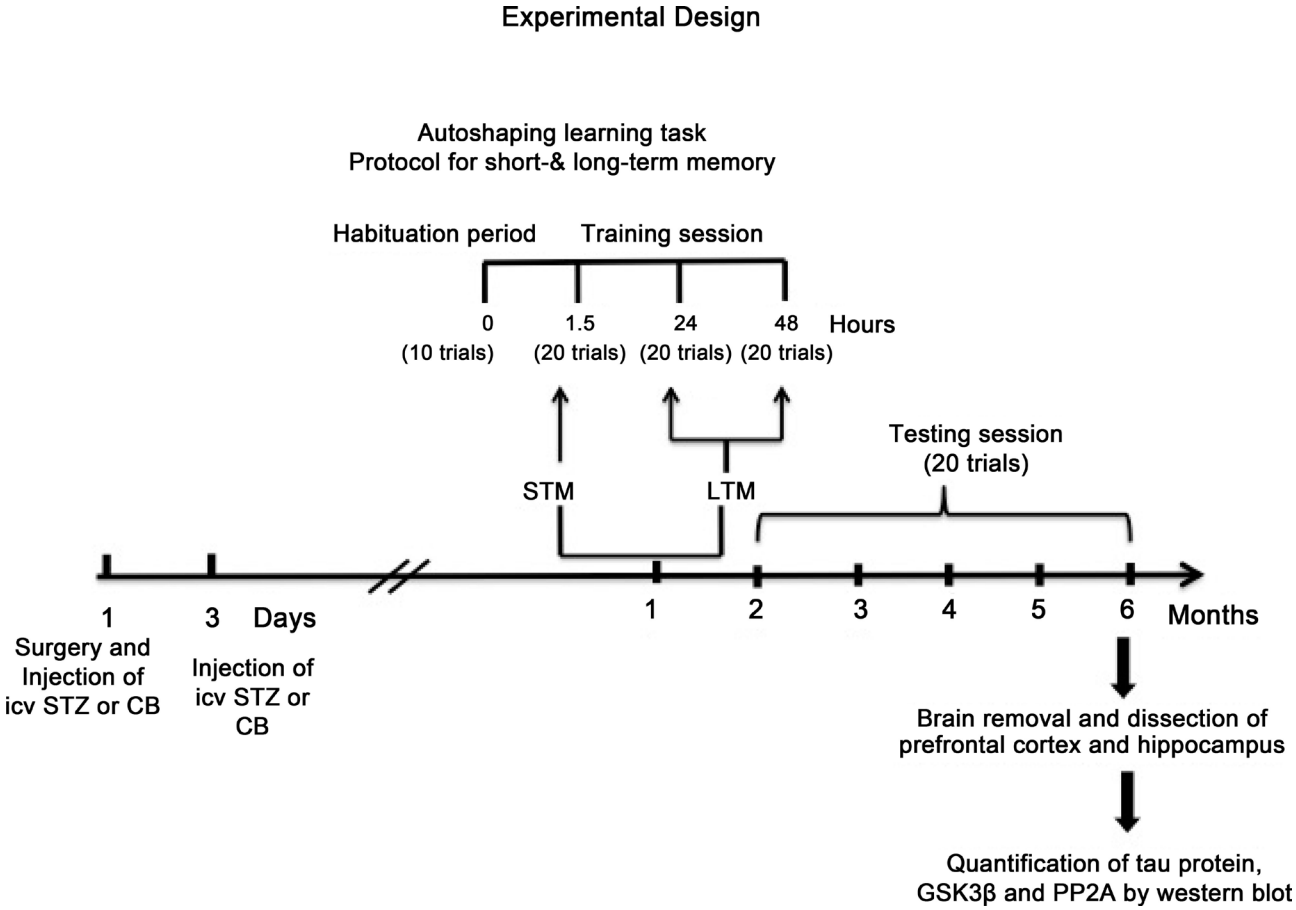

Figure 1. Experimental design. Under anesthesia Wistar rats were bilaterally injected in lateral ventricles with streptozotocin (STZ) or citrate buffer (CB; vehicle) twice on day 1 and 3, after one month short- (STM) and long-term memory (LTM) was assessed in autoshaping, an associative learning task. Subsequently, testing session was conducted each month for 6 consecutive months. The animals were sacrificed at 6 months, the brains were removed and the hippocampus and prefrontal cortex were dissected in order to measure tau protein, GSK3β and PP2A by western blot. ICV: intracerebroventricular.

The concentration was determined by measurement of the absorbance at 595 nm.

2.4.2. Western Blot

Equal amounts of total protein (20 μg per sample for enzyme for analyses) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% polyacrylamide gels and transferred to Polyvinylidenedifluoride (PVDF) membrane [40] [41]. The PVDF membranes were blocked by incubation in 5% non-fat milk added to phosphate buffered saline (PBS-T) containing 137 mM de NaCl, 2.7 mM de KCl, 10 mM de Na_2HPO_4, 2 mM de KH_2PO_4, pH 7.4, 0.5% Tween 20, 1 h at 22˚C for p-GSK3β and total GSK3β. Blocked blots were incubated on the next day with primary antibody anti-phospho-GSK3β (Ser9) rabbit (1:1000; Cell Signaling, Inc.), total GSK3β (27 C10) rabbit (1:1000; Cell Signaling, Inc.), Tau (Tau46) Mouse (1:10,000; Cell Signaling, Inc.), phospho-tau (Ser396) (PHF13) Mouse; (1:1000, Cell Signaling, Inc.), PP2A subunit A (6G3) rat (1:1000, Cell Signaling, Inc.) and anti-GAPDH clone 6C5 (1:5000; Milipore, Inc.) overnight at 4˚C. After incubation, the membranes were washed three times

with PBS-T 1% and incubated for 1 h at room temperature with secondary antibody solution anti-rabbit IgG (1:2000) p-GSK3β, total GSK3β, total tau, phospho-tau and PP2A (1:2000; Milipore, Inc.), and anti-mouse IgG (1:10,000, Milipore, Inc.) for GAPDH. The specificity of the signal was checked on control membranes that were not incubated with primary antibody. After washing three times in PBS, the membranes were immunostained using chemiluminescence western blotting detection reagents (Bio Rad) and exposure to an X-ray film. Relative optical density of bands was analyzed using MCID gel analysis software, Imaging Research Inc.

2.4.3. Statistical Analysis

The values of the conditioned responses (CR) were expressed as a percentage of the total trials (10 or 20) per session (mean ± SEM) in the autoshaping test, meaning that, e.g., 2 - 3 CR corresponded to 20% - 30%. The CR was expressed as means (±SEM) and was analyzed by Student's t test (two groups). The n per group was 6 - 8 animals. The p-tau, total tau, p-GSK3β and total GSK3β values were expressed as means (±SEM) and they were analyzed by one way ANOVA (Three or more groups) followed by Tukey test post-hoc. The PP2A was expressed as means (±SEM) and they were analyzed by Student's t test (two groups). In all comparisons $p < 0.05$ was considered as significant. The n per group was 4 - 5 animals. The statistical software used was GraphPad Prism version 6.00 for Macintosh, San Diego California USA.

3. Results

3.1. Short and Long Term Memory Deficit in Icv STZ-Treated Rats

The results showed that one month after icv injection of STZ did not change conditioned responses (CR) during phase training. Nevertheless, CR significantly $t(18) = 3.128$, $p = 0.0058$ decreased during STM (1.5 h) and $t(18) = 3.16$, $p = 0.0054$; $t(18) = 4.514$, $p = 0.0003$ LTM (24 & 48 h) relative to vehicle animals (Figure 2(a)). Memory was assessed every month for a period of six months, and the memory deficit was significantly maintained $t(14) = 8.804$, $p < 0.0001$; $t(14) = 2.98$, $p = 0.0099$; $t(14) = 5.688$, $p < 0.0001$; $t(10) = 5.179$, $p = 0.0004$; $t(10) = 5.440$, $p = 0.0008$; $t(12) = 4.371$, $p = 0.0009$, respectively (Figure 2(b)).

3.2. STZ Effect on Tau Phosphorylation (P-Tau)

Western blot analysis (Figure 3(a)) demonstrated that p-tau levels were significantly [$F(3, 12) = 31.48$, $p < 0.0001$] higher in STZ groups than the control group at 6 months in the hippocampus (Figure 3(b)). There were no changes in total tau levels (Figure 3(b)). The ratio between p-tau/tau was significantly $t(4) = 2.456$, $p = 0.0494$ increased (Figure 3(c)) in STZ

Western blot analysis (Figure 4(a)) in the PFC p-tau levels significantly [$F(3, 12) = 21.01$, $p < 0.0001$] augmented in STZ groups compared with controls at 6 months (Figure 4(b)). However, total tau levels significantly [$F(3, 8) = 9.154$, $P < 0.0058$] also increased in STZ group compared to control (Figure 4(b)). With

respect to the ratio between p-tau/tau total was not observed significant differences in control and STZ rats (Figure 4(c)).

3.3. STZ Effect on GSK3β Phosphorylation (p-GSK3β)

Western blot analysis (Figure 5(a)) showed the values of p-GSK3β levels significantly [F(3, 12) = 31.81, p < 0.0001] decreased in STZ groups compared with the control group at 6 months in hippocampus. The total GSK3β showed no significant changes in STZ groups in relation to the controls groups (Figure 5(b)). The ratio between p-GSK3β/GSK3β significantly $t(4) = 6.073$, p = 0.0009 dropped in the STZ group (Figure 5(c)).

In the PFC (Figure 6(a)), p-GSK3 levels did not changes at 6 months in rats with STZ in relation to control group. Total GSK3β and the ratio between p-GSK-3β/GSK3β were unchanged compared with control (Figure 6(b) and Figure 6(c)).

3.4. STZ Effect on PP2A Levels

PP2A levels significantly $t(4) = 5.730$, p = 0.0012 decreased at 6 months in the

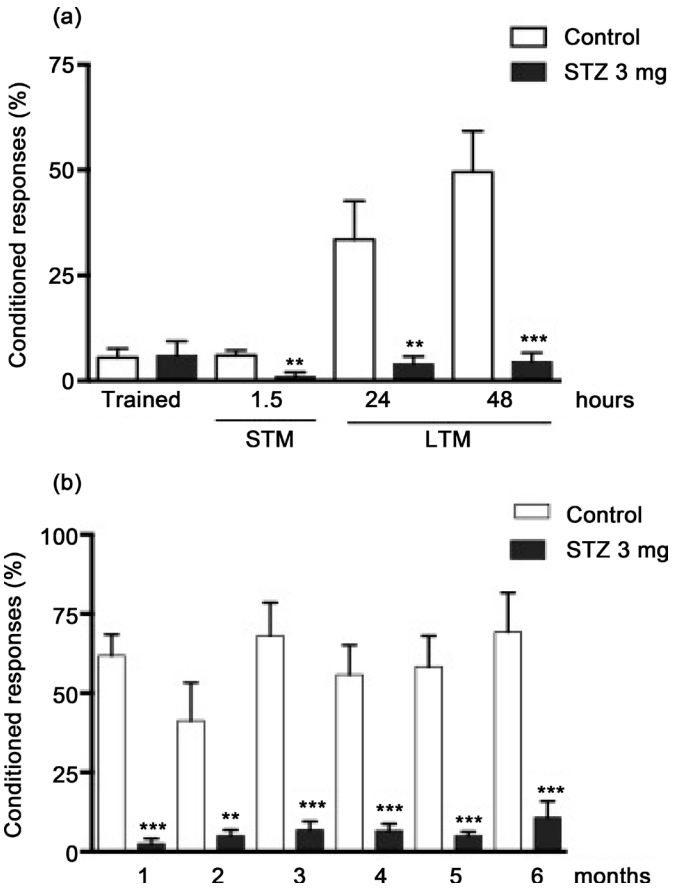

Figure 2. Short and long term memory deficit in icv STZ-treated rats. (a) Short (STM; 1.5 h) and long-term memory (LTM; 24 and 48 h) were evaluated one month after STZ icv injection; (b) Memory was evaluated each month during a period of 6 months. Data are plotted as mean ± SEM of conditioned responses. n = 6 - 8. Student's *t* test, *p < 0.05 control vs. treated groups. STZ: streptozotocin.

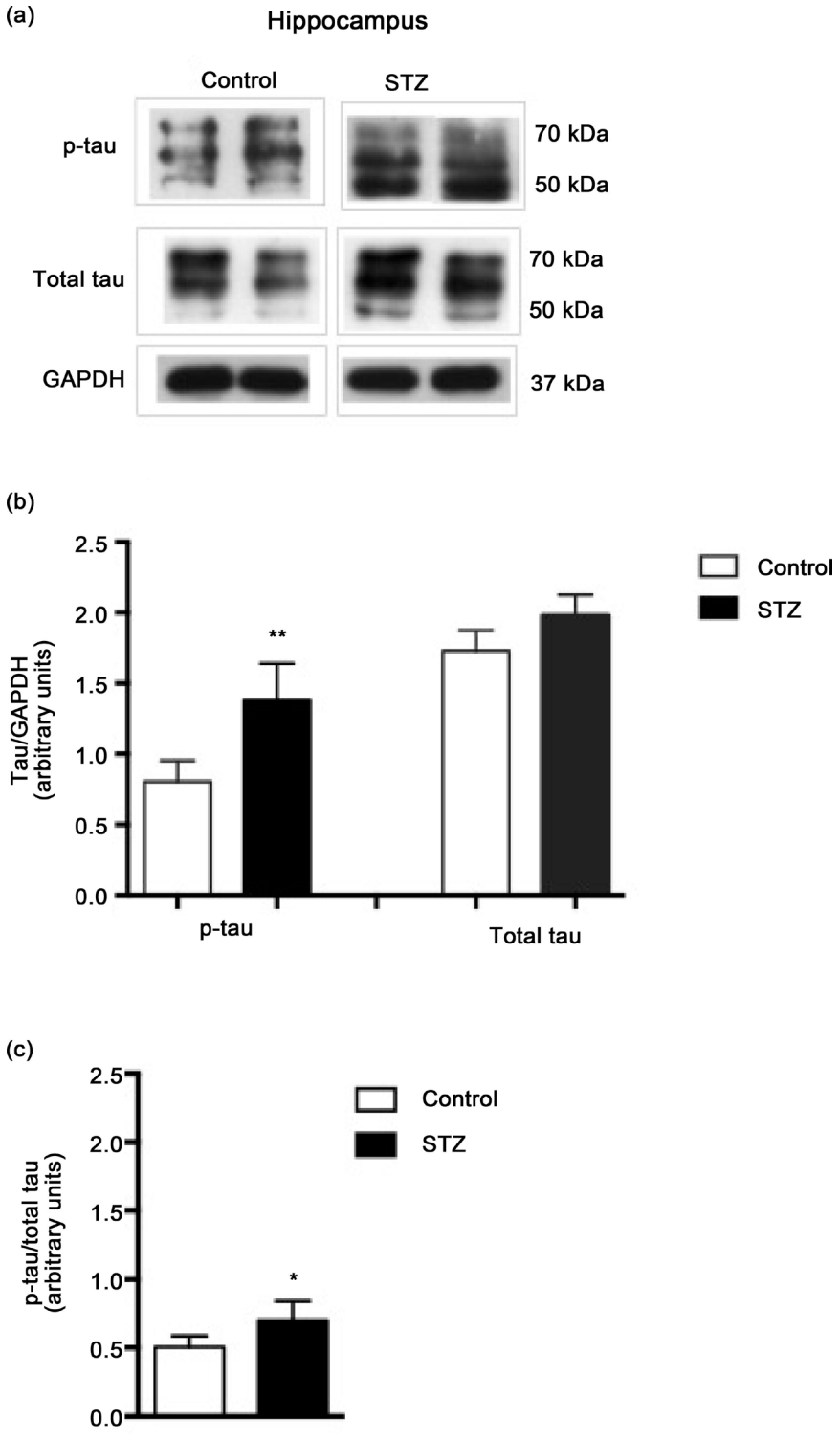

Figure 3. STZ effect on p-tau in hippocampus. (a) Representative immunoblots of p-tau and total tau, (b) tau phosphorylation and total tau and (c) p-tau/total tau ratio. Six months after injection of STZ-treatment, hippocampal protein extracts were immunoblotted for p-tau (Ser396) (PHF13) and total tau (Tau46). Quantification of tau was normalized against GAPDH. (b) Data expressed as mean ± SEM, n = 4 - 5 animals per group. Significant differences (**$p < 0.01$) in STZ vs. control group; one-way ANOVA followed by Tukey test. (c) Data are presented as mean ± SEM, n = 4 - 5 animals per group. Student's *t* test, *$p < 0.05$ relative to control group.

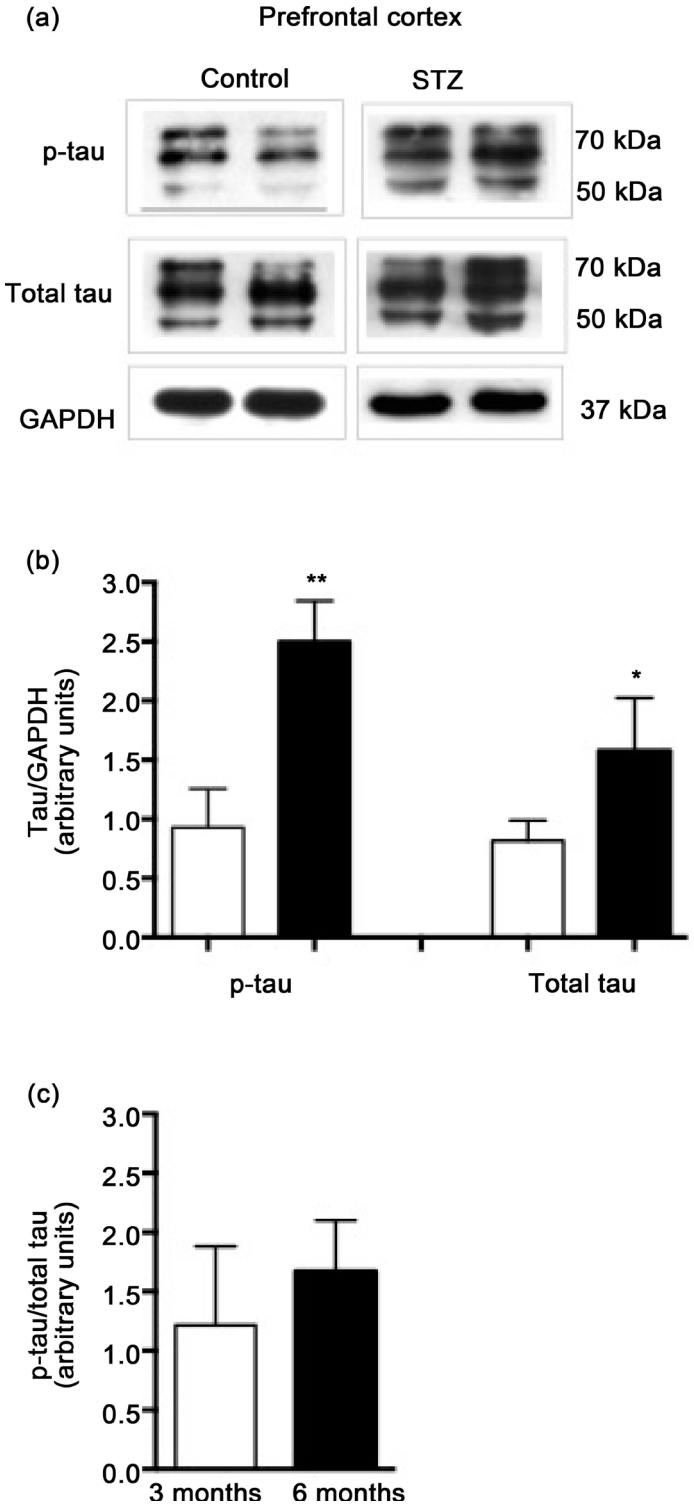

Figure 4. STZ effect on p-tau in prefrontal cortex. (a) Representative immunoblots from a typical experiment, (b) tau phosphorylation and total tau and (c) p-tau/total tau. Six months after injection of STZ prefrontal cortex protein extracts were immunoblotted for p-tau (Ser396) (PHF13) and total tau (Tau46). Quantification of Ser396 p-tau and t-tau were normalized to GAPDH. Data expressed as mean ± SEM, n= 4 - 5 animals per group. Significant differences (*$p < 0.05$, **$p < 0.01$) in STZ vs. control group; one-way ANOVA followed by Turkey test. (c) Data are presented as mean ± SEM, n = 4 - 5 animals per group. Student's *t* test.

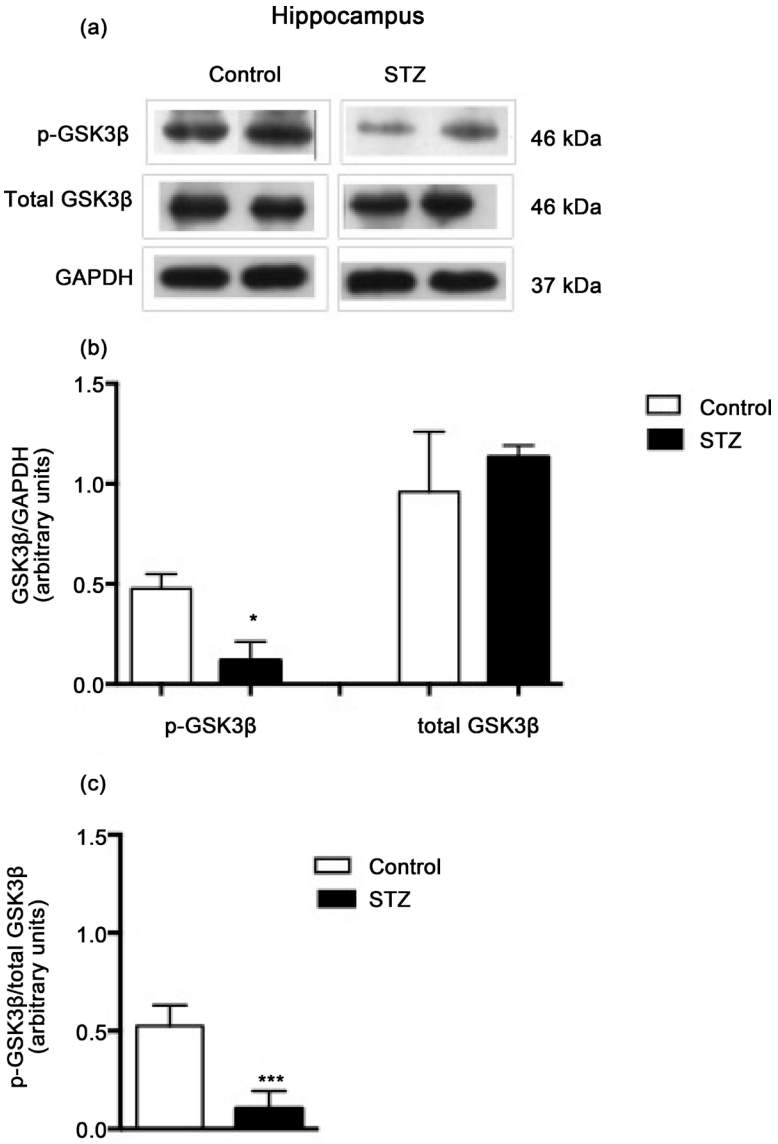

Figure 5. STZ effect on p-GSK3β in hippocampus. (a) Representative immunoblots of p-GSK3β, (b) Total GSK3β and (c) p-GSK3β/total GSK3β. Six months following STZ-treatment, hippocampal protein extracts were immunoblotted for p-GSK3β (Ser9) and total GSK3β (27 C10). Quantification of GSK3β was normalized against GAPDH. (b) Data expressed as mean ± SEM, n = 4 - 5 animals per group. Significant differences (*$p < 0.05$) in STZ vs. control group; one-way ANOVA followed by Turkey test. (c) Data are presented as mean ± SEM, n = 4 - 5 animals per group. Student's *t* test, *$p < 0.05$ relative to control group.

hippocampus of rats with STZ icv compared with the control group (**Figure 7(a)**, **Figure 7(b)**). PP2A levels did not change at 6 months (**Figure 7(c)**, **Figure 7(d)**) in the PFC of rats with STZ in relation to control group.

4. Discussion

The present study found STM and LTM, and progressive memory impairment following one and six months of icv STZ injection, respectively. It is known that insulin and IR are selectively distributed in the brain, including olfactory bulb,

hypothalamus, cerebral cortex, amygdala and hippocampus [42] [43]. The expression of IR in cerebral cortex and hippocampus suggests that insulin is involved in memory process [43]. In this context, it should be noted that the levels of IR and mRNA were increased in hippocampus of rat after a spatial memory task, suggesting that insulin might regulate normal memory function [44]. In accordance with this notion, it has been reported in icv STZ rats' decrease in insulin gene, IR protein and hyperphosphorylated tau protein in the hippocampus and cortex [31], and decrease in IR expression and key proteins of the insulin signaling cascade (e.g. phosphorylation of IRS-1 and Akt) in the CA3 hippocampal region associated with memory damage [45]. Furthermore, the treatment with an

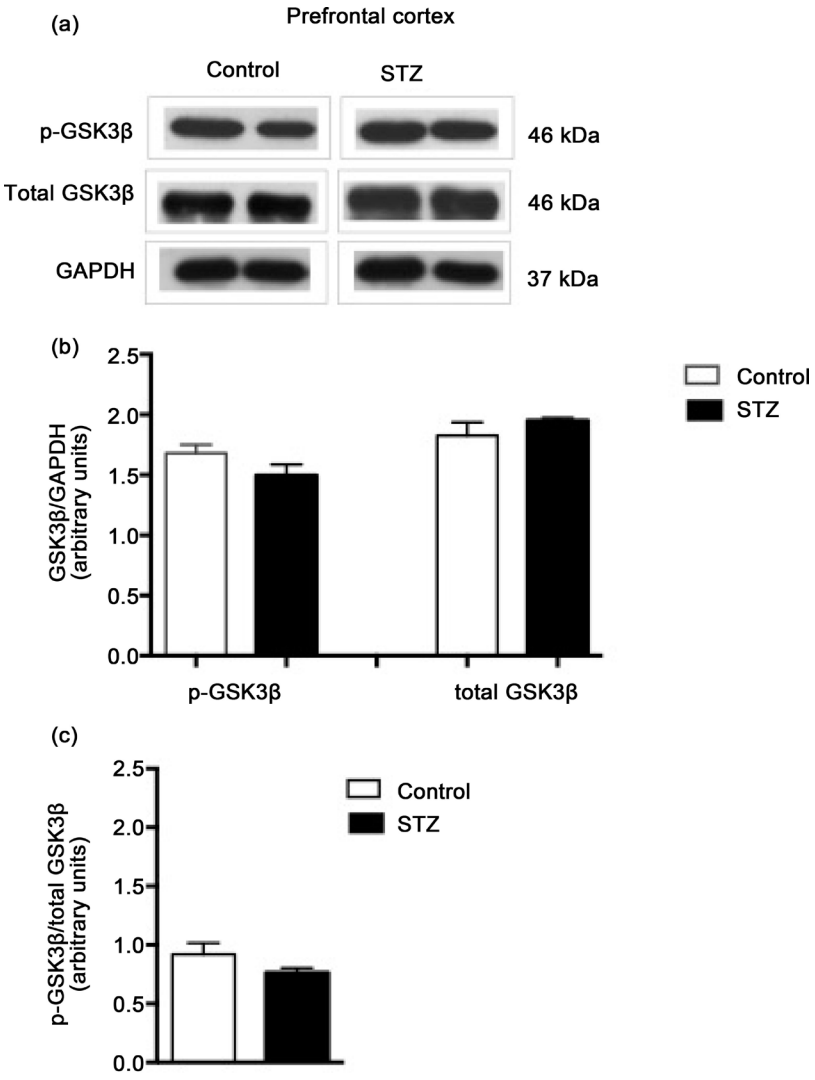

Figure 6. STZ effect on p-GSK3β in prefrontal cortex. (a) Representative immunoblots of p-GSK3β, (b) Total GSK3β and (c) p-GSK3β/total GSK3β ratio. Six months following STZ-treatment, prefrontal cortex protein extracts were immunoblotted for p-GSK3β (Ser9) and total GSK3β (27 C10). Quantification of GSK3β was normalized against GAPDH. (b) Data expressed as mean ± SEM, n = 4 - 5 animals per group. Significant differences in STZ vs. control group; one-way ANOVA followed by Tukey test. (c) Data are presented as mean ± SEM, n = 4 - 5 animals per group. Student's *t* test, *p < 0.05 relative to control group.

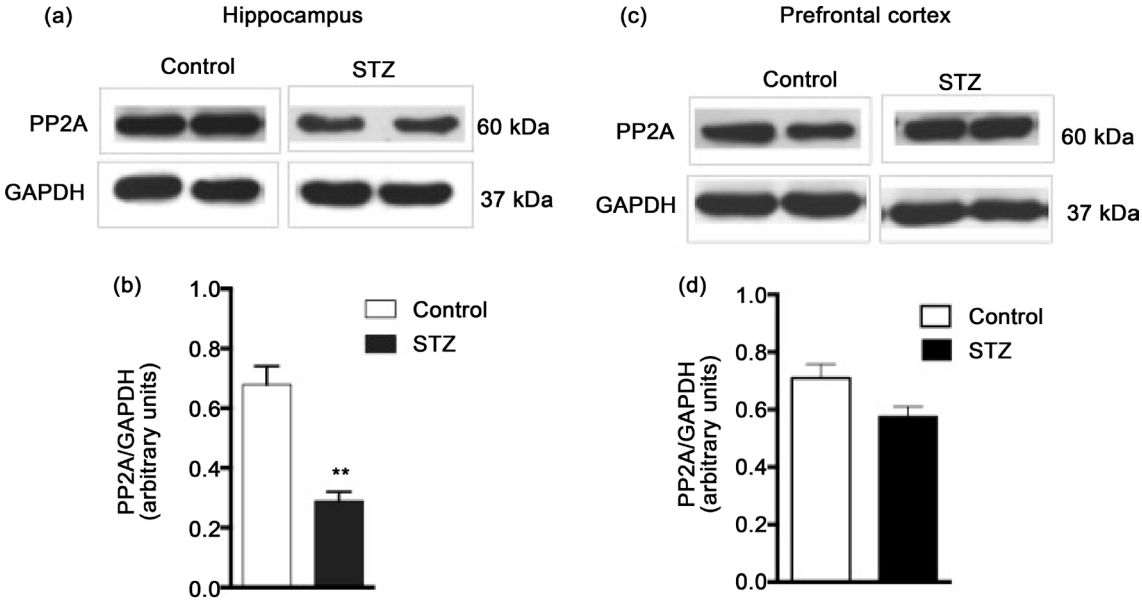

Figure 7. STZ effect on PP2A levels in hippocampus and prefrontal cortex. (a), (c) Representative immunoblots of PP2A and (b), (d) PP2A levels in hippocampus and prefrontal cortex. Six months following STZ-treatment, protein extracts were immunoblotted for PP2A subunit A (6G3). Quantification of PP2A was normalized against GAPDH. Data are presented as mean ± SEM, n = 4 - 5 animals per group. Student's *t* test, *p < 0.01 relative to control group.

insulin sensitizer (e.g., pioglitazone) reversed memory deficit in icv STZ rats [30] [38]. On the other hand, insulin dysfunction (e.g., chronic hyperinsulinemia or diabetes mellitus) has a negative impact on memory and cognitive function [46] [47], and clinical studies demonstrate a beneficial effect of intranasal insulin on memory and cognitive function in AD patients [48].

Interestingly, we observed increased tau phosphorylated, decreased p-GSK3β and PP2A in hippocampus but not in PFC. Hyperphosphorylation of tau is considered as one of the typical pathological changes in AD [49]. In the normal brain, balance between phosphorylation and dephosphorylation of tau results in structural and conformational changes that regulate the stability of the cytoskeleton and axonal morphology [50] [51] [52] [53]. During the development of AD and other neurodegenerative disorders, tau is phosphorylated at multiple sites as a result of the imbalance of numerous Ser/Thr kinases (GSK3β) and phosphatases (PP2A) [50] [51] [52] [53], and integrates paired helical filaments (PHF) leading to NFTs and losing their physiological functions [54] [55] [56]. In this study, the increase observed in p-tau was obtained using a monoclonal phospho-tau (Ser396). Notably, here the total tau did not change in hippocampus; however, increased p-tau and total tau were observed in PFC. The proportion of p-tau/tau total augmented in hippocampus. Hence, the present results might be due to post-translational modifications (increase in phosphorylation) and not by increase in tau protein in hippocampus, but not in PFC.

Regarding GSK3β role, several studies have been shown that insulin could regulate tau phosphorylation in neurons [57] [58] [59]. For instance, binding of insulin to IR induces activating signal transduction cascade of the PI3K pathway

[60]. The activation of the PI3K, in turn activates protein kinase B (Akt/PKB), then Akt/PKB phosphorylates the GSK3, that results in inactivation of GSK3 [61]. Hence, disruption of IR-PI3K-Akt/PKB signaling cascade leads to the dephosphorylation in Ser9 of GSK3β increases in the activity of GSK3β [31]. Notably, the activated GSK3β isoform has been involved in tau-protein phosphorylation [62]. Moreover, it is recognized that GSK3β isoform is the major kinase that phosphorylates *in vivo* tau [63] [64], and, that phosphorylates tau in several sites; hyperphosphorylated in PHF, is present in tangles of brain of AD patients [57] [65] [66]. Our results showed decrease in p-GSK3β and p-GSK3β/total GSK3β ratio; this indirectly suggested an increase in the active GSK3β form, therefore, this might explain the consequent increase of p-tau. Consistent with these results, in our laboratory, we found that insufficient inhibitory GSK3β control in STZ-icv rats was demonstrated by a decrease in p-GSK3β levels in the hippocampus, which was associated to STM and LTM impairment (38). Additionally, lithium (GSK3 inhibitor) and pioglitazone (insulin sensitizer) treatment reversed this memory deficit and restored the inhibitory activity of GSK3β in hippocampus [38]. Similarly, other studies have demonstrated that specific inhibition GSK3 activity by lithium prevents hyperphosphorylation of tau, and spatial memory loss resulting from inhibiting the PI3K and PKC [67]. In addition, transgenic mice (Tet/GSK3β) that conditionally over-expresses GSK3 in neurons of the hippocampus and cortex showed spatial memory deficit [68].

Finally, PP2A diminished in the hippocampus but not in PFC; the former was colocalized with tau and microtubuled in the brain [69], and it was apparently the most active enzyme in dephosphorylating the abnormal tau to a normal-like state [9] [63] [70]. In AD brain, both the activity and the mRNA of PP2A are decreased [71] [72] [73]. Thus, the reduction in the activity of the PP2A promotes the hyperphosphorylation of tau and seems to be an important factor in the progression of the AD [71] [72] [73] [74]. Therefore, the abnormal phosphorylation of tau observed herein in hippocampus, might be as a result of decrease in p-GSK3, but also by diminished dephosphorylating activity of PP2A. In agreement with this, the inhibition of PP2A with okadaic acid (OKA), *i.e.*, a potent and selective inhibitor of PP2A and PP1, produces memory impairment in the Morris water maze and oxidative stress, treatment with memantine (10 mg/kg, po) or donepezil (5 mg/kg, po) for 13 days post-OKA, improves memory and reduces oxidative stress [75].

In brief, a failure in IR signaling pathways (PI3K-Akt-GSK3β) and/or insufficient regulation of phosphorylation by PP2A, might be contributing in the regulation of memory functions and tau protein phosphorylation, hence, the insulin signaling played an important role in AD. These data also suggest that inhibition of GSK3β and activation of PP2A could contribute to inhibiting neurofibrillary degeneration and, which represents an important therapeutic target for AD. The hippocampus is perhaps the brain structure that participates the most notably in the regulation of tau phosphorylation than the frontal cortex. The present evidence supports the experimental approach to sporadic AD, based on the insu-

lin-resistant state induced in the brains of animals following the icv STZ, also, supports the notion that ICV-STZ rat is a suitable model to represent essential features of the SAD and for investigating effective treatments to prevent or reverse memory deficits and neurofibrillary degeneration.

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Cyclic E2 and P4 on Alzheimer's Disease Pathways

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Abstract

In recent years, Alzheimer's disease has been clearly linked to the degradation of microtubules and microtubule-associated tau (τ) and β-amyloid (βA) proteins. Through an examination and evaluation of current literature, we assess the possible effects of the steroid hormones on τ hyperphosphorylation and the regulation of βA proteins and their influence on Alzheimer's dementia and memory loss. We present a mechanism by which Alzheimer's cases may be reduced or perhaps even prevented through the use of non-synthetic, steroid hormones prescribed in a cyclic dosing schedule that mimics the rhythmic, escalating and descending production normally observed in a reproductive female body. Given the ability of estrogen to prevent τ hyperphosphorylation and increase metabolism of the βA precursor protein, we propose the possibility of controlling both protein cycles through the exogenous application of estrogen and progesterone may help those patients with active disease as well as prevent the onset of Alzheimer's and other neurodegenerative diseases.

Keywords

Hormones, Estradiol, Progesterone, Bio-Memtic, Treatment

1. Introduction

Dementia is a chronic and persistent mental disorder that is typically marked by diminished memory, personality changes, and impaired reasoning [1]. A common form of the disease associated with dementia is Alzheimer's disease (AD), which is characterized by neuronal cell loss, amyloid plagues, and vascular damage caused by plaque deposition [2] [3] [4] [5]. While all these factors can describe AD, the underlying cause remains an unknown.

Since the first diagnosis of AD in 1907, there have been a number of theories

about the cause and propagation of the disease [6] [7] [8] [9]. However, the underlying factor or cause has never been discovered. Alzheimer's disease is a chronic neurodegenerative disease that affects an ever-increasing number of the elderly population [6]. With current healthcare costs increasing dramatically, there are a significant number of research groups and organizations examining mechanisms and causes in an attempt to understand and reduce the number of cases. In 2012, R. A. Armstrong presented an extensive review of the many theories about Alzheimer's [6], where the possible causes were broken into eight main categories: aging, degeneration of anatomical pathways, environmental factors, genetics, mitochondrial dysfunction, vascular factors, immune system dysfunction, and infectious agents. The main issue for these theories is that the clinical data makes it difficult to determine whether they are primary causes or secondary effects from another more elusive problem [6] [7] [8] [9]. However, they do provide distinct pathologies that help to pinpoint the potential principal cause. The large number and wide range of possible candidates demonstrate the multifaceted range of factors and components that clinically affect Alzheimer's patients. Therefore, in this manuscript, we look towards connecting all of these aspects to one overarching possibility.

Recent evidence has demonstrated that the microtubule-associated protein tau (τ) and β-amyloid (βA) protein are linked to Alzheimer's as well as other neurodegenerative diseases [10] [11] [12]. The τ protein stabilizes microtubules and is present in neurons located in the central nervous system [13] [14]. Alzheimer's disease is characterized by the breakdown and loss of stability in the microtubules, which has indicated that the τ proteins have malfunctioned in some way to make them ineffective [11]. The impairment of the τ protein can be attributed to the hyperphosphorylation of the protein [12], which results in the tangling of various filaments that affect many neuromechanical pathways and can lead to multiple neurological disease states, including AD.

The most widely studied protein is β-amyloid (βA), due to its connection to the formation of plaques observed in AD patients. However, the function of βA proteins remains fairly unknown. Although, it is suspected that they work to regulate neural plasticity and synapse formation [15]. However, the amino acid residues produced by βA helps to create βA plaques (βAP). The combination of neurofibril tangles due to τ hyperphosphorylation and βAP are both known to contribute to Alzheimer's disease [12]. This occurs when βAP residues produce inflammation and transient ischemic attacks (TIAs) around affected areas of neural tissue.

Since two-thirds of Americans with Alzheimer's disease are women, the steroid hormone contributions to protein production of τ through gene expression controlled by the hormonal fluctuations of the menstrual cycle may be related to steroid hormones [16] [17]. Therefore, through an evaluation of current literature, we assess the possible effects of the steroid hormones (specifically estrogen and progesterone) on τ hyperphosphorylation and βA production and their influence on Alzheimer's, dementia, and memory loss. We present a hypothesis that Alzheimer's cases may be significantly reduced or even prevented with the

use of non-synthetic, bio-identical steroid hormones prescribed in a cyclic dosing schedule that mimics the rhythmic, escalating and descending production created in a young reproductive female body. Given the ability of estrogen to prevent τ hyperphosphorylation and regulate βA precursor protein, as well as the overall effect of all steroid hormones on gene expression and the inflammatory process, the capability of controlling the expression of τ and βA through the exogenous application of estrogen and progesterone has the potential to help those patients with active disease symptoms as well as prevent the onset of Alzheimer's.

The general thesis of this article is to demonstrate that the loss of steroid hormones at menopause, specifically estrogen and progesterone, may provide a critical link between hyperphosphorylation of the τ proteins and the onset of Alzheimer's disease in women. A similar mechanism may also exist in for men when the conversation of testosterone to estrogen falls off later in life. However, the various interactions and biomechanisms remain elusive. To understand this further, we review the literature on the τ and βA proteins and their breakdown, examine current treatments that look to only mask the symptoms, and introduce the idea that steroid hormones may provide a pathway to slowing down and possibly halting the progression of this disease.

2. Current Treatments

Over the past couple of decades, many treatments have become the standard of care for AD patients. Medications range in the treatment for cognitive symptoms, behavioral control and insomnia [18] [19] [20] [21]. This is quite dramatic since there is not current medication or treatment that can reverse or stop AD from progressing. Therefore, treatments can only hope to slow down the appearance and progression of symptoms for a limited time.

Regarding cognitive symptoms, cholinesterase inhibitors (Aricept, Exelon, Razadyne) and memantine (Namenda) have been used to treat the memory loss, confusion, and difficulties with thinking and reasoning of AD [22]. Cholinesterase inhibitors are typically used for mild to moderate case of AD, where they aim to reduce the breakdown of acetylcholine, which is an important chemical messenger that affects learning and memory. Therefore, these medications support communication among nerve cells by keeping acetylcholine levels high. The advantage of this kind of treatment is that it may delay worsening of symptoms for up to 12 months, which has been shown to affect about 50% the people who take them. However, the side effects of the medication have been severe, which include nausea, vomiting, loss of appetite and increased frequency of bowel movements [22].

To treat moderate to severe stages of AD, memantine may be prescribed. This medication affects the regulation of glutamate, which is similar to the cholinesterase inhibitors and works to delay symptoms of memory loss [23]. It is noted that the side effects tend to include a headache, constipation, confusion, and dizziness, at a minimum.

Along with the cognitive symptoms, patients experience mood changes and other issues such as anxiety and depression [24]. This leads the patients to need a variety of antidepressant, anxiolytic, and antipsychotic medications. Overall, patients with AD are typically overwhelmed by so many medications and prescriptions that often interact.

Beyond these pharmaceutical treatments, there are a number of alternative or complementary treatments that "claim" to have an effect on AD with no clinical evidence. These treatments include herbal remedies, dietary supplements, and food regimens. However, the effectiveness and safety of these treatments are also unknown, and they purveyors provide no known mechanisms of action for effectiveness.

3. The τ Hyperphosphorylation and β-Amyloid Plaques

Tau proteins work to stabilize and maintain tubular polymers called microtubules that are abundant in the nerve cells of central nervous system [10]. τ proteins control microtubule stability through the use of its isoforms and phosphorylation [25]. Tau proteins are produced through a process of alternative splicing of a single gene called microtubule-associated protein τ [13]. Hyperphosphorylation of τ proteins can lead to the failure of microtubule stability, which can cause neurofibrillary tangles. When in combination with the formation of β-Amyloid plaques, this breakdown of biomechanisms are widely linked to Alzheimer's disease [14].

Microtubules consist of long tubular structures that are assembled by protofilaments, which produced by tubulin α-β-dimers [26]. While these structures are dynamically stable, changes in the overall environment can affect the microtubules and cause them to breakdown. Since the τ protein controls this process, understanding how external and internal stressors influence the τ protein is relevant. In general, many stressors that could be affecting the τ protein include external (pollutants, electromagnetic fields, etc.) and internal (hormones, chemical intake, etc.) environmental factors [27].

Typically, it is thought that the increase in the τ hyperphosphorylation, which reduces the stability of the microtubules, causes β-amyloid plaques to form. However, recent research has suggested that it is the in βA proteins enhance hyperphosphorylation of the τ protein through glycogen synthase kinase 3 (GSK3) [28]. Therefore, as illustrated in **Figure 1**, the combination of the βA protein build up, and the breaking of the microtubule stability produce the creation of calcium plaques (βAP). This introduces inflammation around the neuron that can lead to transient ischemic attacks in the brain, which could cause permanent damage to memory and cognitive function.

In recent years, AD and degeneration have been connected to the dysfunction of the mitochondria and the production of oxidative stress [7] [8] [15] [29], which is speculated to occur through formation of oxidative and nitrosative stresses, depletion of adenosine triphosphate, and impaired electron transport [7]. However, these subsequent dysfunctions are characteristic of aging effects due to loss

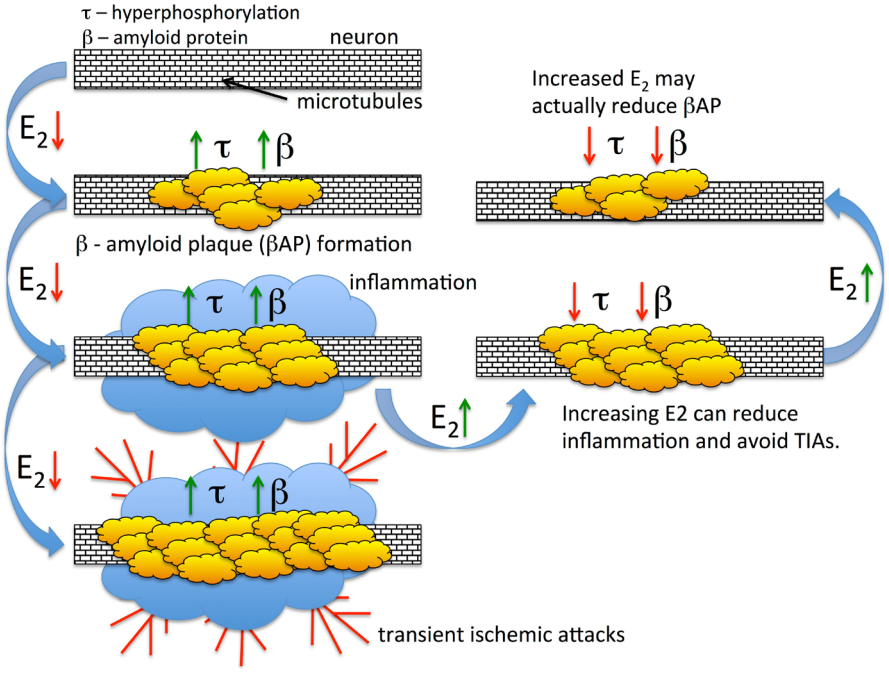

Figure 1. General schematic of the formation of β-amyloid plaques due to decreased estrogen (E2) and τ protein. The right side illustrates the expected response to increased estrogen dosing.

of hormones and its effect on mitochondria in both women and men, which has been widely documented in the literature [30]-[36]. This is why we focus on the effects produced by steroid hormones due to their ability to control and regulate gene protein products.

4. Effects of Steroid Hormones on Protein Expression

The potential for the onset of AD is associated with the time in life when we experience the loss of sex steroid hormones in both women and men [17] [37] [38] [39]. In particular, studies on women show the decline of estrogen (E2) and absence of progesterone (P4) has been suggested to increase susceptibility to AD compared to men. Therefore, there are two possibilities: 1) the sharp decrease in neurological responsiveness in old age is due to the missing steroid hormones, or 2) the loss of steroid neurological feedback to the endocrine function destroys hormone production and brain responsiveness [40]. Experimental evidence has pointed to the promotion of neuron viability and the possible decrease in β-amyloid peptides due to the protective actions of E2 and the immunosuppressive action of P4 [41], which are a critical factor in the origination and advancement of AD and the neurological damage that can ensue. This connection suggests that the use of hormone replacement therapy (HRT) could provide a reduction in the risk of the onset of AD, but also perhaps decreases in AD symptoms. Furthermore, cyclic exposure to P4 helps in the regulation of hippocampal gene expression [42], which indicates the general effects of menopause on the reduction of gene expressions that can lead to diseases of aging.

Over the last decade, there have been many studies that have suggested the re-

levance of HRTs in the fight against and prevention of AD [31]-[36]. While these studies seem to demonstrate a significant reduction in risk of AD in women, other studies have suggested that the protective benefits of HRT may be overstated as some studies indicate little to no associated reduction in AD [43]-[48]. However, the dosing type in all of these studies was static synthetic estrogen and low in dosing amount. This means the women were given enough synthetic estrogen to elevate the overall concentration of hormones in the blood, but not enough to trigger the production of progesterone receptors [48]. Furthermore, they seem to completely ignore the standard bio-rhythmic response of the body that functions to control gene expression [49].

The regular biological rhythm for women has an increase in E2 during the first 12 days of a women's cycle (illustrated in **Figure 2**). This peak in estrogen signals the production of P4 receptors, which, over a 9-day period, E2 is decreased in the body while P4 is increased [50]. Progesterone will peak on day 21, and then the cycle will reset on Day 28. It is this rhythmic pattern that is usually neglected in studies, with adverse outcomes, on HRTs for Alzheimer's patients. We suspected that wide variation between effective and non-effective hormone treatments in the above studies is due to regularity of dosages, the delivery system (orally, transdermally, etc.), and/or whether progesterone is included at all in the treatment.

As discussed in Pike *et al.* [30], estrogen's neuro-protective actions are modulated by progesterone [51] [52], whereas static progesterone exposure always inhibits estrogen's actions, synthetic or otherwise. However, the cyclic delivery of P4 and E2 will allow for the increase of E2 receptors [48] [49]. When low levels of E2 are administered, the potential production of progesterone receptors (P4R) is hindered [53] [54]. This can be improved through a rhythmic pattern of E2 and P4. Furthermore, estrogen is known to increase dendritic spining as well as synaptic plasticity [55] [56].

As observed in most women, the loss or disruption of E2 and P4 has a number

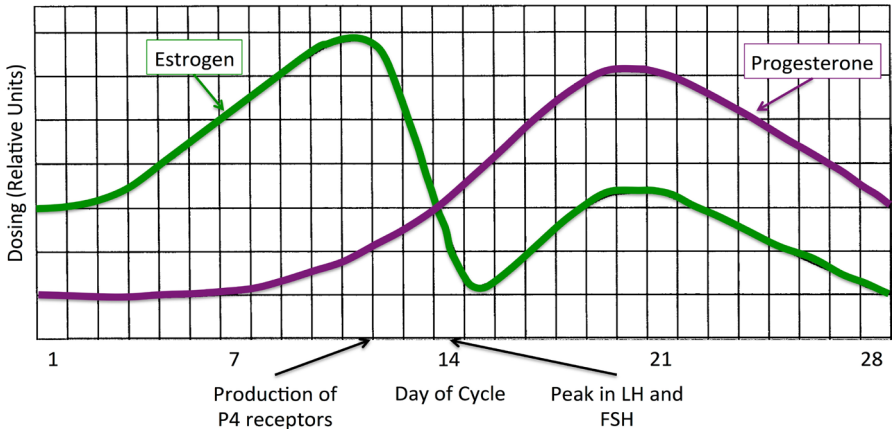

Figure 2. The suggested rhythmic dosing pattern for estrogen (E2) and progesterone (P4) during the normal reproductive cycle for a young woman. As E2 peaks, so does the production of P4 receptors. Around Day 14, women will experience a peak in luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which begin the ovulation process.

of effects on the body and mind. For example, decreased E2 will reduce synaptic plasticity and increase the loss of dendritic spinning [55] [56]. The disruption of the production of progesterone and P4 receptors, via luteinizing hormone (LH), due to estrogen loss produces diminished immunosuppression and vascular endothelial growth factor. Furthermore, it is well documented that the presence of estrogen prevents hyperphosphorylation of the τ protein and helps regulate the metabolism of β-amyloid. Therefore, it is probable that reduction or complete loss of estrogen and progesterone can lead to increased hyperphosphorylation of τ (illustrated in **Figure 3**) and an increase in β-amyloid, which, in turn, also reinforces hyperphosphorylation resulting in the onset of AD. Since the loss of E2 concurrently produces a loss of P4, the body will experience diminished immunosuppression and loss of Vascular Endothelial Growth Factor (VEGF) [57].

Therefore, we propose that the effect of rhythmic dosing of estrogen with dosing levels comparable to those of a reproductive woman may reduce hyperphosphorylation of τ and subsequently inflammation around calcium plaques (illustrated in **Figure 1**), which may decrease the amount of memory loss produced from multiple random transient ischemic attacks (as shown in the right side of **Figure 1**). The effects of E2 restoration should increase the brain's ability to increase dendritic spining and synaptic plasticity, which may lead to a decrease in the formation of plaques; although this would have to be studied clinically. Furthermore, the presence of E2 above the critical threshold produces P4 receptors. The levels of rhythmic P4 will restore that steroid hormone to youthful levels, which will create an increase in immunosuppressive response and VEGF, which has been shown to help restore memory behavior in mice [58],

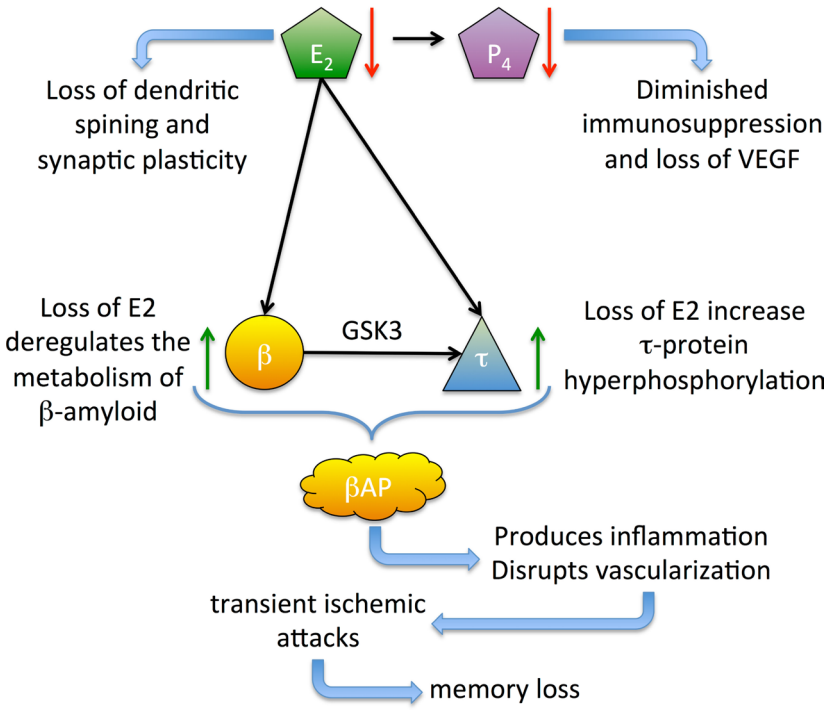

Figure 3. Generalization chain of events for loss of steriods hormones (estrogen-E2 and progesterone-P4) and the effect it has on Alzheimer's disease.

and helps with the reduction of inflammation around calcium plaques. The increasing dendritic spining and loss of τ hyperphosphorylation may also help remove plagues, but this is purely speculative.

5. Conclusions

Through an evaluation of current literature, we examine the possible effects of the steroid hormones (specifically E2 and P4) on the regulation of τ hyperphosphorylation and β-amyloid and their influence on the formation of β-amyloid plaques in Alzheimer's patients. We present a general mechanism that Alzheimer's cases may be reduced or even prevented with the use of non-synthetic steroid hormones prescribed in a cyclic dosing schedule which mimics the rhythmic, escalating, and descending production in a reproductive body to control gene expression. Given the ability of E2 to prevent τ hyperphosphorylation and to regulate βA, there is a possible method for controlling hyperphosphorylation and β-amyloid plaque formation through the application of estrogen and progesterone may help those patients with active disease as well as prevent the onset of Alzheimer's, if begun before menopause. Furthermore, using a bio-mimetic hormone replacement methodology, we examine and propose that modulated hormone dosing and the effects of steroid pulsatility and amplitude at the receptor level are logically a major factor in effectively controlling the expression in the brain.

Future aspects of research include examining how bio-mimetic, bio-identical hormone replacement therapy affects the maintenance of microtubules and the production of τ and β-amyloid. We propose a clinical experiment that will administer bio-identical hormones to women in a manner which mimics the normal rhythmic patterns of young reproductive women. The hormones should be administered using a transdermal cream. This allows for the hormones to be more effective, since they will bypass the liver. As a control, this should be compared to other hormone therapies (variable and static timing), as well as the control group with no hormones (transdermal cream without hormones).

Investigations into the advancement of AD can be determined through standard medical techniques (magnetic resonance imaging and CT scanning) along with physical, diagnostic, and neurological examinations. As per the mechanism above, we expect that there will be a slowing or abatement of AD symptoms due to the restoration of youthful gene products. A reversal of symptoms may be theoretically possible, but other factors such as damage may hinder those possibilities.

Once a satisfactory baseline can be established for women, further investigation into the estrogen derived from testosterone in men may also be studied. While the mechanism for men is not clear, due to the lack of a distinct hormonal pattern, the increase of testosterone will increase the presence of estrogen in the body and may have the same effect overall. However, this exact mechanism is not evident at this time.

Overall, as people age, the only one constant among all people is that we lose

the ability to produce sex steroid hormones and regulate the proper production and regulation of gene products at their behest. It is our conclusion that the manifestation of Alzheimer's disease may be due to this particular problem. In women, the loss of E2 and P4 produces a cascade of events that can result in the growth of calcium plaques on neurons and the subsequent production of transient ischemic attacks that lead to memory loss. The same may also be true for men, although through the loss of testosterone, which typically converts to estrogen. However, when looking at a problem with so many complex interacting parts, it is sometimes needed to step back and acknowledge the loss of something that is basic to human growth and reproduction as the possible cause.

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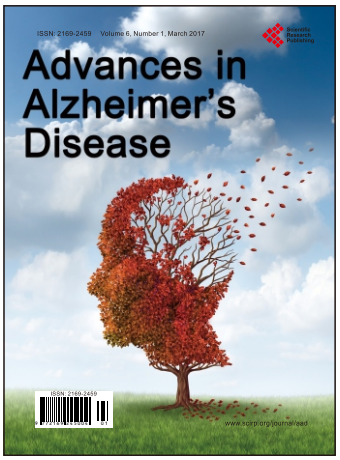

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