Published in final edited form as:

J Alzheimers Dis. 2017 ; 57(4): 1001–1015. doi:10.3233/JAD-160601.

How Studies of the Serotonin System in Macaque Models of Menopause Relate to Alzheimer's

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Abstract

Serotonin plays a key role in mood or affect, and dysfunction of the serotonin system has been linked to depression in humans and animal models. Depression appears prior to or coincident with overt symptoms of Alzheimer's (ALZ) disease in about 50% of patients, and some experts consider it a risk factor for the development of ALZ. In addition, ALZ is more prevalent in women, who also show increased incidence of depression. Indeed, it has been proposed that mechanisms underlying depression overlap the mechanisms thought to hasten ALZ. Women undergo ovarian failure and cessation of ovarian steroid production in middle age and the postmenopausal period correlates with an increase in the onset of depression and ALZ. This laboratory has examined the many actions of ovarian steroids in the serotonin system of non-human primates using a rhesus macaque model of surgical menopause with short or long-term estradiol (E) or estradiol plus progesterone (E+P) replacement therapy. In this mini-review, we present a brief synopsis of the relevant literature concerning ALZ, depression and serotonin. We also present some of our data on serotonin neuron viability, the involvement of the caspase-independent pathway and AIF in serotonin-neuron viability, as well as gene expression related to neurodegeneration and neuron viability in serotonin neurons from adult and aged surgical menopausal macaques. We show that ovarian steroids, particularly E, are crucial for serotonin neuron function and health. In the absence of E, serotonin neurons are endangered and deteriorating toward apoptosis. The possibility that this scenario may proceed or accompany ALZ in postmenopausal women seems likely.

Keywords

Macaque; Estrogen; Menopause; Serotonin; Neuroendangerment

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The work described in the macaque model was performed by the co-authors during their time in the author's laboratory. The addresses of the co-authors are their current addresses.

Introduction

Alzheimer's epidemiology and neuropathology

Of the 5.1 million Americans who currently have Alzheimer's disease (ALZ), almost two-thirds are women who have entered menopause. The most obvious symptoms of Alzheimer's disease (ALZ) are the loss of memory and cognition. The gross neurobiological pathologies of ALZ are tangles of a cytoskeletal protein, tau, and accumulations of toxic protein fragments called amyloid beta ($A\beta$), and referred to as plaques. These anomalies are found in areas of the brain underlying different aspects of cognition. How tangles and plaques develop, and their roles in the decline of cognition, have been the focus of a large number of studies. It has been proposed that deficient transport produces axonopathy and leads to the build-up of $A\beta$, which in turn causes nerve cells in the brain to lose their normal functions and eventually die [1].

Alzheimer's and gender bias

Female birth sex has been associated with increased risk of the development of ALZ, but relatively little research has been performed to date to reveal sex-related dissociations of ALZ onset and symptomology [2, 3]. Women clearly have a greater incidence of ALZ than men [4]. Women go through menopause in midlife and experience a significant drop in estradiol (E) and progesterone (P) secretion. Apparently, the decline in estradiol (E) levels in post-menopausal women makes nerve cells more vulnerable to the damaging effects of $A\beta$, and their brains are less able to remove $A\beta$ before it reaches dangerous levels. Men exhibit a much slower decline in testosterone (T), which has also been correlated with severity of ALZ [5–7]. Populations of women that started hormone replacement therapy (HT) at menopause had fewer new cases of ALZ compared to non-treated women [8–10]. In animal studies, E shows neuroprotective qualities [11, 12] and E has strong anti-inflammatory actions [13, 14].

Depression

Approximately 50% of patients with ALZ have depression of varying degrees [15–17]. Depression associated with ALZ has received significant characterization and investigation [18]. However, the question of whether depression is prodromal for development of overt Alzheimer's symptomology has not been fully answered. In addition, ALZ-depression may occur independently or in concert with apathy, and each may derive from different neurological sources [19]. A number of mechanisms underlying depression appear to be similar to mechanisms implicated in ALZ. For example, a history of major depression appears to be a risk factor for subsequent development of ALZ. Moreover, depressive symptoms can impact the conversion of mild cognitive impairment into ALZ. Plaques and tangles are more prominent in the brains of ALZ patients with depression compared to those without depression [20]. Conversely, neurodegeneration has been observed in various brain regions of patients with a history of depression. Indeed, some investigators have suggested that depression may in fact, be a neurodegenerative disease [21]. Thus, it appears that molecular mechanisms underlying the pathogenesis of depression may be similar to those involved in the pathogenesis of ALZ. In particular, inflammation, oxidative stress, genetic

vulnerability and diminishing neurotrophic factors have been observed in both depression and ALZ [22–26].

Serotonin, Alzheimer's disease and Depression

Although the mechanisms of adult-onset depression can vary, dysfunction of the serotonin system is involved in many depressed patients [27]. Therefore, investigators have examined the serotonin system in ALZ patients as detailed in the excellent review of Rodriquez and colleagues [28]. In brief, postmortem studies of brains from ALZ patients have consistently found a decreased number of serotonergic neurons in the dorsal and median raphe nuclei, which project to most areas of the forebrain including the neocortex, the septum and the hippocampus [29]. These areas are severely affected in ALZ. The raphe nuclei also show enhanced neurofibrillary tangle pathology, which may contribute to the death of serotonergic neurons. Some evidence suggests that neurotoxins are taken up by terminating axons and undergo retrograde transport to the cell body where accumulation triggers cell death. Further studies indicate that the decrease in serotonin neurons is accompanied by a decrease in serotonin neurotransmission. There is increasing evidence for ALZ-related reduction in serotonin receptors including 5-HT1A, 5-HT1B, 5-HT1D, 5-HT2A and 5-HT6. Significant decreases in the serotonin transporter (SERT) have also been reported [30]. Overall, serotonin is involved in a legion of neurological processes through its interactions with cholinergic, GABAergic, glutamatergic, noradrenergic and dopaminergic systems. Thus, it would be surprising if deterioration of the serotonin system were not found in ALZ. However, its role in ALZ *depression* is still questioned by some [18].

With respect to depression in ALZ, the major antidepressant class, selective serotonin reuptake inhibitors (SSRIs) can be helpful [31]. A mega-analysis indicated that antidepressants increase remission of depression in people with ALZ when used for 6–12 weeks [32]. The SSRI, sertraline, and SNRI, milnacipran, showed overall superiority, but monoamine oxidase inhibitors, the acetylcholinesterase inhibitor, donepezil, glutamatergic modulators, and antipsychotics have also lifted depressive symptoms in various random and open-label trials [33].

Serotonin and Cognition

Serotonin also plays an important role in cognition and executive function. It has been suggested that reduced serotonin function could be linked to cognitive disturbances in ALZ [34]. Disorders in cognition have been linked to 5HT2A receptors, particularly in the prefrontal cortex [35]. Tryptophan depletion studies found impairments in declarative episodic memory using a verbal learning task paradigm, and the impairments were larger in women than men [36]. In addition, patients with major depressive disorder (MDD) show reductions in several aspects of cognition [37], and administration of SSRIs improves cognitive function [38, 39].

In addition to pathologies in acetylcholine neurons, ALZ patients exhibit pathologies in the serotonin system and other neural systems, at different phases of the disease. It is hard not to wonder whether there is an underlying, general or unifying disease process that occurs in the

brain, but manifests differently depending on the neuronal type? In other words, is ALZ a whole brain disease?

Neuron health

The notion of neuronal health as a dynamic equilibrium that spans a spectrum between resilient and vulnerable neuronal states is well accepted [40]. Thus, at any given time, neurons may be very healthy and functioning well, including optimal DNA repair, or somewhere on a slippery slope where they are unhealthy and falling farther and farther behind in DNA repair but not dead. The concept of “neuroendangerment” has been proposed for these unhealthy neurons [41]. They are vulnerable and additional stresses could kill them. However, up to the point of no return, recovery may be possible. Many endogenous and external factors could render a neuron vulnerable, including systemic infection and cytokines [42].

Primate models of ALZ and menopause

The major motor neurodegenerative diseases (NDD) have not been observed in non-human primates (NHPs). However, Caribbean vervets (*Chlorocebus pygerythrus*) and rhesus macaques (*Macaca mulatta*) exhibit amyloid plaques in old age [43], but to date, tangles have not been observed. It should be noted that wild caught and research animals eat a diet that is very low in fat and sugar, but high in micronutrients. In depth examination of the brains of old NPHs on a high fat and sugar diet, like that of western humans, has not been accomplished.

Our studies have examined the effects of E, with or without P supplementation, on serotonin function and viability in a macaque model of menopause. These studies impact our concepts of serotonin function that may be relevant to neurodegeneration that leads to cognitive decline or depression in women.

We extensively utilized a non-human primate model of surgical menopause with hormone replacement. Adult female rhesus monkeys (*Macaca mulatta*) were ovariectomized (Ovx) by the surgical personnel of the Oregon National Primate Research Center (ONPRC) according to accepted veterinary surgical protocol. Between 3 to 8 months after Oxv, animals were either treated with placebo (Ovx control group), or treated with E for 28 days (E only group), or treated with E for 28 days and then supplemented with P for the final 14 of the 28 days (E+P group) with Silastic capsules containing, E, P or nothing. This short-term model with Oxv + steroid-treatment has revealed cellular aspects of serotonin function and viability in a cost-effective manner.

Ovarian steroids and serotonin neuron viability

Based upon the observation that ALZ patients exhibited a reduction in serotonin neuron number, and based upon the suggestion that depression may involve neurodegeneration, we further questioned whether ovarian steroids supported serotonin neuron viability (Figure 1). DNA fragmentation and condensation is a marker for cells that are undergoing apoptosis. This process can be visualized with a TUNEL assay (terminal deoxynucleotidyl transferase nick end labeling). TUNEL was applied to the dorsal raphe nucleus, which contains the cell

bodies of serotonin neurons that project to the forebrain. TUNEL staining co-localized in serotonin neurons in the dorsal raphe nucleus; and there were significantly fewer TUNEL-positive neurons after one month of E+P treatment [44]. Type-I and type-II TUNEL staining were observed in serotonin neurons. Type I, with complete dark staining of the nucleus, and type II, with peripheral staining in the perinuclear area, may reflect different stages of the DNA fragmentation process that starts in the periphery and moves inward [45]. Alternatively, the perinuclear, type II staining could indicate DNA leakage from the nucleus [46].

It may be questioned how E and P decrease DNA fragmentation after one month of treatment. That is, are they reversing a process that is underway? We speculate that DNA repair is possible if the fragmentation process has not proceeded to an advanced stage. Indeed, some of the less affected cells with minor type II staining could be rescued with hormone therapy. This speculation means that not all of the TUNEL-positive cells are actively dying, but they are endangered and vulnerable. Therefore, the insults or factors that induce ALZ could also lead to serotonin neuron death, or perhaps vice versa.

Serotonin neurons and apoptotic proteins

A prominent feature of neuroprotection is the inhibition of apoptosis. Apoptosis, also called programmed cell death, is a homeostatic process under genetic control that has been evolutionarily conserved to balance cell replication and optimize cellular organization. It is characterized by condensation of chromatin, cell shrinkage, nuclear fragmentation and formation of apoptotic bodies, and the resulting cells are later ingested by phagocytes [47–49]. Apoptosis may proceed through activation of caspases (caspase dependent pathways) or through translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus (caspase-independent pathway) [50].

Pivotal proteins in the caspase dependent and caspase-independent pathways were examined in microdissected dorsal raphe from adult Ovx monkeys treated with placebo, E or E+P [51]. JNK1 is a pro-apoptotic kinase that can activate both caspase-dependent and independent pathways. The JNK pathway is activated in response to environmental stress and largely, the activation leads to apoptosis. Therefore, expression of JNK1 and phosphorylated JNK1 were measured by western blot in subcellular fractions of the dorsal raphe region to determine the mechanism by which hormone therapy may promote neuroprotection in the absence of global injury [51]. JNK1 protein in E + P-treated monkeys was down regulated compared to the OVX control monkeys. JNK1 is activated by phosphorylation by MAPK kinases MKK4/7. Thus, the phosphorylation status of JNK1 (P-JNK1) was determined, which indicates the activity of JNK1. P-JNK1 was reduced in E + P-treated animals compared to the OVX animals. Because the reduction of P-JNK1 could be a consequence of the reduction in JNK1 protein, we calculated the ratio of P-JNK1 to JNK1. There was no difference between treatment groups in the ratio of P-JNK1/JNK1 suggesting that the decrease in JNK1 protein was producing the decrease in detectable P-JNK1.

Because JNK1 can act through caspase-dependent or independent pathways, we examined both downstream pathways in our different treatment groups. In the caspase-dependent pathways, Bcl-2 family members were not altered by hormone therapy, nor were cytochrome

c, Apaf-1, cleaved (active) caspase 3, or X-linked IAP (inhibitor of apoptosis). However, expression of procaspase-3 was significantly decreased by E+P treatment, but caspase-3 was not detected. Altogether, the role of the caspase dependent pathway was not compelling [51].

Apoptosis-inducing factor is the main mediator in the caspase-independent pathway. Upon apoptotic stimuli, AIF is released from the mitochondria and translocates to the nucleus where it triggers chromatin condensation and large DNA fragmentation. Under non-stressed conditions, AIF is essential for mitochondrial health and function. In healthy cells, AIF is retained in the mitochondria where it is believed to have an oxido-reductase function [52, 53]. However, in response to apoptotic stimuli, AIF becomes an active executioner of the cell. AIF nuclear translocation is thought to be a commitment point to neuronal cell death. There was a marked decrease in mitochondrial AIF and in nuclear AIF proteins in the dorsal raphe with E and E+P treatments, but there was animal-to-animal variation that hindered statistical analysis (Figure 2). We also observed a decrease in AIF mRNA expression with qRT-PCR in the dorsal raphe of OvX macaques treated with E+P. AIF mRNA expression paralleled AIF protein expression. Moreover, dorsal raphe neurons of OVX-placebo treated monkeys were densely stained for AIF with immunohistochemistry (Figure 3). Thus, under normal ovarian conditions, E and P act to maintain mitochondrial integrity, which retains AIF in the mitochondrial membrane, and hormone therapy may decrease total AIF synthesis [51].

Serotonin neurons have a robust tryptophan metabolism, and there are two pathways by which tryptophan may be metabolized. In one pathway, tryptophan hydroxylase 2 (TPH2) is the rate-limiting enzyme in serotonin synthesis. In the other neural pathway, tryptophan is acted upon by indole-0-methyltransferase (IDO) and foraminadase to produce L-kynurenine. Downstream activity of kyurenine mono-oxygenase (KMO) eventually leads to neurotoxic quinolone metabolites. Consequently, we examined the effect of ovarian steroids on KMO. Administration of E or E+P to OvX macaques significantly decreased KMO mRNA expression with qRT-PCR and decreased KMO protein expression on Western blots. In addition, KMO was detected in serotonin neurons with immunohistochemistry. Computer assisted image analysis revealed that E and E+P significantly reduced the KMO-positive pixel area in OvX macaques compared to placebo-controls [54]. Hence, E or E+P treatment increased TPH2 and serotonin synthesis and concurrently decreased KMO and neurotoxic quinolone synthesis. Extrapolating to women, it is clear that long before the onset of overt ALZ, untreated menopause will result in serotonin neuron death by elevated caspase-independent AIF and by increased neurotoxic quinolones. These data indicate that in the absence of ovarian steroids, serotonin neurons are endangered.

Serotonin neuron gene expression in adult monkeys

We first showed that administration of E or E+P to adult OvX monkeys significantly improved gene expression that would lead to increased serotonin neurotransmission in the short-term macaque model [55]. The presence of ovarian steroids increased FEV (PET1) mRNA expression, which governs the serotonin neuron phenotype [56] and increased tryptophan hydroxylase 2 (TPH2) mRNA expression, which codes for the rate-limiting

enzyme in serotonin synthesis [57]. E or E+P also increased mRNA expression for the anxiolytic proteins, CRF-R2 and urocortin 1 (UCN1), but decreased mRNA expression of the anxiogenic protein, CRF-R1 [58].

To further understand the mechanisms underlying DNA fragmentation and endangerment in serotonin neurons, preparations of laser captured serotonin neurons from adult Ovx macaques treated for 1 month with placebo, E or E+P were subjected to Affymetrix microarray analysis. Genes that were involved in aspects of NDD, axon transport, DNA repair, protein folding (chaperones), the ubiquitin-proteasome, dendrite proliferation and synapse assembly, and that showed a 2-fold or greater change between the groups, were further confirmed with qRT-PCR [59]. Based on **KEGG** analysis and **Z**-scores related to NDDs in laser-captured serotonin neurons, there was reason to further validate the effect of E and P on several genes directly related to NDD (Figure 4).

APP (amyloid precursor protein) markedly decreased with E and/or E+P treatment, and decreasing expression of this potentially toxic protein should be beneficial [60]. PSEN1 (presenilin1) was severely suppressed by E and E+P treatment in serotonin neurons. Presenilin is best known for its role in ALZ. Cleaved fragments of presenilin form the proteolytic subunit of γ -secretase. This enzyme is responsible for a toxic cleavage of APP that yields amyloid β 40–42 or A β components of ALZ plaques [61]. The marked inhibition of PSEN1 gene expression by E or E+P further suggests that repression of γ -secretase activity is important for neuronal viability.

ADAM 10 (α -secretase) mRNA was significantly higher with E treatment. Under normal conditions α -secretase cleaves APP within the luminal/extracellular domain to yield soluble APP derivatives and membrane tethered a- or b-carboxyl-terminal fragments that are not toxic. Its expression is elevated during neuronal differentiation and after neural injury. Furthermore, the APP/ α -secretase derivative peptides have proposed roles in cell signaling, long-term potentiation and cell adhesion [62]. The induction of ADAM10 gene expression by E suggests another mechanism by which E is neuroprotective and agrees with *ex vivo* assessments in guinea pigs [63]. In Ovx rats, administration of E before A β injection decreased cholinergic neuron loss and partly prevented fiber degeneration [64]. E and E+P also significantly increased α -synuclein (SCNA1) mRNA, although the normal function of α -synuclein is not well understood. Studies suggest that it has an important role in maintaining a supply of synaptic vesicles in presynaptic terminals [65]. The increase in SCNA1 observed with E indicates a mechanism by which E could facilitate synaptic transmission. Mutations in SNCA1 are associated with early onset of Parkinson's disease, and misfolded α -synuclein is also a sizeable component of Lewy bodies [65].

Axonal transport defects also have a role in neurodegeneration [66–68] and in particular ALZ [1]. Transport-related genes encoding KIF5B (kinesin), DYNLL1 (dynein) and DCTN4 (dynactin) increased with E-treatment (all, $p < 0.03$). These three proteins are critical motors for the transport of vesicles through axons (Figure 5). MAPT (tau) is a component of microtubules and tau is the main component of tangles, but there was no change in MAPT mRNA with E-treatment. The mechanisms underlying tau-tangles is an issue of great interest and appears to involve phosphorylation more so than reduced gene expression. E

administration to Ovx monkeys also markedly elevated gene transcription underlying RhoGTPases and actin remodeling in serotonin neurons [69].

DNA repair minimally requires lesion recognition, single-strand excision, lesion removal, gap-filling synthesis and finally ligation. Repair is divided into single base excision repair (BER), or nucleotide excision repair (NER) with excision of 28 nucleotides around the lesion in one strand and gap repair using the other strand as the template [70]. In adult monkeys with 1 month of E therapy, significant increases were found in four genes that code for proteins involved in repair of strand breaks and nucleotide excision. NBN1 (double strand break repair), PCNA (base excision gap filling), RAD23A (DNA damage recognition) and GTF2H5 (gene transcription factor 2H5) significantly increased with E or E+P treatment (all, $p < 0.01$). Neurons generally do not replicate their genomic DNA, and can therefore dispense with the task of removing DNA damage from the non-essential bulk of their genome, as long as they are able to maintain the integrity of the genes that must be expressed, or transcription-coupled repair [71, 72]. E-induced expression of DNA repair enzymes would increase the viability of serotonin neurons subjected to stresses such as pro-inflammatory cytokines or environmental toxins.

The heat-shock proteins, HSP70, HSP60 and HSP27 are chaperones, and their mRNAs significantly increased with E or E+P treatment (all, $p < 0.05$). Chaperones are pivotal for proper protein folding, without which multiple cellular functions go awry. Of significance to Parkinson's disease, chaperones are capable of preventing α -synuclein misfolding, oligomerization and aggregate formation that leads to Lewy bodies [73–75]. HSP27 particularly modulates intermediate filament organization under conditions of physiological stress and NDD [76]. In addition, several of the HSP family members have important roles in steroid receptor activation, which is needed for mediating the actions of E and P [77].

The modification of proteins with ubiquitin is an important cellular mechanism for targeting mis-folded proteins for degradation [78–80]. Ubiquitination involves at least three classes of enzymes: ubiquitin-activating enzymes, or E1s, ubiquitin conjugating enzymes, or E2s, and ubiquitin-protein ligases, or E3s [81]. Ubiquitinase coding genes UBEA5, UBE2D3 and UBE3A (Parkin) increased with E or E+P (ANOVA $p < 0.003$). UBEA5 encodes a member of the E1-type ubiquitin activating enzyme family. UBE2D5 encodes a member of the E2 ubiquitin-conjugating enzyme family, and UBE3A (Parkin) is an E3 ubiquitin-protein ligase, the third part of the ubiquitin protein degradation system. Parkin is inactivated in Parkinson's disease patients [82], thus preventing degradation of misfolded proteins such as α -synuclein. Thus, E opposes this action.

Much of adult plasticity relies on proliferating dendritic spines and regulated insertion of glutamate receptors. Moreover, the development of new synapses on dendritic spines and the expression of glutamate receptors have been linked [83, 84]. Genes coding for proteins involved in dendritic spine proliferation [69], glutamate receptor signaling, transporters and the glutamate cycle [60] as well as genes encoding proteins involved in synapse assembly [85] were significantly increased with E and/or E+P. Together these actions support the most elementary units of adult neuroplasticity.

The effect of E supplemented with P compared to E alone was variable, and the basis for the different effects of P on different genes needs further study. Nonetheless in general, E or E +P increased gene expression related to DNA repair, protein folding, protein degradation and axonal transport. In addition, E or E+P increased the expression of α -secretase, which prevents the production of toxic A β fragments, and E or E+P decreased the expression of genes that code for potentially toxic proteins.

Serotonin neuron gene expression in old monkeys

The above studies in the short-term Ovx model provided proof of the principle that ovarian hormones can protect serotonin neurons from apoptosis. Nonetheless, it was important to obtain similar information from a more physiologically relevant NHP model. Therefore, older (>18 yrs) female rhesus macaques were Ovx and after 2 months, they were administered placebo, E alone and E with cyclic P (last 11 days of 28 day cycle) for 4 years using Silastic capsules that were replaced as needed [86]. The ratio of serum E to P is critical for physiological results and unfortunately the serum P concentrations were higher than normal and higher than obtained in the short-term model. For comparison of adult and old macaques, only the E-treatment will be considered. Also, a small block of tissue containing the dorsal raphe was used instead of laser captured serotonin neurons. All animals were maintained on standard low fat, low sugar monkey chow. Altogether, there were 4 variables that differed between the old monkey study and the younger adult monkey study: (1) age, (2) length of treatment (3) unintended higher serum P concentrations and (4) preparation extracted for qRT-PCR.

Significant differences between placebo and E treatments were observed in 36 out of 48 genes previously examined in the short-term model. After 4 years of E administration, the majority of genes exhibited the same patterns of expression as found in the younger adult monkeys treated with 1 month of E, relative to the placebo group [86]. That is, E administration caused a significant increase in mRNA expression related to DNA repair, protein folding, degradation, and transport in the old macaques in a manner similar to young macaques [59]. In addition, gene expression related to neurodegeneration was also similar between the adult and old monkeys. Continuing these functions in serotonin and other neurons is vital for healthy ageing.

It should be noted that the ratio's of gene mRNA/GAPDH mRNA differ from 2 to 10-fold between the adult and old monkeys. This is due to the use of a laser capture preparation of serotonin neurons in the adult monkeys versus a small block of the dorsal raphe from the old monkeys that was donated. There was a great deal more mRNA extracted from the block compared to the laser captured neurons. However, the pattern and changes from placebo condition were most important and reflect regulation of gene expression by E.

The common NDDs observed in aging populations have not been observed in old female macaques that do not live much past 30 years of age, and they produce ovarian hormones for almost their entire life. However, macaques accumulate lipofuscin, a product of free radical-induced lipid peroxidation that correlates with cellular aging [87]. In the old Ovx monkeys, E increased ADAM10 and SNCA mRNAs, which would improve correct processing of APP and synapse structure. E also suppressed APP mRNA, the amyloid precursor protein, and

PSEN1 mRNA, the component of γ -secretase that produces A β in the old monkeys (Figure 4). The data are identical to that observed in the younger adult monkeys treated with E for 1 month, and it is consistent with epidemiological observations that HT may delay the onset of Alzheimer's [8].

Gene expression for pivotal transport motors was higher in old Ovx monkeys treated with E than in placebo controls (Figure 5). Kinesin (KIF5B), dynactin (DCTN4) and dynein (DYNCL1) mRNAs were all significantly increased by E administration. These changes may be pivotal in ALZ due to the recognition that decreases in the transport motors promote axonopathy that precedes the accumulation of A β [1].

E alone also increased mRNA expression in 8 of 16 genes coding for DNA repair enzymes. Thus, chronic hormone therapy with E or E+P maintained expression of DNA repair factors in the dorsal raphe of old macaques, as well as in young adult macaques, which is essential for maintaining neural function.

Like the younger adult monkeys, the old monkeys also exhibited increases in HSP90, HSP60 and HSP27 with E treatment. In addition, E-treatment significantly increased ubiquinases UBEA5, UBE2D3 and UBE3A.

Neuroplasticity was likely increased or maintained with E-treatment in the old monkeys. E alone increased the expression of 4 genes (NLGN3, NTRK, SNAP25, NCAM) in old macaques that code for synapse assembly proteins as determined with ANOVA or Cohen's *d*.

NMDA2A and AMPA2 mRNAs increased with E treatment, further indicating that synapses were increased by E replacement in old Ovx macaques. The expression of these two types of receptors is particularly important in the ontogeny of mature dendritic spines [61, 88]. E also stimulated GRM1 mRNA. Altogether, the data support an ongoing supportive effect of E on dendritic spine proliferation and excitatory synapse assembly in old Ovx monkeys.

As shown earlier in adult monkeys, ovarian steroids improved gene transcription related to serotonin neurotransmission. E increased FEV (PET1) and TPH2 mRNAs. Together, their increased expression should promote the serotonin phenotype and increased production of serotonin. E also increased CRHR2 and UCN1 mRNAs. CRHR2 is considered anxiolytic; it binds UCN1 and causes a release of serotonin. CRHR1 is the anxiolytic receptor that binds CRH and decreases serotonin release. E and E+P decreased CRHR1 expression, which would further increase serotonin. Therefore, E supported the serotonin neuronal phenotype [89], increased serotonin synthesis [90] and increased serotonin release via regulation of CRH receptors [91]. Please note that rather than "increase" any of the above expressions, it could be argued that the mRNAs declined with Ovx, but E *maintained* the mRNA levels observed in intact cycling monkeys.

It is essential to keep in mind that diet plays a role in the effects of E in serotonin neurons, and the monkeys in all of the studies mentioned in this review were maintained on low fat, low sugar monkey chow. We found that a high fat diet blocked the positive effect of E on genes coding for proteins that promote serotonin neurotransmission and decrease

norepinephrine transmission in marmosets [92]. This data suggests that clinical populations that are obese and/or eat a high fat and sugar diet may not optimally respond to E therapy, and we are currently testing this hypothesis in old macaques.

In summary, old rhesus macaques that are Ovx and administered E or E+P for ~4 years have improved expression of genes that govern neurodegeneration, transport, synapse assembly, glutamate receptors, DNA repair, chaperones, ubiquinases, transport and serotonin neurotransmission, compared to placebo-treated controls. Jointly, the data reveal mechanisms that could improve serotonin function and decrease neuropathologies in women on low fat and sugar diets, and who initiate low dose bio-identical hormone therapy during the peri-menopause and continue it for at least 4 years.

Conclusions

These studies provide further molecular support to the concept that the pathologies of ALZ may also manifest in serotonin neurons and lead to the onset of depression before, or coincident with overt ALZ symptomology. The data also support the idea that serotonin neurodegeneration has a role in depression, which has been previously proposed [28, 93, 94]. However, the *concept* that the loss of ovarian steroids acts through gene expression and leads to serotonin neurodegeneration, which in turn contributes to postmenopausal and age-associated depression, as well as depression associated with NDDs, provides crucial integration of a loose association of available information. Thus, it makes sense that administration of hormone therapy or antidepressants long after menopause or onset of ALZ would be useless if the target neurons were gone. The key to healthy aging will be to save neurons with early treatments.

Unfortunately, a large clinical trial called the Women's Health Initiative (WHI) has given hormone replacement therapy (HRT) in women a huge setback for approximately 12 years now. This trial did NOT actually examine HRT because it did not use human hormones, and the ligands were administered on average, 10 years after menopause. Patients were administered rather high doses of conjugated equine estrogens (CEE) and medroxy-progesterone acetate (MPA), a synthetic androgen [95]. These compounds are still marketed by Wyeth at lower doses. While the WHI may be condemning of CEE and MPA at higher doses, the WHI was, is, and should not be a general reproach of HRT as presented in the media. Newer data with low doses of human hormones during the perimenopausal period shows multiple benefits, although routes of administration call for improvement and a better progestin is seriously needed. In the meantime, not all but *many* women still struggle with the decision to use HRT (often requiring progesterone to offset E-induced uterine proliferation). While the research presented in this mini-review is brain centric, it should be noted that E has many beneficial actions on peripheral tissues as well. Indeed, the idea that E is a trophic factor has arrived.

Acknowledgments

The authors would like to thank Yukari Tokuyama, PhD for the work on the apoptosis proteins during her postdoctoral tenure in the Bethea laboratory. Dr. Tokuyama currently works for Thermo-Fisher Scientific and she has restrictions on authorship. The authors are very grateful to the staff of the Department of Comparative Medicine at ONPRC for the exemplary care provided to our monkeys and to the staff of the Surgical and Pathobiology Units

for help with all aspects of needed surgeries and necropsies. We also thank Dr. Hemachandra Reddy for the invitation to participate in this issue of the Journal of Alzheimer's Disease. This work was supported by NIH grants MH62677 to CLB, Fogerty Fellowship to FBL, Reproductive Training grant T32 HD07133 support to YT and OD11092 for support of the ONRPC. Current Research for APR is supported by Garrison's Family foundation, a New investigator Research Grant from Alzheimer's Association 2016-NIRG-397187 and Texas Tech Health and Sciences Center of Excellence for Translational Neuroscience and Therapeutics PN-CTNT 2015-12.

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Figure 1.

Photomicrographs of serotonin neurons with TUNEL staining and histograms of TUNEL analysis in the dorsal raphe are shown. Serotonin neurons were immunostained for TPH and developed with alkaline phosphatase (blue). The TUNEL staining was performed with ApopTag Kit-S7100 (Chemicon, Temecula, CA, USA) and developed with H₂O₂, 3% diaminobenzidine tetrahydro-chloride (DAB; Dojindo Laboratories, Kumamoto, Japan). The quantitative analysis was executed on 8 sections of the dorsal raphe from individual animals processed for TUNEL staining with no counterstain (n=5 animals/treatment group). The scale bar applies to all panels.

- A.** Serotonin neuron with no TUNEL staining and a clear nucleus.
- B.** Serotonin neuron with perinuclear, type II, TUNEL staining and a clear nucleus.
- C.** Serotonin neuron with nuclear, type I, TUNEL staining, which obscures the nucleus.
- D.** Serotonin neuron with perinuclear, type II, TUNEL staining and a mostly clear nucleus.
- E.** Serotonin neuron with nuclear, type I, TUNEL staining and advanced degeneration.

F. Serotonin neuron with nuclear, type I, TUNEL staining and advanced degeneration.

G. Histogram of the average total number of TUNEL stained neurons in the dorsal raphe with placebo (OVX), estradiol (E), and estradiol plus progesterone treatment (EP) for one month. The area measured was held constant so that the total number of TUNEL-positive neurons could be obtained. There was a decrease in TUNEL-positive neurons with treatment that reached statistical significance with E+P ($p=0.04$, Mann-Whitney).

H. Histogram of the data in panel G expressed per mm^3 . There was a decrease in TUNEL-positive neurons/ mm^3 with treatment that reached statistical significance with E+P ($p=0.04$, Mann-Whitney).

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Figure 2.

Western blots for pro-apoptotic proteins and quantitative analysis of the optical density of the bands are illustrated. The animals were processed in 4 sets with each set containing one OvX-placebo, one E treated, and one E+P treated. The data are expressed as percent of OvX control for each set and the average is illustrated.

A, B. JNK1 and phopho-JNK1 (P-JNK1) were significantly decreased by E or E+P treatment (n=4; ANOVA $p < 0.05$; post hoc comparison $p < 0.05$).

C,D. Mitochondrial AIF (mAIF) and nuclear AIF (nAIF) are illustrated. There was a significant difference between the groups for nAIF (n=4; ANOVA $p<0.05$). Nuclear translocation of AIF from mitochondria was significantly decreased in E+P-treated animals (post hoc comparison $p<0.05$). The loading control for mAIF was CoxI and the loading control for nAIF was Histone H1 and they indicated that similar amounts of control proteins were loaded on the gels (not shown).

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Figure 3.

Photomicrographs of AIF immunostaining in the dorsal raphe of an OvX-placebo treated monkey and qRT-PCR analysis of AIF expression in dorsal raphe blocks from OvX and treated monkeys.

A. AIF is robustly expressed in the large dorsal raphe neurons. A dashed line surrounds the dorsal raphe.

B. Higher magnification picture of neurons within the box shown in panel A.

C. AIF mRNA expression in the dorsal raphe of treated monkeys (n=3/group). There was a marked decrease in AIF expression, but statistical significance was not achieved due to

variance between monkeys. However, AIF protein expression (Figure 2) reflects AIF mRNA expression.

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Disease Specific Genes

Figure 4.

Neurodegeneration (NDD) related gene expression in serotonin neurons or microdissected dorsal raphe.

Left column - ADAM10, SNCA1, APP, and PSEN1 mRNAs were measured by qRT-PCR (Taqman Microfluidic Custom Array, ABI-Thermo Fisher, Waltham, MA) in laser-captured serotonin neurons from OvX-placebo and E-treated *adult* monkeys (n=3/group). The monkeys were OvX for 5–8 months and then treated for 1 month. There was a significant

increase in ADAM10 and SNCA with E treatment (t-test $p<0.05$). There was a significant decrease in APP and PSEN1 with E treatment (t-test $p<0.05$).

Right column - ADAM10, SNCA1, APP, and PSEN1 mRNAs were measured by qRT-PCR (Taqman Microfluidic Custom Array) in microdissected raphe tissue from Ovx-placebo and E-treated *old* monkeys (*old* >18 yrs + 4 yrs treatment; n=3 or 4/per group). The old monkeys were Ovx for 2 months and then treated for 4 years. There was a significant increase in ADAM10 and SNCA with E treatment (t-test $p<0.05$). There was a significant decrease in APP and PSEN1 with E treatment (t-test $p<0.05$).

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Common Disease Mechanisms: Transport

Figure 5.

Neuronal transport related gene expression in serotonin neurons or microdissected dorsal raphe.

Left column – KIF5B, DCTN4 and DYNCL1 mRNAs were measured by qRT-PCR (Taqman Microfluidic Custom Array) in laser-captured serotonin neurons from OvX-placebo and E-treated *adult* monkeys (n=3/group). The monkeys were OvX for 5–8 months and then treated for 1 month. There was a significant increase in KIF5B, DCTN4 and DYNCL1 with E treatment (t-test $p < 0.05$).

Right column - KIF5B, DCTN4 and DYNCL1 mRNAs were measured by qRT-PCR (Taqman Microfluidic Custom Array) in microdissected raphe tissue from OvX-placebo and E-treated *old* monkeys (*old* > 18 yrs + 4 yrs treatment; n=3 or 4/per group). The old monkeys were OvX for 2 months and then treated for 4 years. There was a significant increase in KIF5B, DCTN4 and DYNCL1 with E treatment (t-test $p < 0.05$).

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