Mitigation of Alzheimer’s Disease Neuropathologies by Human Neural Stem Cell-derived Extracellular Vesicles

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Research

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Abstract

Background: Regenerative therapies to mitigate Alzheimer’s disease (AD) neuropathology have shown very limited success. In the recent era, extracellular vesicles (EV) derived from multipotent and pluripotent stem cells have shown considerable promise for the treatment of dementia and many neurodegenerative conditions.

Methods: Using the 5xFAD accelerated transgenic mouse model of AD, we now show the regenerative potential of human neural stem cell (hNSC)-derived EV on the neurocognitive and neuropathologic outcomes in the AD brain. Two or six-month-old 5xFAD mice received single or two intra-venous (retro-orbital vein, RO) injections of hNSC-derived EV, respectively.

Results: RO treatment using hNSC-derived EV restored fear extinction memory consolidation and reduced anxiety-related behaviors 4-6 weeks post-injection. EV treatment also significantly reduced dense core amyloid-beta plaque accumulation and microglial activation in both age groups. These results correlated with partial restoration of homeostatic levels of circulating pro-inflammatory cytokines in the AD mice. Importantly, EV treatment protected against synaptic loss in the AD brain that paralleled improved cognition. MiRNA analysis of the EV cargo revealed promising candidates targeting neuroinflammation and synaptic function.

Conclusions: Collectively, these data demonstrate the neuroprotective effects of systemic administration of stem cell-derived EV for remediation of behavioral and molecular AD neuropathologies.

Background

The most common form of dementia is Alzheimer’s disease (AD), an irreversible neurodegenerative disease that is characterized by progressive cognitive and functional decline and memory loss [26]. Currently there are no effective therapies to slow or reverse the disease progression. Hallmarks of AD include amyloid-beta (Aβ) plaques, neurofibrillary tangles and neuroinflammation. Microglia, the resident macrophages of the brain, become activated in an effort to engulf and clear the extracellular Aβ plaque deposits. However persistent microglial activation in AD eventually leads to a pro-inflammatory environment that includes increased expression of pro-inflammatory cytokines and reduced expression of anti-inflammatory cytokines [7, 13, 49]. Ultimately, this chronic inflammation creates a neurotoxic microenvironment that leads to loss of dendritic spines and neurons in the AD brain [49].

Similar neuropathologies including persistent inflammation, loss of synaptic integrity, and cognitive impairments have been clearly shown to manifest in the radiation damaged brain [40]. In a series of studies, we demonstrated that hippocampal engraftment of human neural stem cells (hNSC) ameliorated the neurocognitive and neuroinflammatory effects of a clinically relevant radiation exposure using athymic nude rats [2, 3, 5, 6]. These studies showed that stem cell-based approaches improved the functional plasticity of the irradiated host brain, suppressing neuroinflammation, preserving host neuronal morphology and improving cognitive function. Since significant attrition of engrafted cells was
observed, it was hypothesized that the hNSC provided neuroprotective benefits primarily via trophic support rather than proliferation and repopulation [14, 27, 45].

A somewhat similar trajectory involving stem cells has been observed in the search for effective approaches to ameliorate AD neuropathology. Studies have demonstrated that hNSC transplantation can provide at least some neurocognitive benefits in transgenic mouse models of AD [33]. The data from both radiation and AD studies have suggested that the neuroprotective properties and trophic support mechanisms by which NSC act to reduce inflammation and preserve the structural integrity of the injured brain is via the stem cell secretome, more specifically stem cell-derived extracellular vesicles (EV) [27, 28, 31, 45, 53, 59]. In the case of cranial irradiation, this assertion has been borne out through a series of studies, using hNSC-derived EV. In a first study, the efficacy of EV therapy was demonstrated in an irradiated athymic nude rat model [14], and then in studies using immunocompetent rats and mice [27, 45]. Studies by others have similarly indicated, that mouse stem cell-derived EV transplantation can provide neurocognitive benefits in transgenic mouse models of AD [28, 31].

EV are small membrane-bound vesicles that have been shown to be less immunoreactive than their cellular source and to contain bioactive cargo such as proteins, mRNA, and microRNA (miRNA) [19]. Endogenously, EV are recognized as important modulators of biological processes, with specific cargo capable of controlling cell signaling and target cell function to maintain homeostasis or to contribute to disease pathology [9, 41, 46]. Exogenous stem cell derived EV represent a unique paracrine signaling mechanism with great therapeutic potential, particularly in the context of CNS injuries such at traumatic brain injury, stroke and neurodegenerative diseases [28, 31, 46, 57, 61]. These EV not only cross the blood-brain barrier, but also negate concerns regarding the possibility of teratoma formation and immune rejection that confound related stem cell-based approaches [15, 17, 22]. Immune rejection is a particularly significant problem in the case of AD, since the long-term use of immunesuppresants can result in toxicity, particularly in the aged, and may also exacerbate AD-related pathologies [35, 58].

In this study the 5xFAD mouse model of AD that co-expresses five human familial AD mutations was used [38]. The 5xFAD mouse is an accelerated model of AD with measurable Aβ plaque load by 2 months of age, but in the absence of neurofibrillary tangles. These animals also exhibit reductions in synaptophysin, neuronal loss and memory impairments that become evident by 5–6 months of age. The goal of this study was to test the hypothesis that hNSC-derived EV treatment could mitigate AD-related behavioral and molecular neuropathologies when delivered intravenously to early- or late-stage AD mice. Results indicate this strategy to be a promising approach, where EV treatment improved cognition, reduced Aβ plaque load and inflammation, and modulated the expression of genes involved in numerous AD-related pathways.

**Materials And Methods**

Detailed methods and experimental procedures are described in the Supplemental Information section.
hNSC Culture and EV Isolation and Characterization

The use of hNSC was approved by the Institutional Human Stem Cell Research Oversight Committee (HSCRO). The validation, expansion, and characterization of hNSC (ENStem-A; EMD Millipore) followed previously published procedures \[2\]. Before isolation of extracellular vesicles (EV), the conditioned hNSC culture medium was put through a 0.22 µm sterile filter as an initial purification step before centrifugation. EV were then isolated and further purified from the hNSC culture medium by ultracentrifugation \[14\] and characterized using a particle analyzer (ZetaView PMX 110; Meersbusch, Germany). The mean EV size was 147.8 ± 3.9 nm diameter. The purified EV were diluted into sterile hibernation buffer (ThermoFisher Scientific) to deliver \(2.25 \times 10^7\) EV per 50 µl injection in the experiments described below.

Animals and EV delivery

All animal experimentation procedures were performed in accordance with the guidelines provided by NIH and approved by the University of California Irvine Institutional Animal Care and Use Committee. 5xFAD transgenic mice (B6SJL-Tg (APPSwe Fallon, PSEN1*M146L*L286V)6799Vas/Mmjax) and age-matched littermate controls were maintained in standard housing conditions (20 °C ± 1 °C; 70% ± 10% humidity; 12 h:12 h light and dark cycle) and provided ad libitum access to standard rodent chow (Envigo Teklad 2020x) and water. For the early stage AD study, male 5xFAD mice and their wild type littermate controls were divided into the following groups: vehicle-injected AD with sterile hibernation buffer (Gibco) (AD; N = 15), EV-injected AD (AD + EV; N = 16), and vehicle-injected wild type (WT; N = 16). The mice were 1.5–2.5 mo of age at the time of EV treatment and stratified by age to maintain equivalent age distributions among experimental groups. Behavior studies were initiated 1 month post-EV treatment. The late AD study utilized the same treatments and stratification where the AD mice and their WT littermate controls were 5.0-6.5 months of age (N = 14–16 AD, AD + EV, WT) at the first EV treatment. A second EV injection was administered 2 weeks later. One month after the second EV treatment, when the mice reached 6.5-8.0 mo of age, behavior studies were initiated (Fig. 1A).

To administer EV, mice were sedated using 2.5% (v/v) isoflurane/oxygen and \(2.25 \times 10^7\) EV in 50 µl hibernation buffer was delivered into circulation via injection into the retro-orbital sinus (RO). Control AD and WT mice received RO injections on that same schedule using 50 µl of vehicle. Over the years our group, and others, have not included grafted controls (either stem cells or EV) since such transplantation procedures used to treat a variety of pathologies in different rodent models were not found to functionally affect the intact normal brain \[14, 30, 53, 54, 60\]. Importantly, our past work using stem cells \[1\] and from others using EV \[20, 24\], in which grafted controls were included, found that all functional and molecular endpoints were statistically indistinguishable between the control, stem cell-treated control, or EV-treated control groups. Further rationale for excluding controls treated with EV alone, is that inclusion of such cohorts is clinically irrelevant.

Behavioral testing
Behavioral studies began 1 month after EV treatment using N = 15–16 mice per group as described above. Testing occurred over 3 weeks and included the following paradigms in order: novel object recognition (NOR), elevated plus maze (EPM), light-dark box (LDB), and fear extinction (FE) memory tasks. Independent investigators, also blinded to the experimental groups, scored all behavior videos. See Supplemental Information for the details.

**Thioflavin-S (thio-S) staining**

Following behavior, a subset of those same mice were deeply anesthetized using isoflurane and euthanized via intracardiac perfusion using 4% paraformaldehyde (Sigma) in 100 mM phosphate buffered saline (PBS; pH 7.4, ThermoFisher Scientific). Brains were cryoprotected (10–30% sucrose gradient over 2–3 days) and sectioned coronally into 30 µm using a cryostat (Leica Microsystems, Germany). For each endpoint, 4 representative coronal brain sections of the amygdala and medial prefrontal cortex (mPFC) regions from each of the 4 animals per experimental group were selected at approximately 15 section intervals, washed in PBS and stained for thio-S.

**Amyloid-β ELISA**

Fresh frozen brain tissues were isolated from another subset of the behaviorally tested mice. As per the manufacture's protocol, isolated protein samples from fresh frozen brains were applied to a blocked MSD Human/Rodent (4G8) Aβ triplex ELISA plate and incubated for 2 hours at room temperature on an orbital shaker (Aβ1-38, Aβ1-40, Aβ1-42; Meso Scale Discovery, Rockville MD). The plate was then washed and measurements obtained using a SECTOR Imager 2400. N = 7 and 6 mice per experimental group for AD and AD + EV soluble Aβ measurements and N = 7 mice per experimental group for insoluble Aβ measurements, with 1 WT negative control for both measurements.

**Immunohistochemistry, confocal microscopy, image processing and 3D quantification**

Immunofluorescence staining on the PFA fixed brain sections was conducted for the microglial activation marker CD68 and pre-synaptic marker synaptophysin as described in detail in the Supplemental Information section. 3D algorithm-based volumetric quantification was facilitated by AutoQuant and Imaris modules (BitPlane, Inc., Zurich, Switzerland).

**Spleen cytokine analysis and flow cytometry**

Fresh spleens were collected from early and late stage AD, AD + EV and WT mice, 7 mice per group. For cytokine analysis, spleen cells (1 x 10^6/ml) were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml; Sigma) and ionomycin (1 µg/ml; Sigma) in RPMI medium containing 10% FBS. Supernatants collected after overnight stimulation were assayed for IFN-γ, IL-17 and IL-10, IL-1β, using a magnetic bead-based kit (Thermo Fisher Scientific). Single cell spleen suspensions were stained with antibodies specific to B1 cells (CD19^+CD5^+CD43^+; (Biolegend, San Diego, CA). Analysis was done using Flow Jo software (Treestar, Ashland, OR).
Gene expression analysis

Total mRNA was purified from the fresh frozen hippocampus of each of 4 mice per group, WT, AD, and AD + EV, from the late stage AD cohort and multiplexed using the nCounter Mouse Neuroinflammation Panel that includes 757 genes that cover core pathways that define neuroinflammation processes and 13 internal reference genes (NanoString, CA).

MicroRNA microarray and validation

MiRNA isolated from EV analyzed on a miRNA microarray chip (Exiqon, Denmark; Genomics Shared Resource at the University of Texas South Western Medical Center). Validation of miRNA array data was performed using qPCR as described in the Supplemental Information section.

Statistical Analysis

Statistical analyses were carried out using GraphPad Prism (v6). One-way analysis of variance (ANOVA) was used to assess the normal distribution of data and the significance for the AD, AD + EV and WT groups of mice. When overall group effects were found to be statistically significant, the Bonferroni multiple comparisons test was used to compare the AD group with each of the other experimental groups. A $P$ value of $\leq 0.05$ was considered to be statistically significant.

Results

Effects of disease progression and EV treatment on cognitive function

One month after RO injection of EV, the mice were habituated and tested on the NOR task that engages hippocampal and medial prefrontal cortex (mPFC) function [11, 12] that may be impaired in AD. For this task, the total exploration time during the familiarization phase for each object was not different between any of the experimental groups for either the early or the late AD studies, suggesting that there were no AD-related alterations in locomotion or exploratory behavior (Supplemental Table 1). While there were no significant differences between WT, AD and AD + EV mice during the test phase of the NOR task for either the early or late AD studies there was a trend for decreased novel object exploration in the AD mice as compared to both WT and AD + EV cohorts of late AD mice (Fig. 1B and E, respectively).

Because altered mood frequently manifests in AD [25], we also used the EPM and LDB tests as a measure of anxiety-like behavior. Early AD male mice spent significantly less time in the open arms of the maze as compared to both WT and AD + EV mice ($P< 0.05$), suggesting increased AD-related anxiety that could be ameliorated at early disease times by a single EV treatment (Fig. 1C). However, these mice did not exhibit any reluctance to transition between light and dark compartments during LDB testing (Fig. 1D). Conversely the late AD males exhibited no anxiety-like behavior on the EPM (Fig. 1F), but did show fewer transitions between compartments on the LDB test that were again mitigated by EV treatment ($P< 0.05$ and $P< 0.01$, respectively; Fig. 1G).
AD mice can successfully learn the aversive association during the conditioning phase of the FE test, but are impaired in dissociative learning [16], thus we evaluated the extinction of fear memory, the active process of memory consolidation [18]. Indeed, during the fear acquisition phase of testing all mice from both the early and late AD cohorts showed similar percent times freezing during the 3 tone-shock pairings (Fig. 2A, C; T1-3 Conditioning). During the subsequent fear extinction training in a new context, early WT and AD + EV mice demonstrated gradual decreases in freezing behavior over the course of the day 2 and day 3 trials as compared to the AD mice that continued to freeze at a significantly higher level (Fig. 2A; Day 1–3 Extinction Training). The results of the final day of extinction testing showed that the WT and AD + EV mice had equivalent fear extinction as illustrated by reduced percent times freezing as compared to the AD mice that continued to freeze for a significant percent of the test time (Fig. 2B; \(P<0.05\)). This AD-related impairment of fear memory extinction suggests dysfunction of neural circuitry in the hippocampus, mPFC and amygdala [16, 34] that could be ameliorated by EV treatment. While not statistically robust, similar trends were seen in the late AD study (Fig. 2C, D).

**Stem cell-derived EV reduce Aβ plaques in the AD brain**

To determine whether EV treatment reduced amyloid pathology observed in AD, we stained for dense-core plaques using Thio-S (Fig. 3A-D). In the early stage AD mice that underwent EV treatment at ~2 months of age, the plaque load was still very low. Nonetheless, EV treatment reduced the number of plaques in the mPFC region of the brain (Fig. 3E; \(P<0.05\) compared to AD). In the late stage AD mice that received EV treatment at ~5.5 months of age, where the plaque load was significantly higher, EV treatment significantly reduced the number of Aβ plaques in both the amygdala and the mPFC regions of the brain (Fig. 3F; \(P<0.01\) and \(P<0.05\), respectively). We then evaluated the levels of both soluble and insoluble Aβ\(_{1-42}\), Aβ\(_{1-40}\), and Aβ\(_{1-38}\) in the brain using triplex ELISA. While no difference between groups was observed for soluble Aβ\(_{1-42}\) and Aβ\(_{1-38}\), Aβ\(_{1-40}\) levels were significantly reduced in the brains of late stage AD mice that had received EV treatment (Fig. 3D; \(P<0.05\)). No differences were observed between AD and AD + EV mice for any species of insoluble Aβ (Fig. 3E). Together, these data suggest that just one to two EV treatments can contribute to either a reduced production of Aβ or aid in clearance.

**Effect of EV treatment on activated microglia**

Microglia, the innate immune cells of the CNS, have a protective function in modulating the accumulation of Aβ plaques in the early stages of AD. However, evidence suggests that once activated, the microglia become a source of damaging inflammation and synaptic loss as the disease progresses. Using CD68 staining as a marker for microglial activation in the brain, we observed significantly increased CD68 immunoreactivity in the amygdala of the early AD mouse brain that was not significantly reduced by EV treatment (Fig. 4A; \(P<0.05\) for AD vs WT groups), however no significant differences among experimental groups were observed for CD68 in the mPFC region of the brain at that same time (Fig. 4B). As with the early AD mice, increased CD68 immunoreactivity was observed in the amygdala region of the brains of late AD mice, with only a trend for modulation by EV treatment (Fig. 4C, E-G; \(P<0.01\) AD vs WT groups). Unlike the early AD mice, though, late AD mice exhibit significant increases in CD68 immunoreactivity in
the mPFC that were attenuated to control levels by EV treatment (Fig. 4D, H-GJ; P < 0.01 for AD vs either WT or AD + EV groups).

**EV treatment restored synaptophysin in the AD brain**

Significant loss of pre- and post-synaptic proteins, such as pre-synaptic density protein-95 and synaptophysin, respectively, has been linked to the cognitive impairments associated with AD. Evaluation of synaptophysin immunoreactivity by confocal microscopy and volumetric quantification revealed significant decreases in this pre-synaptic marker in both the amygdala and the mPFC in the early AD brain that were restored to WT control levels in the EV treated AD brain (Fig. 5A, B; P < 0.05 for AD vs either WT or AD + EV groups). Similar, reductions were observed in the amygdala of the late AD brain that were also restored to WT levels by EV treatment (Fig. 5C, E-G; P < 0.05 for AD vs either WT or AD + EV groups). While no significant differences in synaptophysin were observed in the comparison of the mPFC region of the brain for the WT and AD groups of late AD mice, the EV treatment increased the levels of synaptophysin to greater than that of either WT or AD mice (Fig. 5D, H-J; P < 0.05 for AD vs AD + EV groups).

**The effect of EV on peripheral inflammatory responses in AD**

The increased neuroinflammation observed in AD is frequently associated with significant peripheral immune responses that correlate with adverse outcomes in human AD patients [13]. Therefore, the levels of inflammatory and anti-inflammatory cytokines in the plasma were evaluated. Interferon-γ (IFNγ) is a pro-inflammatory cytokine that has been shown to prime microglia under pathological conditions including AD. While it remained unaffected in the early AD spleen, IFNγ was upregulated in the late stage AD spleen, and EV treatment significantly reduced those level (Fig. 6A; P < 0.05 for AD vs AD + EV groups). IL-17 overexpression has similarly been implicated in neuropathologies and AD. It was also found to be unaltered in early stage AD, but significantly elevated in the late AD mice, and reduced WT levels by EV treatment (Fig. 6B; P < 0.05 for AD vs AD + EV groups). Conversely, the anti-inflammatory cytokine IL-10 that downregulates the expression of inflammatory cytokines was found to be dramatically reduced in AD mice as compared to either WT or EV + AD mice (Fig. 6C; P < 0.01 and P < 0.05, respectively for AD vs WT and AD + EV groups, respectively). It has been shown that the ratio of IgM to IgG is significantly decreased in human AD patients, and in the 5xFAD mouse model of AD [13]. A specialized subset of B1 cells produce IgM, the percentage of which was shown to be reduced in the AD mouse. Similarly, in this study, while not significant a trend for decreased B1 cells was observed in AD mice as compared to WT or AD + EV mice (Fig. 6D).

**Effect of EV treatment on mRNA levels in the hippocampus of the AD brain**

Significant changes in transcription levels of genes implicated in AD pathology have been observed previously. In the current study, increases in expression for pro-inflammatory and immune response genes, as well as genes involved in microglial activation were noted when comparing WT and late stage AD groups of mice (Fig. 7). Analysis of the AD + EV mice demonstrated a subtle trend for effectiveness of
the EV treatment in certain animals, while one mouse remained a consistent non-responder to EV treatment for all AD-related genes evaluated.

**miRNA microarray analysis reveals potential therapeutic EV cargo**

In order to investigate potential functional components of hNSC-derived EV, miRNA cargo was evaluated using a targeted human miRNA array [27] (Supplemental Table 2). Candidate miRNA implicated in AD-related pathways including learning, memory, neuroinflammation were identified by cross-referencing the array data with the literature, which vary widely in opinions regarding the pathways influenced by a particular miRNA and whether the effect is beneficial or detrimental to CNS homeostasis or pathology. Among the miRNA identified in this analysis were miR-134-3p, miR-125b-5p, miR-124-3p, and miR-125a-5p. Of these four select candidates, all except miR-134-3p were confirmed to be present in EV RNA samples using TaqMan Advanced miRNA Assays, providing three candidate miRNA for potential follow up studies that could influence CNS function (miR-125b-5p, miR-124-3p, and miR-125a-5p; Supplemental Table 3) [32, 51, 52].

**Discussion**

Our past work has defined the effects of clinically relevant radiation exposures on the brain, that include cognitive impairment, neuroinflammation, dysregulation of pre- or post-synaptic protein levels, and loss of neuronal structure [2, 39]. Work then demonstrated that cranially grafted hNSC and then hNSC-derived EV were effective in mitigating radiation-induced neurodegenerative events. Here, we have demonstrated that the same hNSC-derived EV therapy is effective in ameliorating AD-related neurodegenerative pathologies. Single or duplicate EV treatments were found to reverse cognitive impairments in AD mice, including anxiety-like behaviors and fear memory consolidation.

One of the hallmarks of AD pathology is progressive accumulation of Aβ plaques in the brain. While plaque density is not directly linked with dementia [37, 43], the neurodegenerative effects of plaques in triggering neuroinflammation and loss of neurons is well documented [42, 48, 49]. To delineate the regenerative effects of stem cell-derived EV on pathology, whole brain ELISA on late stage AD brains was employed and revealed significant reductions in the levels of soluble amyloid-β$_{1-40}$, a particularly robust predictor of AD related synaptic loss. Concurrently, thio-S staining for dense core Aβ plaques confirmed that EV treatment provided some reductions in plaque load. These data indicate that the neuroprotective effects of EV are exerted, in part, by reducing the plaque burden in the AD brain at early and late stages of disease progression.

Persistent activation of microglia in the AD brain is detrimental for neuronal and cognitive function. Our past data have shown anti-inflammatory effects of stem cells or stem cell-derived EV in clinically relevant irradiation [2, 5, 14, 27] and chemobrain [4, 8] models. Substantiating the neuroprotective impact of EV in the AD brain, we found significant reductions in the levels of CD68, a marker of microglial/myeloid cell activation. While we did not evaluate neuron structure in the current study, persistent microglial reactivity...
has been linked to synapse loss in AD [42, 44, 48]. Mouse models of AD, including the 5xFAD model, have also been found to have reduced levels of pre- and post-synaptic protein levels. Our data demonstrated significant reductions in synaptophysin immunoreactivity in the AD brain that were restored by EV treatment. Together, our data suggest dysfunction of neural circuitry in the hippocampus, mPFC and amygdala that could be ameliorated by EV treatment [16, 34].

Inflammatory markers from the peripheral immune response indicate a highly pro-inflammatory environment in AD mice, that could be mitigated in part, by EV treatment. IFNγ and IL-17 are highly inflammatory cytokines and both implicated in CNS related disorders such as multiple sclerosis. Reduction in IL-17 is especially important as IL-17 has been shown to cause neuronal cell death in human Parkinson's disease iPSC model [47]. Decreased levels of IL-10 levels, an anti-inflammatory cytokine in early AD also indicates an impairment in controlling inflammation. Reductions in B1 cells in late stage AD may also contribute to AD-related accumulation of Aβ plaques as these cells contribute significantly to removal of cell debris and self-proteins. Restoration of B1 cell frequencies and reduced IFNγ and IL-17 by EVs at late stage AD combined with increased IL-10 levels at early stage indicates that EV treatment is affecting multiple pathways to dampen inflammation.

The cognitive and molecular observations from this study are supported by gene expression analysis from the late stage AD brain that demonstrated consistent increases in mRNA levels for genes involved in microglial activation, inflammation and other AD pathologies. Critically, the two EV treatments to the late stage AD mouse resulted in strong trends for reductions in that gene overexpression as compared to AD only mice.

An important part of this study is the analysis and identification of potentially beneficial EV cargo. Array analysis identified at least 3 strong candidate miRNA, miR-125a, miR-125b, and miR-124. While overexpression of any of these candidate miRNA can be damaging in specific cases [50], literature has also suggested they serve beneficial roles in the CNS. MiR-125a and miR-125b have been shown to maintain PSD-95 levels in dendrites and regulate synaptic structure and miR-125b is an important regulator of synaptic structure and function [21, 36]. MiR-124 expression has been demonstrated to reduce neuroinflammation and promoting neurite outgrowth in traumatic brain injury models [23, 29, 55, 56]. Further, miR-124 has been suggested to regulate glycogen synthase kinase 3β and glucocorticoid receptor levels in the context of AD (GSK3β and GR, respectively) and regulate anxiety as well as impulse control disorders that are indicative of poor decision making in humans [10]. The reported downregulation of miR-124 in the AD brain may, at least in part, contribute to the neuropathological phenotypes associated with AD. Significantly, we have recently demonstrated that AAV9-mediated over-expression of miR-124 alone was able to reduce the neurodegenerative consequences of cranial irradiation on cognition and microglial activation [27]. We hypothesize that similar pathways may be at play in the AD brain, where miR-124 helps to resolve a wide range of neuropathologies.

Similar observations regarding the efficacy of EV in treatment of AD have been made by other laboratories as well. Li and colleagues used embryonic mouse NSC-derived EV to treat 9 month old B6C3-
tg mice via stereotactic injection into the lateral ventricles [28]. Five weeks later, AD mice treated with EV were shown to have improved performance on the Morris water maze as compared to untreated AD littermates. Similarly, the EV treatment increased the levels of synaptic proteins, including synaptophysin and PSD-95, improved synaptic morphology, and mitochondrial function. While the EV treatment also reduced the levels of pro-inflammatory cytokines, it did not reduce the Aβ plaque load, supporting the assertion that plaques might not drive the cognitive phenotype of AD. Using mouse mesenchymal stem cell derived EV, Losurdo and colleagues administered two intranasal doses to 3xTg AD mice and found a shift towards an anti-inflammatory phenotype and reduced activation of microglia, as well as improved dendritic spine density [31]. These examples using two distinct models of AD corroborate our data on the beneficial effects of systemic injections of EV on disease pathology.

**Limitations**

While we do not see complete remediation of cognitive function, one or two injections of EV were able to partially restore cognitive indices. Our current observations suggest that additional, ongoing treatments with the EV could improve efficacy of the therapy, forestalling or even reversing AD pathologies. Importantly, our studies provide indications of at least one of the mechanisms by which these hNSC-derived EV may repair the AD brain, delivering miRNA capable of reducing inflammation and protecting neuronal structure, possibly through epigenetic regulation of gene expression. We acknowledge that other cargo are likely to play a role in the effectiveness of these EV and future studies will focus not only on the miRNA cargo and mechanisms, but also on the other potentially beneficial EV cargo such as protein.

**Conclusion**

AD is an irreversible neurodegenerative disease affecting millions of people worldwide and regenerative therapies to mitigate AD neuropathology have shown very limited success. The findings of this study demonstrate the neuroprotective efficacy of systemic administration of stem cell-derived EV for remediation of behavioral and molecular AD neuropathologies. Further, these data suggest that EV-contained miRNA may represent a potential, specific mechanism for follow up studies to develop therapeutic strategies to meet this critical unmet medical need.

**List Of Abbreviations**

Ab (amyloid beta)

AD (Alzheimer’s disease)

Basal lateral amygdala (BLA)

CNS (central nervous system)

ELISA (enzyme linked immunosorbant assay)
EPM (elevated plus maze)
EV (extracellular vesicle)
FE (fear extinction)
HSCRO (human stem cell research oversight committee)
hNSC (human neural stem cell)
Infralimbic cortex (IL)
IFNg (interferon gamma)
IL (interleukin)
Light-dark box (LDB)
MiR, MiRNA (microRNA)
mPFC (medial prefrontal cortex)
NIH (national institute of health)
NOR (novel object recognition)
PFA (paraformaldehyde)
PSD-95 (post synaptic density protein-95)
RO (retro-orbital)
Syp (synaptophysin)
Thio-s (thioflavin-s)
WT (wild type)

Declarations

Ethical approval and consent to participate
Not applicable

Consent for publication
Not applicable
Availability of data and materials

Correspondence and request for data or materials should be addressed to JEB.

Competing interests

The authors declare no competing interests.

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Author contributions

Conception and design: JEB, MMA.

Development of methodology: AA

Acquisition of data: LAA, AADB, CG, LA, EG, NR

Analysis and interpretation of data: LAA, AADB, AA, MMA, JEB

Writing, review and/or revision of the manuscript: AA, JEB, MMA

Administrative, technical, or material support: EG, NR, MMA

Study supervision: JEB, MMA

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**Figures**
Figure 1

A) Experimental design. Early AD male mice were treated with a single dose of human neural stem cell derived extracellular vesicles (EV) via retro-obital (RO) injection at ~1.5-2.5 mo of age and behaviorally tested 1 month later. Late stage AD male mice received 2 RO injections of EV at 2 week intervals and began behavior testing 1 month after the second injection. B-D) Early stage AD behavioral testing indicates no impairments on Novel Object Recognition (NOR) or Light-Dark Box (LDB) tests, but AD mice
show increased anxiety-like behavior on the Elevated Plus Maze (EPM) test that was mitigated by EV treatment. E-G) Late stage AD mice exhibit no significant impairments on NOR or EPM tests, but increased anxiety-like behavior on the LDB test that was mitigated by EV treatment as shows by restored number of transitions between the light-dark compartments. Data are presented as mean + SEM where N=14-16 mice/group. P values are derived from ANOVA and Bonferroni’s multiple comparisons test. *P<0.05, **P<0.01.
AD mice exhibit impairments in fear extinction memory and enhanced memory recall. All mice show elevated freezing following a series of 3 tone-shock pairings (0.6 mA, T1-T3). Subsequently, fear extinction training was administered every 24 hours (15 tones) for 3 days. These data are presented as the average of 5 tones (A, C). All A) early and C) late study mice exhibit a gradual decrease in freezing behavior (Day 1-3), however AD mice spend significantly more time freezing during the extinction training compared to controls. Twenty-four hours after extinction training B) early stage AD mice exhibit enhanced fear recall (elevated freezing, P<0.05), while AD mice receiving EV treatment exhibit successful extinction equivalent to that of WT control mice (reduced freezing). D) Late stage AD mice exhibit enhanced fear recall as compared to WT control mice. Data are presented as mean + SEM where N=14-16 mice/group. P values are derived from ANOVA and Bonferroni’s multiple comparisons test. *P<0.05 as compared to WT and AD+EV, +P<0.05 as compared to WT.
Figure 3

Dense core Aβ plaque number is reduced in AD mice that received EV treatment. A) Early stage AD mice exhibit increased numbers of Aβ plaques in the medial prefrontal cortex (mPFC) that was reduced by EV treatment and B) late stage AD mice exhibit increased numbers of Aβ plaques in both the amygdala and the mPFC that were reduced by EV treatment. Representative images of Thio-S staining for AD and AD+EV mice qualitatively demonstrates late AD neuropathology as shown by the accumulation of Aβ
plaques in the C) amygdala and E) mPFC that is reduced in both the D) amygdala and F) mPFC regions of the AD brain by EV treatment (basal lateral amygdala, BLA; infralimbic cortex, IL; AβP, green). G) Aβ multiplex ELISA indicates elevation of soluble Aβ40 in brain lysates that is significantly reduced by EV treatment, H) while no changes are observed between AD and AD+EV mice in levels of insoluble Aβ. Data are presented as mean + SEM (Thio-S, N=4 mice/group; ELISA, N=7 mice/group). P values derived from unpaired Student’s t tests. *P<0.05, **P<0.01. Scale bar = 70 μm.

Figure 4
Microglial activation is reduced in AD mice that received EV treatment. Early stage AD mice exhibit significantly increased numbers of CD68+ microglia in the A) amygdala that were not significantly altered by EV treatment, and B) no effect of disease or treatment is observed in medial prefrontal cortex (mPFC). Late stage AD mice show significant increases in CD68+ immunoreactivity in the C) amygdala and D) mPFC that are ameliorated by EV treatment. Representative images of CD68 staining qualitatively demonstrates these relative changes in the E-G) amygdala of wild type, AD and AD mice treated with EV (WT, AD, AD+EV, respectively) and H-J) the mPFC similarly. (basal lateral amygdala, BLA; infralimbic cortex, IL) (red, CD68; blue, DAPI nuclear counterstain). Data are presented as mean + SEM (N=4 mice/group). P values are derived from ANOVA and Bonferroni’s multiple comparisons test. *P<0.05, **P<0.01. Scale bar = 40µm.
Levels of the presynaptic protein synaptophysin are reduced in the brains of AD mice. Early stage AD mice exhibit significantly decreased levels of synaptophysin (Syp) in the A) amygdala and the B) medial prefrontal cortex (mPFC) that were ameliorated by EV treatment. Late stage AD mice show significant reductions in Syp in the C) amygdala that were ameliorated by EV treatment. D) AD-related changes in Syp were not observed in the mPFC. Representative images of Syp staining qualitatively demonstrates these relative changes in the E-G) amygdala of wild type, AD and AD mice treated with EV (WT, AD, AD+EV,
respectively) and H-J) the mPFC similarly. (basal lateral amygdala, BLA; infralimbic cortex, IL) (red, Syp; blue, DAPI nuclear counterstain). Data are presented as mean + SEM (N=4 mice/group). P values are derived from ANOVA and Bonferroni’s multiple comparisons test. *P<0.05. Scale bar = 40µm.

Figure 6

Elevated levels of inflammatory cytokines AD mice are reduced by EV treatment. Levels of cytokines secreted by PMA, ionomycin stimulated spleen cells were measured in WT, AD, and AD+EV mice. A, B)
While unaffected in early stage AD mice, Interferon-γ and IL-17 pro-inflammatory cytokines are significantly elevated in late stage AD mice are significantly reduced by EV treatment. C) Alternatively, reduced levels of the anti-inflammatory cytokine IL-10 in early stage AD mice are restored to nearly control levels in the early stage AD mice that received EV treatment. No changes among groups was observed for IL-10 in the late stage animals. D) Spleen cells were also stained for B1 cells (CD19+, CD5+, CD43+) and analyzed by flow cytometry. While not statistically significant, a trend for a reduced percentage of B1 cells was observed for AD mice and improved by EV treatment. Data are presented as mean + SEM (N=7 mice/group). P values are derived from ANOVA and Bonferroni’s multiple comparisons test. *P<0.05, **P<0.01.
Gene expression in the hippocampus of late stage AD mice. Analysis of A) microglial, B) pro-inflammation, C) immune response and other mRNA levels demonstrate increased gene expression in the hippocampus of late stage AD mice as compared to WT controls. Those elevated mRNA levels were reduced in some, but not all AD+EV treated mice. Data are presented as mean + SEM (N=4 mice/group).
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