

## Review

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# Neuro-Immuno-Gene- and Genome-Editing-Therapy for Alzheimer's Disease: Are We There Yet?

Sudhanshu P. Raikwar<sup>a,b</sup>, Ramasamy Thangavel<sup>a,b</sup>, Iuliia Dubova<sup>a</sup>, Mohammad Ejaz Ahmed<sup>a,b</sup>, Govindhasamy Pushpavathi Selvakumar<sup>a,b</sup>, Duraisamy Kempuraj<sup>a,b</sup>, Smita Zaheer<sup>a</sup>, Shankar Iyer<sup>a,b</sup> and Asgar Zaheer<sup>a,b,\*</sup>

<sup>a</sup>*Department of Neurology, Center for Translational Neuroscience, School of Medicine, University of Missouri, Columbia, MO, USA*

<sup>b</sup>*U.S. Department of Veterans Affairs, Harry S. Truman Memorial Veteran's Hospital, Columbia, MO, USA*

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**Abstract.** Alzheimer's disease (AD) is a highly complex neurodegenerative disorder and the current treatment strategies are largely ineffective thereby leading to irreversible and progressive cognitive decline in AD patients. AD continues to defy successful treatment despite significant advancements in the field of molecular medicine. Repeatedly, early promising preclinical and clinical results have catapulted into devastating setbacks leading to multi-billion dollar losses not only to the top pharmaceutical companies but also to the AD patients and their families. Thus, it is very timely to review the progress in the emerging fields of gene therapy and stem cell-based precision medicine. Here, we have made sincere efforts to feature the ongoing progress especially in the field of AD gene therapy and stem cell-based regenerative medicine. Further, we also provide highlights in elucidating the molecular mechanisms underlying AD pathogenesis and describe novel AD therapeutic targets and strategies for the new drug discovery. We hope that the quantum leap in the scientific advancements and improved funding will bolster novel concepts that will propel the momentum toward a trajectory leading to a robust AD patient-specific next generation precision medicine with improved cognitive function and excellent life quality.

**Keywords:** AAV, Alzheimer's disease, CRISPR, gene editing, gene therapy, regenerative medicine, stem cells

## INTRODUCTION

Human brain is a highly complex vital organ comprising of various cell types including neurons,

astrocytes, oligodendrocytes, tanycytes, and microglia. These various cell types are specifically arranged in a highly complex architectural network, work together in a highly ordered and orchestrated fashion, and are regulated spatiotemporally. During the course of the normal development, the molecular mechanisms underlying normal homeostasis are very robust at young age. However, with age decline these molecular mechanisms undergo gradual senescence thereby causing a wide variety of neurodegenerative

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\*Correspondence to: Asgar Zaheer, PhD, Professor, Department of Neurology, Director, Center for Translational Neuroscience, M741A Medical Science Building, University of Missouri–School of Medicine, 1 Hospital Drive, Columbia, MO 65211, USA. Tel.: +1 573 882 5386; E-mail: zaheera@health.missouri.edu.

disorders and progressive cognitive decline and dementia. One of the most prevalent neurodegenerative disorder with huge socioeconomic burden is Alzheimer's disease (AD). Although AD was discovered by Alois Alzheimer in 1906, the precise molecular mechanisms underlying AD pathogenesis are still not very clear. As a result, despite significant efforts, an effective treatment for AD remains elusive. Currently, there are an estimated 5.5 million Americans suffering from this devastating disease with an annual healthcare burden of \$259 billion. The worldwide incidence of AD is rising at an alarming pace and it is expected that by 2050 the number of Americans with AD will surpass 16 million with ~\$1.1 trillion in healthcare costs. These alarming statistics are an eye opener, and with the race against time, it is absolutely essential to develop novel therapeutic strategies to delay the onset, halt the progression, and improve the cognitive decline in AD patients.

Here we describe the recent developments and efforts in the emerging field of neuro-immunogene therapy and gene editing and their potential applications to develop precision patient-specific regenerative therapy for the treatment of AD. Since there are excellent review articles on gene therapy vectors as well as gene editing, the major emphasis of our review will be on the AD preclinical or clinical gene therapy studies. We describe AD therapeutic targets that are excellent candidates for developing novel genome editing as well as immune-gene therapies. Further, we address the most desirable features in AAV that will enhance the gene transfer efficiency to maximize the therapeutic efficacy of novel AD gene therapies.

## TARGETING AMYLOID- $\beta$ (A $\beta$ )

Extensive research has shown that AD is characterized by amyloid plaques which are produced as a result of sequential cleavage of the amyloid- $\beta$  protein precursor (A $\beta$ PP) by the  $\beta$ - and  $\gamma$ -secretases [1]. There are multiple forms of A $\beta$  including A $\beta$ <sub>34</sub>, A $\beta$ <sub>40</sub>, A $\beta$ <sub>42</sub>, etc. Although A $\beta$ <sub>40</sub> is the most abundant physiological form, A $\beta$ <sub>42</sub> that is produced at lower levels is the main culprit and is critically important for A $\beta$  accumulation in the brain. Aberrant accumulation of A $\beta$  aggregates acts as a trigger for AD pathogenesis. To disrupt this crucial pathological pathway, multiple attempts have been made with varying degrees of success. Autophagy plays a crucial role in neurodegeneration and AD

pathogenesis. Pickford et al. have shown that an autophagy related protein *Beclin1* is reduced in AD patients during the early phase of the disease and regulates A $\beta$  accumulation in mice [2]. Their studies using *APP<sup>+</sup>Beclin1<sup>+/-</sup>* mice indicate that *Beclin1* deficiency caused altered microglial response to A $\beta$ , reduced autophagy, increased extracellular and intraneuronal A $\beta$  deposition, synaptodendritic degeneration, and neuronal loss. Lentivirus-mediated overexpression of *Beclin1* in the frontal cortex and hippocampus led to decreased amyloid pathology. These interesting findings suggest that *Beclin1* is an attractive AD therapeutic target and that improving autophagy by restoring *Beclin1* may lead to a novel AD therapy.

Targeting A $\beta$  is an attractive approach to ameliorate AD pathogenesis. One of the earliest attempts was made by Zhang et al. wherein they developed a recombinant adeno-associated virus (AAV) expressing CB-A $\beta$ <sub>42</sub> (cholera toxin B subunit and A $\beta$ <sub>42</sub> fusion protein) [3]. A single administration of AAV-CB-A $\beta$ <sub>42</sub> vaccine in PDAPP<sup>V7171</sup> transgenic mice induced high levels of anti-A $\beta$ <sub>42</sub> antibodies either by the intranasal, intramuscular, or oral routes. High levels of anti-A $\beta$ <sub>42</sub> antibodies led to a significant reduction in cerebral A $\beta$  burden, decreased plaque-associated astrocytosis and improved memory as well as cognitive function. Subsequently, Fukuchi et al. developed an AAV encoding anti-A $\beta$  single-chain antibody and injected it into the corticohippocampal regions of Tg2576 mice [4]. Their results indicate sustained expression of scFv for one year without neurotoxicity. Although, there was a reduction in the A $\beta$  deposits in the brain of Tg2576 mice, there were no functional cognitive studies performed thereby making the data interpretation little challenging.

Hara et al. have developed an oral vaccine for AD using recombinant AAV vector expressing A $\beta$ <sub>1-43</sub> [5]. Oral administration of AAV $\beta$ <sub>43</sub> or AAV/A $\beta$  in the Tg2576 mice led to marked reduction in A $\beta$  deposition. However, the mechanism by which the antibodies clear A $\beta$  deposits from the brain tissue remain unresolved and no cognitive function studies were performed. Later on Mouri et al. have reported on the histological studies and cognitive function in the Tg2576 mice that received oral vaccination of AAV/A $\beta$  [6]. Their results suggest that cognitive impairments were attenuated without lymphocytic infiltration or microhemorrhage in the brain as compared to non-treated group. Levites et al. have evaluated the therapeutic potential of three recombinant anti-A $\beta$  single-chain variable fragments

(scFvs) in APP CRND8-transgenic mice [7]. Recombinant AAV1 expressing either A $\beta$ <sub>1-16</sub>, A $\beta$ <sub>x-40</sub>, or A $\beta$ <sub>x-42</sub> were injected into the ventricles of postnatal day 0 (P0) APP CRND8-transgenic mice. Their data suggest widespread neuronal delivery as well as expression of the anti-A $\beta$  scFv as well as 25–50% decrease in A $\beta$  deposition. Building upon the success of these exciting findings, Levites et al. have generated a monoclonal antibody 3H3 which is capable of binding to a pan-amyloid epitope and when delivered using an AAV in TgCRND8 and ADan mice decreased parenchymal A $\beta$  deposition.

Taking a different approach, Carty et al. investigated the effects of an intracranial administration of AAV5 expressing endothelin-converting enzyme (ECE) on amyloid deposition in 6 months old APP+presenilin-1 (PS1) mice [8]. Their results suggest that upregulation of ECE causes reduction in total amyloid deposition. Similarly, Liu et al. have reported that intramuscular injection of a recombinant AAV8 expressing neprilysin in 3X-Tg-AD mice led to decline in brain A $\beta$  burden [9]. However, both the studies did not report on the cognitive function tests in the treated mice. In a subsequent study, Jiang et al. reported that upregulation of TREM2 ameliorates neuropathology and rescues spatial cognitive impairment in APP<sup>swe</sup>/PS1<sup>dE9</sup> transgenic AD mouse model [10]. They showed that A $\beta$ <sub>1-42</sub> stimulation leads to upregulation of TREM2 in microglia *in vitro* as well as *in vivo* in wild type mice. Further, they showed that TREM2 directly inhibited microglia-mediated proinflammatory responses after A $\beta$ <sub>1-42</sub> stimulation. Lentivirus mediated-overexpression of TREM2 in the brain of APP<sup>swe</sup>/PS1<sup>dE9</sup> led to amelioration of A $\beta$  deposition and this was attributed to enhanced microglial phagocytosis rather than attenuation of A $\beta$  production. Overexpression of TREM2 in the brain significantly attenuated neuroinflammation as measured by reduced proinflammatory cytokine expression. These findings suggest that TREM2 could be a potential therapeutic target for developing novel AD therapies.

Most recently, short A $\beta$  peptides have been shown to attenuate A $\beta$ <sub>42</sub> toxicity *in vivo* [11]. In these comprehensive studies, Moore et al. initially expressed multiple copies of A $\beta$ <sub>36-40</sub> and A $\beta$ <sub>42-43</sub> in *Drosophila melanogaster* using PhiC31-mediated transgenesis to evaluate inherent toxicity and potential immunomodulatory effects on A $\beta$ <sub>42</sub> toxicity. Their data revealed that as compared to A $\beta$ <sub>42</sub> the short peptides are not toxic, do not induce locomotor dysfunction and do not accumulate as amyloid.

They further demonstrated that AAV2/1-mediated expression of A $\beta$ <sub>38</sub> and A $\beta$ <sub>40</sub> in nontransgenic mice did not cause abnormal deposition or behavioral alterations. However, the effects of A $\beta$ <sub>38</sub> from the rAAV-expressed BRI2 fusion proteins in APP TgCRND8 mice were rather complex since BRI2 expression can modulate A $\beta$  deposition *in vivo*. In a recent study, intramuscular delivery of AAV-p75NTR (a receptor for A $\beta$ ) ectodomain has been shown to attenuate cognitive deficits and AD-like pathology in APP/PS1 transgenic mice [12]. However, further research is warranted to gain a better understanding of the role of p75NTR-mediated improvement in AD pathogenesis.

Sevigny et al. have generated Aducanumab, a human monoclonal antibody that selectively targets aggregated A $\beta$  [13]. Aducanumab has been shown to enter the brain, bind parenchymal A $\beta$ , and reduce soluble and insoluble A $\beta$  in a dose dependent manner in Tg2576 mice. It significantly increased the recruitment of Iba-1-positive microglia to A $\beta$  plaques thereby suggesting Fc $\gamma$ R-mediated phagocytosis of antibody-A $\beta$  complexes as a possible clearance mechanism. In the PRIME study involving AD patients, Aducanumab was shown to penetrate brain, decrease A $\beta$  levels in a time and dose-dependent manner, and confer clinical benefit. Although, a human monoclonal antibody Aducanumab that selectively targets aggregated A $\beta$  has shown promising results in the ongoing phase III clinical trials, many other clinical trials have witnessed major setbacks including the recent trials of the humanized monoclonal antibody Bapineuzumab and Solanezumab designed to increase the clearance of soluble A $\beta$  peptides from the brain of the AD patients [14–18]. These major setbacks necessitate the development of the next generation of precision-targeted and AD patient-specific therapies.

## TARGETING TAU

Tau plays a critical role not only in AD pathophysiology but also in various other neurodegenerative disorders [19–23]. In order to successfully treat AD it is critical to simultaneously target both A $\beta$  as well as tau. There have been numerous attempts to eliminate the toxic tau forms either using anti-tau antibodies or by employing gene silencing.

Most recently, Tan et al. have reported the generation of a new tau knockout strain (tau $\Delta$ ex1)

using CRISPR/Cas9-mediated genome editing of intron1/exon1 of *Mapt* gene in C57Bl/6J mice [24]. To introduce a small deletion at the intron-1/exon1 junction of *MAPT* by CRISPR/Cas9 genome editing, a pair of guide RNAs 115 bp up and 18 bp downstream of the transcriptional start codon were used. The CRISPR/Cas9 protein as well as the two guide RNAs were directly injected into the cytoplasm of the fertilized oocytes. Traditionally generation of  $\tau^{-/-}$  has been achieved by ES cell gene targeting which requires introduction of large selectable marker cassettes in the *MAPT* coding sequence. The drawback of such an approach is that these selectable markers can create undesirable and inconsistent results. These mice did not display the overt phenotype but showed a significantly reduced susceptibility to excitotoxic seizures with normal memory formation in young mice.

Utilizing CRISPR-dCas9-activator transgenic SPH mice, Zhou et al. have achieved simultaneous transcriptional activation of multiple genes to directly and efficiently convert astrocytes into functional neurons *in vivo* [25]. In a series of ambitious experiments, they were able to simultaneously control the expression of ten different genes or ten genetic elements including eight genes and two lncRNAs into the brain by multiplexing different ten sgRNAs into a single AAV vector. The different genes were simultaneously activated to different extents due to their differential basal expression levels, epigenetic status or sgRNA target sites. Their unique approach is potentially applicable to multiple fields including disease modeling, cellular reprogramming as well as gene therapy.

Due to safety concerns and the development of neurovascular side effects and other adverse events, many immunotherapies targeting A $\beta$  were discontinued. Holtzman et al. have previously shown that passive immunization with an anti-Tau antibody HJ8.5 decreases the accumulation of pathological tau in a human P301S tau-expressing transgenic (P301s-tg) mouse model of FTD/tauopathy [26]. To further improve upon their results, they developed an AAV2/8 vector expressing anti-Tau-scFv and demonstrated the expression of anti-scFvs by the neurons and astrocytes [27]. These anti-Tau-scFvs retained their antigen binding specificity and effectively reduced pathological tau accumulation in the hippocampus of P301S-tg mice. These studies highlight the potential of AAV-mediated gene transfer for the therapeutic delivery of not only scFvs but also neurotrophic growth

factors, microRNAs, CRISPR/Cas9, or any potential combinations.

## TARGETING BACE1

BACE1 overexpression contributes to pathogenesis. BACE1 which acts upstream of  $\gamma$ -secretase in amyloid processing is an attractive AD therapeutic target for reducing the cerebral A $\beta$  levels. There are several BACE1 inhibitors currently being explored for AD therapy. However, recently Merck and Co has halted a pivotal phase II/III trial of its BACE1 inhibitor Verubecestat in more than 2,000 patients with mild to moderate AD [28]. Besides there are multiple BACE1 inhibitor trials including phase III trials for early AD using Elenbecestat (Biogen/Eisai) early and mid AD using AZD3293 (AstraZeneca/Eli Lilly), phase II/III trial of JNJ-54861911 (Johnson & Johnson) for asymptomatic at-risk patients with family history, APOE4 or biomarker positivity and phase II trial of CNP520 (Novartis) for asymptomatic at-risk patients (APOE4) [28]. Atwal et al. have developed a high affinity, phage-derived human antibody that targets BACE1 [29]. Anti-BACE1 antibody blocks the activity of BACE1 *in vitro* as well as *in vivo*. Five-month-old Tg2576 mice treated with anti-BACE1 antibody led to a 70% reduction in A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub> at doses of 30 and 100 mg/kg. However, when tested in the PS2APP mouse model, it showed reduction in A $\beta$  in the periphery but no reduction in brain A $\beta$ . Further testing in cynomolgus monkeys revealed ~50% A $\beta$  reduction. Recently Hu et al. have demonstrated that deletion of BACE1 in the adult mouse reverses preformed amyloid deposition and improves cognitive functions [30]. They generated BACE1 conditional knockout BACE1<sup>fl/fl</sup> and bred these mice with ubiquitin-Cre<sup>ER</sup> to induce deletion of BACE1 at adult stage. Interestingly, BACE1 deletion in adult AD mouse model (5xFAD) led to complete reversal of amyloid deposition and led to significant improvement in gliosis and neuritic dystrophy and improvement in cognitive function. These exciting results will hopefully lead to an improved understanding of BACE1 inhibitors in AD patients.

## NGF THERAPY

AD is a highly complex neurodegenerative disorder and the current treatment strategies are largely ineffective leading to progressive cognitive decline

in AD patients. There are multiple therapeutic targets that could potentially be utilized for either halting or delaying the progression of AD. Tuszynski et al. performed a phase I trial of *ex vivo* NGF gene delivery in eight individuals with mild AD by implanting autologous fibroblasts genetically modified to express human NGF into the forebrain [31]. These studies led to improvement in the rate of cognitive decline without long term adverse effects. In an interesting preclinical study, Bishop et al. have evaluated the therapeutic potential of AAV2-NGF (CERE-110) for targeted, stable, and sustained NGF delivery and trophic activity in rodent basal forebrain cholinergic neurons. Their studies indicated that NGF transgene delivery to the targeted brain region is reliable, accurate and leads to sustained long-term NGF expression with no loss or buildup of protein. Further, the results indicated that CERE-110 was neuroprotective and neurorestorative to basal forebrain cholinergic neurons in the rat fimbria-fornix lesion and aged rat models as well as had bioactive effects on young rat basal forebrain cholinergic neurons. These and additional studies in the primates led to the initiation of a phase I gene therapy study in which Rafii et al. stereotactically injected CERE-110 in the nucleus basalis of Meynert in AD patients. The AAV2-NGF vector utilized in this study has an expression cassette comprising CAG promoter, human NGF cDNA and a human growth hormone polyadenylation signal. The study demonstrated the safety and tolerability of the approach and led to least cognitive decline, which was dose dependent over a two-year period. Positron emission tomographic imaging and neuropsychological testing revealed no evidence of accelerated cognitive decline. The brain autopsy revealed long term, targeted, gene-mediated NGF expression and bioactivity. These phase 1 studies have paved the way for the future AAV based gene therapy trials for AD as well as other neurodegenerative diseases.

More recently, Eyjolfssdottir et al. have utilized a second-generation encapsulated cell biodelivery device to achieve delivery of nerve growth factor to the cholinergic basal forebrain of AD patients in a first in human study [32]. In their study they engineered a clonal NGF-secreting ARPE-19 cell line using a sleeping beauty transposon expression system. These implants released NGF at the levels ranging from 24.4 ng to 148.8 ng/24 hours than prior to implantation. Although the levels of neurofilament light chain protein increased and GFAP remained stable, the levels of A $\beta$  t-tau and p-tau

did not change significantly. A few patients showed improved cognitive performance and EEG. The ECB technology offers a number of advantages including the following: 1) the grafted cells are encapsulated and isolated from the host cells thereby making it easier to remove or replace the graft, 2) unlike viral vectors, the host cells do not undergo any direct genetic manipulation thereby avoiding the risk of mutation and carcinogenesis, and 3) the transgene expression is very stable thereby allowing sustained long term controlled release of NGF. However, these advantages need to be weighed with caution because a follow up study has revealed that MRI results in responders indicated more brain atrophy and neurodegeneration, increase in cerebrospinal fluid (CSF) levels of T-tau and neurofilaments at baseline despite better clinical status, and reduced pathological levels of CSF A $\beta$ <sub>1-42</sub> [33]. A larger clinical trial will be able to address any further potential concerns.

## BDNF THERAPY

BDNF levels have been shown to decline in AD and therefore restoring BDNF levels may offer potential benefits in the AD therapy [34–36]. Chen et al. have investigated the effects of AAV-ProBDNF in APP<sup>swE</sup>/PS1<sup>dE9</sup> mice [37]. Their results indicate that proBDNF enhances memory and learning impairment and also exacerbates brain A $\beta$  burden. However, it is not clear from these studies as to how proBDNF aggravates learning and memory impairment. Osborne et al. have developed a novel gene therapy vector to achieve sustained BDNF signaling in neurons [38]. BDNF signaling occurs through TrkB and p75. However, overexpression of BDNF has been shown to cause downregulation of TrkB receptors. To overcome this limitation, they have designed a novel gene sequence simultaneously coding for BDNF and TrkB receptor that are separated by a short viral-2A sequence. AAV-mediated expression of this novel gene sequence in HEK293 cells and SH-SY5Y cells led to increased BDNF/TrkB-mediated intracellular signaling. It will be interesting to evaluate the therapeutic efficacy of this vector *in vivo* in multiple AD models. Caccamo et al. found that A $\beta$  deposition interferes with the activity of the cAMP-response element binding protein (CREB) and have reported that lentiviral-mediated CREB binding protein gene transfer increased BDNF levels and ameliorated learning and memory deficits

in the 3xTg-AD mouse model [39]. Most recently, Nagahara et al. have evaluated BDNF gene delivery to the entorhinal cortex to treat AD and reduce neurodegeneration and associated memory loss [40]. Using MRI-guided and convection enhanced delivery they were able to achieve accurate AAV2-BDNF delivery to entorhinal cortex of non-human primates.

## TARGETING APOE4

ApoE, a lipid binding protein, is produced in the liver and abundantly expressed in the brain especially in astrocytes and microglia where it facilitates neuronal plasticity and synaptogenesis, A $\beta$  fibrillogenesis, and deposition and is also involved in the clearance as well as degradation of A $\beta$  [41–45]. ApoE has three different isoforms, ApoE2, ApoE3, and ApoE4, which differ by only two residues. ApoE2 is neuroprotective, ApoE3 is neutral, while ApoE4 is the most important genetic risk factor for AD. The ApoE mediated influence on AD pathogenesis is a subject of intense investigation. Huang et al. have used embryonic stem cell-derived neurons to show that ApoE secreted by glia stimulates neuronal A $\beta$  production with an ApoE4>ApoE3>ApoE2 potency rank order [46]. They further demonstrated that ApoE binding to ApoE receptors activates dual leucine-zipper kinase (DLK), a MAP-kinase kinase that then activates MKK7 and ERK1/2 MAP kinases. Activated ERK1/2 induces cFos phosphorylation thereby stimulating AP-1, which in turn enhances the transcription of APP and thereby increases A $\beta$  levels. To prove that this mechanism of action operates *in vivo*, they stereotactically injected AAVs that express a control protein or DN-cFos (Dominant-negative form of cFos) or AAVs that express dCas9 with a control guide RNA or the guide RNA directed to the AP-1 binding sequence of the APP promoter to achieve CRISPRi *in vivo*. Their results indicate that AP-1 dependent regulation of APP expression operates physiologically *in vivo*.

To investigate whether ApoE independently influences tau pathology in AD, Shi et al. have developed new P301S/E4 tau transgenic mice, which have significantly higher tau levels [47]. These mice have significantly higher tau levels in the brain and a greater extent of somatodendritic tau redistribution by 3 months of age as compared to P301S/E2, P301S/E3, and P301S/KO mice. By 9 months, P301S mice with different ApoE genotypes display distinct

phosphorylated tau protein (p-tau) staining patterns. Interestingly, P301S/E4 mice develop markedly more brain atrophy and neuroinflammation than P301S/E2 and P301S/E3 mice while P301S/KO mice are largely protected from these changes. Overall, their data suggest that ApoE affects tau pathogenesis, neuroinflammation, and tau mediated neurodegeneration independently of A $\beta$  pathology. Thus in order to develop the next generation of precision-targeted AD therapy, it will be necessary to co-target A $\beta$  as well as tau and ApoE.

Dodart et al. have investigated the potential of direct intracerebral administration of lentiviral vectors expressing three common human ApoE isoforms to alter hippocampal A $\beta$  and amyloid burden in the PDAPP mice lacking ApoE model of AD [48]. They demonstrated that intracerebral gene delivery of the lentiviral vectors expressing human ApoE4 constructs results in an increased A $\beta$  burden in PDAPP-apoE<sup>-/-</sup> mice. They further confirmed that gene delivery of a lentiviral vector expressing human ApoE2 for 5 weeks reduced brain A $\beta$  burden and insoluble A $\beta$ <sub>1-42</sub> by 30–50% in PDAPP mice expressing endogenous mouse ApoE. In contrast they did not observe a significant reduction in brain A $\beta$  burden 5 weeks post treatment with lenti-ApoE3 or lenti-ApoE4.

Rosenberg et al. have developed AAVrh.10 hAPOE2-HA and tested its potential therapeutic efficacy following direct intraparenchymal, intracisternal, and intraventricular delivery in the African Green non-human primates [49]. Their data demonstrated that while all the three routes tested were capable of mediating high level ApoE2 expression in AD relevant regions, intracisternal delivery of AAVrh.10hAPOE2-HA led to wide distribution of ApoE2 with the least invasive surgical intervention. ApoE2 expression was detected in the ependymal cells of the choroid plexus in the ventricles, as well as around the frontal and mid-brain, including the hippocampal region, as well as around the posterior of the brain and the spinal cord. Besides the targeted areas, ApoE2 staining was also observed in the anterior olfactory nucleus, thalamic reticular nucleus, lateral hypothalamus, pineal body in the epithalamus, stria terminalis, pontine gray fibers in the brain stem, Purkinje cells and deep cerebellar nuclei in the cerebellum and gray nuclei of the spinal cord. ApoE2-HA levels in the CSF were quantified by ELISA and were found to be consistently >1  $\mu$ g/ml at day 28 and day 56 post vector delivery.

## CYTOKINE GENE THERAPY

Neuroinflammation plays a critical role in the pathogenesis of multiple neurodegenerative disease including AD [50, 51]. Therefore, it would be beneficial to develop strategies to counteract neuroinflammation by utilizing anti-inflammatory cytokines. Targeting neuroinflammation presents an attractive paradigm for developing novel gene therapy strategies for AD. In this regard, earlier attempts were made by Kiyota et al. who reported that AAV serotype 2/1 hybrid-mediated neuronal expression of 7ND gene; a dominant-negative CCL2 mutant in APP/PS1 mice reduced astro/microgliosis and improved spatial learning [52]. In subsequent studies, they evaluated AAV2/1 mediated delivery of the mouse IL4 and IL10 genes to ameliorate cognitive dysfunction in APP+PS1 mice [53, 54]. The hippocampal neurons transduced with AAV2/1-IL4 led to a sustained expression of IL4 reduced astro/microgliosis, enhanced neurogenesis, improved cognitive function, and restored NR2B phosphorylation and cell surface expression in the neurons. IL4 promotes A $\beta$  degradation in mononuclear phagocytes, monocyte-derived macrophages as well as reduces the levels of monomeric and oligomeric A $\beta$ . IL4-mediated enhanced neurogenesis including enhanced neuronal progenitor cell proliferation as well as neuronal differentiation are potentially mediated by the activation of extracellular signaling-regulated kinases, p70 ribosomal protein S6 kinase-survival signaling pathways, and insulin-response substrate 1 and 2 pathways and through protection of neurons from apoptosis through the activation of PI3 kinase and Akt. Further hippocampal injection of AAV-IL10 in APP+PS1 mice led to sustained expression of IL10 and led to significantly reduced astrogliosis and microglial accumulation around thioflavin-S (TS)+ compact plaques and enhanced clearance of A $\beta$  from the brain to the vascular circulation. Surprisingly IL10 expression did not reduce A $\beta$  burden in the mouse brain. It remains to be seen whether AAV-mediated simultaneous co-expression of IL4 and IL10 in the hippocampus of APP+PS1 mice might have a potentially synergistic effect by significantly reducing A $\beta$  burden and robustly improving cognitive function.

In a separate study, Chakrabarty et al. have investigated the potential therapeutic efficacy of AAV2/1-IL10-mediated intracranial expression of murine IL10 in TgCRND8 and Tg2576 AD mouse models [55]. Their studies revealed that IL10 expression leads to increased amyloid loads, decreased

levels of immediate early genes and synaptic markers, worsened cognitive behavior, reduced microglial A $\beta$  phagocytosis, and increased ApoE expression and its sequestration within insoluble amyloid plaques. Except for the fact that IL10 did not reduce A $\beta$  burden, these results are contradictory to those reported by Kiyota et al. but consistent with the findings by Guillot-Sestier et al. who have reported that IL10 signaling is elevated in AD patient brains and IL10 deficiency in APP/PS1+IL10<sup>-/-</sup> mice mitigates cerebral amyloidosis and partially rescued synaptic toxicity and behavior impairment.

Prior studies have shown that the IL2 knockout mice exhibit impaired learning and memory formation and altered hippocampal development [56]. Alves et al. observed decreased IL2 expression in the hippocampus of AD patients and have therefore developed and tested the potential therapeutic efficacy of an AAV8-IL2 vector in APP/PS1 $\Delta$ E9 mice [57]. Administration of AAV8-IL2 intraperitoneally led to IL2 secretion, rescued impaired synaptic plasticity and spatial memory impairment, caused an expansion and activation of systemic and brain regulatory T cells and activated the JAK/STAT3 pathway in the hippocampus. Further, IL2 induced astrocytic activation and recruitment of astrocytes around amyloid plaques, decreased amyloid- $\beta$ 42/40 ratio amyloid plaque load and significantly reduced spine density.

Chakrabarty et al. have developed an AAV2/1 vector expressing murine IL4 (AAV2-mIL4) [58]. Surprisingly, stereotactic injection of AAV2-mIL4 into the CA layer of hippocampus at 4 months of age in TgCRND8 mice led to 33.5% increase in amyloid plaques and 41% increase in A $\beta$ <sub>42</sub> levels and 55% increase in A $\beta$ <sub>40</sub> levels in the SDS extractable A $\beta$  levels, respectively, and 76% increase in A $\beta$ <sub>42</sub> levels and 62% increase in A $\beta$ <sub>40</sub> levels in the formic acid extractable A $\beta$  levels, respectively. However, APP, APP CTF, BACE, ApoE, and A $\beta$  degrading enzyme levels did not get altered. These results are contradictory to those reported by Kiyota et al. [54] even though both these studies have used AAV2/1, with similar expression cassettes. The only differences include a dose of  $2 \times 10^9$  viral particles and the use of APP+PS1 Tg mice by Kiyota et al. [54] whereas Chakrabarty et al. [58] have used a slightly higher titer  $2 \times 10^{10}$  viral genomes and TgCRND8 mice. There is a need to evaluate these AAV2/1-mIL4 vectors in multiple AD models to resolve the controversial nature of the results. Most recently, Vogelaar et al. have shown that intrathecal IL-4 treatment during the

chronic phase of several experimental autoimmune encephalomyelitis models reversed disease progression without affecting inflammation [59].

Chakrabarty et al. have explored the effect of murine tumor necrosis factor alpha (mTNF $\alpha$ ) in regulating A $\beta$  accumulation [60]. Recombinant AAV2/1-mTNF $\alpha$ -mediated expression of mTNF $\alpha$  in the hippocampus of 4-month-old APP transgenic TgCRND8 mice resulted in significant reduction in hippocampal A $\beta$  burden. However, no changes in A $\beta$ PP levels or A $\beta$ PP processing were observed either in mTNF $\alpha$  expressing APP transgenic mice or in the nontransgenic littermates. Furthermore, analysis of A $\beta$  plaque burden in mTNF $\alpha$  expressing mice showed that even after substantial reduction compared to EGFP expressing age-matched controls, the A $\beta$  plaque burden levels of the treated mice do not decrease to the levels of 4-month-old unmanipulated mice.

In a separate study, Chakrabarty et al. have evaluated the role of IFN $\gamma$ -mediated neuroinflammation in pathology in transgenic mice. They expressed murine IFN $\gamma$  in the brains of APP transgenic mice using a recombinant of AAV2/1-mIFN- $\gamma$  virus [61]. Their results demonstrate that the expression of IFN $\gamma$  in the brains of 4-month-old APP TgCRND8 mice leads to robust noncell autonomous activation of microglia and astrocytes, upregulation of MHCII, CD11b, CD11c and complement system components, infiltration of Ly-6c positive monocytes and a significant suppression of A $\beta$  deposition. When AAV2/1-mIFN- $\gamma$  virus was injected into the cerebral ventricles of 36–48 hours old (neonatal day P2) TgCRND8 mice much prior to the onset of plaque deposition, it led to expression largely localized in the choroid plexus and ependymal cells lining the ventricles, along with a few neurons in the hippocampus, cortex, and cerebellum. Quantification of A $\beta$  plaques in the forebrain and hippocampus of AAV2/1-mIFN- $\gamma$  injected TgCRND8 mice revealed 73% and 70% reduction in plaque burden respectively. When the AAV2/1-mIFN- $\gamma$  virus was injected in the 4-month-old TgCRND8 mice, A $\beta$  plaque burden was higher than the pretreatment levels. Further, cognitive studies did not reveal a beneficial effect in mIFN $\gamma$  expressing mice. Therefore, newer strategies are required to simultaneously reduce A $\beta$  plaque burden and improve cognitive function.

To assess the effects of IL-6 on A $\beta$  deposition and A $\beta$ PP processing, Chakrabarty et al. have developed AAV-mIL-6 [62]. Overexpression of mIL-6 in the brains of APP transgenic TgCRND8 and Tg2576

mice led to extensive gliosis and concurrently attenuated A $\beta$  deposition in TgCRND8 mouse brains. They also found that there was an upregulation of glial phagocytic markers and enhanced microglia-mediated phagocytosis. Surprisingly, mIL-6-induced neuroinflammation had no effect on A $\beta$ PP processing in TgCRND8 or Tg2576 mice. These results suggest that mIL-6 mediated reactive gliosis may be beneficial during the early AD phase by potentially enhancing A $\beta$  plaque clearance rather than creating a neurotoxic feedback loop, which might exacerbate amyloid pathology.

Intraneuronal accumulation of A $\beta$  peptides is responsible for driving the neurodegenerative disease pathology in AD. Sudol et al. have developed A $\beta$ <sub>42</sub>-specific intracellular antibodies with and without an intracellular trafficking signal using AAV2 [63]. Testing of these intrabodies in doxycycline inducible A $\beta$ <sub>42</sub> expressing cells revealed that only endoplasmic reticulum targeted anti-A $\beta$ <sub>42</sub> intrabody was effective in significantly reducing the secreted form of A $\beta$ <sub>42</sub>. Subsequent testing of rAAV-scFvA $\beta$ <sup>IB</sup> and rAAV-scFvA $\beta$ <sub>KDEL</sub><sup>IB</sup> by intrahippocampal injections into the CA1 layer in 3xTg-AD mice revealed that the rAAV-scFvA $\beta$ <sub>KDEL</sub><sup>IB</sup> treatment significantly reduced both extracellular A $\beta$ <sub>42</sub> and phospho-Tau. These interesting results support the feasibility of using tropism modified and transcriptionally targeted AAV-mediated expression of A $\beta$ -specific intrabodies to significantly reduce and possibly halt the progression of neurodegeneration in AD patients.

The most popular AD transgenic models utilize overexpression of mutant human A $\beta$ PP and PS1 to increase A $\beta$  production and mimic AD cognitive impairment and AD pathogenesis [64–67]. However, a major caveat is that overexpression of A $\beta$ PP causes increased production of not only A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> but also other A $\beta$ PP moieties that may have either neurotoxic, neuroprotective, or other signaling functions, which may positively or negatively influence the cognitive function. Therefore, to gain an improved understanding of the role of A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> *in vivo*, Lawlor et al. have developed AAV1 vectors encoding BRI-A $\beta$  cDNAs to achieve high level and specific A $\beta$  expression in the absence of A $\beta$ PP overexpression [68]. Their results indicate that AAV-mediated gene transfer in adult rats leads to elevated A $\beta$  levels and AD like phenotype with learning and memory deficits when both AAV-BRI-A $\beta$ <sub>40</sub> and AAV-BRI-A $\beta$ <sub>42</sub> are co-injected. Subsequent gene transfer studies in APPCRND8 transgenic mice using AAV1 expressing truncated BRI2 protein lacking the

BRI<sub>2-23</sub> peptide have revealed that BRI<sub>2-23</sub> sequence is required to suppress A $\beta$  deposition *in vivo* [69]. In another study, they have compared CNS transduction profiles of AAV serotypes bb2, cy5, rh20, rh39, and rh34 with AAV8 [70]. Their results suggest that as compared to AAV8, more widespread neuronal transduction was observed by employing cy5, rh20, and rh39 while preferential transduction of astrocytes was observed with rh43. Prior results by Klein et al. had suggested that AAV8 has superior transgene expression in the adult rat hippocampus as compared to AAV2 or AAV5 [71]. They also tested an AAV8 vector expressing the P301L form of tau, which led to profound loss of dopamine neurons and caused significant amphetamine-stimulated rotational behavior. These research findings are significant because it will become possible to engineer novel AAV capsids to achieve high-level tissue specific expression.

Independent studies have shown that IL-33 transcript and protein levels are reduced in the brains of late onset AD patients as compared to healthy individuals [72, 73]. To investigate the role of IL-33 in AD pathophysiology Fu et al. injected IL-33 in APP/PS1 transgenic mice and examined the cognitive function as well as measured soluble A $\beta$  and amyloid plaque deposition. Their results indicate that stimulation of IL-33/ST2 signaling reverses synaptic plasticity, rescues contextual memory deficits and reduces A $\beta$  accumulation. Further, IL-33 treatment causes an increase in the anti-inflammatory genes including Arg1 and Fizz1 in the microglia and the macrophages in the brain causing alternative activation state with enhanced A $\beta$  phagocytosis. Their finding that IL-33 mobilizes innate immunity to prevent and clear established A $\beta$  accumulation even at late stage AD potentially represents an attractive approach for the treatment of AD. These exciting results suggest that targeting neuroinflammation has significant benefits and could potentially lead to development of a multi-target AD gene therapy.

### AAV9 TOXICITY

Prior studies by Gray et al. have demonstrated that AAV9 is able to transduce approximately twice as many neurons as astrocytes in the adult rodent CNS at doses of  $1.25 \times 10^{12}$ ,  $1 \times 10^{13}$ , and  $8 \times 10^{13}$  vg/kg body weight. Further, they reported that self-complementary vectors were >10 fold more efficient when compared with the single stranded vectors. However, when tested in the non-human primates,

they observed reduction in peripheral organ and brain transduction with a preferential glial transduction. Their results indicate that high peripheral tropism, limited neuronal transduction in non-human primates, and the pre-existing neutralizing antibodies are the major caveats to human translation of intravascular AAV9 based gene delivery. Recently, the results from a first-in-human trial of high-dose i.v. AAV9 in infants with SMA were reported [74]. In these trials the patients demonstrated significant improvement in survival and motor function in comparison to the controls. However, in a subset of the patients (4/15) there was an asymptomatic transaminase elevation [35 times the upper limit of the normal range for alanine aminotransferase (ALT) and 37 times for aspartate aminotransferase (AST)] potentially due to T-cell response against capsid-derived peptides presented by the transduced hepatocytes. To better understand the efficiency and safety of this approach, Hinderer et al. have reported severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an AAV9 variant AAV9hu68 expressing human SMN [75]. In yet another study, Hordeaux et al. have reported that the neurotropic properties of AAV-PHP.B are specifically limited to C57BL/6J mice and not to other commonly used mouse strain BALB/cJ or non-human primates [76]. They further reported the potential for serious acute toxicity in the non-human primate after systemic administration of high dose of AAV. Therefore, significant caution needs to be exercised during pre-clinical testing to thoroughly evaluate the safety and toxicity profiles of all the gene therapy vectors in male as well as female subjects.

### AAV IMMUNOGENICITY

AAV serotype comparison for CNS transduction [77, 78]. The most commonly used AAV serotypes for the CNS gene therapy clinical trials include AAV5, AAV9, and AAVrh.10 [79–82]. However, one of the major caveats of the current gene therapy strategies is AAV-specific adaptive immune response. More specifically AAV-neutralizing antibodies will interfere with AAV transduction causing suboptimal transduction rates. Further, cytotoxic CD8<sup>+</sup> T cells directed against the AAV capsid would lead to untimely rejection of AAV transduced cells. Since some of the epitopes are common between different serotypes, it may not be possible to use different serotypes to bypass the immune response. Therefore,

it is important to develop suitable strategies to overcome vector directed immune responses. In this regard, CpG-depleted AAV vectors have been shown to evade immune detection [83]. In their pioneering studies, Faust et al. initially tested the immunogenicity of AAVrh32.33 in wild type as well as Tlr9 knock out mice. They found that in Tlr9-deficient mice, IFN $\gamma$  T cell responses toward capsid and transgene antigen were suppressed resulting in minimal cellular infiltrate and stable transgene expression. Recently Hudry et al. have shown that AAV can associate with exosomes (exo-AAV) when the vector is isolated from conditioned media of producer cells and it is more resistant to neutralizing antibodies [84]. Systemic injection of exo-AAV was capable of efficient transduction of the CNS at low vector doses compared to conventional AAV. The ability of exo-AAV to evade neutralizing antibodies while retaining excellent CNS gene transfer is clinically relevant.

#### AAV NOVEL SEROTYPES AND AAV CAPSID ENGINEERING

One of the major caveats of the current gene therapy vectors is limited CNS gene transfer. CNS gene transfer efficiency and the gene therapy efficacy can be significantly improved by rationally engineering AAV capsid. There have been several studies to maximize the *in vivo* gene transfer efficiency. Zhong et al. have previously shown that protein tyrosine kinases phosphorylate specific residues on the AAV2 capsid surface resulting in the ubiquitination and degradation of virion prior to reaching the nucleus [85]. Specific mutation of surface exposed AAV2 capsid tyrosine residues to phenylalanine resulted in improved intracellular transport thereby significantly improving AAV-mediated transduction *in vitro* as well as *in vivo* [86]. Yang et al. have compared CNS transduction efficiencies of 12 different rAAVs (rAAV 1, 2, 5, 6, 6.2, 7, 8, rh.8, rh.10, rh.39, and rh.43) in 10-week-old mice following systemic delivery. Their results suggest that rAAVrh.8 robustly achieves global CNS transduction with minimal peripheral tissue dissemination as compared to other vectors and may be very useful for the treatment of AD.

Deverman et al. have developed novel AAVs by using CREATE (Cre recombinase-based AAV targeted evolution) for widespread gene transfer to the brain [87]. The CREATE system utilizes a rAAV capsid genome (rAAV-Cap-in-cis-lox) along with a full-length AAV cap gene, controlled by regulatory

elements from the AAV rep gene with a Cre-invertible switch. They developed AAV capsid libraries by inserting 7 amino acids of randomized sequence between amino acids 588 and 589 of the AAV9 capsid within the rAAV-Cap-in-cis-lox backbone and delivered the virus libraries to animals with Cre expression in selected cell population. Selective amplification and recovery of sequences that transduced the target population yielded multiple AAV variants. They developed an AAV variant AAV-PHP.B with a 40-fold higher gene transfer efficiency (80% transduction of astrocytes and 50–100% transduction of neurons) in various brain regions as compared to the standard AAV9 [88, 89]. Chan et al. have subsequently developed two more variants AAV-PHP.eB and AAV-PHP.S that efficiently transduced the central and peripheral nervous system, respectively [90]. Morabito et al. have demonstrated AAV-PHP.B-mediated global scale physiological expression of GBA1 gene as an approach for wide protection from synucleinopathy in A53T-SCNA transgenic mice [91]. However, whether the selective neurotropism is also preserved in nonhuman primates or human CNS remains to be seen and needs to be investigated in future studies. In this regard, very recently, Matsuzaki et al. have tested and compared the potential gene transfer efficiency of AAV9 as well as AAV-PHP.B in marmosets using intravenous injection [92]. Their results demonstrate limited transduction of neurons (0–2%) and astrocytes (0.1–2.5%) in both AAV-PHP.B and AAV9 but marked transduction of the peripheral dorsal root ganglia neurons.

Murlidharan et al. have reported on CNS restricted transduction and CRISPR/Cas9-mediated gene deletion with an engineered AAV [93]. They reported the development of a chimeric AAV by replacing the viral protein 3 amino acid residues on AAV9 capsid required for galactose binding on the AAV2 capsid by site directed mutagenesis. The capsid engineered AAV2g9 had a superior CNS transduction efficiency due to preferential neurotropism, CNS restricted biodistribution and minimal transgene expression in off-target peripheral organs. They subsequently demonstrated the utility of AAV2g9 for targeted disruption of the MIR137 locus in CRISPR/Cas9 transgenic mouse. Tervo et al. have used directed evolution approach to develop a designer AAV variant rAAV-retro that exhibits robust retrograde access to projection neurons and has superior genome editing capabilities [94]. This rAAV-retro vector holds significant promise for the therapeutic intervention for neurodegenerative diseases especially AD and

Parkinson's disease wherein multiple AAV injections pose a safety risk and may be insufficient to achieve widespread transduction. This is due to the fact that early intervention by targeting subcortically projecting neurons in the mesocortical regions in the AD and Parkinson's disease could have the potential to significantly halt or stabilize the disease progression and possibly improve or restore cognitive function. It would be interesting to determine whether rAAV-retro vector is capable of efficiently transducing the neural stem cells.

More recently, Choudhury et al. have used directed molecular evolution to construct AAV capsid library by DNA shuffling of AAV1, 2, 4, 5, 6, 8, rh8, rh10, rh39, and AAVrh43 capsid genes [95]. They have developed a novel AAV-B1 capsid, which has superior transduction in the neuronal, glial, and endothelial cell population within the CNS. This AAV-B1 capsid is also highly effective for gene transfer of various peripheral organs including skeletal muscle, heart, lung, pancreas, and retina. Further, AAV-B1 is significantly resistant to neutralization than AAV9 and therefore represents an important improvement over AAV9 for CNS gene therapy. They have also generated a novel AAV-AS vector by insertion of a poly-alanine peptide, which imparts 6 and 15 fold more efficient transduction than AAV9 in the spinal cord and cerebrum, respectively [96]. Kanaan et al. have investigated the efficacy of CNS transduction using a variety of tyrosine and threonine capsid mutants based on AAV2, AAV5, and AAV8 capsids as well as AAV2 mutants incapable of binding heparin sulfate [97]. They found that simultaneously mutating multiple tyrosine residues on the AAV2 capsid significantly enhanced neuronal transduction in the striatum and hippocampus and the ablation of heparan sulfate binding also increased the volumetric spread of the vector *in vivo*.

### **CRISPR/CAS9 HOLDS SIGNIFICANT PROMISE FOR THE DEVELOPMENT OF NEW AD MODELS AND PRECISION TARGETED AD THERAPY**

Clustered regularly interspaced short palindromic repeat (CRISPR)-Cas nucleases have revolutionized the field of gene editing and have tremendous application in the field of molecular medicine [98–102]. Despite a significant surge in CRISPR/Cas9-mediated genome editing in various disease models, the progress in the field of AD has lagged behind

substantially. We believe that genome editing can significantly improve the development of AD models and also create novel opportunities for the development of the next generation precision targeted AD gene and stem cell therapies. Since there are several excellent review articles on CRISPR/Cas9-mediated genome editing, here we will limit our focus on select recent articles that are noteworthy.

CRISPR/Cas9 system can be engineered to either activate transcription (gain-of-function) or achieve gene silencing (Loss-of-function). Dahlman et al. have developed a CRISPR-based system that uses catalytically active Cas9 and distinct single guide (sgRNA) constructs to activate and knockout different genes in the same cell [103]. Konermann et al. have used structure-guided engineering of a CRISPR-Cas9 complex to mediate efficient transcriptional activation at endogenous genomic loci [104]. Using crystallographic studies, they have engineered a combination of sgRNA2.0, NLS-dCas9-VP64 and MS2-p65-HSF1 to develop one of the most effective transcription activation system.

Hu et al. have used phage-assisted continuous evolution (PACE) to evolve an expanded PAM spCas9 variant (xcas9) that can recognize a broad range of PAM sequences including NG, GAA and GAT [105]. The engineered xCas9 has broadest PAM compatibility and much greater specificity than the regular SpCas9 and substantially lower genome-wide off-target activity. Kramer et al. used CRISPR/Cas9 genome-wide gene knockout screens for suppressors and enhancers of C9ORF72 DPR toxicity in human cells [106]. They discovered potent modifiers of DPR toxicity whose gene products function in nucleocytoplasmic transport, endoplasmic reticulum, proteasome, RNA-processing pathways and chromatin modification. Their research findings highlight the potential of CRISPR-Cas9 screens in uncovering novel molecular mechanisms underlying neurodegenerative diseases.

Paquet et al. have used CRISPR/cas9-mediated scarless CORRECT (Consecutive re-guide or re-Cas Steps to Erase CRISPR/Cas-blocked targets) genome editing to develop knock-in model of AD mutations in human iPS cells [107]. Their gene editing approach allows selective introduction of mono- and bi-allelic sequence changes with high efficiency and accuracy. Using this approach, they were successful in generating human iPS cells with heterozygous and homozygous dominant early onset AD causing mutations in amyloid precursor protein (APP<sup>Swe</sup>)<sup>10</sup> and presenilin 1 (PSEN1<sup>M146V</sup>)<sup>11</sup> and

derived cortical neurons. This unique strategy has the potential to introduce specific sequence changes using CRISPR/Cas9 to develop disease in a dish model especially for AD using human ES or iPS cells.

Platt et al. have developed a Rosa26-LSL-Cas9 knock-in mice in which a Floxed-STOP polyA stop cassette prevents the expression of the CAG promoter driven downstream bicistronic Cas9 and EGFP [108]. Treatment with Cre recombinase leads to deletion of the STOP cassette thereby resulting in widespread Cas9 and EGFP expression. These Rosa26-LSL-Cas9 knock-in mice can be used to generate either single or multiple specific mutations using an AAV vector expressing Cre under the control of a tissue specific promoter along with single or multiple sgRNAs. To demonstrate the potential utility of the Rosa26-LSL-Cas9 knock-in mice for neuronal gene editing, they developed a chimeric AAV1/2 vector in which a CBh promoter drives the expression of Cre recombinase and U6 promoter drives the expression of RNA-splicing factor NeuN. Stereotactic injection of the AAV into the prefrontal cortex of the Rosa26-LSL-Cas9 knock-in mice followed by the Deep sequencing of the NeuN locus revealed indel formation as well as NeuN protein depletion.

Zheng et al. have utilized dCas9-mediated CRISPR interference-based specific and efficient gene inactivation in the brain [109]. In their studies, they demonstrated the efficiency of CRISPRi in cultured hippocampal neurons to successfully knock down (90%) the expression of *Syt1*, *Vamp2*, *Stx1a*, and *Snap25*. Further, they modified their lentiviral vectors either to coexpress dCas9-KRAB and 2-3 sgRNAs that were driven by different RNA Pol III promoters in an all-in-one lentiviral vector or used a dual vector CRISPRi system in which one lentiviral vector solely expressed the dCas9-KRAB and another lentiviral vector contained a tandem array of 5 U6::sgRNA cassettes against *Syt1*, *Vamp2*, *Snap25*, *Stx1a*, and *Stx1b*. They were successful in knocking down the expression of the five genes in the mouse hippocampus using the multiplexing approach. These studies suggest that multiplex CRISPRi can be successfully used to abrogate gene expression in multigeneic neuronal disorders. Prior studies have shown that genetic fusion between dCas9 and the transcriptional repressor KRAB (dCas9-KRAB) successfully blocks transcription leading to excellent gene knockdown [110–115].

Swiech et al. have utilized a dual AAV system to achieve efficient SpCas9-mediated gene editing of MeCP2 (methyl CpG binding protein) in

neurons [116]. They observed significant MeCP2 gene editing 7 days post transduction. Stereotactic injection of a (1:1 ratio) of AAV-SpCas9 and AAV-SpGuide (MeCP2 or LacZ targeting sgRNAs) into the hippocampal dentate gyrus of adult male mice led to ~70% reduction in MeCP2 nuclei and >60% decrease in MeCP2 protein levels. They further used sgRNAs targeting the family of DNA methyltransferases (DNMTs: Dnmt1, Dnmt3a, and Dnmt3b) which are highly expressed in the adult brain and are required for synaptic plasticity, learning and memory formation. Stereotactic injection of a mixture of AAV-SpCas9 and AAV-SpGuide (targeting Dnmt3a, Dnmt1, and Dnmt3b) into the dentate gyrus of adult mice led to indels in all three loci and revealed ~75% modification rate in Dnmt1 and Dnmt3a and ~50% modification in Dnmt3b. CFC behavior tests were used to investigate the effect of SpCas9-mediated knockdown of DNMTs on memory acquisition and consolidation. Their data revealed that triple DNMT knockdown mice had impaired memory formation.

We have recently developed recombinant AAV and lentiviral vectors to achieve CRISPR/Cas9-mediated genome editing of glia maturation factor in microglia [117]. We investigated if CRISPR-Cas9-mediated GMF gene editing leads to inhibition of GMF expression and suppression of microglial activation. Confocal microscopy of murine BV2 microglial cell line transduced with an adeno-associated virus AAV co-expressing *Staphylococcus aureus* (Sa) Cas9 and a GMF specific guide RNA (GMF-sgRNA) revealed few cells expressing SaCas9 while lacking GMF expression, thereby confirming successful GMF gene editing. To further improve GMF gene editing efficiency we developed lentiviral vectors (LVs) expressing either *Streptococcus pyogenes* (Sp) Cas9 or GMF-sgRNAs. BV2 cells co-transduced with LVs expressing SpCas9 and GMF-sgRNAs revealed reduced GMF expression and the presence of indels in the exons 2 and 3 of the GMF coding sequence. Lipopolysaccharide (LPS) treatment of GMF-edited cells led to reduced microglial activation as shown by reduced p38 MAPK phosphorylation. We will be testing GMF gene editing in 5xFAD mice. We believe that targeted *in vivo* GMF gene editing has a significant potential for developing a unique and novel AD therapy.

As compared to the complete gene knockout mouse models, CRISPR/Cas9 based gene editing provides only a partial knockout in a fraction of targeted cells. Therefore, it is important to improve the current

gene editing efficiency of the CRISPR/Cas9 systems. Another caveat is that using a ubiquitous promoter to drive the expression of CRISPR/Cas9 can lead to gene editing in the non-target cells. This can be achieved using a highly tissue specific promoter to drive the expression of CRISPR/Cas9. However, despite utilizing a tissue specific promoter, it is still not desirable to have constitutive expression of CRISPR/Cas9 as it may lead to off-target effects. This potential drawback can be overcome by utilizing a conditional expression system wherein the expression levels as well as the duration of CRISPR/Cas9 can be tightly regulated. This has been recently demonstrated by de Solis et al. who have developed a doxycycline-inducible AAV based system for gene editing [118]. Their strategy involved generating two separate AAV/DJ vectors such that the vector harbors a TRE Tight promoter driving the expression of CRISPR/Cas9 while the second vector contains a U6 promoter driving Tet2 sgRNA and a CMV promoter driving the expression of rtTA (Tet-On Advanced and an IRES driven GFP. Surprisingly, their results indicate doxycycline-inducible expression of CRISPR but Tet2 gene editing in a doxycycline independent manner due to leakiness. To overcome the issue of leakiness, they have significantly modified their vectors by utilizing a combination of hybrid H1/TO promoter to drive the expression of Tet2-sgRNA and a CMV promoter controlling the expression of TetR in frame with a self-cleaving P2A sequence followed by a GFP ORF fused to a KASH domain. In this system in the absence of doxycycline, TetR binds to H1/TO promoter and represses the gRNA transcription. However, addition of doxycycline inhibits TetR binding and induces gRNA expression. This system allowed doxycycline dependent genome editing of Tet2 in N2A cells *in vitro*. Besides, doxycycline inducible system there are several other inducible systems available including rapamycin, mifepristone, tamoxifen, and ecdysone inducible systems that can be engineered to overcome the leakiness of the inducible system.

The currently used genome editing approaches are inefficient and induce significantly higher random insertions and deletions (indels) at the target locus. Recently, Nishiyama et al. have shown that precise genome editing via homology-directed repair is possible in post mitotic neurons as well as mitotic cells in mouse brain by combining CRISPR-cas9-mediated DNA cleavage and the efficient delivery of donor template with AAV [119]. Using this approach of virus-mediated single-cell labeling of endogenous

proteins via HDR (vSLENDER), they were able to achieve highly efficient tagging of endogenous proteins in dissociated and organotypic slice cultures *in vitro* and various brain areas and cell types in developing and adult brains *in vivo*.

In a pioneering study, Komor et al. have recently described programmable base editing that enables the direct irreversible conversion of one target DNA base into another in a programmable fashion without requiring dsDNA backbone cleavage or a donor template [120]. They engineered fusions of CRISPR/Cas9 and a cytidine deaminase enzyme that retain the ability to be programmed with a guide RNA, do not induce dsDNA breaks and mediate the direct conversion of cytidine to uridine thereby effecting a C→T (or G→A) substitution. They successfully demonstrated the potential of programmable base editing to convert a target base in APOE4 into APOE3r in immortalized mouse astrocytes by replacing the endogenous ApoE gene with human APOE4. These exciting new developments in the field of genome editing have the potential to revolutionize the development of next generation patient-specific precision medicine for a wide variety of genetic diseases as well as neurodegenerative diseases especially AD.

## EPIGENETIC EDITING

Bustos et al. have analyzed the role of Dlg4 gene which encodes post-synaptic density protein 95 (PSD95), a major synaptic protein that is diminished in neurodegenerative disorders including AD [121]. They engineered a Dlg4/PSD95 zinc finger DNA-binding domain and fused it to the effector domains to either repress (G9a, Suvdel76, SKD) or activate (VP64) transcription thereby generating Herpes simplex viral, lentiviral and AAV-PHP.B vectors expressing artificial transcription factors or epigenetic factors (methylating H3K9). Their *in vitro* data revealed that PSD95-6ZF fusion constructs induce epigenetic reprogramming and bidirectionally control *Dlg4/PSD95* gene expression in N2a cells. Further, *in vivo* gene transfer led to modulation of Dlg4/PSD95 gene expression, regulation of synapse and spine maturation of hippocampal neurons and improved learning and memory deficits in aged and AD mouse model AβPP<sup>swe</sup>/PS-1 *in vivo*. These exciting results suggest that epigenetic editing could be utilized for developing novel gene therapies for the treatment of AD.

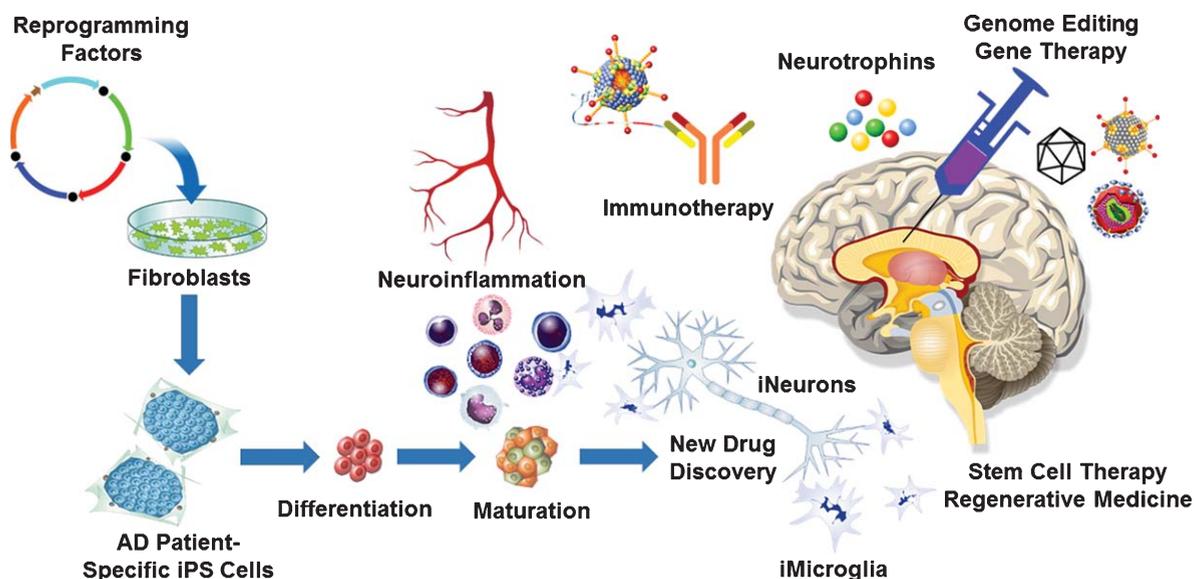


Fig. 1. Neuro-immuno-genome-editing-stem-cell-therapy for Alzheimer's disease (AD). Development of novel AD therapies would involve harnessing the latest technological advancements in the fields of neurology, immunology, molecular biology, virology, molecular medicine as well as stem cell biology. Neuroinflammation plays a significant role in the development and progression of AD. Targeting neuroinflammation using CRISPR/Cas9-mediated gene editing and gene therapy approaches will delay the onset as well as potentially halt the progression of AD. Genetically engineered recombinant viral vectors with enhanced neurotropism due to novel capsid engineering will maximize targeted gene editing and gene therapy efficacy. Latest advancements in the field of stem cell biology and regenerative medicine will enable the development of disease in a dish model using AD patient derived iPS cells to generate 3D organoids, which can be used for the new drug discovery. Such an approach will lead to the development of AD-patient specific neuro-immuno-genome-editing-stem-cell-therapy.

### *Pu.1*

In a large scale genome-wide survival analysis of 14,406 AD cases and 25,849 controls Huang et al. have identified 8 previously reported AD risk loci (*CR1*, *BIN1*, *SPI1*, *MS4A*, *PICALM*, *SORL1*, *FERMT2*, and *APOE*) and 14 novel loci (*C1orf112*, *CMC1*, *SYNPR*, *PDE6B*, *LINC00290*, *PCDHA1*, *LINC00951*, *NT5C3A*, *CSMD1*, *VLDLR*, *IPMK*, *SLC6A15*, *IQCK*, and *SUN2*) associated with age at onset [122]. They discovered an association with delayed AD onset and lower expression of *SPI1* in monocytes and macrophages. The *SPI1* gene encodes for the PU.1 transcription factor that plays a critical role in the myeloid and B cell development and function and binds to the cis-regulatory elements of several AD-associated genes. PU.1 was shown to be bound to cis-regulatory elements of many AD-associated genes including *ABCA7*, *CD33*, *MS4A*, *MS4A6A*, *PILRA*, *PILRB*, *TREM2*, *TREML2*, and *TYROBP* but surprisingly not *APOE*. PU.1 overexpression led to increased phagocytosis while PU.1 knockdown led to diminished phagocytic activity in BV2 cells. Their study revealed that lower *SPI1* expression might reduce AD risk potentially by

modulating microglial and/or myeloid cell gene expression and function. Overall, these interesting studies highlight the potential of CRISPR/Cas9 mediated *SPI1* genome editing as an approach to develop novel therapeutic strategies to treat AD.

### STEM CELLS AND REGENERATIVE MEDICINE

There is evidence linking AD related pathology with increased hippocampal neurogenesis in AD patients. Jin et al. examined the expression of neurogenesis marker proteins in hippocampus brains from AD patients and neurologically normal subjects [123]. They found that the expression of several neurogenesis markers was increased in the hippocampus of severely affected AD patients. The upregulated proteins included DCX, PSA-NCAM, TUC-4, and NeuroD. These findings are in contrast to the findings in AD transgenic mice, which show impaired hippocampal neurogenesis [124–126]. Baglietto-Vargas et al. have now investigated the effect of A $\beta$  on neuronal and glial cell proliferation by using APP/PS1 transgenic model and *in vitro* assays [127].

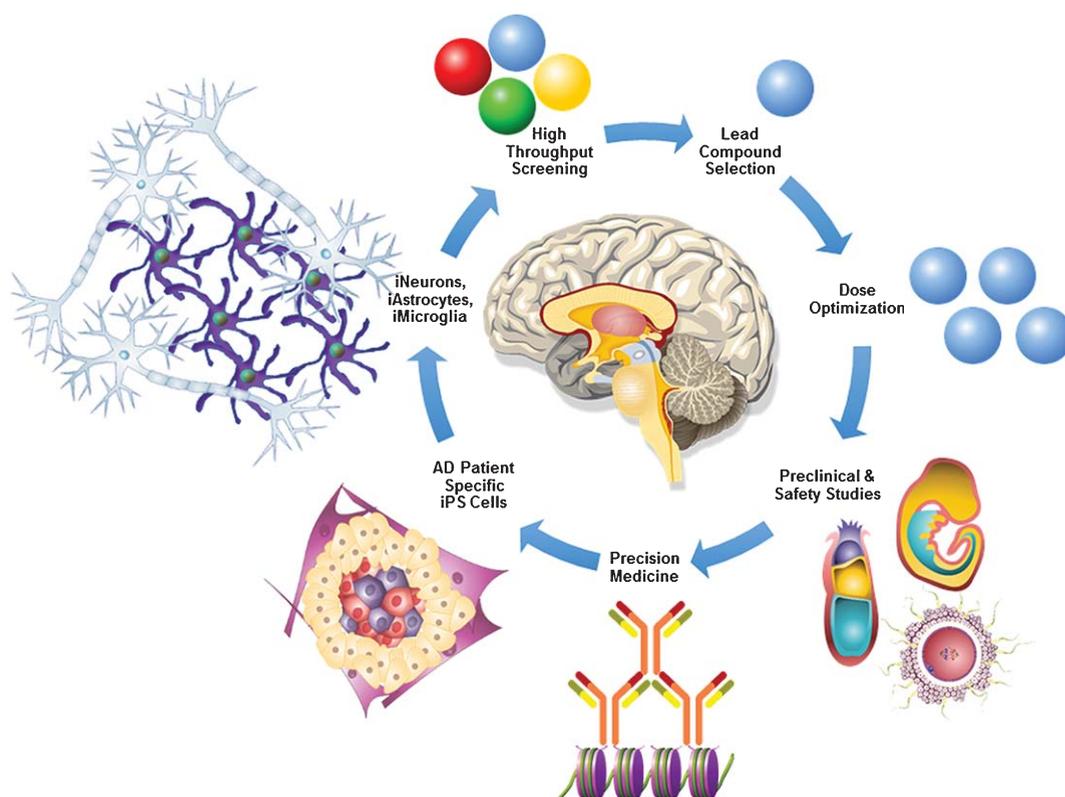


Fig. 2. AD in a dish model for precision medicine. AD patient-specific fibroblasts can be reprogrammed into iPS cells. Progressive step-wise differentiation and lineage commitment of AD patient-specific iPS cells will generate iNeurons, iAstrocytes, and iMicroglia which play a crucial role in AD pathogenesis. Advancements in biomedical engineering have made it possible to generate 3D brain-like mini organoids derived from iPS cells, which can be very valuable in robotic high throughput screening of novel AD therapeutic targets. Once a lead compound with significant therapeutic potential is identified it can be used for the dose optimization, preclinical, pharmacokinetic, pharmacodynamic, toxicology, and safety studies in AD murine and non-human primate models. Finally, utilizing crystallography, mass-spectrometry, bioinformatics tools and latest advancements in precision medicine, AD patient-specific novel therapies can be successfully developed.

They showed that neurogenesis is affected early in the APP/PS1 hippocampus due to reduced number of both radial glia-like neural stem cells and intermediate progenitor cells. There is a need to investigate the role of hippocampal neurogenesis in AD pathology as well as cognitive function.

Han et al. have developed an antibody that induces the differentiation of bone marrow cells into microglia cells that traffic to the brain where they self-organize into typical networks [128]. They created a human ScFv lentiviral intracellular combinatorial antibody library with  $10^8$  unique antibody clones. Total bone marrow cells were harvested from mice, transduced with the lentiviral library and then transplanted into lethally irradiated mice. Seven days post transplantation, various organs were harvested and the cells assessed for

migration and the DNA was subjected to PCR to amplify and sequence human ScFv sequences. They were able to identify an antibody B1 that induced migration of the cells to the brain. This antibody was also able to differentiate mouse bone marrow or human  $CD34^+$  cells into microglia-like cells that expressed mRNA for the microglial markers CX3CR1, IBA1, CD11b, CD68, TMEM119, and HEXB but failed to express mRNA for established oligodendrocyte (Olig1, Olig2, and MOG) and astrocyte (GFAP, SLCA2, and ALDH1LA) gene markers. The induced microglia-like cells exhibited an anti-inflammatory M2-like phenotype and phagocytosed  $A\beta$  peptide *in vitro*. These microglia-like cells when injected in APP/PS1 mice were capable of lowering  $A\beta$  deposition in the brain. These exciting results could potentially lead to the

development of novel therapeutic strategies to successfully treat AD.

## CONCLUSION AND FUTURE PERSPECTIVES

There has been a significant interest in developing novel AD therapies. However, despite significant global efforts, successful AD therapy remains elusive. There are multiple hurdles and roadblocks that need to be overcome before a precision-targeted patient-specific AD therapy becomes a reality. With the latest multi-disciplinary advancements including single-cell whole-genome sequencing [129], transcriptomics [130], metabolomics [131], proteomics [132, 133], CRISPR/Cas-mediated genome and base editing [134, 135], gene therapy [136], stem cell-based brain organoids [137–139], direct neuronal reprogramming and transdifferentiation [140–142], nanotechnology [143], biomolecular 3D organ printing [144], molecular imaging [145, 146], artificial intelligence [147], machine learning [148], robotics [149], and optogenetics [150] there is tremendous excitement and renewed vigor to win the war against AD. Toward our commitment to our long-term goal of developing targeted gene-editing and stem cell-based patient-specific therapies against AD and diabetes, we have very recently proposed a novel paradigm wherein tanycytes have been implicated to play a crucial role in diabetes and AD pathogenesis [151]. Although, the molecular mechanisms linking AD and diabetes are poorly understood, recent studies suggest that elevated glucose and oligomeric A $\beta$  disrupt synapses via S-nitrosylation [152]. Further, we believe that there is critical need to focus our efforts on the deciphering the nexus between traumatic brain injury, post-traumatic stress disorder and AD as it will enable us to uncover potentially novel molecular mechanisms underlying AD initiation and progression [153, 154]. We are cautiously optimistic that, multi-faceted approaches simultaneously targeting neuroinflammation [117, 155, 156], blood-brain barrier [157, 158], robust A $\beta$  processing and clearance by targeted immunotherapies [13], tau phosphorylation [159], APOE4 [160–164], improving mitochondrial function [165–167], iPS cell-based disease modeling [168], development of neural organoids [169, 170], stem cell trials [171, 172], neuroepigenomics [173, 174], microRNAs [175–177], improving CRISPR/Cas9 genome editing [178, 179], designer AAV vectors with

enhanced neurotropism [87, 90, 180], improved drug delivery using nanotechnology [181], targeting exosomes [182], development of large animal AD models [183, 184], next generation nutraceuticals [185] as well as advances in microbiome-gut-brain axis [186, 187], neuroimaging [188] and robust, reproducible and reliable biomarker discovery [189, 190] hold significant promise and potential for the development of the next generation patient-specific neuro-immuno-gene and genome-editing as well as exosome and stem cell-based AD therapies (Figs. 1 and 2).

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