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Neurodegenerative Diseases

What Is to Be Done?

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Abstract

Neurodegenerative diseases, including Alzheimer disease, Parkinson disease, and many others, lead to significant morbidity and mortality. As medical care for other disorders (e.g., cardiovascular disease and cancer) has improved and people are living longer, neurodegenerative diseases have become more prevalent because age is a major risk factor for most of them. There have been tremendous advances in our understanding of the scientific underpinnings of neurodegenerative diseases over the last thirty years. Nonetheless, with a few exceptions, very few effective treatments are available to delay the onset or affect the course of these diseases. This Forum brought together leaders in the field of neurodegeneration from different disciplines and tasked them with defining areas in need of more attention—areas where focused work is needed to reveal a better understanding of these disorders. A time frame of 5–20 years was the goal within which to develop more effective diagnostics and treatments. This chapter identifies eight areas to address:

1. Major specific pathologies and circuit dysfunction in neurodegenerative diseases need to be pinpointed over the life span, and dysfunctional circuits require identification.
2. Utilization of genetically well-defined patient populations will likely offer a better chance for therapeutic success.
3. Therapies affecting neurotransmitter systems and signaling pathways should be further explored utilizing defined patient populations and disease-affected nodes.

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4. Better ways need to be developed to understand protein aggregation processes, from the formation of misfolded proteins to the critical clearance pathways that regulate their levels and toxicity, including understanding the mechanisms which underlie protein aggregate spreading in the brain as this could lead to novel therapeutics.
5. Better understanding is needed on the role of human apolipoprotein E (apoE), lipoproteins, and lipid biology under normal conditions and in neurodegenerative diseases.
6. To increase understanding of disease and facilitate drug/biological delivery, more information on the blood-brain barrier, the neurovascular unit, and other barriers separating CNS from non-CNS compartments is required.
7. The role of the innate immune system and other immune mechanisms that contribute to progression of neurodegeneration must be better understood.
8. Regardless of the upstream processes, it may be possible to activate neuroprotective mechanisms using defined factors, signaling pathways, or via cell-based methods.

With significant progress in each of these eight areas, substantial changes in the diagnosis and treatment of neurodegenerative diseases should be possible over the next twenty years. Given the current cost of these diseases to society and the increase in their prevalence with no additional progress, a major worldwide effort should be made to address these issues immediately, with the highest of priorities.

Introduction

Neurodegenerative diseases, including Alzheimer disease (AD), Parkinson disease (PD), dementia with Lewy bodies, frontotemporal dementia (FTD), amyotrophic lateral sclerosis (Xia et al. 2013), and Huntington disease (HD), have devastating effects on the nervous system that lead to progressive cognitive, behavioral, and motor dysfunction. With improvement in overall medical care, the importance of these age-related diseases to society is rising. In the United States and Europe, it is estimated that the cost of dementia care alone is already equal to or greater than that of cardiovascular disease and cancer (Hurd et al. 2013). The number of affected individuals and the cost of caring for them are expected to triple over the next forty years in the absence of effective disease-modifying treatments. Thus, developing a better understanding and treatment for these disorders has become paramount. While several useful treatments exist to treat PD and multiple sclerosis, no treatments have been shown to delay the onset of these diseases, and few have significantly affected disease progression. In our discussions, several major areas emerged which we believe are important to make progress over the next 5–20 years, if we are to begin to decrease the incidence of these diseases and develop better treatments for those affected. Eight specific topics are discussed in this chapter, which we believe are critical in making progress.

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Need to Pinpoint Major Specific Pathologies and Circuit Dysfunction in Neurodegenerative Diseases over the Life Span and to Identify Dysfunctional Circuits

There is clear data that aggregation of specific proteins is a signature feature of most neurodegenerative diseases (Caughey and Lansbury 2003): amyloid-β (Aβ) and tau in AD; α-synuclein and Aβ in dementia with Lewy bodies; α-synuclein in PD; tau in various forms of FTD, progressive supranuclear palsy, corticobasal degeneration, and chronic traumatic encephalopathy; TDP-43 in amyotrophic lateral sclerosis (Xia et al. 2013); and huntingtin in HD. For most of these disorders, however, there is strong data that the proteins found in aggregates play major roles in disease pathogenesis. Importantly, in AD, PD, and HD (and probably in the other diseases), these protein aggregates begin to accumulate many years prior to the onset of symptoms—at least 15 years, for example, in the case of Aβ and AD (Jack and Holtzman 2013). By the time symptoms appear, there is already significant neuronal cell loss in certain regions of the CNS as well as synaptic, axonal, and dendritic loss and dysfunction. These findings argue that if effective treatments are going to be developed, identification of pathology and regional brain dysfunction *in vivo* prior to the onset of symptoms and signs of these diseases is critical to the initiation of primary or secondary prevention. In addition, specific neurological syndromes can result from different underlying pathologies. Thus, in individuals with different symptomatic stages of a disease process, identifying the actual underlying pathology is critical to an accurate diagnosis, enrollment in clinical trials, and ultimately treatment. To establish the exact pathological process that is taking place, as well as staging of the disease, an assessment of what is occurring in the CNS (e.g., by imaging, biofluid, and electrophysiological analysis) needs to begin during the asymptomatic period (e.g., at middle age), to identify preclinical disease, as well as once symptoms and signs emerge for both prognosis and to monitor disease progression (Holtzman et al. 2011; Sperling et al. 2011a). In addition to genetic factors, aging plays a big role in disease risk and thus needs to be accounted for in the assessment and interpretation of the biomarkers for neurodegenerative diseases. As testing of asymptomatic individuals for the presence of disease becomes more widespread, in both research and for clinical use, appropriate education and informed consent will be required.

In regard to the detection of protein aggregates, use of molecular imaging has been very useful. There are now good ways to detect the presence of fibrillar Aβ by both imaging and by measuring cerebrospinal fluid Aβ42 (Klunk et al. 2004; Fagan et al. 2006). In recent studies, tau aggregates are now detectable with molecular imaging (Maruyama et al. 2013; Xia et al. 2013). For the other major aggregates that occur in these diseases, including α-synuclein, huntingtin, and TDP-43, molecular imaging is not yet possible and should be developed. Cerebrospinal fluid or blood tests for the presence of any of these

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aggregates in the CNS are also not yet possible and would be very helpful. In addition, for both molecular imaging and biofluid assessment, it would be extremely helpful to be able to detect oligomeric forms of these proteins, which may be the forms that mediate toxicity (Benilova et al. 2012). There is strong evidence that many of these proteins that aggregate form different types of aggregates in different diseases. For example, aggregated tau has both different ratios of 3 or 4 repeat isoforms as well as different structure in AD versus progressive supranuclear palsy (Mandelkow and Mandelkow 2012). For each of the proteins that aggregate, development of methods to detect the specific type of aggregate as well as the cell type within which they are present (glia versus neurons) would help classify the specific molecular features that are present for each proteinopathy. Other types of biomarkers that would be potentially very helpful to assess and develop, to add to the information about protein aggregates, involve the status of microRNA in biofluids, given recent data showing their role in disease pathogenesis (Mendell and Olson 2012). It is also worth determining if fluid biomarkers or the earliest cellular triggers precede protein aggregation.

Important issues to consider in biomarker development include the necessity to develop reproducible, reliable, and cost-effective tests. Before widespread use, biomarkers need to be well validated in large populations in longitudinal studies. As effective treatments emerge, the risk and cost-benefit ratio of the test versus the treatment will emerge. The more invasive the test, the more demand there will be for having effective treatments. In non-Mendelian neurodegenerative disorders, it will be necessary to develop sensitive measures for population screening which can then be paired with more specific secondary tests to identify persons for treatment. A measure that is not as costly (e.g., a blood test) is certainly preferable, but there is already a precedent for more invasive measures (e.g., colonoscopy for colon cancer screening); dementia is certainly in a similar category in terms of burden of illness. Of course, a less invasive test will increase acceptance and utilization. Along those lines, assessments of plasma, serum, peripheral blood cells, and skin cells should be further assessed with the more advanced "–omics" and other technologies. Finally, as the cost of complete genome sequencing has reduced dramatically, the assessment of each person's genome in combination with the other imaging and biofluid tests could prove very useful: the integration of genetic and biochemical information from an individual offers greater predictive value.

In all the neurodegenerative diseases under discussion, specific regions and circuits in the brain are selectively vulnerable. It is thus critical to both utilize and expand upon current methods to detect synaptic and circuit dysfunction, as well as to pick up changes that are occurring prior to, during, and after the development of protein aggregation in the CNS, but still prior to the onset of clinically detectable symptoms and signs of disease. Both structural and functional connectivity magnetic resonance imaging combined with assessments of brain metabolism with FDG-PET are showing changes in some of these

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diseases in asymptomatic individuals (Jack and Holtzman 2013). It would thus be very useful to optimize both of these methods, especially their quantitative aspects. Data coming from the Human Connectome Project may provide new methods for quantitative tract tracing, which would prove useful (Van Essen et al. 2013). Using these and other imaging methods, it will be important to determine if any changes occur in brain regions that precede protein aggregation. This will permit a better understanding of pathogenesis as well as early detection of disease-related cause or dysfunction (e.g., precuneus/posterior cingulate cortex in AD; Greicius et al. 2004).

In addition to current neuroimaging methodologies for assessing brain function, assessment of synaptic and circuit function via other methods might provide important new ways to get at pathogenesis and diagnosis. For example, neuroimaging ligands that would permit detection of specific synaptic markers would be very useful. This has been developed for the dopaminergic system (Varrone and Halldin 2012) but not to similar degrees for other major neurotransmitter systems or for proteins present in excitatory versus inhibitory synapses. Other methods that would detect activity within specific neuronal populations would also be very useful. Knowledge from methods currently utilized in animals that allow for expression profiling of specific neuronal populations (e.g., BAC-TRAP; Heiman et al. 2008) may provide information to allow assessment of specific neuronal populations *in vivo*. Electrophysiological testing with qEEG during alert and sleep phases as well as other methods should be further studied to determine how early circuit and synaptic changes might be detectable. In many cases, current application of neuropsychological testing in humans may not be able to detect some of the earliest functional changes. Longitudinal cognitive assessments and cognitive tests in areas such as spatial memory and attention in presymptomatic populations might permit earlier changes to be detected (Sperling et al. 2011a).

To achieve the aspirational goal of disease prevention, a number of steps should be taken in preparation for the testing of promising therapies. To demonstrate prevention benefit, it is necessary to be able to identify clear, quantifiable end points, often of conversion from presymptomatic to symptomatic stages. It is also critical to be able to identify a cohort for study in whom there is high probability of conversion within the time period of a study, generally 1–5 years. Natural history studies are therefore required ahead of a trial to construct and validate end points and formulae that accurately predict expected time to reach these end points in a specific population. The PREDICT and PHAROS studies in Huntington disease provide a model for this exercise: imaging and neuropsychological changes have been followed over time in individuals with known cytosine-adenine-guanine expansion who are close to expected time of conversion to symptomatic HD (Biglan et al. 2013). Given the time frames needed to follow individuals in a prevention trial, the current patent lifetimes are a major disincentive and these need to be revisited by policy makers.

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Utilization of Genetically Well-Defined Patient Populations May Increase Chance for Therapeutic Success

Once we are able to diagnose and assess neurodegenerative diseases better, which populations should clinical trials focus on over the next 5–20 years? The negative results obtained through the testing of drugs for the treatment of neurodegenerative disorders from the general non-enriched population, in which genetics or biomarkers have not been utilized for enrollment criteria, has been both disappointing and discouraging. This continuing approach reflects the standard conventional practice of testing of neurological drugs and market-driven decisions: to seek as broad an indication as possible, if successful, and to position the drug in ways that differentiate it from competing drugs. The recent testing of the anti-Aβ monoclonal bapineuzumab in apoE4 carriers and apoE4 noncarriers provided very useful information (Salloway et al. 2014a). It was one of the first times that a potential Alzheimer treatment was tested in genetically defined groups (by apoE status). If the treatment had been successful in one but not both subgroups, this would have set the precedent that a treatment is not intended for the affected population at large. While still novel in the Alzheimer field, this practice follows the lead from oncology, where the genetic makeup of a particular tumor can show predictable outcomes to targeted therapies. The recent start of several secondary prevention trials in individuals with various rare hereditary mutations in the *APP* and *Presenilin* genes (Moulder et al. 2013; Ayutyanont et al. 2014) as well as in apoE4 homozygotes continues this new trend to examine genetically defined populations to test mechanism-based therapies in subjects who may either benefit most from the treatment or offer insights not available from unselected populations.

Genetically well-defined populations can offer a clearer route to demonstrate pathophysiological mechanisms or provide proof-of-concept for the mechanism of drug action. For example, the recent positive response to either tafimidis (Coelho et al. 2012) or diflunisal (Berk et al. 2013) in the rare familial amyloid polyneuropathy, due to transthyretin accumulation in peripheral tissues, was a seminal success in a previously untreatable neurodegenerative disease. This trial offers a number of instructive lessons:

1. Use of objective measurements (in this case, tests of nerve and muscle function) can provide meaningful assessment of disease progression.
2. Treatment can be initiated after onset of disease and still demonstrate a positive effect.
3. Early treatment demonstrated greater benefit than when treatment was instituted later in disease course.
4. Results represent a dramatic proof-of-concept of the proposed underlying disease mechanism (inhibition of aggregation of transthyretin).

This benefit may, in the future, extend to similar success in the more common systemic amyloidosis, which does not have a genetic basis. Therefore, this

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example suggests that targeting a "purer" population can provide more clear-cut answers, albeit in a very select group of individuals. This idea could be expanded to a much larger nongenetic disease-affected group of individuals. Following the genetic enrichment lead, one can envision similar approaches applied to genetic forms of other neurodegenerative diseases, such as with the *LRRK2* and *GBA* mutations associated with PD and mutations in various genes in FTDs. In the latter, progranulin mutations are particularly interesting as disease pathophysiology appears to be related to haploinsufficiency (Cenik et al. 2012). Thus, one can readily envision boosting progranulin levels as a therapeutic approach.

Genetically well-defined groups offer quick validation of surrogate markers that track disease stages or progression. They also provide confirmation of validity of various end points used to assess treatment effects. Further, new biomarkers could be discovered that represent changes in brain circuits. The latter are particularly informative as they may represent new clinical measures of disease symptomatology useful for quantification of treatment outcomes.

One shortcoming of this approach is the rarity of individuals carrying various mutations. Mounting a successful biomarker or treatment study will require a consortium of national or international sites, a precedent established by the Dominantly Inherited Alzheimer Network (DIAN) study (Bateman et al. 2012; Moulder et al. 2013). In the DIAN study, a number of *PSEN* and *APP* mutations were included, since any one mutation is exceptionally rare. Fortunately, the different mutations all appear to induce perturbations in Aβ metabolism, aggregation, or production as the underlying disease pathophysiology. This may not be the case with other neurodegenerative diseases, such as in the ALS-FTD spectrum, where mutations have been identified in different genes that seemingly appear to affect very different cellular pathways (Perry and Miller 2013).

In AD, in addition to apoE4 homozygotes, trisomy 21 (Down syndrome) offers another genetically well-defined population to study Alzheimer pathophysiology that arguably is a resource that has not been taken advantage of fully. As these individuals invariably develop Alzheimer brain changes by the fourth decade of life and dementia in later years, they offer the possibility to correlate markers of disease initiation and progression as well as to provide another genetically defined group for treatment trials. Further, drugs affecting other neurotransmitter pathways, such as pentylenetetrazol acting as a GABA-A antagonist, may also provide cognitive benefit in these individuals, perhaps distinct from an effect on dementia (Fernandez et al. 2007). Of course, use of different instruments to assess cognition will likely be required, given that all individuals suffer mental retardation to varying degrees at baseline.

Finally, many neurodegenerative disorders have both genetic and nongenetic causes and are likely to share multiple disease mechanisms. Complicating this is the tendency for the diseases to be age related. As such, comorbidities are frequently present in the elderly, and these concurrent systemic disorders

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complicate drug testing. These comorbid conditions can contribute to disease risk, confound treatment outcomes (as these conditions can be nonresponsive to the treatments) and, in so doing, contaminate the treatment pool or decrease diagnostic accuracy. The use of genetically well-defined populations can overcome some of these complicating factors. On the other hand, some of these comorbid diseases (e.g., hypertension, cardiovascular disease, or diabetes) can be effectively treated, and treatment may well reduce risk of, for example, dementia or AD (Schrijvers et al. 2012). Thus, for multiple reasons, comorbidities should not be ignored.

Explore Therapies That Affect Neurotransmitter Systems and Signaling Pathways through Defined Patient Populations and Disease-Affected Nodes

Over the past few years, efforts have been directed toward the development of strategies to address the pathogenic mechanisms underlying diseases such as AD and PD. Strategies to prevent or remove accumulation of Aβ, tau, and α-synuclein are in the early stages of testing. Utilizing these approaches, a clear-cut success in humans has not yet been obtained, but newer trials of agents directed against these targets have identified potentially more appropriate populations of patients earlier in the course of disease, optimizing chances for success. The reason that previous trials of potential disease-modifying therapies targeting Aβ in individuals with mild to moderate dementia have not yet been successful may be due to a fundamental problem with targeting Aβ accumulation in patients with overt manifestations of disease—the intervention may be already too late. However, there is reason to believe that delaying disease is possible in individuals at early clinical stages of diseases such as AD (mild cognitive impairment, very mild dementia) or in asymptomatic subjects with preclinical AD. Progress has been made in targeting tau pathology in animal models (Boutajangout et al. 2011; Yanamandra et al. 2013), which may be applicable to a variety of neurodegenerative disorders. Similar strategies are thought possible for PD, and common approaches include antibodies to prevent the spreading of protein aggregates in brain.

In addition to potential therapies that target molecules involved directly in disease pathogenesis, several other possibilities address symptoms of disease in AD, PD, and other disorders, and have the potential to delay disease progression and improve quality of life, even in patients where disease is already advanced. Arguably, these can be considered "disease-modifying" therapies. Delaying or ameliorating disease progression can produce substantial benefits. Historically, symptomatic treatments that have been developed and widely utilized include cholinergic/dopaminergic agents. While useful in AD, cholinergic agents have only been moderately effective (Birks 2006). New formulations and combinations with other therapeutics might increase effectiveness. Testing

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early symptomatic treatments in patients without overt disease manifestations might offer better chances for success. In particular, delaying the onset of disease by targeting early synaptic dysfunction might significantly delay other major disease symptoms. Some of the major neurotransmitter systems in the CNS that have not been fully explored therapeutically in neurodegenerative diseases include agents to modify the glutaminergic, GABAergic, serotonergic, and noradrenergic systems. In addition, development of better cholinergic and dopaminergic agents is likely still possible. One potential example of a novel type of neurotransmitter/excitability modulator comes from the use of levetiracetam, a drug used for treatment of epilepsy. At lower doses, it has shown cognitive benefits in mouse models of Aβ-amyloidosis that have epileptiform features and brain network abnormalities (Sanchez et al. 2012). Here, the difference between symptomatic and disease-modifying therapies, if similar effects are seen in humans, would only become apparent when neurodegenerative processes progress beyond the manifestations of the initial synaptic dysfunction observed. Symptomatic treatments might also be directed to early symptoms which are dependent or independent of disease, such as sleep, which could optimize memory performance (Ju et al. 2014). Critical to a research roadmap is careful attention to the problems that are most important to quality of life in affected individuals and their caregivers. More effective means of ameliorating symptoms is a critical area for research. Symptomatic trials could be performed more quickly and potentially at lower cost. For instance in PD, nonmotor symptoms have been identified as most troublesome in the modern age of effective treatments for bradykinesia (Chaudhuri et al. 2006). Cognitive impairment, gait disturbance, and dyskinesias rise to prominence. In AD and HD, disorders of circadian rhythm and behavioral disorders frequently exhaust a family's ability to care for an affected individual at home. Pharmacologic or nonpharmacologic means of addressing these concrete problems should be a focus of research and could lead to success in the short term. It will also be important to determine whether responses to treatments are seen only in patients with specific characteristics, so as not to miss effects.

Another major area for investigation that can offer a strong therapeutic prospective is the modification of *APOE*. This area is largely underfunded and understudied, despite it being recognized as the major genetic risk factor for AD and other diseases (see below). Inflammation and the innate immune system offer another symptomatic/disease-modifying treatment target in both AD and PD. Inflammation appears to be involved in disease progression in AD, PD, ALS, FTD, and HD at different disease stages. Identifying the window of opportunity to intervene with drugs that reduce or prevent activation of infection-related inflammation or innate immunity could also prove fruitful. Finally, treatments that would enhance or restore plasticity including transcranial magnetic stimulation, deep brain stimulation, or nonpharmacological interventions (exercise, diet, sleep) have the potential to produce significant benefits and should be further explored.

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Some limitations to the development of new symptomatic or disease-modifying therapies include:

- Current design of clinical trials (which have so far been considering individuals with overt disease over presymptomatic individuals with high risk).
- Poor outcome measures (e.g., better tools to assess cognition).
- Lack of multiple biomarkers: Better and more molecular, pathogenesis-linked biomarkers need to be identified and followed over time during, before, and after disease onset to detect dynamic changes. To this end, new useful biomarkers need to correlate with existing biomarkers related to disease progression.
- Funding agencies need to support areas of research which, although not widely represented within the scientific community, offer strong potential for therapies (e.g., *APOE*). Regulatory agencies need to be more open to the acceptance of preventive trials and new functional biomarkers.

Understand Protein Aggregation Processes and Spreading Mechanisms to Spur Novel Therapeutics

In terms of ultimately developing disease-modifying therapies for neurodegenerative diseases, it is critical to develop a better understanding of the normal metabolism as well as the pathophysiology of key proteins that form aggregates and accumulate in the brain. Most proteins implicated in the pathogenesis of neurodegenerative disorders can exist in a dazzling array of different assemblies and conformational states, and for most, if not all, it remains to be determined which conformation has the greatest impact on neuronal function and survival. To fill this important knowledge gap, there is an urgent need to develop methods that can reliably quantitate distinct assemblies of pathogenic proteins in brain tissues of humans and experimental animal models, ideally through noninvasive means. Relevant protein species include soluble oligomers, which have emerged as potentially critical but rather elusive culprits in a number of diseases (Benilova et al. 2012). With such methods, it should be possible to define not only how many of these forms of proteins are present but also how much the most pathogenic subspecies have to be reduced to improve significantly brain function at the synaptic, network, and clinical levels. This information could have an important impact on the design and interpretation of clinical trials. For example, none of the anti-Aβ trials completed to date have shown whether treatment significantly lowered Aβ oligomer levels in relevant brain regions, nor is it clear whether, and by how much, the levels of these assemblies would have to be lowered to improve synaptic function or slow down neuronal loss.

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Based on a large body of published data, it is likely that pathogenic protein assemblies or conformations can trigger diverse molecular cascades (Hayden and Teplow 2013). The most proximal downstream mechanisms may involve both receptor-dependent and receptor-independent processes. This diversity of protein species and downstream pathways makes it unlikely that targeting any one of them by itself will have a major impact on the disease overall. Blocking the production of the abnormal proteins, inhibiting their aggregation, or enhancing their clearance may be more effective strategies. Far greater efforts appear to have been invested to date in inhibiting production of specific proteins, such as Aβ, as opposed to enhancing their clearance. Indeed, modulation of clearance mechanisms clearly deserves greater attention as this may be one of the main overall mechanisms by which aging leads to elevated risk of protein aggregation. Some of the abnormalities in clearance pathways which may give rise to disease and be targetable include degrading enzymes, chaperone-mediated clearance, autophagy, uptake into glial cells, better utilization of interstitial fluid or cerebrospinal fluid flow pathways, and transfer across the blood-brain barrier into the circulation. Determining which of these mechanisms are most effective, most druggable, and most affected by disease could result in the identification of novel entry points for therapeutic interventions.

Closely related to this quest is the issue of whether the spread of pathogenic proteins, such as tau and α-synuclein, from one cell to another, might involve unique processes that are amenable to specific therapeutic interventions. In cellular and animal models, cell-to-cell spread appears to involve converting the normal, non-aggregated form of a protein into an aggregated form with high β-sheet content by interacting with an already seeded/aggregated form of the same protein (Frost and Diamond 2010; Lee et al. 2014). In the case of tau and α-synuclein, this process appears to move from cell to cell in what some scientists call a prion-like manner. However, to what extent this type of spread via template seeding contributes to the progression of neurodegenerative disorders in both animals and humans remains uncertain and is currently under intense investigation. One condition that may be a particularly good example to test more extensively is chronic traumatic encephalopathy, where there appears to be spreading of tau aggregates over many years between different brain regions (Stein et al. 2014). There also is a need to understand the mechanisms by which pathogenic proteins impair the function of brain cells and the networks they form. In particular, in AD and dementia with Lewy bodies, there is strong evidence that Aβ accumulation leads in some way to further accumulation and spread of tauopathy in AD (Musiek and Holtzman 2012) and synuclein in dementia with Lewy bodies (Masliah et al. 2001). The mechanisms (e.g., cellular signaling pathways) that underlie these effects are unknown and if sorted out, could lead to new ideas for treatment. From a treatment standpoint, it would also be valuable to complement ongoing efforts to detect the accumulation of abnormal proteins in the brain with intensified efforts to develop and

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incorporate novel methods into clinical trials to detect the emergence, progression, and potential therapeutic reversal of synaptic and network dysfunction.

Understand the Role of ApoE, Lipoproteins, and Lipid Biology under Normal Conditions and in Neurodegenerative Diseases

Of the common diseases in humans, one of the strongest genetic risk factors is the link between *APOE* and AD. ApoE4 dose dependently increases risk for AD and cerebral amyloid angiopathy whereas apoE2 protects against AD (Strittmatter and Roses 1996; Kim et al. 2009). *APOE* genotype may also modulate risk for other CNS disorders, such as recovery after head injury, stroke, and other forms of neurodegeneration (Mahley and Huang 2012; Wolf et al. 2013). Despite the strong effects of *APOE* genotype on AD, our understanding of exactly how apoE has these effects is incomplete, and this has limited development of ways to target apoE-related pathways. Despite the importance of apoE in AD risk, there is a paucity of academic and industry-related efforts and investment in this important topic. Significantly more effort and resources need to be prioritized here, given the strong impact of this gene which appears to be mediated by the protein product.

There are two general categories by which apoE proteins appear to modulate AD and neurodegeneration: Aβ-dependent and Aβ-independent. For Aβ-dependent mechanisms, there is overwhelming evidence that apoE isoforms influence whether and when Aβ aggregates and accumulates in the brain and contributes to its toxicity (E4 promotes Aβ accumulation, E2 retards it relative to E3) (Holtzman et al. 2012). The general mechanisms appear to be due to the ability of apoE to interact directly with Aβ to influence its aggregation as well the ability of apoE to influence soluble Aβ clearance. In terms of which receptors modulate apoE-related Aβ clearance, this has not been entirely resolved. However, there is strong genetic and biochemical evidence that increasing apoE lipidation (Koldamova et al. 2010) as well as decreasing levels of apoE3 and apoE4 can decrease Aβ accumulation (Kim et al. 2011; Bien-Ly et al. 2012). ApoE lipidation may be targetable by liver X receptor and retinoid X receptor agonists (Cramer et al. 2012), although side effects from this approach would need to be overcome. Decreasing apoE levels can be accomplished by increasing activity of apoE receptors, such as low-density lipoprotein receptor (LDLR) and LDLR-related protein (LRP1) (Holtzman et al. 2012). There may be ways to increase function of these receptors using a biological or small molecule approach. Knocking down apoE3 and apoE4 via an antisense oligonucleotide approach should also be considered. Experiments with anti-apoE antibodies show promise in decreasing Aβ deposition (Kim et al. 2012) and should be further explored and developed. Inhibiting the interaction between apoE and Aβ is another target that has been attempted successfully in animal models, using peptides that might be approachable with small molecules. In

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addition to apoE's effect on Aβ, it may also influence tauopathy via direct interactions with tau or via an indirect mechanism. This needs further exploration as a biological mechanism as it offers new ways to target this interaction.

In regard to other non-Aβ-related mechanisms, apoE has been shown in various *in vitro* and animal models to have the following effects, which may be relevant to neurodegeneration (Mahley and Huang 2012; Wolf et al. 2013):

- effects on neurite outgrowth and synaptic plasticity,
- formation of apoE fragments in neurons that can be toxic,
- effects on mitochondrial function,
- effects on neuroinflammation, and
- effects on reverse cholesterol transport and lipid scavenging.

Further exploration of these effects *in vivo* in different models of neurodegeneration and in different laboratories will be critical to determine how important each mechanism is. Investigators at the Gladstone Institute (Chen et al. 2012) suggest that small molecules act as "structure correctors" to convert an apoE4 structure close to that of apoE3 or apoE2. This should be further explored in regard to the influence on both Aβ- and non-Aβ-dependent effects of apoE.

Two areas of apoE biology should be fruitful: further characterization of apoE structure and neuroprotective effects of apoE2. Although the structure of apoE has been determined, it was done in the nonlipidated state. Since almost all apoE *in vivo* is lipidated, understanding apoE structure on high-density lipoprotein-like lipoproteins, as it occurs in the brain, should assist in modeling its various interactions. ApoE2 is strongly protective against AD, yet our understanding of the mechanism underlying this effect is poor. ApoE2 results in lower Aβ deposition; increased expression of apoE2 in the brain (via a viral vector approach) decreases Aβ deposition (Hudry et al. 2013; Dodart et al. 2005). Further experiments utilizing such an approach should be pursued in animal studies and potentially taken into humans if successful.

To Facilitate Disease and Drug/Biological Delivery, More Information Is Needed on the Blood-Brain Barrier, Neurovascular Unit, and Other Barriers Separating CNS from Non-CNS Compartments

In AD, PD, NeuroAIDS, and in other neurodegenerative disorders, there is evidence of dysfunction of the neurovascular unit (NVU), which is composed of neurons, astrocytes, pericytes, and endothelial cells that surround blood vessels in the brain (Iadecola 2004). This dysfunction may contribute to CNS dysfunction as well as accumulation of neurotoxic molecules. It has been suggested that NVU abnormalities might contribute or even trigger the neuropathology of AD and other diseases (Zlokovic 2008). The NVU plays a key role in regulating the permeability of the blood-brain barrier, cerebral blood flow,

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and the activity of neuronal circuits. Better understanding of how the NVU and blood-brain barrier normally function as well as how alterations in the NVU are involved in clearance of misfolded proteins, neuroinflammation, and other aspects of degeneration will provide important new insights. In addition, the brain's intrinsic drainage pathway, the recently named gliolymphatic system (Iliff et al. 2012), needs to be better understood both under normal and disease conditions, in relation to all the issues just mentioned. How the neurons are interacting with endothelial cells and astroglia at the NVU is very important and a key area for further research. Clarifying the nature of the alterations in the NVU in neurodegeneration is likely to be important in the elucidation of treatments designed to target the NVU as well as in understanding how this alters the traffic of drugs and biologics into and out of the CNS. The concept that the blood-brain barrier is damaged in CNS disorders has been around for a while, but whether this contributes to disease pathophysiology remains controversial. Still, recent evidence has shown that AD changes affect pericytes and the blood-brain barrier (Zlokovic 2008), because vascular amyloid is a known association that leads to hemorrhage and potentially to impaired autoregulation. In this context, there is a need for better tools to quantify blood-brain barrier leakage in both animals and humans.

The traditional drug release systems that deliver drugs systemically fail to transport biologics and drugs effectively into the brain. As most therapeutic agents for neurodegenerative disorders need to likely reach the CNS to be effective, approaches that will selectively target the CNS more efficiently need urgent development. Growth factors, neuroprotective and anti-inflammatory peptides, antibodies directed against neurotoxic protein aggregates, and enzymes are some examples of biologics with potential application in the treatment of neurodegenerative disorders. Major challenges when considering the delivery of such biologics into the CNS are how to target them specifically to the brain. This has the potential to reduce off-target effects by lowering the drug dose as well as by other mechanisms. Trafficking of proteins and peptides into the CNS involves the interactions of the macromolecules of interest with receptors located on the luminal and/or abluminal surfaces of the brain endothelial cells. Both endocytosis and transcytosis play key roles in the trafficking of such macromolecules across the blood-brain barrier.

Multiple strategies deliver proteins of therapeutic interest into the CNS, including the use of peptides or antibodies that bind to endothelial cell receptors as well as nanoparticles, liposomes, and other container-type of carriers and viral vectors (e.g., adeno-associated virus, rhabdovirus). All have advantages and disadvantages. In the case of charged nanoparticles, ensuring specificity has been a challenge. With viral vectors, questions have been raised as to side effects and long-term toxicity. Overall, each strategy needs to be categorized according to specificity, capacity, mechanism of action, and application. The so-called "one-fit-for-all" approach is unlikely to work; thus we recommend that each strategy be considered independently and specific

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applications developed. We also recommend that combinations of strategies to carry biologics into the CNS be considered. For example, nanoparticles can be combined with selected peptides to improve specificity. Given the potential complications with rabdoviruses and some nanoparticles, other strategies have been considered, in particular those involving receptor-mediated delivery.

The main receptors that mediate endocytosis and transcytosis of antibodies, peptides, and proteins across the blood-brain barrier include LDLR, LRP1, transferrin receptor (TfR), Fc receptor (FcRn), as well as the insulin receptor (IR) and insulin growth factor-1 (IGF-1) receptor. Compared to other strategies, the use of targeted small peptides or antibodies to transfer macromolecules into the CNS has the advantage of being minimally invasive. One of the most advanced strategies involves the use of monoclonal antibodies against TfR or against IR. An interesting application of this strategy for the potential treatment of AD has been the development of a divalent antibody in which one arm of the antibody targets TfR (to facilitate blood-brain barrier permeability) while the other arm targets β-secretase-1 (BACE1), a protease involved in the processing of amyloid precursor protein (APP) into Aβ (Atwal et al. 2011). These types of antibodies have been shown to facilitate the blood-brain barrier with great efficacy and to reduce Aβ levels in both mice and nonhuman primates. Antibodies against TfR have also been used to enhance the trafficking of antibodies in which one arm targets Aβ. This approach has been tested in APP/PS1 transgenic models that develop Aβ deposition and was more effective than using a standard anti-Aβ antibody in reducing Aβ levels (Niewoehner et al. 2014). More recently, utilizing phage display, two single domain antibodies, FC5 and FC44, were identified that display high permeability across the blood-brain barrier (Haqqani et al. 2013). Both have been proposed for development as vectors for brain delivery. Overall, utilizing this or analogous approaches offers great hope that one can increase the entry of proteins such as antibodies in the brain that will be able to bind to and target specific proteins and cells in the CNS.

Another approach is to use selective targeted small peptides that bind lipoprotein receptors present on the surface of endothelial cells in the blood-brain barrier. For example, in recent years small peptides derived from apolipoproteins (B and E) that bind LDLR have been shown to have the capacity of increasing peptides and antibody entry into the CNS. Through the fusion of the LDLR-binding domain of proteins, such as apoB (38 amino acids) and apoE (19 amino acids), to cargo proteins, therapeutic promise has been shown in various neurodegeneration models for AD, PD, Gaucher, and Sly disease. For example, by coupling the LDLR-binding domain of apoB to neprilysin (a metalloprotease that degrades Aβ), it has been shown that the systemically administered fusion protein crosses the blood-brain barrier and accumulates in the neocortex and hippocampus, thus reducing Aβ levels in the brain as well as behavioral deficits and neurodegeneration (Spencer et al. 2011).

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Other molecular Trojan horses, which use a lipoprotein receptor, are peptides derived from the apoE sequence, Angiopep-2, and receptor-associated proteins that target LRP1 (Demeule et al. 2008). This lipoprotein receptor has been shown to mediate the endocytosis of Aβ peptides across the blood-brain barrier (Zlokovic 2008). Aprotinin, a pancreatic trypsin inhibitor that contains the Kunitz protease inhibitor (KPI) sequence, is a ligand for LRP1 and can cross the blood-brain barrier. Aligning the sequence of aprotinin with the KPI domain, a family of peptides (named Angiopeps) was developed. Angiopep-2, in combination with micelles, has been tested to increase the CNS penetration of drugs such as the antifungal amphotericin B (Shao et al. 2010). The amphoterocin B-incorporated, angiopep-2 modified micelles showed highest efficiency in penetrating into the CNS. Another peptide has been tested as a receptor-specific carrier, namely cross-reacting material 197 or CRM197, which is a nontoxin mutant of diphtheria toxin. CRM197 increased pinocytotic vesicles and vacuoles in brain microvascular endothelial cells and enhanced caveolin-1 protein expression in brain microvessels (Wang et al. 2010b). Regarding the apoE strategy, peptides of this molecule have been fused to α-L-iduronidase (IDUA), a lysosomal enzyme being evaluated for efficacy in a mouse model of mucopolysaccharidosis type I (Miller et al. 2013b). After 5 months of treatment, apoE-IDUA was found to have normalized brain glycosaminoglycan and β-hexosaminidase in an mucopolysaccharidosis type I model (Wang et al. 2013a). These strategies offer great promise as they are further developed. Targeting receptor-mediated transport via receptors such as TfR has potential side effects. For example, long-term use can theoretically trigger deficits in iron transport. Thus, due to the potential side effects for chronic use, other receptors should be considered. For example, LRP1 may have a higher capacity, better trafficking abilities, and be more efficient than TfR. Some controversy has emerged over whether LRP1 is present in endothelial cells in the blood-brain barrier; further investigation is thus needed.

Nonreceptor-mediated approaches are also being considered for targeting the CNS. The use of the HIV protein Tat as a carrier has been tested alone as well as in combination with liposomes to deliver antibodies, peptides, and growth factors. In addition, approaches that involve nanoparticles and liposomes are being tested to deliver small molecules into the CNS. In terms of delivering biologics to the brain, it is important to consider how the biologic will be delivered to the correct compartment, how specific brain regions can be targeted, and how the trafficking of the biological can be measured.

It will be crucial to develop imaging tools that allow measurement of the trafficking of the therapeutically administered bioactive proteins more effectively. Some work has shown that selectively tagged (coded) methods using RNA sequences can be used to target neuronal populations selectively. Once a therapeutic bioactive protein is targeted to the correct brain region and cellular population, the next problem is for the protein to find the correct cellular compartments. Using molecules that target a receptor, such as LRP1, has a

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unique advantage: once the protein is transcytosed, it is targeted to the lysosome, which may be useful for specific metabolic diseases. This approach has also been used with antisense oligonucleotides tagged for delivery into the CNS to treat neurodegenerative disorders.

Other questions to consider concern the advantages and disadvantages of invasive versus noninvasive approaches. Is it preferable to deliver a biological or medicinal therapy into one specific brain region site or diffusely into the brain? For example, for some years, intranasal administration has been considered a potential approach for getting certain compounds into the CNS effectively. An intranasal trial of insulin in AD is currently in progress: the advantage here is that it appears to influence solely the CNS and does not change blood glucose levels. Is intranasal administration still a valid approach for drug delivery? Alternatives include considering whether compounds can be delivered into the brain more effectively in areas lacking a blood-brain barrier (e.g., hypothalamus). It may also be possible to utilize cell-mediated delivery of material into the brain via cells such as monocytes to deliver drugs and biologics in the CNS. Finally, direct CNS delivery via the lumbar or ventricular cerebrospinal fluid as well as into the brain parenchyma via convection-enhanced delivery under appropriate circumstances may be very effective and, while more invasive, may still provide a good risk-benefit ratio. This approach is being explored for gene delivery via adeno-associated virus (AAV) as well as administration of antisense oligonucleotides (Miller et al. 2013b). Overall the NVU, blood-brain barrier, and gliolymphatic systems are being actively investigated under normal conditions and in the setting of neurodegeneration. Clearly, more work needs to be done in this area.

Developing a Better Understanding of the Role of the Innate Immune System and Other Immune Mechanisms That Contribute to Progression of Neurodegeneration

The occurrence of microglial activation in AD and all of the neurodegenerative diseases has been observed for many years. Over the course of these diseases, microglia change their phenotype, retract their ramified processes, and adopt an amoeboid cell shape. These morphological changes are accompanied by the accumulation of microglia in areas of injury as well as in and around AD amyloid-β plaques. Microglial activation is accompanied by secretion of pro-inflammatory cytokines (Meyer-Luehmann et al. 2008; Prinz et al. 2011b). However, questions remain as to whether and how the main innate immune effector cells, microglia, contribute to disease progression. In addition to activated microglia and their potential role in neurodegenerative diseases, there is a striking increase in certain complement proteins. This is interesting given the emerging role that complement proteins such as C3 appear to play in synaptic

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pruning during development (Schafer et al. 2012), suggesting that increased complement in disease may contribute to synaptic damage. This area needs further exploration as complement may prove to be an attractive target for intervention.

Further highlighting the role of microglia, specifically in AD, are recent genetic data. Genome-wide association studies as well as whole genome sequencing have led to the identification of additional confirmed genetic risk factors for AD: among them are CD33 (Bertram et al. 2008; Hollingworth et al. 2011; Naj et al. 2011) and TREM-2 (Guerreiro et al. 2013; Jonsson et al. 2013). Both receptors are specifically expressed by microglia in brain. These may offer future specific insights into both mechanism and treatment opportunities.

There are a number of additional questions that will be important to ask regarding the role of the innate immune system and microglia in neurodegeneration:

- We need to understand the inefficient role of microglia in amyloid clearing in AD. While antibodies to Aβ and other stimuli can enhance microglial clearance of Aβ *in vivo*, can other methods be utilized to activate this pathway?
- How do molecules released by microglia, such as cytokine and chemokines, influence CNS function? These may be good therapeutic targets.
- What is the time course of inflammation? When is the right time to intervene? Magnitude of the anti-inflammatory intervention as well as the specific inflammatory target will be important to resolve.
- Recent evidence suggests the importance of microRNAs in modulating microglial function. Can this be better understood to develop therapies?

In the healthy brain, microglia continuously scan their microenvironment for pathogens and inflammatory stimuli by sending out membrane processes. This surveillance activity is thought to support tissue homeostasis. Moreover, microglia play a role in monitoring synaptic activity and assist in synaptic pruning, perhaps via complement receptors which they express (Prinz et al. 2011b; Tremblay and Majewska 2011). Recent animal data show that the chronic accumulation of Aβ within the brain drives inflammation. This effect appears to be mediated by the activation of the microglial NALP3 inflammasome and the subsequent release of interleukin-1β that leads to the shift of microglia toward a pro-inflammatory M1-type phenotype (Heneka et al. 2013). In turn, Nalp knockout animals in the APP/PS1 model background show reduced interleukin-1β activation as well as enhanced Aβ clearance and a shift of the microglial phenotype toward the anti-inflammatory M2-like phenotype (Heneka et al. 2013). Moreover, it was shown that inflammation not only results in a gain of microglial function, the protective function that microglia exert in tissue is impaired in mouse models of amyloidosis (Krabbe et al. 2013). These findings raise the following questions:

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- Is it possible to shift microglia back toward a phagocytic, surveillance M2-like phenotype that would improve neurodegeneration and postpone disease onset?
- If we understand the transition between phenotypes, what are further trigger events that could be targeted?
- Is NALP3 a good target for intervention?
- How do we target microglia? Can nanoparticles be used to deliver therapeutics?

Other important questions to be addressed in the future in this area include:

- In addition to microglial activation, astrocytes are activated in neurodegenerative diseases. What is the role of this activation? Do astrocytes contribute to the pathology? Can this activation be harnessed in a protective fashion?
- Is there any role for an adaptive immune system in neurodegenerative disease outside of multiple sclerosis? If so, how does this occur?

Since most of the mechanistic data on the effects of the immune system in neurodegeneration has been generated through mouse models, it will be important to understand the differences in innate and adaptive immunity of mice and humans.

Neuroprotective Mechanisms through Defined Factors, Signaling Pathways, or Cell-Based Methods

Extensive dendritic atrophy and synapse loss correlates with the severity of cognitive and memory impairment in neurodegenerative diseases. Neuronal damage (e.g., the loss of synapses) is believed to be the pathophysiological consequence of neurotoxic agents, such as oligomeric and other forms of Aβ, tau, and α-synuclein in AD, tauopathies, and synucleinopathies. Since these early deficits are likely reversible, strategies targeting the stabilization and potential restoration of dendritic arbors and spines are expected to modify disease progression. Based on current understanding of the pathways and regulatory mechanisms that are disrupted by such neurotoxic agents, a variety of therapeutic approaches may activate neuroprotective mechanisms in the brain.

Neurotrophic factors (e.g., BDNF, NGF, GDNF, Neurturin, IGF1, and BMP9) have been shown to be supportive of dying and injured/stressed neurons in animals. Some of them are also effective in reducing Aβ and plaque burden in animal models of AD. However, direct delivery of neurotrophic factors into the human brain has proved to be very challenging. To date, two main approaches have been used: (a) the factors were infused into brain as recombinant proteins and (b) viral vectors carrying the genes encoding the trophic factors were directly injected into specific brain areas. Mixed results

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have been obtained in human studies, and there is much to learn from these past experiences if we are to improve future clinical trial design. For example, GDNF was found to be beneficial for PD in a small, open-label study (5 patients). However, Amgen conducted a systematic study (34 patients) where no clear activity was found (Lang et al. 2006). It is important to improve the technology for delivery of GDNF and other growth factors. Importantly, in these and other trials, the patients treated had advanced clinical disease. Conducting studies at earlier ages, when there is less cell death, would seem more likely to yield benefits. More recently, gene therapy has been used to treat PD by introducing the neurturin gene via a viral vector approach. A Phase II trial by Ceregene did not show a statistically significant benefit; however, further work with this type of approach seems warranted (Bartus et al. 2014). AAV-NGF is also being tested in AD patients and appears to show some promise (Rafii et al. 2014). In addition to direct brain delivery, intranasal trophic factor administration has been tested for decades using NGF, BDNF, FGF-2, IGF-1, and insulin. Outcome measures using nasal delivery of trophic factors has been modest and acceptance limited and controversial. Overall, optimization of protein delivery, better understanding of pharmacokinetics/pharmacodynamics, and beginning trials early in disease course seem to be critical factors for future trials. Further, implementation of larger trials with such approaches need to be justified by careful Phase I results that show strong target engagement.

Attempts have also been made to design or identify small molecules from screening that activate neurotrophic factor receptors, such as TrkB receptor, or their signaling pathways (Obianyo and Ye 2013). For some small molecules, efficacy data were not reproducible, calling for a need to use unified protocols. While the ability of these small molecules to mimic the neurotrophic factors remains to be validated, some have been orally effective and show promise as a basis to develop novel therapeutics for neurodegeneration. Rigorous pharmacological profiling of these small molecules is warranted. In addition to classical growth factor signaling, other intracellular signaling pathways have the potential to be exploited to prevent axonal, dendritic, and synaptic degeneration. These include pathways involving Nmnat and the sirtuin pathways (Araki et al. 2004). Further exploration of their role in preventing degeneration in the CNS seems warranted.

Cellular transplantation offers an alternative to provide a source of growth or other trophic factors as well as a potential cell replacement strategy. Further experiments to sort out how iPS cells differentiated into neurons or glia might best serve in this role should be explored. Use of neural precursor cells that can differentiate into specific neural cell types, such as interneurons, may prove useful in that they can migrate to the appropriate layers within regions such as cortex as well as modulate regional neuronal activity.

The relevance of nonpharmacological manipulations (e.g., physical exercise, sleep, cognitive activity, and specific lipids) to neuroprotection needs further exploration and may be better understood as neuroprotective strategies.

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Identification of genes/factors, such as apoE4 homozygotes, which allow high-risk individuals to escape disease, might provide insight into new neuroprotective mechanisms (Jonsson et al. 2012). In addition, the use of iPS cells coupled with genomic analysis of such subjects could provide a way to explore this area.

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