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Aging in a Dish: iPSC-Derived and Directly Induced Neurons for Studying Brain Aging and Age-Related Neurodegenerative Diseases

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Abstract

Age-associated neurological diseases represent a profound challenge in biomedical research as we are still struggling to understand the interface between the aging process and the manifestation of disease. Various pathologies in the elderly do not directly result from genetic mutations, toxins, or infectious agents but are primarily driven by the many manifestations of biological aging. Therefore, the generation of appropriate model systems to study human aging in the nervous system demands new concepts that lie beyond transgenic and drug-induced models. Although access to viable human brain specimens is limited and induced pluripotent stem cell models face limitations due to reprogramming-associated cellular rejuvenation, the direct conversion of somatic cells into induced neurons allows for the generation of human neurons that capture many aspects of aging. Here, we review advances in exploring age-associated neurodegenerative diseases using human cell reprogramming models, and we discuss general concepts, promises, and limitations of the field.

Keywords

aging; induced neurons; iNs; disease modeling; age-associated neurodegeneration; induced pluripotent stem cells; iPSCs; rejuvenation

INTRODUCTION

Humans are exceptionally long-lived in comparison to other primates, with a projected maximum lifespan of approximately 115 years (24). This extreme longevity presents unique challenges to many of the postmitotic cells that compose our bodies and makes us highly susceptible to age-associated diseases (AADs) such as cancer, diabetes, and various neurodegenerative diseases (NDDs). Although many different research approaches are being pursued to better understand the process of aging (81), as well as to assess the interface

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between aging and disease, there is no unifying concept that fully tackles the complexity of the biology of aging (36). As the global mean population age continues to rapidly increase, AADs constitute an enormous burden on societies' health care systems. Further complicating the issue is the fact that many AADs lack effective therapies, due in large part to failed drug discovery efforts in recent years. These shortfalls are united by the common thread that our basic understanding of AADs has been limited by our ability to successfully generate workable human models in the laboratory. Although the animal models we rely on so heavily to model AADs are useful tools to grasp generalized concepts related to aging and the genes involved in age-related decline, they may not reflect specific mechanisms of human disease. In order to bridge this knowledge gap, better understand the process of aging in humans and its contribution to disease, and translate our findings into new therapeutic advances, we must develop better models that are more reflective of our biology (108).

To understand AADs, many laboratories have adopted an approach that relies on modeling diseases in a dish with induced pluripotent stem cells (iPSCs) (7). Primary human cells can be collected directly from patients, and the cells can then be reset to an embryonic-like state with the transient expression of the Yamanaka transcription factors, OCT4, KLF4, SOX2, and C-MYC (OKSM) (127). These cells can then be coaxed to differentiate into various cell types such as neurons, myocytes, or hepatocytes, thus allowing for cell-autonomous features to be probed in the context of the disease of interest (90). In recent years, this iPSC approach has expanded to the development of organoids—collections of cells that reflect features of whole tissues (71). However, the iPSC approach is fundamentally limited in its ability to model aging because expression of the OKSM transcription factors acts as a developmental reset, erasing many of the desired features required for AAD modeling (121).

As an alternative to iPSC reprogramming and differentiation, so-called direct conversion or transdifferentiation strategies to maintain the age-associated features of donor cells are being employed (20, 90). Transcription factors, microRNAs, and chemical cues are used to encourage terminally differentiated cells to directly transform between lineages and thereby bypass the rejuvenating embryonic state. Although many induced cell models are now available, this review largely focuses on the modeling of aging in induced neurons (iNs). Neurons are among the longest living cells in the human body; most are generated in embryonic development, with few new neurons being produced after birth (29). Neurons from primary human sources are also exceptionally difficult to obtain and maintain, making direct study highly challenging. Thus, iNs generated from human donors present a unique opportunity to begin to understand how the aging process impacts these cells and can give rise to various NDDs. This review addresses the key features of aging that appear to drive neurodegeneration, compares the iPSC and direct conversion approaches for next-generation human cellular modeling, and discusses the extent to which iNs reflect aged neurons. We hope the review provokes a thoughtful examination of the nature of neuron-specific aging in humans and of the power of this model to address human disease.

AGING AND NEURODEGENERATION

Aging is the progressive loss of cellular homeostasis, leading to an overall decline of organismal fitness. Over the past 30 years, the contributions of numerous molecular

mechanisms and pathways that define the etiology of the aging process have been elucidated (81). Many of these aging pathways are also involved in NDDs such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis/frontotemporal dementia (ALS/FTD). The risk of developing these diseases highly correlates with age, and it, is difficult to predict susceptibility because the vast majority of cases are sporadic, i.e., lacking a well-defined genetic component (35, 59). Although many of the key features that define aging are associated with these diseases, we wish to primarily address features of age that are highly challenging to neurons (Figure 1).

Genomic Integrity and Metabolism

As the longest living cells in the human body, neurons are faced with the unique problem of maintaining their genetic material for decades of life (21). In the process of becoming postmitotic cells, neurons inactivate or transcriptionally downregulate many of the pathways associated with the DNA damage response (DDR) and focus the bulk of their DNA repair efforts on transcriptionally active genes (40). As neurons age, they accumulate lesions in these neuronal genes, resulting in decreased transcriptional output and a shift in cellular metabolism to maintain cell viability (55, 141). Additionally, mitochondria become more compromised with age, leading to an increase in free reactive oxygen species (ROS) production, which is a potent DNA damaging agent (31). DNA repair can often be compromised further in neurodegeneration (88). In ALS/FTD, mutations in the protein FUS are known to disrupt genomic integrity, and the C9orf72 repeat expansion RNA and proteins promote the formation of R-loops, leading to DNA damage (83). Neurons also appear to employ induced DNA breaks to trigger transcription of immediate early genes, the repair of which can be delayed by the extracellular amyloid β (Aβ) found in AD (83, 122). Most interesting, however, is the strong link found between persistent DNA damage and altered metabolism in neurons.

Our understanding of this process is largely viewed through the lens of developmental neurodegeneration models such *ERCC1*$^{-/\Delta 7}$ mice and human patients with Cockayne syndrome (CS). *ERCC1*$^{-/\Delta 7}$ mice are deficient for the nucleotide excision repair complex formed by ERCC1–XPF, leading to a progeria phenotype, a progressive loss of neurons, and greater risk for PD development (117). As the neurons in these mice accumulate unrepaired DNA lesions, the persistent DDR signaling then subsequently suppresses insulin-like growth factor-I signaling, leading to decreased function of these cells but greater longevity (141). The effects can be further augmented by dietary intervention through caloric restriction. Similarly, in CS, the key DNA repair helicase ERCC6 is less than optimally functional, leading to unrepaired DNA lesions or unresolved G-quadruplex secondary structures in ribosomal DNA (115). These act as persistent sources of damage that induce poly (ADP-ribose) polymerase (PARP) activity and consume nuclear NAD^+ (114). Depletion of NAD^+ locally in the nucleus decreases SIRT1 activity, and it, along with DNA-PK and ATM kinase, downregulates mitochondrial activity and biogenesis (30). Finally, telomeres in aging neurons present a potentially unique source of persistent damage, as the shelterin complex protecting them can prevent repair of lesions and lead to persistent signaling (34).

Transcription and Epigenetics

There is no unifying measure for the age of a cell or tissue, and the deviation between chronological age and different measures of biological age is currently a matter of debate and ongoing research (46, 161). Neurons must maintain their cellular identity for a considerably longer period of time than do most other postmitotic cells. In large part, cellular identity can be defined by the appropriate transcription of neuronal genes and the maintenance of appropriate epigenetic marks on the chromatin. Aged tissues display a general increase in transcriptional noise and a loss of regulation, contributing mechanistically to features of the aging process (6, 42). Cellular aging has been further refined through newer single-cell sequencing techniques, showing that the accumulation of mutation and transcriptional noise drives loss of cellular identity (28, 80). As part of the aging transcriptome, global hypomethylation of the genome occurs, but certain key regions become hypermethylated (10, 46). This altered methylation can be calibrated to accurately predict the age of cells, allowing for accurate age typing of iNs.

Nuclear Pores and Proteostasis

Nuclear pore complexes are composed of nucleoporins that control the flow of information between the nucleus and the cytoplasm of eukaryotic cells (45). These pores fenestrate the nucleus and appropriately traffic various transcription factors and RNAs, allowing cells to respond to signals in their local environment (14). Furthermore, these nuclear pore complexes appear to act as platforms for gene regulation, transcription, and global nuclear organization (18). A recent surprising discovery is that nuclear pore permeability becomes altered with age (22, 113, 136). Pores get more permeable to cytoplasmic proteins entering the nucleus and are increasingly leaky for nuclear proteins with advancing age. This leakiness is in part due to the low turnover of extremely long-lived nucleoporins that form the scaffold and core of the channel. These proteins are being efficiently incorporated only during mitosis; therefore, it is likely that many nucleoporins are as old as the neurons themselves. Thus, nuclear pore-associated damage and functional defects are a prime example of age-related protein damage that likely has tremendous downstream effects on the subcellular localization of transcription factors and other regulatory proteins and directly impacts chromatin structure and transcription (51). Finally, in ALS/FTD, low-complexity proteins such as poly-PR, FUS, or TDP-43 often further compromise pores by blocking them (57, 62). These and other disease-related proteins coaggregate with FG nuclear pore proteins in the center channel of pores (39, 44, 118). Collectively, these observations suggest a unique nexus of age-related dysfunction that may arise in neurons.

INDUCED PLURIPOTENT STEM CELL MODELS FOR AGE-ASSOCIATED DISEASE—SOMETHING MISSING?

The study of NDDs has been hindered by the inability to access living human brain tissue for research purposes. Further, postmortem samples from patients reflect only the end stage of the disease, making it particularly difficult to unravel the specific pathogenic mechanisms involved in initiating the disease. The advent of human iPSC technology and refined differentiation strategies have provided well-defined patient-derived neural cells, including

various types of neural stem cells (NSCs), dopaminergic neurons, glutamatergic neurons, GABAergic neurons, motor neurons, astrocytes, oligodendrocytes, and many others, to model NDDs (5, 19, 47, 67, 68, 76, 86, 159). Although many NDDs can be caused by genetic mutations, the majority of cases arise sporadically and exclusively at advanced ages. This age dependency highlights the need for patient-specific models that not only reflect the genotype of the patients but also reflect molecular aging signatures. Here iPSC-based models face a major challenge: iPSCs are, as advertised, reprogrammed to an earlier epigenetic state (101, 110). This iPSC rejuvenation has been demonstrated at the level of epigenetic methylated DNA (mDNA) and the transcriptome (33, 46, 79, 89, 91), as well as in telomeres (84, 124) and several functional phenotypes, including mitochondrial function, cellular senescence, DNA damage, and others (72, 100, 107, 125). Although iPSC reprogramming can be seen as an advantage for cell replacement strategies and rejuvenation-directed research (101), it represents a major challenge for modeling AADs.

Primary cell samples obtained from donors have unique features that have accumulated throughout the lifespan and disease development of the individual. These features are important for understanding AAD mechanisms. Because iPSC reprogramming rejuvenates cells on many levels, and because clonal selection is typically used to further select and screen the fittest cells, neurons from typical iPSC clones may not be the most characteristic cells to model AADs. In fact, most iPSC-based disease models for NDDs have demonstrated a requirement for additional stressors applied to normal culture conditions to elicit disease-specific phenotypes. For example, studies using iPSC-derived dopaminergic neurons to model familial PD consistently used ROS or mitochondria-related stressors such as H_2O_2, 6-OHDA, valinomycin, or CCCP to detect PD neuron-specific malfunctions (98, 109, 111, 116). Similarly, proteasome inhibition using compounds such as MG132 was required to obtain protein aggregates in Huntington's disease (HD) iPSC neurons (97), and excitotoxic glutamate exposure was needed to stimulate the formation of ataxin-3-containing microaggregates in iPSC neurons from Machado-Joseph disease patients (64). Further, overexpression of pseudohyperphosphorylated tau in human embryonic stem cell (hESC)-derived neurons induced neurite transport defects and cell death only when antioxidants were removed from the culture (92). Interestingly, familial AD-causing mutations in PS1 or APP, which can lead to early onset of AD symptoms at the age of 40, were shown to consistently alter Aβ production without the need for additional stressors (54, 66, 93, 148, 151). However, excitotoxic glutamate exposure was needed to induce cell death (26). These findings reflect how aberrant Aβ is already present in very young familial AD patient neurons but fails to induce significant neuronal malfunctions unless features of aging also come into play. Though not inconsistent with the amyloid hypothesis of AD, these data are in line with the paradigm shift in the AD field where more and more focus is dedicated to alternative disease mechanisms, including age-related pathways (21, 36). Taken together, even if application of stressors helps reveal disease-specific phenotypes in iPSC neurons, it remains unclear whether they elicit a type of stress that is adequate to better understand the disease in phenotypically and epigenetically young neurons.

A more systematic and comprehensive strategy to induce broad aspects of aging in iPSC models was first introduced in a study utilizing expression of a premature aging-associated protein variant in iPSC neurons (94). Expression of the Hutchinson-Gilford progeria

syndrome (HGPS)-causing variant of the *LMNA* gene progerin resulted in the induction of a series of aging markers, including γH2AX and H2K9me, in otherwise rejuvenated neurons. Progerin also triggered pathological phenotypes in PD patient-specific dopamine neurons, such as dendritic degeneration and impairment of AKT signaling (94). Another study showed that telomerase inhibition at the iPSC stage using the compound BIBR1532 could also induce age-related phenotypes in neurons derived from treated iPSCs (140). Although telomere shortening with age in postmitotic neurons is an up-and-coming field of research (27), more work needs to be done to clarify the role of overall telomere length, as well as active telomere shortening, in mature postmitotic human neurons. The mere existence of telomerase activity in iPSC-derived neurons warrants further investigation. Although both age-inducing strategies represent significant steps forward in modeling AADs with iPSCs, it remains unclear whether the latter approach imitates normal human aging or represents only a pathological type of aging. Clearly, these strategies to induce aging in iPSC derivatives can recapitulate only certain aspects of aging; it will be interesting to determine to what extent different strategies can be combined to provide a more complete representation of actual cellular aging in neurons. This review focuses on direct iN conversion, an alternative strategy for generating human neurons to study aging and AADs that allows circumvention of the pluripotent stem cell stage.

DIRECT LINEAGE CONVERSION INTO INDUCED NEURONS

Despite the rapid progress in the directed differentiation of iPSCs (96, 126), extensive effort has also gone into the development of alternative strategies for obtaining functional cells. Direct reprogramming, which is defined as the direct conversion of cells from one lineage to another without going through the pluripotent stage (38), holds great promise for biomedical applications. The first study to demonstrate the possibility of this strategy was the conversion of fibroblasts into myoblasts by overexpressing MYOD1 (23). The concept that lineage conversion could be achieved by cell type-specific transcription factors and was possible between distant cell types led to protocols for the direct conversion of many cell types, including blood cell lineages, neurons, cardiomyocytes, hepatocytes, pancreatic cells, and macrophages (49, 52, 70, 144, 163).

The reprogramming of mouse fibroblasts into functional iNs was first achieved by overexpressing a set of proneural transcription factors, BRN2, ASCL1, and MYT1L, also known as BAM (144). A year later, a study from the same group used BAM together with NEUROD1 to convert fetal human fibroblasts into functional neurons (103). A number of other studies also reported successful conversion of other cell types into functional neurons (60, 85). The field has since progressed rapidly to investigate the mechanisms involved in direct conversion and pursue the best strategy, including use of novel transcription factors and compounds, for generating neurons that have comparable properties to in vivo or iPSC-differentiated counterparts (Table 1).

Conversion Factors for Induced Neurons

The reprogramming of one cell type to another requires appropriate changes in epigenetic status, which are mediated by transcription factors. During development, cell fate is

determined by stepwise changes in the cellular epigenetic profile mediated by a genetically defined cascade of transcription factors. In the process of reprogramming, the pioneering factors play a crucial role in initiating lineage conversion. Pioneer factors are capable of accessing the closed region of chromatin just by themselves and can help other factors to initiate cell fate change. In BAM iN conversion, ASCL1 acts as a pioneer factor, accessing the closed chromatin region and recruiting BRN2 to ASCL1 target sites (146). This mechanism suggests that, although simultaneously expressed, the transcription factors still coordinate in a hierarchical manner. Neurogenin2 (NGN2) is another well studied proneural transcription factor that has been widely reported to initiate iN reprogramming in human fibroblasts with the aid of small molecules (78). Several research groups have used a dual-pioneer factor strategy (ASCL1 and NGN2) that yields high conversion efficiencies, even in adult and aged human fibroblasts (69, 160). This approach indicated the feasibility of using adult donor-derived fibroblasts as a starting cell source for the study of AADs, including NDDs. Importantly, several studies showed that the transient expression of transcription factors is sufficient to induce neural conversion; prolonged expression is not necessary. In most studies, an inducible expression system is used (69, 91, 103, 134); however, it is also possible to employ an endogenous neuron-specific miRNA to negatively regulate the factors and achieve the optimal timing for factor expression (73).

In addition to transcription factors, alternative factors such as miRNAs, short hairpin RNA (shRNA), and small molecules can facilitate direct fate conversion. For iN, strategies include the overexpression of the neuron-specific miRNAs miR-9/9* and miR-124, which, however, still requires co-overexpression of transcription factors (4, 156). Furthermore, mouse iNs have been generated by small interfering RNAs to inhibit PTB, which blocks miRNA-mediated activity of the RE1-silencing transcription factor (REST) complex (150), or a shRNA targeting the REST complex directly (25). Both studies showed that inhibition of the REST complex triggers the expression of epigenetically silenced proneural genes in fibroblasts and therefore facilitates the neuron induction process, at least in the mouse system.

Although most iN methods are based on the use of lentiviral vectors, these viruses raise safety concerns with regard to clinical applications and potential integration-induced artifacts (see also the section titled Induced Neuron Models for Age-Associated Diseases). Recently, cocktails of small molecules have been reported to promote iN conversion and reduce the requirement for exogenous factors (69, 78, 105). Inhibition of TGFβ/SMAD signaling with Noggin and SB431542, together with GSK3β inhibitor, forskolin, and cyclic-AMP, has been shown to improve iN conversion. Notably, recent studies have demonstrated that it is possible to induce neurons with only a cocktail of small molecules and without exogenous transcription factors. One study induced mouse neurons with SMAD and GSK3β inhibition and the addition of the proneurogenic molecules ISX9 and I-BET151, which inhibit fibroblast-specific gene expression (74). In another study, human neurons were induced with histone deacetylase inhibitor VPA, GSK3β inhibitor RepSox, JNK inhibitor SP600125, PKC inhibitor GO6983, and ROCK inhibitor Y-27632 (48). Both studies marked important steps in replacing exogenous factors with small molecules, which have several major advantages such as cost effectiveness, comparatively easy synthesis and preservation, and possible standardization. However, identifying small molecules that reproducibly and

completely replace exogenous transcription factors during lineage reprogramming remains a major challenge for iN conversion.

Subtype-Specific Induced Neurons

The originally identified BAM iNs often exhibited glutamatergic neuronal properties (144), and NGN2/ASCL1 iNs yielded a major fraction of glutamatergic neurons, a minor fraction of GABAergic neurons, and single dopaminergic and serotonergic neurons (91). Since the neural lineage is constituted of a vast number of neuronal subtypes, researchers are actively investigating the induction of different functional neuronal subtypes such as dopaminergic, cholinergic, GABAergic, serotonergic, medium spiny, and motor neurons (Table 1) (16, 77, 78, 106, 120, 138, 139, 142, 149, 152). These iN subtypes have all been shown to possess the required basic neuronal functional properties, and they also exhibit subtype-specific functions such as dopamine release or responsiveness to selective serotonin reuptake inhibitors (SSRIs), for example (106, 139). iN induction involves hierarchical coordination between the pioneer factor and other transcription factors. The strategies used to generate subtype-specific iNs involve pioneer factors such as ASCL1 and NGN2, which are used alongside a collection of factors that have been shown to be important in neural subtype determination. Combinations include NGN2 and SOX11 for cholinergic neurons; NGN2 with SOX11, ISL1, and LHX3 for motor neurons (77); the pioneer factors alongside LMX1A, LMX1B, NURR1, PITX3, OTX2, and FOXA2 for dopaminergic iNs (16, 106); the pioneer factors alongside CTIP2, DLX1, and DLX2 for GABAergic medium spiny neurons (142); the pioneer factors alongside LMX1B, FEV with FOXA2, or NKX2.2 and GATA2 for serotonergic neurons (139, 149); and NGN2 alongside NGN1 and BRN3A for sensory neurons (8). In general, the induction of functional lineage subtypes indicates that iN conversion is highly plastic and holds great application potential in modeling subtype-specific neurological disorders. However, identifying the cocktail of subtype-determining factors that faithfully convert fibroblasts into mature and fully functional neurons that are comparable to their in vivo counterparts remains a major focus.

INDUCED PLURIPOTENT STEM CELLS VERSUS INDUCED NEURONS

It is known that both iPSC differentiation and direct conversion can generate functional neurons, but it is important to choose the best method for the questions being asked. Differentiation protocols for human pluripotent stem cells, including human iPSCs and hESCs have been developed and optimized over a much longer period of time than for those of iNs, but the relatively new technology of direct conversion is rapidly developing, with many new protocols being published in recent years. There are several technical and conceptual differences between differentiation and direct conversion protocols.

Cell Source

The starting cell sources described for iNs are limited. Although iN conversion has been reported successfully with multiple starting cell types such as astrocytes, NG2 glia, hepatocytes, and adipocytes (43, 85, 112, 134, 155), most of these cell types represent, or are derived from, fetal rather than adult murine or human tissues. Currently, reports on successful iN conversions with human adult cells, use of which is a necessity for modeling

AADs with iNs, have been limited to pericytes and skin fibroblasts. However, pericytes are relatively hard to obtain from donors, so skin fibroblasts remain the most feasible option as a cell source at the moment. In contrast, iPSC reprogramming protocols using many human cell types, including skin fibroblasts, blood cells (102), keratinocytes (1), and renal epithelial cells (162), are successful and robust. It is important to mention that pluripotent stem cells are also important cell sources for direct reprogramming. Since hESCs and iPSCs are epigenetically naïve, they possess the full potential to faithfully convert into the desired lineage by expressing appropriate transcription factors that determine cell fate commitment. iPSCs have been successfully induced into mature neurons (iPSC-iNs) by overexpressing NGN2 (132) and into subtype-specific iNs (95, 130).

Time and Cost Efficiency

Because research budgets are unfortunately limited, time and money play important roles when deciding between differentiation and conversion protocols. The time to generate stable iPSC clones is typically 2–3 months, including basic characterization. Once the clone is successfully established, it can be expanded indefinitely and provide a stable supply of cells. The differentiation of iPSCs to neurons takes around 6–15 weeks, depending on the protocol and desired level of maturity (99). Many iPSC differentiation protocols, involving a stable and expandable progenitor cell stage that can be generated from iPSCs within 2–3 weeks, are used by many laboratories as stable intermediates (65). The iPSC differentiation protocol requires application of several different recipes of media over a precise timeline to mimic molecular signaling changes in their niche during development.

iN conversion from fibroblasts generates neurons that express neuronal markers in 2–4 weeks and show basic functional properties such as evoked action potentials. It takes another 5–6 weeks coculturing with astrocytes before iNs typically show mature physiological features such as spontaneous action potentials and postsynaptic currents (91, 103). The conversion procedure is relatively simple, usually involving one conversion medium for the conversion phase and one maturation medium for the maturation phase.

Expandability

iPSCs, once derived, can theoretically expand infinitely. The progenitors derived during differentiation also possess reliable self-renewing ability. Furthermore, iPSCs and NSCs are comparably small cells and grow at very high density. These properties make iPSC differentiation suitable for the generation of large numbers of neurons for subsequent applications like transplantation and metabolomic, proteomic, transcriptomic, and genomic analyses that require relatively large amounts of material. Direct iN conversion does not involve a highly expandable intermediate stage, and the neurons generated are postmitotic. Therefore, the expandable phase of iN conversion depends entirely on the proliferation of fibroblasts. As fibroblasts cannot infinitely expand and will go senescent after multiple passages, there is a limitation of starting materials even if a proliferating “iN-ready” transgenic cell line can be established using antibiotic-selectable vectors.

INDUCED NEURON MODELS FOR AGE-ASSOCIATED DISEASES

The fundamental challenge in the generation of human cellular disease models is the ability to represent the diseased cell in the body in a meaningful and predictive way. For studies using directly reprogrammed or iPSC-derived neuronal cells to study cellular and molecular mechanisms in the human system, the cellular composition of the cultures, neuronal subtype identity, and maturation state of the cultures are of central importance. When it comes to harnessing generated neurons as model systems to study aspects of monogenic or multigenic neurological diseases, the genetic identity of the generated cells needs to adequately reflect the genetic identity of the donor's cells in the body. Specifically, as mutations are acquired through tissue culture and the reprogramming process (123), and due to the potential importance of human somatic mosaicism (2, 87), issues associated with the clonality of the derived cell lines emerged as a focus of researchers. Additionally, in the case of using reprogrammed neurons for modeling AADs, the biological age of the generated neurons is thought to be a most influential risk factor for a variety of modeled diseases and thus deserves special attention. Among the various risks for human diseases, age stands out as being a special multifaceted factor. Unlike other risk factors, biological age interacts with other risk factors to drive NDDs, many of which we have mentioned previously. Below, we summarize how different iN-based studies have addressed aging and disease-related phenotypes in iNs (see also Table 2).

Age-Related DNA Lesions are Retained in Induced Neurons and, with Exceptions, also in Induced Pluripotent Stem Cells

The accumulation of DNA lesions that become somatic mutations is thought to play a role in aging and AADs. Cumulative DNA mutations are believed to be the central age-dependent component in the development of cancers (21), but they might also play an instrumental role in the initiation of AD (15). Neither iN conversion nor iPSC reprogramming generally alters the genetic identity, including somatic mutations, of reprogrammed cells. However, there are several notable exceptions. First, the use of retro- and lentiviral vectors results in the irreversible integration of DNA sequences into the genome, even if tetracycline-regulated or self-silencing strategies are employed. Whereas many improved systems to minimize reprogramming-associated integrations have been applied in iPSC reprogramming (61, 147), and the use of commercially available nonintegrating Sendai virus has become almost a standard in the iPSC field, efforts to generate integration-free iNs has received some, but markedly less, attention (3, 73). One reason clearly is that iN generation is by far a less widespread and younger technology, and Sendai viruses for iN factors such as ASCL1, BRN2, NGN2, NEUROD1, and others are not yet commercially available. Another more specific reason is that iN conversion, in contrast to iPSC reprogramming, is not clonal, meaning that each converted cell harbors a different pattern of integrations, thereby minimizing integration-related artifacts when bulk cultures or larger numbers of single iNs are assessed. This concept clonality versus random multicellular integration extends from studies of nuclear DNA to mitochondrial DNA (mtDNA), where elegant work has described the transmission of age-related mtDNA mutations in different hetero- and homoplasmic iPSC clones from aged human donors (53, 58). Importantly, the existence of endogenous somatic mosaicism, age-related or not, is an important issue to consider when interpreting

and comparing results from iPSC-based studies (2, 87). Further, genomic artifacts have been widely reported in iPSCs and have made the careful karyotype analysis of all generated iPSC clones obligatory. However, it is not clear to what extent clonal artifacts are truly the result of the reprogramming process or if they are caused by cell culture in general, or if they actually reflect a mosaic genotype and just become visible in clonal cell lines.

More important for the sustained functionality and viability of neurons than the number of accumulated DNA mutations is the error-free functionality of DDR pathways that, according to calculations, repair over 100,000 DNA lesions per cell every day (75). Thus, it would be interesting to measure not only mutation load in aging iNs but also the efficiency of DDR pathways; however, their functionality is technically difficult to measure. Indications that DDR might be compromised in aged human neurons comes from an elegant study that found increased numbers of γH2AX foci in aged donor fibroblast-derived iNs, whereas iPSC-derived neurons from the same aged donors did not show more γH2AX foci than their young donor-derived counterparts (128).

Induced Neurons Retain Long-Lived Protein Damage; Induced Pluripotent Stem Cell Reprogramming Erases it

As opposed to DNA mutations, protein damage generally does not persist in the numerous cell divisions and anabolic requirements of iPSC reprogramming. In fact, even during the first days of iPSC reprogramming, the cells appear to largely reset their proteome, demonstrating the extremely high protein turnover and anabolic need associated with acquiring iPSC identity (41). Interestingly, the nucleoporin NUP210, which is part of the oldest protein complex of the cell forming the nuclear pore scaffold structure, undergoes dramatic protein expression changes during early reprogramming, illustrating the rejuvenating character of iPSC reprogramming on the level of damaged proteins of the nuclear pore (41). In contrast, iN conversion does not involve cell division and conserves nuclear pore-associated aging features. Fibroblast-derived glutamatergic/GABAergic iNs from 1- to 89-year-old donors showed a marked decline in nucleo-cytoplasmic compartmentalization and nuclear shuttling rates with age, which is in part caused by the age-related loss of the importin-β family transport factor RanBP17 (91). Consistently, induced motor neurons from aged donors also showed changes in the nuclear envelope structure, as assessed by LaminB1 (128). As expected, protein compartmentalization in iPSCs as well as in iPSC-derived neurons from the same donors did not show age-related differences, thus rendering iN the model system of choice for studying age-related changes that might involve nuclear pore function and protein localization (91, 128). Indeed, glutamatergic/GABAergic iN cultures from middle-aged ALS patients carrying the disease-causing *C9orf72* repeat expansions were shown to possess faulty localization of the nuclear transport regulator Ran guanine nucleotide exchange factor (RanGEF/RCC1) (56). These results using patient-specific iNs confirmed data from yeast and *Drosophila* showing that nuclear transport factors were major modulators of C9orf72 toxicity (32, 56, 157). Further, induced motor neurons from 37- to 50-year-old ALS patients carrying FUS mutations were shown to possess impaired nucleo-cytoplasmic localization of the low complexity protein FUS, and they were unable to form neuromuscular junctions, a phenotype that could be ameliorated, however, by a kinase inhibitor known to inhibit CDK1, 2, and 5 as well as

GSK-3β (77). Similarly, unlike iPSC neurons (97), iNs from HD patients showed age-related proteostatic impairments and spontaneously developed aggregates composed of mutated huntingtin (mHTT) with peri- and intranuclear localization (143). Together, these studies suggest that both aging-related and ALS/HD-genotype-related stressors might converge in parallel at the nuclear pore level to promote protein mislocalization, neuronal dysfunction, and eventually neurodegeneration.

Another important example of age-related cumulative protein damage is mitochondria; a major pillar of the theory of age-related mitochondrial dysfunction is that increased production of ROS damages mtDNA and oxidizes mitochondrial proteins, which in turn further increases mitochondrial dysfunction (12). Indeed, data from healthy aged donor iNs that showed neurite beading and a decrease in synaptic transmission genes also revealed stark age-related mitochondrial defects, including suppression of tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS)-related genes and proteins, increased levels of oxidized proteins, mitochondrial morphology defects, loss of mitochondrial membrane potential, and decreased ATP production (63). Interestingly, the fibroblasts from which the iNs were derived showed only mild mitochondrial aging defects that, however, could be artificially induced by forcing the fibroblasts from their typical glycolytic bioenergetics into a neuron-like OXPHOS-dependent bioenergetic profile. Affirmation of these observations also comes from other studies where iNs from aged donors showed increased levels of ROS (50). ROS levels were found to be even higher in HD patients' iNs, where they were also accompanied by signs of DNA damage and neuronal degeneration (143).

Epigenetic and Transcriptional Aging in Induced Neurons

The epigenome can be regarded as a convergence platform for the features of many intrinsic and extrinsic factors that confront a cell over its lifetime. Age-dependent epigenetic changes (also referred to as epigenetic drift) are among the best-studied features of aging research (46, 129, 161). Although iPSCs possess only minimal epigenetic memory of their tissue of origin, epigenetic (mDNA) profiling using the aging clock has demonstrated that iPSCs even from very old donors reset to a prenatal epigenetic age (46, 79). In contrast, iNs possess the same epigenetic age as their parental fibroblasts, demonstrating that the epigenetics can be transferred from fibroblasts to neurons (50). This is an important finding also because it shows that, even though iN conversion reorganizes large parts of the human epigenome (119), it apparently leaves the most age-related marks virtually untouched. Further, comparative studies between iNs and iPSCs from young and aged donors showed that iNs derived from aged donors possessed age-related differences in the levels of H3K9me3 in humans (128), as well as other epigenetic marks (154). Consequently, even on the transcriptome level, iNs possess stark age-related signatures that share similarities with the transcriptomic aging signatures detected in postmortem human brain samples (11, 82, 91). Notably, transcriptomic aging signatures between iNs and their parental fibroblasts differ dramatically with only a small overlap, thus suggesting a model where cell type–independent epigenetic aging changes that remain untouched during iN conversion lead to distinct transcriptomic changes in different aging cell types. Unlike iNs, iPSCs were shown to be reliably erased of transcriptomic aging information (see the section titled Induced

Pluripotent Stem Cell Models for Age-Associated Disease—Something Missing?). It will be interesting to explore to what extent aging iNs display transcriptional noise and transcriptional identity drift on the single-cell level (28) and if this erosion of epigenetic marks leads to transcriptional changes that relate to AADs.

A CONCEPT FOR AGE-PRESERVATION IN LINEAGE CONVERSION

Studies employing direct lineage conversion have demonstrated that features of age can be directly transferred from one cell type to another (Table 2). Importantly, these findings demonstrate that the functional manifestation of old age in a cell is not just a hodgepodge of different dysfunctional pathways but that aging follows a certain hierarchical degree of organization. All the way upstream, at the top of the hierarchy, reside aging regulator pathways (ARPs) that are mainly tissue- and cell type-independent and that have the power to induce different cell type-specific downstream aging features in different cell types. This is good news, because this hierarchical concept of aging suggests that a limited number of target features of cellular aging control most other aging phenotypes. Currently, it still appears too early to point to one master ARP, but data seem to focus attention on epigenetic (mDNA) aging as well as nuclear pore defects. Epigenetic changes directly control the transcriptome output of a cell, which, however, differs largely between cell types (i.e., fibroblast aging genes are different from iN aging genes). Surprisingly, epigenetic (mDNA) signatures that define age were shown to be left untouched during iN conversion (50) and were not overwritten during the major conversion-related epigenetic overhaul (119,145, 146). Similarly impactful is control by the nuclear pore over subcellular protein and RNA localization, transcription factor activation and deactivation, chromatin structure, and gene expression (51,133). Age-dependent damage to the long-lived proteins of the nuclear pore is a common observation in aging and appears to be specifically relevant to postmitotic neurons (22, 113, 135, 136). Several iN aging studies have consistently described nuclear transport-related defects in aging and AAD iNs (56, 77, 91). Another strong argument for the nuclear pore being an upstream ARP in the hierarchy stems from the fact that the expression of progerin, which can be regarded as a general insult to the nuclear envelope and embedded pores, is sufficient to trigger AAD-related phenotypes in otherwise rejuvenated iPSC-derived neurons (37, 94).

In the quest to generate the perfect model of aging human brain neurons in vitro, iN conversion represents a major step forward, but it also has limitations. Many technical limitations of the iN technology would be solved by having a proliferative intermediate; for instance, it will be interesting to explore to what extent directly induced NSCs retain aging features (131). Further, due to their postmitotic state and the absence of neural progenitors, iNs face technical challenges in adapting into organoid-like 3D culture systems (71). Conceptual limitations will be even more challenging to address. A simplified view of what can be expected from iNs and iPSC models in general is illustrated in Figure 2, which shows that fibroblasts partially contain the relevant aging information for modeling aged brain neurons that is completely encoded by ARPs. This information is mostly preserved in iNs but largely erased in iPSCs—yet there are strategies to artificially induce age in the latter (Figure 2). In the future, it will be important to explore the mechanisms that define the most impactful ARPs, to identify the best conversion strategies to maintain maximum ARP

information, to develop new methods of inducing aspects of aging in iPSC models, and to harness the tools at hand for navigating the interface between aging and disease at the disease-, age-, and patient-specific levels.

OUTLOOK

Going forward, it behooves us to ask what the future holds for the iN technology and other direct conversion strategies. An interesting and potentially incredibly valuable difference between iPSC differentiation and direct conversion stems from the fact that direct conversion skips certain intermediate developmental stages (13). By combining differentiation and direct conversion, this concept might help to identify disease-related phenotypes that require the intermediate neural progenitor cell stage to unfold in mature neurons. Although iNs are a valuable model for studying aging human neurons in a dish, they remain challenging to generate economically at scale. This is in part due to the choice of dermal fibroblasts as the primary starting material for generating iNs. These cells do not grow very densely, and usually most good patient cohorts are limited to collections of fibroblasts and peripheral blood monocytes. We will need to improve our ability to generate more materials and achieve higher conversion efficiencies before the induced cell model is translatable to large-scale pharmacological drug discovery in the treatment of AADs. For this reason, many investigators are working to better understand how direct conversion of neurons takes place at the single-cell level (137). Many advances in this technology are on the horizon, offering us an improved understanding of what it means to be not just an old neuron but also an old region-specific neuron. Further, although neurons steal much of the limelight, many of the other cells in the nervous system are also extremely old, though quiescent as opposed to postmitotic. These other cell types play support roles in the brain and are often associated with the dysregulation of various pathways in neurodegeneration. Many of the risk alleles for AD, such as TREM2 and APOE4, relate directly to astrocytes and microglia and their ability to destroy Aβ in the extracellular space. Some efforts have been made to demonstrate in animal models the direct reprogramming of fibroblasts to either astrocytes or oligodendrocytes (17, 153). As these studies are translated to the human system, they might provide valuable tools to model AD and ischemic demyelination. Collectively, iNs and related nervous system direct conversion models will eventually allow us to build models for the aging human brain, potentially in the form of induced cell organoids that may recapitulate disease. By directly studying both the cell-autonomous features of each type and the collective cell-nonautonomous disease pathology of the resulting organoids, we will gain a deeper understanding of neurodegeneration and potentially develop treatments for disorders that replace cells using in vivo reprogramming to create new neurons in patients with AD, PD, ALS, stroke, and other age-associated conditions.

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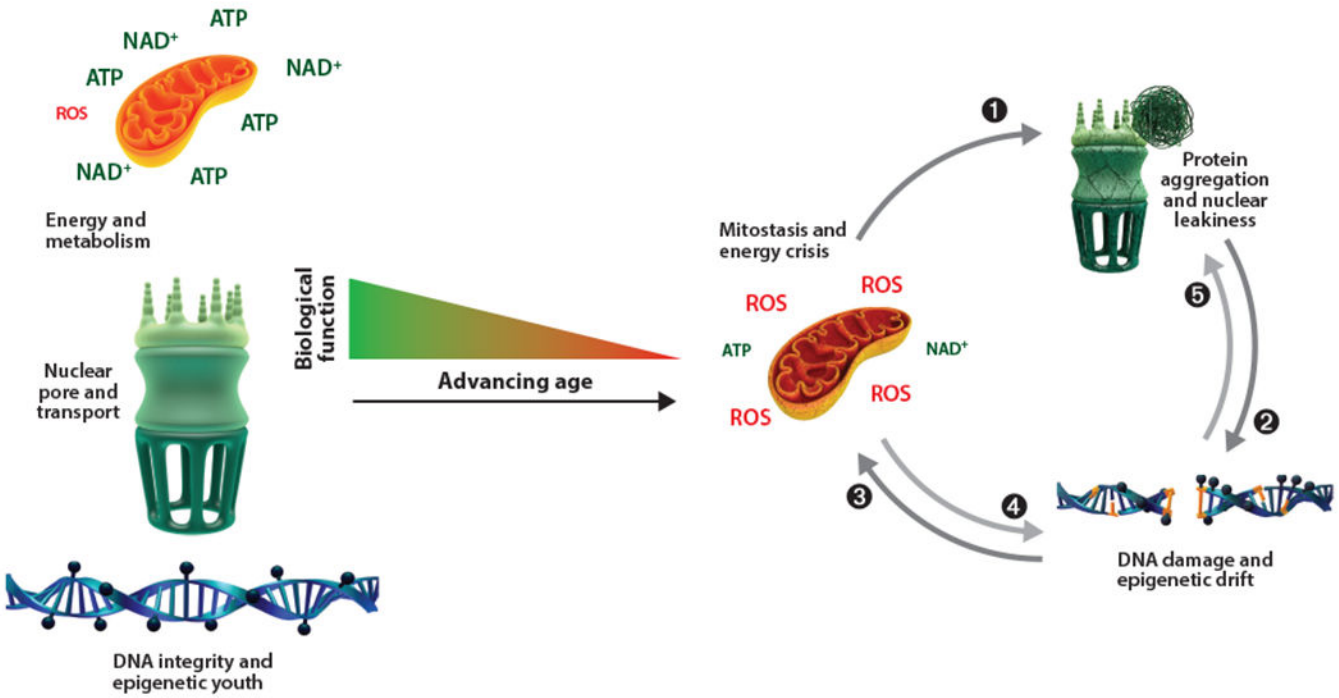

Figure 1.

Intersection of age-related dysfunction. Most age-related pathways are often thought of as independent with little overlap between the various components. ❶ In neurons, as advancing age occurs, nuclear transport becomes altered and pore leakiness increases. ❷ Damaged nuclear pores lose their capability to regulate nuclear homeostasis, genome architecture, DNA modification, and transcription. ❸ Additionally, DNA lesions can accumulate via errors in transcription or in the removal of epigenetic methylation markers, and persistent lesions fundamentally alter metabolism due to bioenergetic stress; ❹ this leads to changes at the mitochondrial level, which in turn increase the amount of reactive oxygen species (ROS) produced and damage the genome further. ❺ DNA damage triggers global stress responses in cells and leads to proteostasis disruption, potentially further destabilizing nuclear pores.

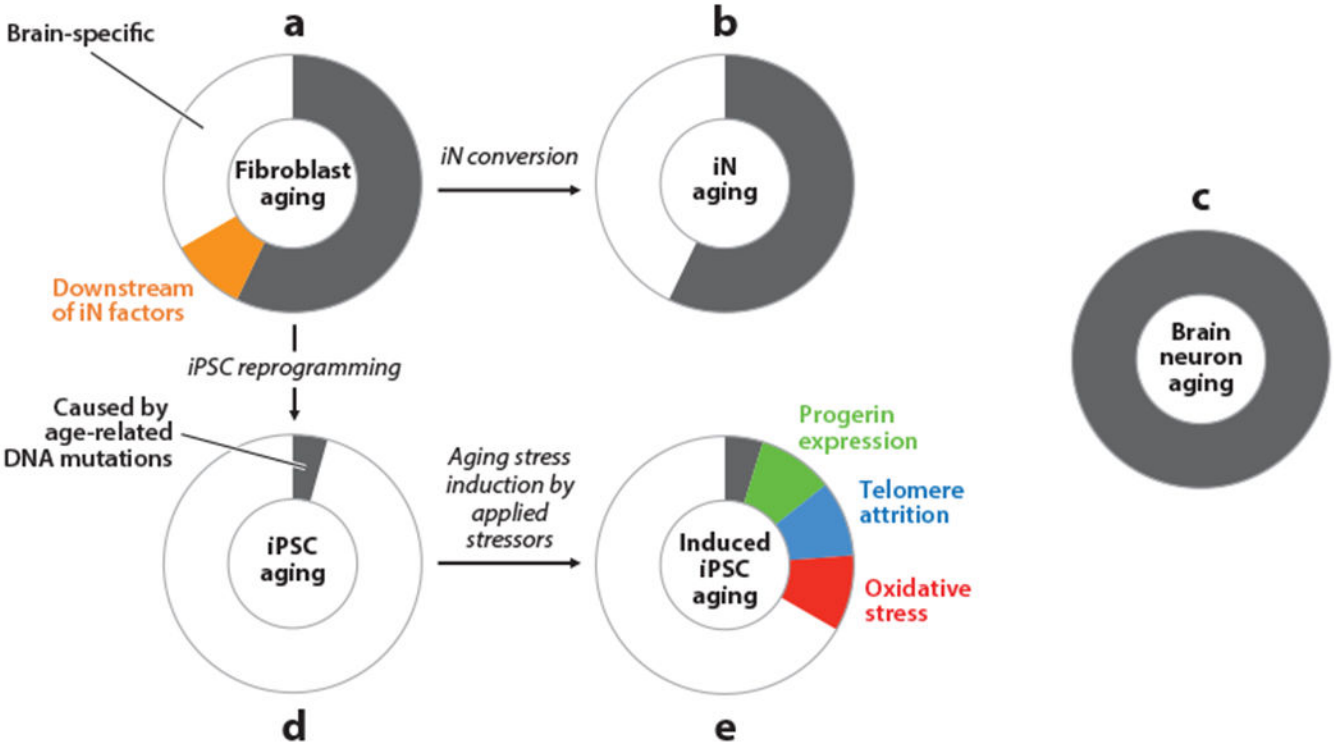

Figure 2.

Conceptual model of relevant aging information encoded in upstream aging regulator pathways (ARPs) reflected in induced neurons (iNs) and induced pluripotent stem cells (iPSCs) as compared with those of the brain. (*a*) Fibroblasts contain a large portion of ARP-encoded aging information (*dark gray*) that is relevant also for (*c*) brain aging. (*b*) After iN conversion, the cells preserve a large part of this aging information within ARPs (*dark gray*), but some information (*orange* in panel *a*) has been lost. This would be the case for information that results from neuron–glia interactions for example, which might not be present without adding glial cells to the culture. In contrast, (*d*) iPSC reprogramming erases the vast majority of aging information in ARPs, leaving only DNA-encoded aging lesions. (*e*) Approaches to induce aging in rejuvenated iPSC derivatives such as progerin expression (*green*), telomere attrition (*blue*), or oxidative stress (*red*) can be used to fill the glass, but likely also fall short in mimicking major parts of the aging information encoded in the ARPs.

Table 1

Neuronal reprogramming of human cells without going through the pluripotent stage

Neuronal subtype(s)	iN method(s)	Cell source(s)	Developmental stage of source	Reference
Dopaminergic neurons	ASCL1, BRN2, MYT1L, LMX1A, FOXA2	Fibroblasts	Fetal	106
Dopaminergic neurons	ASCL1, LMX1A, NURR1	Fibroblasts	Fetal, adult	16
Glutamatergic neurons	ASCL1, BRN2, MYT1L, NEUROD1	Fibroblasts	Fetal	103
Glutamatergic neurons	miR-124, BRN2, MYT1L	Fibroblasts	Adult	4
Glutamatergic neurons, GABAergic neurons	miR-9/9*, miR-124, ASCL1, MYT1L, NEUROD2	Fibroblasts	Fetal, adult	156
Motor neurons	ASCL1, BRN2, MYT1L, LHX3, HB9, ISL1, NGN2	Fibroblasts	Fetal	120
Glutamatergic neurons	ASCL1, SOX2	Pericytes	Adult	60
Glutamatergic neurons, GABAergic neurons	ASCL1, NGN2 plus small molecules	Fibroblasts	Neonatal, adult	69
Cholinergic neurons	NGN2 plus small molecules	Fibroblasts	Fetal, neonatal	78
Dopaminergic neurons	ASCL1, BRN2, MYT1L, LMX1A, LMX1B, FOXA2, OTX2	Fibroblasts	Fetal	134
Retinal pigment epithelium	PAX6, RAX, CRX, MITF-A, OTX2, NRL, KLF4, C-MYC	Fibroblasts	Adult	158
Neurons, unspecified subtype(s)	PTB inhibition	Multiple cell lines	Embryonic and adult-derived cell lines	150
Neurons, unspecified subtype(s)	ASCL1, BRN2, MYT1L, miR-124 negative regulation	Fibroblasts	Fetal	73
Glutamatergic neurons, GABAergic neurons	ASCL1, BRN2, MYT1L	Fibroblasts	Fetal	105
GABAergic, medium spiny neurons	miR-9/9*, miR-124, CTIP2, DLX1, DLX2, MYT1L	Fibroblasts	Adult	142
Sensory neurons	NGN1, NGN2, BRN3A	Fibroblasts	Embryonic, adult	8
Glutamatergic neurons plus GABAergic neurons and other subtypes	NGN2, ASCL1 plus small molecules	Fibroblasts	Adult	91
Glutamatergic neurons and other subtypes	VPA, CHIR99021, Repsox, Forskolin, SP600125, GO6983, Y-27632, Dorsomorphin	Fibroblasts	Adult	48
Serotonergic neurons	NGN2, ASCL1, LMX1B, FEV, NKX2.2, GATA2	Fibroblasts	Adult	139
Serotonergic neurons	ASCL1, LMX1B, FEV, FOXA2	Fibroblasts	Fetal, neonatal, adult	149
Spinal motor neurons	NGN2, SOX11, ISL1, LHX3	Fibroblasts	Adult	77
Neurons, unspecified subtype(s)	ASCL1, BRN2, REST inhibition	Fibroblasts	Fetal, adult	25
Dopaminergic neurons	ASCL1, LMX1A, NURR1, NEUROD1, miR-218	Astrocytes	Fetal (immortalized), embryonic	112

Abbreviations: iN, induced neuron; REST, RE1-silencing transcription factor.

Table 2

Studies that employed induced neuron (iN) conversion to study aging and age-associated diseases (AADs)

Donor age(s)	Disease-related mutation(s)	iN methods	Neuronal subtype(s)	Phenotypes	Compared to iPSCs	Reference
1 year (n = 1 mouse)	None	ASCL1, BRN2, MYT1L	Glutamatergic neurons and GABAergic neurons	Age-related gene expression, ROS, cell death	Yes	154
0–89 years (n = 18)	None	NGN2, ASCL1	Glutamatergic neurons, GABAergic neurons, and other	Transcriptomic aging, RanBP17 loss, compartmentalization defects, nuclear transport rates	Yes	91
43–71 years (n = 5)	Healthy and ALS C9orf72 repeat expansions	NGN2, ASCL1	Glutamatergic neurons, GABAergic neurons, and other	ALS-specific: nucleo-cytoplasmic RanGEF/RCC1 mislocalization	No	56
37–71 years (n = 6)	Healthy and ALS FUS mutations	NGN2, SOX11, ISL1, LHX3	Spinal motor neurons	ALS-specific: nucleo-cytoplasmic FUS mislocalization, defects in morphology, physiology, survival, and impaired NMJ formation	No	77
0–96 years (n = 15)	Unknown	miR-9/9*, miR-124, CTIP2, DLX1, DLX2, MYT1L	GABAergic striatal neurons	Epigenetic (mDNA) age, transcriptomic aging, ROS, DNA damage (comet assay)	No	50
0–71 years (n = 6)	None and ALS FUS mutations	NGN2, SOX11, ISL1, LHX3	Spinal motor neurons	DNA damage (γH2AX), dystrophic nuclear envelope, loss of epigenetic markers	Yes	128
0–89 years (n = 12)	None	NGN2, ASCL1	Glutamatergic neurons, GABAergic neurons, and other	Mitochondrial dysfunctions (gene expression, morphology, membrane potential, ATP), oxidized proteins, neurite beading	Yes	63
6–71 years (n = 29)	mHTT (CAG repeat expansions)	miR-9/9*, miR-124, CTIP2, DLX1, DLX2, MYT1L	GABAergic striatal neurons	HD-related gene expression, peri- and intranuclear mHTT aggregates, impaired proteostasis, ROS, DNA damage, neuronal cell death	Yes (proteostasis phenotype only)	143

Abbreviations: ALS, amyotrophic lateral sclerosis; HD, Huntington's disease; iPSCs, induced pluripotent stem cells; mHTT, mutated huntingtin, NMJ, neuromuscular junction; RanGEF, Ran guanine nucleotide exchange factor; ROS, reactive oxygen species.