Induced pluripotent stem cell-based modeling of neurodegenerative diseases: a focus on autophagy

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Received: 25 January 2017 / Revised: 30 March 2017 / Accepted: 10 April 2017 / Published online: 7 June 2017
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Abstract The advent of cell reprogramming has enabled the generation of induced pluripotent stem cells (iPSCs) from patient skin fibroblasts or blood cells and their subsequent differentiation into tissue-specific cells, including neurons and glia. This approach can be used to recapitulate diseasespecific phenotypes in classical cell culture paradigms and thus represents an invaluable asset for disease modeling and drug validation in the framework of personalized medicine. The autophagy pathway is a ubiquitous eukaryotic degradation and recycling system, which relies on lysosomal degradation of unwanted and potentially cytotoxic components. The relevance of autophagy in the pathogenesis of neurodegenerative diseases is underlined by the observation that disease-linked genetic variants of susceptibility factors frequently result in dysregulation of autophagic-lysosomal pathways. In particular, disrupted autophagy is implied in the accumulation of potentially neurotoxic products such as protein aggregates and their precursors and defective turnover of dysfunctional mitochondria. Here, we review the current state of iPSC-based assessment of autophagic dysfunction in the context of neurodegenerative disease modeling. The collected data show that iPSC technology is capable to reveal even subtle alterations in subcellular homeostatic processes, which form the molecular basis for disease manifestation.

Keywords Disease modeling · iPSC cells · Neurodegenerative disease · Autophagy

Introduction
“"We have colonies." and "We realized we had almost the entire pathway in our hands." Two statements that changed the world of life sciences within less than a 15-year-timespan [1, 2].

The first statement refers to the colonies discovered by Kazutoshi Takahashi in Shinya Yamanaka’s small research group at Kyoto University in Japan back in 2006. The colonies were the result of 24 carefully chosen genes introduced via retroviruses into skin fibroblasts from mice, reprogramming them into what we now call induced pluripotent stem cells (iPSCs). In the weeks to come, the essential genes for the reprogramming process could be narrowed down to the four transcription factors Oct4, Sox2, Klf4, and c-Myc [3]. A few months later, this revolutionizing approach was successfully translated to human cells [4, 5].

Pluripotent stem cells (PSCs) have the potential to differentiate into derivatives of all three germ layers, thus providing a route to generate any somatic cell type in limitless numbers in vitro. Unlike pluripotent embryonic stem cells, iPSCs can be derived from any patient or healthy donor, thereby opening unprecedented prospects for generating autologous donor cells for regenerative medicine, patient-specific disease models, and drug discovery (Fig. 1). In 2012, not even a decade after the first description of the first iPSCs, Shinya Yamanaka was awarded the Nobel Prize for this revolutionizing breakthrough together with Sir John B. Gurdon, who had shown reprogramming by nuclear transfer in Xenopus oocytes decades before [1].

The second statement refers to the discovery Yoshinori Ohsumi and his group in Tokyo made in 1993 when they discovered 15 genes in yeast that were essential for the autophagic process [6] which is induced in yeast mainly upon starvation. The term “autophagy” (from Greek οὐτός/autós = „self-“and φαγεῖν/phageîn = „eating“) was introduced by Christian de Duve in 1963 [7] as an umbrella term for the delivery of cytoplasmic material to lysosomes (or the vacuole in plants or fungi) for degradation. (For clarity on terminology used in this review, Textbox 1 provides an overview on autophagy-related terms,
definitions, and methodology.) Today autophagy is subclassified into four variants: microautophagy [8], chaperone-assisted selective autophagy (CASA) [9], chaperone-mediated autophagy (CMA) [10, 11], and macroautophagy. The best-studied subclass in the mammalian system, macroautophagy (hereafter referred to as autophagy), is a process where a double membrane cup-shaped structure, the phagophore, forms to engulf a portion of the cytosol including protein aggregates, entire or parts of organelles and intracellular pathogens, and after closure delivers the cargo as mature autophagosome by fusion to lysosomes for degradation [12]. The genes discovered by Ohsumi and colleagues, nowadays called Atgs (Autophagy related genes), cover essentially the whole molecular machinery required for this process. In the years to follow, his group deciphered the precise mode-of-action of a large variety of previously uncharacterized Atgs, which turned out to form a ubiquitin-like conjugation and a lipidation system. Specifically, Atg12 was found to be a ubiquitin-like protein that is activated at its C-terminus by the E1 enzyme Atg7 and transferred to the E2 enzyme Atg10 before being covalently linked to Atg5 [13]. This Atg12-Atg5 conjugate, together with Atg16, then forms a complex essential for autophagy [14, 15], a process also conserved in mammalian cells [16, 17]. The second system for lipidation is composed of Atg8 as a precursor that is cleaved by the cystein protease Atg4, then activated and transferred by the E1 enzyme Atg7 to the E2 enzyme Atg3 and finally covalently bound to the lipid phosphatidylethanolamine (PE) [18]. Again, this system was found to be conserved in mammals, and the mammalian homolog of Atg8, LC3, is also cleaved by ATG4 to LC3-I and further processed by ATG7 and ATG3 to form lipidated LC3-II that is stably integrated into the membrane of the growing autophagosome [19] (Fig. 2). Detection of LC3-II provided the first reliable marker for mammalian autophagosomes, and its abundance correlates with autophagosome number [20]. The importance of autophagy in neuronal homeostasis was demonstrated only a few years later by Noboru Mizushima, a former postdoc of Ohsumi, and Komatsu et al. by CNS-specific conditional targeting of Atg5 and Atg7, respectively. Affected mice showed neurodegeneration, motor impairment, and formation of ubiquitin-positive intraneuronal protein inclusions [21, 22]. Since then, the role of defective autophagy as a key player in neurodegeneration [23] (Fig. 3), cancer [24], and aging [25] gained more and more attention. In 2016, Yoshinori Ohsumi was awarded the Nobel Prize for his seminal discovery.
In this review, we focus on the interface between these two Nobel Prize-awarded advances and present recent progress in using iPSCs for studying autophagy-related phenotypes in neurodegenerative disease.

**Parkinson’s disease**

Parkinson’s disease (PD) is the second most common neurodegenerative disorder affecting ~2% of the population over the age of 60 [26]. Prominent symptoms of PD include motor deficits like tremor, bradykinesia, and limb rigidity. The neuropathology underlying these symptoms is a progressive preferential loss of ventral dopaminergic (DA) neurons in the pars compacta of the substantia nigra. A histopathological hallmark is the occurrence of intra-cytoplasmic ubiquitin-positive inclusions in surviving neurons known as “Lewy bodies,” which are mainly composed of the neuronal protein α-synuclein [27]. Approximately 90% of all PD cases are sporadic with no family history but there are several inherited or de novo mutations that cause PD [28, 29]. For most of them, patient samples have already been used to generate iPSC-derived neurons. Here, we will present the results regarding autophagy and PD chronologically in the background of the inherited mutation.
LRRK2

PD-causing mutations in the leucine-rich repeat kinase 2 (LRRK2) are autosomal dominant and manifest as a late-onset PD that is clinically and pathologically indistinguishable from the common idiopathic form [30, 31]. For that reason, LRRK2 mutations represent an interesting target for iPSC-based research into the pathomechanisms underlying PD. While there are over 50 different variants involving various LRRK2 domains in PD patients, the G2019S mutation is the most prevalent one, being detectable in up to 2% of sporadic PD cases [32]. Interestingly, an increased kinase activity of LRRK2 has been proposed to mediate the neurotoxic effect of this mutation [33].

In the study from Sanchez-Danes and colleagues [34], iPSCs from 4 LRRK2^{G2019S} PD patients, 7 patients with sporadic (non-LRRK2^{G2019S}-related) PD, and 4 healthy control donors were generated. The differentiation into midbrain dopamine (DA) neurons was achieved by overexpression of LMX1A in a 30-day protocol [35], and no significant difference in the yield of neurons was detected, although the fraction of DA neurons was only 9–29%. Analysis of LC3 in the neuronal cultures by immunoblot showed increased LC3-II levels in PD samples compared to controls, and inhibition of autophagosome-lysosome fusion [20] revealed an impairment of autophagic flux in all PD lines. In addition, electron microscopy (EM) showed accumulation of lipid droplets as well as more autophagosomes compared to...
autophagolysosomes in PD lines, indicative for a defect in autophagic flux through inhibited fusion of autophagosomes with lysosomes.

Su and Qi found that LRRK2<sup>G2019S</sup> DA neurons exhibit excessive autophagic and lysosomal activity, and mitochondrial dysfunction compared to control DA neurons could be detected at day 30 of differentiation in a protocol based on patterning cues for the ventral midbrain [36]. Interestingly, these phenotypes could be partially reverted by inhibition of mitochondrial fission, pointing to a highly intertwined functional crosstalk between mitochondrial function and autophagy.

Orenstein and colleagues analyzed whether chaperone-mediated autophagy (CMA) in LRRK2<sup>G2019S</sup> DA neurons is compromised by the interaction of α-synuclein with lysosome-associated membrane protein type 2A (LAMP-2A), the essential component for CMA [10, 37]. They found elevated α-synuclein levels in 30-day-old DA neurons from two LRRK2<sup>G2019S</sup> lines compared to two controls and reported a dramatically increased co-localization of α-synuclein with LAMP-2A [38]. As α-synuclein is considered a substrate of CMA by shuttling through the lysosomal channel protein LAMP-2A into the lysosome, these findings suggest impaired activity of the CMA pathway in this experimental system [38]. Interestingly, the increase in the number of α-synuclein<sup>+</sup> DA neurons between day 30 and day 75 in culture could be further increased by knockdown of LAMP-2A.

Reinhardt and colleagues generated isogenic LRRK2<sup>G2019S</sup> iPSCs from a healthy control line and compared both lines with respect to their LC3-II flux. In this system, a reduced autophagic flux could be detected in the engineered PD line under starvation conditions [39].

Very recently, light was shed on the mechanism how the LRRK2<sup>G2019S</sup> mutation affects the turnover of damaged mitochondria [40]. Hsieh et al. could show that in LRRK2<sup>G2019S</sup> iPSC-derived DA neurons, the mitochondrial protein Miro persisted longer on the outer membrane of mitochondria after treatment with the complex III inhibitor antimycin A than on control DA neurons. This was accompanied by delayed recruitment of LC3 and the autophagy receptor optineurin to damaged mitochondria, further arguing for an impairment of mitophagy. In addition, a failure in LC3 recruitment and a delayed turnover of damaged mitochondria could be detected in DA neurons of two sporadic PD lines, thereby implicating a general role of impaired mitophagy in PD.

Ohta et al. investigated another PD-causing LRRK2 mutation, P2020T, located in the LRRK2 kinase domain, using iPSC-derived neurons [41]. They discovered slightly increased levels of p62/SQSTM1, a generic autophagy substrate [42], and elevated levels of LC3-II in LRRK2<sup>P2020T</sup> neuronal cultures compared to controls.

Taken together, the data from PD iPSC models relating to autophagy and LRRK2 mutations point to a model where defective autophagosome-lysosome fusion and delayed turnover of damaged mitochondria might conspire with impaired CMA, thus favoring α-synuclein accumulation, increased sensitivity to oxidative stress, and eventually demise of DA neurons.

**GBAI**

In recent years, heterozygous mutations in the β-glucocerebrosidase (GBA1) gene, which encodes the lysosomal enzyme β-glucocerebrosidase (GCase), have been associated with a higher risk of developing PD, thereby challenging the role of LRRK2 mutations as most common monogenic risk factors for PD [43–45]. GCase is a lysosomal enzyme that hydrolyzes glucosylceramide (Glc-Cer) to ceramide and glucose. Homozygous mutations of GBA1 are causing Gaucher’s disease (GD), the most prevalent lysosomal storage disorder [46]. GD patients have a 20-fold increased lifetime risk for developing PD [47]; for heterozygous GBA1 carriers, the lifetime risk is still five times higher than in the general population [43]. Considering these numbers and the fact that GBA1 mutations are also associated with Lewy body dementia [48], understanding the downstream disease mechanisms resulting from these mutations is of great importance.

Exploiting the reprogramming technology, Schöndorf and colleagues generated two control iPSC lines, four GBA1 PD lines, and two GD lines, and even genetically corrected two of the GBA1 PD lines [49]. In both GBA1 PD and GD, there was an accumulation of LAMP1, a lysosomal marker, in DA neurons at day 65, which was reverted to control levels in gene-corrected cells. In addition, LC3 and LAMP1 co-localized less in GBA1 mutant DA neurons, and LC3-II levels where elevated compared to control and gene-corrected cells. Assessment of autophagic flux revealed impairment of autophagosome-lysosome fusion in GBA1 DA cultures that was not present in control cells and reduced in gene-corrected cells. The authors also reported dysregulated calcium signaling (a mechanism which may contribute to autophagy impairment [50]) and elevated α-synuclein as well as Glc-Cer levels that conform to the histopathological phenotype in patient brains.

More recently, Fernandes et al. made use of three control and three unrelated GBA1 PD iPSC lines to generate DA neurons and analyzed them at day 31–35. Their data revealed elevated levels of LC3-II, p62, LAMP1, LAMP-2A, and beclin-1 in GBA1 PD DA neurons compared to controls. These observations strongly suggest that autophagosomal-lysosomal turnover is impaired in the mutant lines, although autophagic flux was not directly investigated [51]. Strikingly, EM analysis confirmed an increased number of autophagosomes and lysosomes with undigested cargo. Taken together, the experimental evidence emerging from...
these studies points to an impaired autophagic flux as a contributing factor for GBA1-linked PD.

**PINK1 and parkin/PARK2**

PTEN-induced putative kinase 1 (PINK1) and parkin (PARK2) exert essential functions in mitochondrial homeostasis and quality control [52–54]. Recessively inherited mutations in both genes are related to PD [55, 56]. Parkin functions as an E3 ubiquitin ligase that ubiquitinates PINK1 on the outer membrane of damaged and aged mitochondria [54, 55] to enable their removal by p62-mediated autophagy, a process referred to as "mitophagy" [57].

For the investigation of PINK1, Seibler et al. generated one iPSC line each from one PINK1 PD patient and one healthy family member, derived DA neurons and, at day 60 of differentiation, analyzed the recruitment of parkin to mitochondria upon treatment with the potassium carrier and antibiotic valinomycin [58]. They observed that PINK1 PD DA neurons—in contrast to controls—showed no recruitment of parkin to mitochondria. Furthermore, no reduction of mitochondrial DNA, an indicator of damaged mitochondria removal, could be detected. Both defects, lack of parkin recruitment and impaired mitochondrial removal, were restored by overexpression of wild-type PINK1. This phenotype of defective parkin recruitment to mitochondria in PINK1 PD DA neurons upon valinomycin treatment could be recapitulated by Rakovic and colleagues using one PINK1 PD iPSC line and one control [59].

To investigate the effect of parkin mutations, Shatouli et al. used 4-week-old DA neurons derived from four PARK2 PD patients, one healthy control along with hetero- and homozygous PARK2 knockout iPSC lines [60]. Although parkin localization or direct mitophagy was not assessed, all parkin-deficient lines, hetero- and homozygous, generated less DA neurons, with PARK2 patient neurons containing less mitochondrial mass than control neurons. Collectively, these data suggest that impairment of mitochondrial turnover is a contributing factor in PINK1/parkin-related PD.

**Alzheimer's disease**

Alzheimer’s disease (AD) is the most frequent form of dementia and the most common neurodegenerative disorder. AD is clinically characterized by progressive loss of memory and neuropsychologically by the presence of amyloid plaques and neurofibrillary tangles [61]. Intriguingly, functional abnormalities of autophagosomes and lysosomes were found to precede these paradigmatic pathological changes in AD brains [62]. Interestingly, presenilin 1 (PS1), the most commonly affected gene in early onset familial AD [63], is essential for lysosomal and autophagy function [64] with AD-linked PS1 mutations impairing these pathways [65]. In addition, elevated levels of acid sphingomyelinase (ASM) are associated with AD [66], and sphingomyelin metabolism is regulated by presenilins [67]. Alterations of ASM activity could therefore be a downstream effect of presenilin mutations in AD.

Addressing this relationship, Lee and colleagues reprogrammed cells from one PS1 AD patient and one control subject to iPSC and assessed the ASM levels in the clonally derived PS1 lines and their neuronal derivatives [68]. In the PS1 iPSC line with the highest ASM levels, LC3-II and p62 levels in iPSC-derived neurons were elevated whereas the levels of LAMP1 and the basic helix–loop–helix transcription factor EB (TFEB), a transcriptional master regulator of autophagy [69, 70], were decreased. EM analysis of iPSC-derived neurons revealed an accumulation of autophagosomes in PS1 AD neurons. Knockdown of ASM by siRNA in PS1 AD neurons normalized the levels of LC3-II, p62, LAMP1, and TFEB comparable to control neurons, reduced autophagosome number detected by EM, and further partially restored the impaired expression of TFEB target genes such as cathepsin B. These findings argue for a defect in autophagosomal-lysosomal fusion mediated by ASM activity in PS1 AD neurons.

Using two PS1 AD and two control iPSC lines, Reddy et al. found that PS1 AD neurons exhibit decreased nuclear calcium signaling compared to control neurons [71]. Moreover, knockdown of PS1, cAMP responsive element binding protein (CREB) or calcium/calmodulin dependent protein kinase IV (CaMKIV) in control neurons resulted in decreased expression of sestrin2, LC3 and p62. Noteworthy, PS1 overexpression could not rescue the observed gene expression alterations in the CREB/CaMKIV knock-down setting, thus placing dysregulated nuclear calcium signaling downstream of the PS1 mutation. Upon application of a calcium ionophore increasing the cytosolic calcium level, LC3-GFP punctae were increased in PS1 AD neurons; in the same setting, PS1 depleted neurons showed a calcium dependent increase of sestrin2 with the decreased autophagic flux being reestablished. Taken together, these findings suggest that PS1 AD iPSC-derived neurons show a deficiency in autophagic flux, which is probably mediated by altered nuclear calcium signaling as well as altered sphingomyelin metabolism and subsequent downregulation of TFEB and its target genes.

**Frontotemporal dementia/amyotrophic lateral sclerosis**

The neurodegenerative disorder manifesting clinically as frontotemporal dementia (FTD) is genetically a pleiotropic group of sporadic cases and identified different mutations [72, 73]. Some of the mutations linked to FTD also manifest as
amyotrophic lateral sclerosis (ALS) [74]. FTD is characterized by focal but progressive neuronal atrophy in the frontal and temporal cortices as well as astrogliosis, inflammation, and prominent intracellular protein inclusions, mostly positive for ubiquitin, tau protein, p62, and TAR DNA-binding protein 43 (TDP43) [75, 76]. Given the heterogeneity of FTD, this section will focus on distinct genetic changes that have been addressed using iPSC models.

**C9ORF72**

The hexanucleotide repeat expansion GGGGCC in the non-coding region of the C9ORF72 gene is the most common known pathogenic mutation underlying FTD and ALS [77–79]. A number of different pathogenic mechanisms have been proposed, including C9ORF72 haploinsufficiency, RNA toxicity, and repeat-associated non-ATG (RAN) translation of neurotoxic dipeptides [80, 81].

In their search for neuropathological phenotypes in C9ORF72 iPSC-derived neurons from two patients compared to one control, Almeida and colleagues stressed the neuronal cultures around day 35 with the lysosomal inhibitor chloroquine and the autophagy induction via forming a complex with SMCR8 and WDR41, two proteins involved in the early steps of autophagosome formation [83]. C9ORF72 neuronal cultures displayed a higher vulnerability and increased cell death upon inhibitor exposure than control cultures and showed increased levels of p62, although autophagic flux was not assessed. These findings could point to a contribution of autophagic impairment to the pathogenesis of C9ORF72-related FTD and also ALS. Interestingly, a recent study established a link between loss of C9ORF72 and impairment of autophagy, as C9ORF72 is involved in regulating autophagy induction via forming a complex with SMCR8 and WDR41, two proteins involved in the early steps of autophagosome formation [83].

**GRN**

Progranulin (PGRN) is a secreted glycoprotein involved in cell survival, inflammation, and neuroprotection [84, 85]. In the vast majority of cases mutations in the GRN gene encoding PGRN result in haploinsufficiency and decreased expression of the protein. The complete loss of PGRN is one genetic cause for early-onset neuronal ceroid lipofuscinosis, a pleiotropic multiformalysosomal storage disorder, implicating a role in lysosome homeostasis [86].

To treat the haploinsufficiency of PGRN, Holler et al. screened for potential inducers of PGRN expression and went on to validate their hit, the autophagy inducer trehalose [87], in 25-day-old iPSC-derived motor neurons generated from one control and one patient-derived GRN line [88]. Application of trehalose elevated PGRN and LC3-II in GRN neurons, but there was no increase of LC3-II in control neurons. Unfortunately, autophagic flux was not assessed in this study, which will be required to determine whether trehalose represents a therapeutic option for treating PGRN-associated FTD.

**MAPT**

The tau protein, encoded by the MAPT gene, is ubiquitously expressed in the brain with a predominant localization in axons, where it is involved in microtubule polymerization and organelle transport [89]. There are many different mutations in MAPT linked to FTD [87], including the A152T mutation [90, 91].

Characterizing their iPSC-based FTD model comprising two patient lines, two control lines and also a MAPT knockout line, Silva et al. discovered elevated levels of LC3-II, p62, LAMP1, LAMP-2A, ATG12-5 and also an accumulation of ubiquitinated proteins in 35-day-old cortical neuronal cultures derived from MAPTA152T iPSCs [92]. Interestingly, the detected susceptibility to rotenone, NMDA or amyloid-β toxicity as well as elevated levels of tau and phospho-tau could be attenuated by treatment with rapamycin. Although autophagic flux was not directly investigated, the observed reduction of tau protein, a known substrate of autophagy [93], could suggest that the effect is elicited by an increase in autophagic flux, thereby depicting rapamycin as potential candidate for the treatment of MAPT-linked FTD.

**Niemann-Pick type C disease**

Like GD, Niemann-Pick type C disease (NPC) is an inherited autosomal recessive lysosomal storage disorder where >95% of cases are caused by loss-of-function mutations in the NPC1 gene, leading to severe neurodegeneration and liver dysfunction [94, 95]. The result of lost NPC1 function is impaired cholesterol homeostasis that mediates damage in liver and brain [96]. Interestingly, autophagy is involved in lipid metabolism [96] and, conversely, cellular lipid content affects autophagosomal membrane turnover [97]. Therefore, alterations in lipid composition are likely to affect the autophagy pathway.

As NPC is a recessive disorder, Maetzel et al. reprogrammed four NPC patients, one heterozygous healthy control and one homozygous control, and even genetically corrected one NPC line to a homozygous wild type genotype [98]. Neuronal cultures from NPC patients showed elevated LC3-II and p62 levels compared to control cells and the isogenic control at day 28, and the autophagic flux in NPC neurons was reduced. A compound screen for normalization of p62 levels revealed carbamazepine as a potential drug for NPC treatment.

Using one NPC iPSC line and one healthy control, Lee and colleagues detected also elevated LC3-II and p62 levels in NPC neurons compared to control cultures. In addition, they assessed...
autophagosome-lysosome turnover by electron microscopy and a fluorescence mCherry-eGFP-LC3 reporter assay (where eGFP fluorescence is quenched in the acidic lysosomal environment, thereby labeling fused autolysosomes purely red [99]). Both EM and LC3 reporter analysis revealed an accumulation of autophagosomes in NPC neurons compared to control cells. The detected autophagosomal-lysosomal turnover impairment could be rescued by application of vascular endothelial growth factor (VEGF).

The third study that reported an elevation of LC3-II and p62 in NPC iPSC-derived neural cells compared to control cultures was performed by Soga et al. using two NPC iPSC lines (two clones each) and two control iPSC lines [100], though only neural progenitor cells were analyzed. Nevertheless, treatment of NPC neural progenitors with 2-hydroxypropyl-cyclodextrin, a substance that induces release of cholesterol from late endosomes and lysosomes [98], decreased LC3-II and p62 levels without affecting viability. All three NPC iPSC-based studies presented here point to a reduced autophagic flux as pathogenic mechanism underlying NPC. They further exemplify the potential usefulness for iPSC-derived neurons for validating drug candidates that may restore autophagic flux.

**Appropriate controls and cell types matter**

A closer look at studies with different patient lines reveals that a disease-specific phenotype can vary markedly amongst iPSC lines derived from different subjects and even between clones generated from the same subject, although there might still by a significant difference to control lines [68]. To avoid false conclusions in either direction that are solely based on genetic variability and not disease-related, the generation of isogenic iPSC pairs is strongly recommended whenever possible. Generating isogenic iPSC lines is greatly assisted by emerging genome editing techniques such as the CRISPR/Cas9 system [101].

One of the major puzzling questions associated with the pathogenesis of neurodegenerative diseases is why ubiquitously expressed mutant proteins exert their main detrimental effects specifically in neurons and even in defined neuronal subtypes. This basic observation calls for model systems which enable pathogenetic studies in distinct human neuronal subtypes, a scenario which has become palpable with the advent of cell reprogramming and recent progress in the in vitro differentiation of iPSC towards a number of neural sublineages. Indeed, some of the phenotypes observed in the PD-related iPSC studies discussed in this review were reported to be visible only when DA neurons were assessed [38, 51,58]. Along the same line, in a recently reported ALS iPSC study, a neurofilament phenotype could only be delineated in motoneurons but not in interneurons [102]. Thus, choosing the right differentiation protocol and neuronal subtype for each disease can be of major importance, although it might be possible to discover disease-related alterations in other neural subtypes as well.

**Flux or no flux**

Frequently, controversies in the field of autophagy arise from the different interpretation of observed changes as either activation or inhibition of autophagy. This phenomenon underscores the necessity to differentiate between the induction of the autophagic process and the concept of autophagic flux [20]. For example, elevated levels of LC3-II can result either from increased induction of autophagic activity (e.g., by inhibition of the mTOR pathway) or by blockade of autophagic flux via impairment of lysosomal degradation [20]. The majority of the studies revisited in this review took this caveat already into consideration, but some results could still faithfully be interpreted the opposite way the authors initially suggested. To avoid such ambiguity, future studies should assess autophagic flux whenever possible, e.g., by introduction of tandem fluorochrome reporter systems for measurement of direct turn-over of autophagy substrates, or by the use of lysosomal inhibitors (such as Bafilomycin A, or the combination Leupeptin/E64) to block the endpoint of degradation. In addition, the investigation of additional parameters such as p62 or BECN1 turnover in addition to analysis of LC3 conversion is recommended. It is important to realize that such studies can still be confounded by, e.g., context-dependent transcriptional (up-) regulation of LC3 and p62, or spontaneous aggregation of reporter fusion proteins such as p62-GFP [103, 104].

**Outlook**

While autophagy and iPSC cell-based disease modeling have both become key topics in neurodegeneration research (see summary in Table 1), there is still a lot of room for synergizing these two fields. The studies presented here show that iPSC models can be used successfully to identify autophagy-related phenotypes associated with different neurodegenerative diseases. This approach is not only suitable for the detection of new autophagy-related pathomechanisms but also for corroborating or dismissing presumptive disease-related disturbances in autophagy described in other, less authentic model systems.

Of particular interest will be the further investigation of iPSC models of diseases where a pathologically aggregating protein is a known autophagy substrate such as, e.g., in the polyglutamine (polyQ) disorders Spinal and Bulbar Muscular Atrophy (SBMA) [105], Huntington’s Disease (HD) [106], and Machado-Joseph Disease (MJD) [107]. Experiments in
this direction are bound to provide more insight into the pathogenic role of autophagy as exemplified by a recent study showing impairment of TFEB-mediated autophagy already in iPSC-derived neural precursors derived from SBMA patients [105]. Such an early manifestation of a phenotype raises the question whether autophagy impairment exerts its pathogenic role already during development.

Further key questions to be addressed are whether and to what extent autophagy is differently regulated in neuronal subtypes and why general modulation of autophagy elicits specific effects in individual cellular subtypes [108, 109]. iPSC-derived neurons could also be used to gain insight into the role of autophagy during age-associated progression of a neurodegenerative phenotype [110] and potentially open new avenues towards slowing down the aging process by precise modulation of autophagic pathways [25].

Future studies may also address a potential disease-related role of autophagy in glial cells. While autophagy appears to be particularly relevant in neurons due to the unique biology of this cell type (postmitotic state, high energy demands, extreme life span), there is growing evidence for specialized functions of autophagy pathways in glial cells (reviewed in [111]). Examples include a contribution to myelination in oligodendrocytes, a possible role in synaptic pruning in astrocytes, and autophagy-mediated cell death in microglia [111]. Furthermore, there is evidence that oligodendroglial α-synuclein inclusions in multiple system atrophy (MSA) patients are co-localizing with LC3, p62 and ubiquitin [112, 113]. It will be interesting to decipher the role of autophagy in the generation of these glial inclusions using iPSC-derived oligodendrocytes and whether a dysregulation of autophagy contributes to the pathogenesis of MSA and other neurological disorders.

Taken together, while both, cell reprogramming and autophagy, have already been subject of Nobel Prizes, the amalgamation of both research lines towards understanding and treating neurodegenerative disorders has just begun.

### Table 1

| Disease | Disease-linked gene | Cell type analyzed | No. of patient/control lines | Proposed autophagy-related pathophenotype | Reference |
|---------|---------------------|--------------------|------------------------------|------------------------------------------|-----------|
| PD      | LRRK2 (G2019S)     | Midbrain DA neurons| 11/4a                        | Defective autophagosome clearance        | [35]      |
|         | LRRK2 (G2019S)     | DA neurons         | 1/1                          | Excessive mitochondrial fission → exacerbated autophagy induction | [36]      |
|         | LRRK2 (G2019S)     | DA neurons         | 1/2                          | LRRK2G2019S-dependent impairment of CMA  | [38]      |
|         | LRRK2 (G2019S)     | Neurons            | 1/1b                         | Autophagic defect under starvation conditions | [39]      |
|         | LRRK2 (G2019S)     | DA neurons         | 3/3c                         | Impaired mitochondria turn-over by Miro stabilization | [40]      |
|         | LRRK2 (I2020T)     | Neurons            | 2/2                          | Decreased autophagic flux                | [41]      |
| GBA1    | DA neurons         | 4/4d               | Impaired autophagosome-lysosome fusion | [49]      |
|         | GBA1                | DA neurons         | 3/3                          | Impaired lysosomal degradation           | [51]      |
|         | PINK1               | DA neurons         | 1/1                          | Defective Parkin-mediated mitophagy      | [58]      |
|         | PINK1               | DA neurons         | 1/1                          | Defective Parkin-mediated mitophagy      | [59]      |
| PARK2   | DA neurons         | 5/1e               | Defective mitophagy          | [60]      |
| AD      | PS1                 | iPSCs and neurons  | 1/1                          | Lysosomal depletion and defective autophagic degradation | [68]      |
|         | PS1                 | Neurons            | 2/2                          | Impaired autophagy                       | [71]      |
| FTD     | GRN                 | Motor neurons      | 1/1                          | Impaired autophagosomal-lysosomal turnover | [88]      |
|         | MAPT                | Cortical neurons   | 3/2f                         | Impaired autophagosomal-lysosomal turnover | [92]      |
| ALS & FTD | C9ORF72  | Neurons            | 2/1                          | Impaired autophagy                       | [82]      |
| NPC     | NPC1                | Neurons            | 4/3b                         | Impaired autophagic flux                 | [98]      |
|         | NPC1                | Neurons            | 1/1                          | Impaired autophagosomal-lysosomal turnover | [99]      |
|         | NPC1                | Neural progenitors  | 2/2                          | Impaired autophagy                       | [100]     |

*Table 1* iPSC-based models of neurodegenerative diseases with focus on the autophagy-lysosomal system

*a 7 sporadic PD + 4 LRRK2-mut
*b Engineered/parental
*c 3 controls (2 healthy +1 gene corrected)
*d 4 controls (2 healthy +2 gene corrected)
*e 4 iPSC disease lines +1 PARK2—knock out line/1 control
*f 2 iPSC disease lines +1 MAPT—knock out line/1 control
*g C9ORF72 (polyGGGGCC)
*h 3 controls (2 healthy +1 gene corrected)
Textbox 1. Definition of autophagy-related terms.

| term | definition / comment |
|------|----------------------|
| autophagy induction | Term describing the entire turnover of material through the autophagic system, from sequestration of cargo to degradation in the lysosomes/vacuoles and recycling of molecules. The autophagic flux must be distinguished from induction of the system by engagement of upstream signaling events, e.g. inhibition of the TOR pathway. Common methods to determine autophagic flux include the use of tandem-fluorochrome assays and the analysis of cargo turnover (e.g. LC3-II, p62) in combination with blockade of lysosomal degradation (e.g. by E64/Leupeptin, chloroquine or bafilomycin A1). |
| autophagy receptors | A group of proteins that are capable to target specific cargo for selective autophagic degradation by physically linking cargo (or its tags on its cytosolic surface) to LCI family members. Typically, autophagy receptors (such as p62/SQSTM1, NBR1, OPTN, NDP52) themselves become degraded and thus can serve as markers for autophagic activity. |
| autophagic flux (or ‘autophagic flow’) | Term describing the entire turnover of cytoplasmic material (‘cargo’) to lysosomes/vacuoles, resulting in degradation of the cargo and recycling of the respective molecular building blocks. |
| autophagy | Umbrella term describing the delivery of cytoplasmic material to lysosomes/vacuoles for degradation and recycling of the respective molecular building blocks. |
| autophagophagy | A non-degradative cytosolic compartment characterized by a double membrane that is often decorated with LCI-II (or its orthologues, e.g. Atg8). Autophagosomes sequester cytosolic components (‘cargo’) and mediate their lysosomal degradation by transport towards and fusion with lysosomes. |
| autophagy receptors | A non-degradative cytosolic compartment characterized by a double membrane that is often decorated with LCI-II (or its orthologues, e.g. Atg8). Autophagosomes sequester cytosolic components (‘cargo’) and mediate their lysosomal degradation by transport towards and fusion with lysosomes. |
| autophagy receptors | A group of proteins that are capable to target specific cargo for selective autophagic degradation by physically linking cargo (or its tags on its cytosolic surface) to LCI family members. Typically, autophagy receptors (such as p62/SQSTM1, NBR1, OPTN, NDP52) themselves become degraded and thus can serve as markers for autophagic activity. |
| LC3 | Abbreviation for ‘microtubule-associated protein 1 light chain 3’, an evolutionarily conserved family of proteins that are primarily involved in autophagosome formation and fusion events. During autophagy induction, the cytosolic LC3 (then called LC3-I) is protonated, lipidated and conjugated to phosphatidylethanolamine (PE) which enables its insertion into the growing autophagosomal membrane. The membrane-anchored PE-conjugated form of LC3 is termed LC3-II. During autophagy execution, LC3 is removed from the cytosolic face of the autophagosomal membrane but remains inside the vesicle and thus is degraded along with the cargo within the lysosomal compartment. While the conversion of LC3-I to LC3-II is often used to analyze autophagy induction and flux, it is important to note that the result of this conversion is highly dependent on the cell type, the treatment and the experimental context. Blocks of lysosomal degradation during a treatment period are recommended in order to stabilize LC3-II and thus to use the LC3-II/1 ratio as a measure of autophagic flux. |
| macroautophagy | The autophagic sequestration of cytoplasmic cargo into a double-membrane bound vesicle (autophagosome), followed by degradation by the lysosomal/vacuolar system. Importantly, some proteins and organelles may be selectively degraded via macroautophagy while other cytosolic components (e.g. cytoskeletal elements) are selectively excluded from degradation. |
| microautophagy | An autophagic process involving direct uptake of cytosolic components, inclusions and organelles (e.g. ribosomes) at the lysosome/vacuole by invagination of the sequestering organelle membrane. |
| mitophagy | A specific type of selective autophagy, involving autophagic sequestration and degradation of mitochondria. Mitophagy is induced by organelle aging or damage accumulation (e.g. due to membrane depolarization). In mammalian cells, at least two mitophagy pathways exist, i.e. the PINK1/parkin and the NIX pathway. |

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**Acknowledgments** Work at the Institute of Reconstructive Neurobiology and LIFE & BRAIN GmbH has been supported by the EU (FP7- HEALTH-F4-2013-602278-Neurostemcellrepair; FP7-HEALTH-F5-2010-266753-SCR&Tox, COLIPA, IMI-115582-EBISC, Horizon2020-667301-COSYN, IMI-115975-2-ADAPTED, IMI-115975-2-PHAGO), BMBF (grants 13N11830-LANDCEM, 01EK1603A-Neuro2D3 and a grant within the framework of the e:Med research and a funding concept 01ZX1314A-IntegraMen), the North Rhein Westphalian Ministry of Innovation, Science and Research Programmes (grants 005-1403-0102, 005-1403-0106 StemCellFactory II), the North Rhein Westphalian Programme LifeSciences.NRW, European Regional Development Fund (EFRE-0804047, EFRE-0804048 NeuRoWeg), the Stem Cell Network North Rhein Westphalia and BONFOR.

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