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UPDATE ARTICLE

Studying neurodegenerative diseases in culture models

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Neurodegenerative diseases are pathological conditions that have an insidious onset and chronic progression. Different models have been established to study these diseases in order to understand their underlying mechanisms and to investigate new therapeutic strategies. Although various in vivo models are currently in use, in vitro models might provide important insights about the pathogenesis of these disorders and represent an interesting approach for the screening of potential pharmacological agents. In the present review, we discuss various in vitro and ex vivo models of neurodegenerative disorders in mammalian cells and tissues.

Keywords: Neurodegenerative diseases; in vitro models; ex vivo models; neurons; neuroglia

Background

Neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, affect millions of people around the world. Unfortunately, the pathogenesis of these chronic neurodegenerative diseases is not fully understood, and current treatments do not stop or slow down progression of these pathological conditions. Therefore, different in vivo, ex vivo, and in vitro models have been generated. In vitro models of these pathological conditions offer advantages over in vivo models in several aspects. First, it is possible to study the role of isolated cells of one particular type in an environment that simulates the disease and to investigate mechanisms of a possible deleterious or protective role of specific molecules and compounds. Second, screening for potential actions of drugs is also facilitated. In this sense, in vitro models of neurodegenerative processes have been used to provide important clues about mechanisms of the diseases and potential pharmacological targets. In the present review, we discuss in vitro and ex vivo models of chronic neurodegenerative diseases using cells or tissues.

Parkinson's disease

PD is a slowly progressive neurodegenerative disease clinically characterized by motor impairment, namely bradykinesia, rigidity, resting tremor, and postural instability.[1] Synaptic and axonal degeneration within the striatum followed by loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) leads to reduced levels of dopamine in the nigrostriatal circuitry.[2] Besides dopaminergic cell loss, intracellular formation of Lewy bodies and Lewy neurites, consisting predominantly of aggregated alpha-synuclein (αSyn), has been suggested to be crucial in the pathogenesis of this disease.[3] Moreover, genetic factors contribute to the pathogenesis of PD.[4] To date, more than 16 loci and 11 associated genes have been identified. Among these, mutations in the gene for αSyn were the first ones to be mapped.[5] On the cellular level, research in PD focuses on protein aggregation, neurotoxicity, increased oxidative stress, excitotoxicity, mitochondrial dysfunction, and defects in the protein degradation machinery (including the ubiquitin-proteasomal system and autophagy pathways).[6]

Several cell culture systems have been employed to study these possible disease processes. But what would be the perfect cell? A homogeneous cell culture system that is easy to handle would be preferable. Cells should be easy to expand in order to generate large numbers of neuronal precursor cells. Next, these cells should be able to be transferred from a proliferative into a post-mitotic state. Finally, these cells should be easily directed towards a post-mitotic state in a synchronized manner with a mature neuronal (dopaminergic) phenotype. One cell culture model that will surely play an important role in PD research and that already combines many of the aforementioned aspects consists of dopaminergic neurons derived from human induced pluripotent stem cells redirected from human fibroblasts.[7] However, we will not include human induced pluripotent stem cells in this review, because their usage is still hampered by very labor-intensive and costly procedures. Some problems, such as the low absolute yield of differentiated dopaminergic neurons and low homogeneity, are of high research interest and we would like to refer the interested reader to the very comprehensive review by Studer.[8]

Primary midbrain dopaminergic neurons are suitable to study dopaminergic cell survival and neurite retraction as well as regeneration. Usually, embryonic midbrain

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neurons from embryonic day 14 (E14) are dissected.[9] A high yield of dopaminergic neurons can be obtained, which can be exposed to various neurodegenerative stimuli. Several neurotoxins are employed to study neurodegeneration. In particular, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP^+) are widely accepted to induce neurotoxicity. Both neurotoxins are thought to induce dopaminergic toxicity by intra- and extracellular oxidation, hydrogen peroxide formation, and direct inhibition of the mitochondrial respiratory chain.[10] On the one hand, this cell model is very suitable to study methods of neurodegeneration and neurite retraction; on the other hand, possible neurorestorative capacities by pharmacological compounds and the underlying mechanisms can be nicely dissected. For example, inhibition of rho kinase mediated by fasudil promotes the survival of rat primary midbrain neurons after addition of MPP^+ to the culture dish via the Akt survival pathway.[11] As a read-out for cell survival, cell number can be counted in this cell culture system. Other possibilities include, for example, the MTT assay, determination of adenylate kinase in the supernatant, or fluorescence-activated cell sorter (FACS) analysis for annexin V and propidium iodide.

Since axonal loss seems to be an early event in PD pathogenesis, analysis of the neurite network can also be performed and may be suitable as a good read-out for neurite preservation. An interesting approach to study neurite regeneration has been described by Tönges et al. Neurite processes were mechanically transected with a thin silicone scraper, treated with MPP+, and finally the length of neurites was determined using ImageJ software.[11,12] However, primary midbrain neurons are not easy to prepare; they are time-consuming and hard to transfect. Therefore, different cell lines were generated.

A commonly human cell line used in PD research is the HEK293 (human embryonic kidney 293) cell line. These cells can be easily transfected (e.g., via calcium phosphate, liposome based, electroporation). In one paper, the kinetics of αSyn aggregation was studied with respect to aggregation formation. Increased expression of wild-type αSyn was shown to result in the formation of cytoplasmic aggregates.[13] Time-lapse imaging illustrated how cells form and accumulate aggregates of αSyn in HEK293 cells.[14] HEK293 cell line is also a suitable model system to study the effect of αSyn mutations and other PD associated genes. The expression of a mutant A53T form of αSyn caused an increased susceptibility to dopamine.[13] Recently, it could be demonstrated that overexpression of leucine-rich repeat kinase 2 (LRRK2) does not result in altered gene expression in HEK293 cells.[15] Mutations in LRRK2 are strongly associated with late-onset autosomal dominant PD, and HEK293 may be suitable to go for candidate pharmacological screening for LRRK2 inhibitors.[16] Moreover, mechanisms of possible in vitro transfer of αSyn and its modified species may be studied in this cell line.[17]

Despite the common usage of HEK293 cells in PD research, there are some drawbacks, including the fact that these cells lack a neuronal phenotype. Another cell line that is widely used in the field of PD research is the SH-SY5Y cell line, which is derived from human neuroblastoma cells. These cells are widely used to study mechanisms of neurodegeneration. For example, overexpression of wild-type human αSyn was shown to promote inclusion formation in SH-SY5Y cells.[18] Moreover, extracellular addition of αSyn oligomers caused transmembrane seeding of αSyn aggregation in a dose- and time-dependent manner.[19] However, SH-SY5Y cells are hard to differentiate into a post-mitotic mature dopaminergic state.[20]

Several other human cell lines mainly derived from embryonic teratocarcinomas (NT2, hNT) are currently used, and they can be directed towards a post-mitotic neuronal phenotype.[21,22] The human H4 neuroglioma cell line has been used to study the oligomerization of intracellular αSyn by fluorescence lifetime imaging (FLIM) for the first time.[23] Moreover, the role of αSyn in the autophagy pathway has been addressed in this cell line.[24] It could be shown that dysfunction of the autophagy pathway may lead to exosome-mediated release of αSyn oligomers in order to clear these toxin αSyn species.[25] However, all these cell lines are derived from tumorous cells and only moderately show a distinct neuronal phenotype. Thus, we would finally like to address here the Lund human mesencephalic (LUHMES) cells. LUHMES cells were derived from 8-week-old human fetal ventral mesencephalic cells. To induce immortalization and thereby continuous proliferation, these cells were transformed based on the LINXv-*myc* vector with tetracycline-regulated v-*myc* expression.[26] This vector also contains a tetracycline transactivator that enhances the expression of v-*myc* from a minimal promoter from human cytomegalovirus (CMV) fused to the tetracycline operator sequence. Addition of tetracycline inactivates the transactivator and thereby abolishes v-*myc* expression. Supplementation with GDNF and cAMP induces a dopaminergic phenotype after 5 days of differentiation.[27] Differentiated LUHMES cells showed a high degree of dopaminergic phenotype, including release of dopamine and neuronal electric properties.[28,29] The LUHMES cell line has been widely used to study dopamine-related cell death mechanisms.[27,29,30] A drawback of this cell line is that classical transfection methods showed very low transfection efficiency. Thus, a lentiviral approach to efficiently transfect these cells is necessary.

Recent reports support the hypothesis that extracellular αSyn plays an important role in PD-associated neurodegenerative processes.[31,32] These findings suggest that extracellular αSyn released by neurons may also modulate microglial and astrocytic activity. Both glial types may respond to extracellular αSyn by increased expression of inflammatory mediators. In particular, inflammation in PD has been recognized recently not only as a mere bystander in the disease process but also as an important disease modifying or even accelerating factor. There is accumulating evidence for inflammatory processes in the progression of PD derived from 1) serum and cerebrospinal fluid (CSF) analyses, 2) genetic analyses, and 3) epidemiological studies.[33] In post-mortem studies of PD

patients, expression of pro-inflammatory cytokines was elevated in the striatum of PD patients, and activated microglia was observed within the SNpc, respectively.[33,34] Astrocytes and microglia cultures will be addressed in a separate topic below.

Alzheimer's disease

AD is a slowly progressive neurodegenerative disorder and the most common cause of dementia in the elderly. The neuropsychological profile of AD includes deficits in episodic memory, language, semantic knowledge, visuospatial abilities, executive functions (i.e., panning, organization, etc.), and apraxia.[35] The brain regions involved early in the course of the disease are the entorhinal cortex and the CA1 region of the hippocampus, followed by limbic structures and, at later stages, all isocortical areas.[36] The neurodegenerative process is characterized by early damage to synapses with retrograde degeneration of axons and eventual atrophy of the dendritic tree. In fact, loss of synapses is the best correlate of the cognitive impairment in patients with AD.[37,38] Neuropathological changes include abundant extracellular amyloid plaques and neurofibrillary tangles, comprised of hyperphosphorylated tau.[39] Deciphering mechanisms leading to neuronal dysfunction and cell loss are the main advantages of in vitro model systems. Different neuronal cell lines are commonly used for neuronal in vitro culture system, such as PC12, HEK293, and SH-SY5Y cell lines. These cells can be transfected with wild-type amyloid-precursor proteins, tau, or mutant forms of these molecules. In addition to cell lines, primary cortical and hippocampal cultures play a valuable tool in AD research. The addition of amyloid-beta to the medium of primary neuronal cells induces apoptosis.[40,41]

It is widely accepted that glial cells also contribute to the pathogenesis of AD. It has been shown that, besides neuronal loss, reactive astrocytes and activated microglial cells can be associated with amyloid plaques and neurofibrillary tangles.[42-44] Although amyloid-beta itself can be toxic to neurons, it also activates microglia, leading to neuronal damage.[45] Below, we discuss the role of glial cells in AD and PD and make a brief discussion of how these cells might be used for the investigation of these two pathological conditions.

Microglia and astrocytes in AD and PD

Microglia, the phagocytic innate immune cells of the central nervous system (CNS), continuously survey the local microenvironment. Activated microglia can be morphologically distinguished from "resting" microglia, because activated microglia have larger cell bodies as well as thicker and shorter processes.[46] Detection of pathogens or adverse patterns is accomplished by a vast array of highly conserved pattern-recognition receptors, including Toll-like receptors (TLRs). Stimulation of TLRs results in the activation of well-characterized signaling pathways, e.g., nuclear factor κB, and eventually leads to subsequent transcriptional activation of pro-inflammatory genes and to the production of reactive oxygen species.[45]

Primary microglial cells from rat or mice are commonly used to study inflammatory processes. For instance, primary microglial cells can be isolated from cerebral cortices of 1-day-old Wistar rats.[47-51] It is important to take extreme care to avoid lipopolysaccharide contamination, thus to keep microglia in a resting or "surveying" state instead of an activated state. Floating microglia can be harvested from 10- to 14-day-old mixed astroglial and microglial primary cultures. Finally, the purity of the microglial culture should be determined. Several microglial markers can be obtained to perform immunocytochemistry or FACS analysis, e.g., Iba1, CD68 (ED1), CD11b (OX-42), tomato lectin, or isolectin-B4. Primary microglial cultures have been used to study whether and by what means extracellular αSyn can activate microglial cells. Indeed, consistent and permanent microglial activation and subsequent production of pro-inflammatory cytokines have been shown in primary microglial cells.[52-58] In particular, TLR4 may be crucial in activating microglial cells and may be involved in phagocytosis.[54] Another recently published study showed that oligomeric αSyn may interact and activate TLR2 in microglial cells.[59]

The production of inflammatory mediators might contribute to the formation of amyloid-beta plaques.[60] Also, microglia of PS1-APP transgenic mice, a mouse model for AD, express increased amounts of cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF) α in comparison to their WT littermates, probably contributing to increased neurodegeneration.[61] Moreover, primary microglial cells may be used to study inflammatory processes and anti-inflammatory approaches. For example, the role of prostaglandins and underlying cell signaling after activation of lipopolysaccharide led to novel insights.[62-66]

Since AD and PD are age-related disorders and microglia may change their functional properties in the aging brain,[67] protocols are in demand for the isolation of microglia from adult rodents. A few protocols exist; however, presently, literature is scarce on this topic.[68-70]

Besides the primary microglia cell system, one microglial cell line is widely used, i.e., the BV-2 cell line. Microglial cells from C57Bl/6 were immortalized with v-*myc*.[71] The BV-2 cell was recently characterized, and transcriptome and proteome analysis revealed a high similarity to primary microglial cells.[72] Since BV-2 are easy to culture, they are a valuable tool to study not only inflammatory processes,[72] but also phagocytosis.[73] In addition, astrocytes may contribute to the activation of microglial cells and vice versa.[74]

Reactive astrogliosis, characterized by hypertrophy of astrocytic processes and soma as well as increased proliferation accompanied by progressive changes in gene expression, is generally moderate in human post-mortem tissue of PD patients. Astrocytes are the most abundant cells in the CNS, and show a wide variety of functions including regulation of blood flow and synaptic function, but may also play an important role in mediating neuroinflammation in neurodegenerative diseases.

Indeed, astrocytes play an important role in initiating and regulating CNS immune response through the release of pro-inflammatory cytokines and chemokines.[75] Recently, it could be shown that αSyn is directly transferred from neurons to primary astrocytes in vitro. Interestingly, αSyn was uptaken by astrocytes via endocytosis and showed an increase in TNFα gene expression.[76]

Primary astrocytes cultures are relatively easy to prepare. Astrocytes can be obtained from every region of the CNS and at any age, although the optimal time point would be in rodents from 2-3 days postnatal when astrogenesis is at its peak.[77] Several astrocytes isolation protocol exist.[78-80] However, a caution needs to be taken when dealing with astrocytic cultures because these cells may be ''contaminated'' with a high amount of microglia, oligodendrocytes, neurons, and endothelial cells.[81] Thus, it is important to use specific markers for the cell types. Commonly employed astrocyte markers are GFAP, GLAST, vimentin, glutamine synthetase, glutamate transporter 1, aldehyde dehydrogenase 1 L1, and S100beta.[82-84] To determine the percentage of microglial cells, immunocytochemical or FACS analysis for common microglial markers should be performed. Several methods can be used to reduce the number of microglial cells. First, frequent medium changes, shaking, and subculturing all reduce the number of microglial cells. Secondly, laminin enhances astroglial growth and inhibits microglial growth.[85,86] Also, application of cytosine arabinoside (Ara-C) or L-leucine methyl ester may effectively deprive the astrocytes cultures from microglial cells. In addition to primary cell cultures, a few astroglial cell lines exist, such as the human U373 astrocytoma cell line.[87-89]

Huntington's disease

HD is an autosomal dominant inherited neurodegenerative disease characterized by progressive motor abnormalities, psychiatric symptoms, and cognitive decline. The cause of the disease is accepted as a CAG repeat expansion in the huntingtin gene, resulting in a long stretch of polyglutamine (PolyQ) in the encoded protein, huntingtin (Htt).[90] This mutant huntingtin (mHtt) contains more than 40 glutamine repeats. Thirty-six to 40 glutamine repeats are associated with an increased risk for developing HD and a slower progression of the pathology.[91] HD is characterized predominantly by degeneration of striatal and cortical neurons, although other regions can also be affected.[91]

Few in vitro models have been developed to study important hallmarks of HD, allowing the investigation of key intracellular mechanism involved in the disease, as well as the identification of novel pharmacological targets. Considering the role of mHtt in the pathogenesis of HD, this protein has been used as a main tool for the study of HD in vitro. Increased frequency of aggregates is associated with toxicity in in vitro models of HD.[92,93] It has been shown that expression of the truncated mHtt in HD models resembles the disease process at a delayed stage of PolyQ toxicity. Conversely, expression of the full-length mHtt would be more representative of the entire process observed in the disease.[94]

Many aspects of the pathological features observed in HD, such as the role of mHtt protein, can be studied in neuronal cells. Examples of these cells are the rat pheochromocytoma (PC-12), the mouse Neuro2a (N2a), and the human SH-SY5Y.[95] PC-12 cells can be transfected with different PolyQ-expanded huntingtin constructs.[96,97] For example, transfection of these cells with HD exon-1 protein with expanded polyglutamine (150Q) reveals mHtt localization in the nucleus, as well as altered morphology, multiple gene expression, and decreased viability.[98] Moreover, these cells can also be transfected with a construct (pCDNA3-1-GFP-HtEx1-104Q) that expresses HtEx1 with 104 glutamines fused with GFP under the control of a cytomegalovirus-based promoter.[99,100] Various other transfections of PC12 have been performed, e.g., with the exon 1 region of the Htt gene with 109 CAG repeats[101] and with an ecdysone-inducible protein comprising the first 17 amino acids of huntingtin plus 103 glutamines fused with enhanced GFP (htt103Q-EGFP).[102,103] N2a neuroblastoma cells can also be transfected with different types of mHtt. For example, N2a stably expressing truncated htt with expanded 150Q tracts lead mainly to cytoplasmic aggregates formation.[104]

The ST14A cells are derived from E14 rat striatum primordial cells that exhibit characteristics of medium-size spiny neurons and can also be transfected with mHtt.[105-108] Another important cell model is the mouse-rat neuroblastoma-glioma hybrid cell line NG108-15 that exhibits neuronal properties after differentiation, allowing the expression of mHtt over many days.[109-111] Besides that, the immortalized rat hippocampal neuronal cell line (HN33) is another type of cell used because the hippocampus is one of the brain regions affected in HD.[112-114] Expression of PolyQ-expanded huntingtin in these cells has been shown to induce apoptosis.[112]

Although many cell lines have been used, they might reveal different aspects in comparison with primary cells. Therefore, primary neurons prepared from HD transgenic mice are frequently used: neocortical or striatal cultures from HdhQ111 mice that have 111 CAG repeats in exon 1 of the mHtt gene[115-119]; neostriatal cultures of the YAC46 (668 line) and YAC72 (2511 line) mice, which express the full-length mutant huntingtin containing 46 or 72 glutamine repeats (46Q or 72Q)[120]; YAC128 (line 55) mice expressing full-length human mHtt containing 128 CAG repeats[121]; transgenic BACHD mice that express a full-length mHtt with 97 glutamine repeats.[122]

Besides the neuronal cells, mHtt may also be transfected to non-neuronal cells,[95] such as HeLa cells,[123-126] human embryonic kidney cell-line 293T (HEK293T),[93,123,126,127] and monkey kidney cell lines (COS-7).[107,128,129]

An interesting approach has been obtained with acute transfection of rat corticostriatal brain slices with DNA constructs derived from the human mHtt.[130] This model has an advantage in comparison with the isolated cells since it maintains the resident interaction between the cells, which is important for the pathogenesis of HD.[130]

Importantly, this model might be used for the screening of potential compounds for the treatment of HD.

Non-genetic animal models of HD, which use chemical substances, have also been used. The 3-nitropropionic acid and quinolinic acid (QA) are used as excitotoxic agents in animal models of HD. The first compound is a mitochondrial toxin that induces neurotoxicity by irreversible inhibition of succinate dehydrogenase, a key enzyme located at the internal mitochondrial membrane and responsible for succinate oxidation to fumarate. Conversely, QA is an agonist of the N-methyl d-aspartate type glutamate receptors.[131] The excitotoxicity induced by these agents are studied in organotypic striatal, corticostriatal or sagittal hypothalamic slice cultures,[131-133] as well as hippocampal slices from the transgenic mice R6/2.[134,135]

Amyotrophic lateral sclerosis

ALS, also known as Charcot's or Lou Gehrig's disease, is characterized by a degeneration of cortical motor neurons and anterior horn cells of the spinal cord. This leads to muscle atrophy, loss of muscle control, and death resulting from respiratory failure, generally within 3-5 years of diagnosis.

Different studies have shown that oxidative stress plays a major role in the pathogenesis of this disease, classified as a rare familial form, which frequently exhibits mutations of the superoxide dismutase 1 (SOD1) gene.[136,137]

Considering that the disease affects motor neurons, different cell lines with the characteristics of these neurons can be used to study ALS. Moreover, these cells can be transfected with mutant SOD-1. Examples of cell lines include mouse neural hybrid cell line (MN-1), which expresses motor neuron features and high affinity glutamate transporters,[138] and mouse motor neuron hybridoma line NSC-34, a hybridoma cell line derived from the fusion of neuroblastoma cells with mice spinal cord cells.[139-143] Moreover, primary cells, like the mouse primary spinal cord culture,[144,145] are also used.

The pathogenesis of ALS involves not only neurons, but also other cell types, such as microglia and astrocytes.[146] Therefore, cell lines can be transfected with mSOD1, or primary cultures can be produced from transgenic animals. Interestingly, it has been shown that expression of mSOD1 in microglia enhances the release of inflammatory mediators, augmenting its potential to induce neurotoxicity in comparison with wtSOD1.[139,147-152] It has also been shown that transfection of astrocytes with mSOD1 induced toxicity to motoneurons in a co-culture model.[153,154]

Similar to other in vitro models of neurodegenerative disorders, organotypic rat spinal cord slice cultures,[155-158] as well as post-mortem samples of brain and spinal cord from ALS patients,[143,155] are frequently used.

Conclusion

In the present review, we discussed the possibilities of using cells and tissues in the investigation of neurodegenerative disorders. Importantly, these models might offer advantages in various aspects discussed along the text. Moreover, they complement in vivo studies that investigate the mechanisms involved in the pathogenesis of neurodegeneration.

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Disclosure

The authors report no conflicts of interest.

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