How do neurons die in neurodegenerative diseases?

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Given that neurons are post-mitotic cells, their life span is generally long enough to reach that of humans. However, sometimes neurons die without recognizable causes, as a result of a process called neurodegeneration. Apart from when gene mutations can be correlated with disease, it is difficult to pinpoint molecules that are responsible for neuronal death. Therefore, neurons living in a "sick state" for many years might reveal important information about neuronal death. Systematic and extensive single-neuron analysis of 'sick' neurons is expected to provide clues to the mechanisms of neurodegeneration. Moreover, the elimination of putative triggering and promoting factors involved in neurodegenerative disease might prevent disease progression.

Neurodegenerative disorders are characterized clinically by insidious onset and slowly progressive course, and are frequently hereditary. Pathologically, these diseases share a common feature: the selective loss of a particular subset of neurons for unknown reasons [e.g. cerebral cortical neurons in Alzheimer's disease (AD)] (see Glossary), substantia nigra neurons in Parkinson's disease (PD), spinal motoneurons in amyotrophic lateral sclerosis (ALS), and striatal small neurons in Huntington's disease (HD)].

Apoptosis has recently been implicated as a possible mechanism for neuronal death in neurodegenerative diseases. However, there is no direct and convincing evidence of apoptosis in human brains, and the mechanisms of neuronal death in neurodegenerative diseases are still unknown. Here, I will discuss several proposed mechanisms of neuronal death in individual diseases. In addition, I will put forward the concept of a long-standing 'sick state' of remaining neurons and the possible underlying mechanisms of neuronal 'sickness'.

Neuronal loss in neurodegenerative diseases is an extremely slow process

Each neurodegenerative disease has its own clinical course. AD, PD and HD, for example, begin gradually and progress slowly for more than 10–20 years. By contrast, ALS progresses rapidly and the disease process usually lasts only 2–3 years. Corresponding to the clinical course, the time course of neuronal loss is slow in AD and PD, and relatively rapid in ALS (Refs 1–3) (Fig. 1). The speed of neuronal loss in neurodegenerative diseases is much slower than that of apoptotic neuronal loss in developing nervous systems⁴. It is, therefore, unlikely that neuronal loss in neurodegenerative diseases is solely accomplished by apoptosis. Any proposed mechanisms of neuronal death should explain this extraordinarily slow time course.

Morphological and molecular hallmarks of individual neurodegenerative diseases

Each disease has its own hallmarks. These hallmarks are the obvious choice as parameters for the study of specific neural death.

Senile plaques and neurofibrillary tangles in cortical neurons in AD

Extracellular senile plaques (SP) and intraneuronal neurofibrillary tangles (NFT) are cardinal pathological hallmarks of AD. The main chemical component of the core of SP is amyloid β protein (Aβ), a mixture of Aβ40 and Aβ42 proteins, which are produced by cleavage of an amyloid β protein precursor (APP) by secretases⁵. Presenilins, which have recently been identified as γ-secretases, are mutated in a subset of early-onset familial AD (Refs 6, 7). Given that Aβ added to cultured neurons is toxic to the cells⁶, neuronal death is expected to occur by intracellular accumulation of Aβ. The second hallmark of AD is the intracellularly deposited NFT, which is a polymerized, argyrophilic abnormal structure composed of hyperphosphorylated tau protein⁷. By analogy with studies of Down's syndrome, AD pathology might begin with the formation of SP and years later proceeds to the formation of NFT (Ref. 10). In this respect, it is worth noting that the tau-phosphorylating enzyme (tau phosphokinase, TPKI) could be one of the missing links between SP and NFT. Indeed, the activation of intracellular TPKI is induced by the extracellular Aβ (Ref. 11). Neuronal loss in the superior temporal gyrus of AD patients exceeds the number of NFT-positive neurons by more than sevenfold¹. Therefore, the majority of neurons can die without developing NFT. Thus, although Aβ in SP and phosphorylated tau in NFT are key molecules for the understanding of the AD pathogenesis, there is still a gulf between hallmarks of AD and neuronal death.

Lewy bodies in nigral neurons in PD

The cardinal pathological hallmark of PD is the appearance of hyaline-like intracytoplasmic inclusions, Lewy bodies (LB). LB are found in the remaining dopaminergic neurons in the substantia nigra and other nuclei. Following the identification of an α-synuclein gene mutation as the cause of dominantly inherited rare PD (Ref. 12), α-synuclein has also been established as the major component of LB in sporadic PD. Indeed, a mutation in the gene encoding α-synuclein leads to the loss of dopaminergic neurons and intra-neuronal inclusions in a Drosophila model of PD (Ref. 13). Normal α-synuclein is localized in the presynaptic terminals,

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binds to synaptic vesicles, and is transported by the axonal flows. The mutation in the α-synuclein gene renders the protein devoid of vesicle-binding activity and promotes accumulation-forming β-sheet structure\textsuperscript{14}. However, the precise role of this protein in neurodegeneration is still unclear. Recently, the parkin gene was identified as being responsible for an autosomal-recessive form of juvenile-onset PD. Parkin is thought to be a substrate for ubiquitination and promotes accumulation-forming aggregates of a truncated form of protein containing a polyglutamine stretch, is now thought to cause neuronal death. The mutation in the \textit{IT15} gene was identified as being responsible for a rare subset of ALS, SOD might not have a role in neuronal death in sporadic ALS.

\textbf{Intracellular inclusions in spinal motoneurons in ALS}

The Bunina body in spinal motoneurons is the most well known intracellular inclusion body associated with ALS, but is still not fully characterized. Other intracellular bodies in motoneurons of ALS are, unlike LB in PD, not uniform and are described by various names such as argyrophilic, hyaline, conglomerate and skein-like inclusions. Most of them are, however, an accumulation of phosphorylated neurofilaments\textsuperscript{16}. These findings, and the presence of axonal spheroids suggest that ALS might be strongly related to the disturbance of neurofilament function. Recently, mutations in the intracellular Cu\textsuperscript{2+}–Zn\textsuperscript{2+}-dependent superoxide dismutase (\textit{SOD1}) gene were discovered in rare familial ALS (Ref. 17). Spinal motoneurons from familial ALS patients frequently bear intracellular inclusions that are immunoreactive for an antibody against SOD1. Because SOD1 acts as a detoxifier of free radicals, a mechanism of neuronal death is expected to be related to the loss of SOD activity. However, there is no positive correlation between the gene defect and motoneuron loss, not only in patients but also in transgenic mice containing the mutated \textit{SOD1} gene\textsuperscript{18}. Moreover, given that the familial type is a rare subset of ALS, SOD might not have a role in neuronal death in sporadic ALS.

Intraneuronal inclusions in striatal small neurons of HD

HD is caused by an expansion of \textit{CAG} repeats located in the coding region of the \textit{IT15} gene, whose product is named huntingtin. Abnormal huntingtin, therefore, has an unusually long glutamine tract\textsuperscript{19}. Intraneuronal inclusion bodies were found in the striatal neurons of transgenic mice expressing the \textit{CAG} repeat containing exon 1 of the huntingtin gene\textsuperscript{20}. The inclusion bodies are also found in the HD striatum and cortex. Inclusion bodies are aggregates of a truncated form of protein containing a polyglutamine stretch, ubiquitin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and many other proteins. However, there does not seem to be a correlation between the formation of inclusion bodies and neuronal death in cultured neurons that express abnormal huntingtin\textsuperscript{21}. By analogy with other polyglutamine diseases, the incorporation of truncated proteins with a polyglutamine stretch into the nucleus itself, rather than the formation of aggregates, is now thought to cause neuronal death through disturbing normal functions of transcription factors\textsuperscript{22}. The actual relationship between the disturbed gene expressions and the consequent neuronal death is still a matter of debate. In summary, hallmarks of neurodegenerative diseases are valuable clues for understanding the pathogenesis of the disease. However, it is important to recognize that disease hallmarks are not necessarily parameters for neuronal death.
In PD patients, less than 20% of nigral neurons remain 20 years after onset of the disease. Because they are destined to die, these remaining neurons might provide important insight into neuronal degeneration. Some of the remaining neurons show ‘degenerative changes’ in terms of size, shape and morphology of neuronal soma and dendrites. Although these changes are not specific, they might represent definite signs of ‘sickness’ of neurons. For example, the density of dendritic branches of most cortical neurons becomes coarse even in the early stage of AD (Ref. 23). In addition, significantly reduced numbers of dendritic spines and synaptic terminals were noticed in AD cortex24. These findings support the decreased synaptic function in AD brain.

In the substantia nigra of PD patients, the remaining neurons show condensation of cytoplasm and nuclear indentation25. Quantitatively, 4–40% of dopaminergic nigral neurons in PD were reported to remain 20 years after onset of the disease. Because aging proceeds insidiously from middle life (for a review see Ref. 29), similar to neurodegenerative diseases, one might hypothesize that neurodegenerative diseases are caused by an accelerated aging process. Indeed, a certain number of neurons are lost with age30. nigral neurons are most severely affected, cortical neurons next and spinal motoneurons least. However, the speed of neuronal loss in AD and PD is notably faster than normal aging. Biochemically, levels of 4-hydroxynonenal (nonenal), dolichol and 8-hydroxy-2-deoxyguanosine (8-OHdG) are significantly increased in the brains of elderly people. Nonenal might contribute to membrane damage and increased susceptibility to free radicals and consequently lead to serious disturbance of neuronal structures and functions31, and 8-OHdG might be a marker of DNA oxidation. Moreover, inactive enzymes, oxidized proteins and structurally altered proteins increase with age in the brain29. The amino acid groups of proteins non-enzymatically react with glucose or other monosaccharides, a form of post-translational modification. The products of this reaction further produce, through oxidations or dehydrations, more complex protease-resistant large molecules, for example, advanced glycation end-products (AGE), which increases with age. AGE promotes inter- and intra-molecular crosslinking, and disturbs normal protein function32. It is possible, however, that the aging process itself is not sufficient to kill neurons, but acts as a maintaining factor of the ‘sick state’ of neurons.

Molecular mechanisms of ‘sick state’ of neurons
Whatever the initial trigger of neuronal death in neurodegenerative diseases is, consequent events can proceed insidiously, gradually or episodically. Each neuron has its own optimal intraneuronal biochemical conditions such as intracellular pH, water content, concentrations of oxygen, glucose, ATP, second messengers and Ca2+ ions. In ‘sick’ neurons, these conditions might deviate slightly from the optimum, without exceeding the life-threatening limit for neurons. It is possible, therefore, that a long-standing unfavorable living condition might make neurons consume their energy insidiously and after many years lead to the neuronal death. Possible mechanisms of ‘sickness’ of neurons are summarized in Fig. 3.

Aging process
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Oxidative stress
Oxidative stress (reviewed in Refs 33,34) is caused by the enhanced production of harmful cellular oxidants: free radicals (e.g. hydroxyl radical (•OH), superoxide (O2•−), hydrogen peroxide (H2O2), nitrogen oxide (NO) and peroxynitrite (ONOO−)), or a failure of protective mechanisms, including superoxide dismutase (SOD) or glutathione peroxidase. Free radicals can enhance membrane permeability to various molecules through
peroxidation of membrane lipid, and lower the level of neuronal activity. Of course, there are two protective systems against free radicals in living cells, (1) enzymes converting radicals into harmless compounds (e.g. SOD and glutathione peroxidase), and (2) non-enzymatic antioxidants (e.g. ascorbic acid or tocopherol). If processes for free radical production are somehow enhanced and protective processes reduced, neurons could die. Indeed, there is evidence that free radicals play a role in neuronal death not only in ischemic brain lesion but also of AD, PD, ALS and HD. In PD, free radicals are easily produced with the help of Fe²⁺ in the course of the metabolism of dopamine. Therefore, dopaminergic neurons are always exposed to free radicals. Evidence of a role of oxidative stress in AD and HD is also accumulating³⁵,³⁶.

**Excitotoxicity**

Glutamate is the most abundant excitatory neurotransmitter in the brain, and almost every neuron expresses glutamate receptors, either permeating ions directly (AMPA/KA and/or NMDA types) or indirectly (metabotrophic type). An increase of extracellular glutamate produces prolonged depolarization of neurons, inducing prolonged Ca²⁺ influx into glutamate-receptive neurons, which then leads to neuronal death (i.e. excitotoxicity)³⁷,³⁸. The role for excitotoxicity in neuronal degeneration has been extensively studied in ALS and HD. Although the concentrations of glutamate in the spinal cord and the brain of sporadic ALS patients are not increased³⁹, the predominant high-affinity glutamate transporter (EAAT2) expressed...
specifically in astrocytes is lost in the ALS spinal cord. This might be a result of aberrant mRNA caused by splicing errors\(^4^0\), causing an increase of available glutamate in the peri-motoneuronal environment. mRNA for the glutamate receptor 2 (GluR2) subunit, which strongly regulates Ca\(^{2+}\) conductance of the AMPA/KA receptor, is edited normally at the Gln/Arg residue in the subunit assembly. Recently, in the ventral horn of ALS patients, GluR2 mRNA editing was shown to be significantly reduced\(^4^1\). This can lead to a continuous Ca\(^{2+}\) influx through AMPA/KA receptors, thereby making motoneurons vulnerable to various endogenous or exogenous adverse insults. Indeed, in a mouse model of cerebellar ataxia, a causative mutation in the gene (GluR\(\delta^2\)) in the lurcher mouse leads to continuous Ca\(^{2+}\) influx and cerebellar neuronal death\(^4^2\).

Reduced protein synthesis
Using DNA microarray techniques, a recent study with a mouse model of HD revealed that in the early stage of the disease, the brain expresses reduced levels of mRNA of certain receptors and second messengers, but not of mitochondrial proteins or apoptosis-related proteins\(^4^3\). Indeed, the dopamine-synthesizing enzyme tyrosine hydroxylase (TH) and its mRNA has been reported to be reduced in the remaining nigral neurons of PD (Ref. 44).

Preliminary single neuron analysis also showed that the remaining nigral ‘sick’ neurons in PD patients definitely express GAPDH mRNA at normal levels but express less than normal levels of TH, dopa decarboxylase and \(\alpha\)-synuclein mRNAs (Fig. 4).

These lines of evidence suggest that it is important to know the overall expression profile of neurons in ‘sick state’ to clarify the mechanism of ‘sickness’.

Therapeutic implications of ‘sick’ neurons
Although the ‘sick state’ of neurons is generally regarded as irreversible, one could speculate that ‘sickness’ of neurons could go back to the normal state, if triggering or promoting factors for neuronal damage were eliminated. This assumption is based on a recent report of HD transgenic mice model using a tet/off system\(^4^5\). The system makes it possible to turn off the expression of a transgene with oral administration of tetracycline analogs at any age after birth. The damage of the striatal neurons of this particular mouse model is definitely reversed by inhibition of the continuous expression of the mutant HD-causing gene. If extrapolated to HD and other polyglutamine diseases in humans, the ‘sick’ neurons could go back to normal by inhibiting the expression of the mutant gene. In addition, vaccinations with A\(\beta\) peptide were reported to reduce amyloid deposition in a transgenic mouse model of AD (Ref. 46). Moreover, the learning ability of A\(\beta\)-vaccinated mice was found to be superior to that of non-immunized mice\(^4^7\). Therefore, it is possible to speculate that the cognitive dysfunction in AD could be successfully reversed by this treatment. Apart from the possible reversal of ‘sickness’ of neurons, there are several lines of therapeutic trials, either experimental or clinical, for neurodegenerative diseases. First, the fetal nigral tissues were transplanted to the striatum of PD patients and a part of dopaminergic function was improved. Second, a glial cell line-derived neurotrophic factor (GDNF) introduced directly into substantia nigra or indirectly by vectors provided protection of nigral neuronal death and functional recovery of the nigra in the experimental model of PD (Ref. 48). Third, a recent experimental study demonstrated that grafts derived from human fetal striatal tissue can survive, develop, and are unaffected by the disease process after transplantation into a patient with HD (Ref. 49). Finally, using mesenchymal cell-derived factor(s), mammalian ES cells successfully differentiated into neurons, particularly dopaminergic neurons\(^4^6\). This achievement opens the door towards a cure of PD, and hopefully this therapeutic principle can be applied to the other neurodegenerative diseases.