

Neurobiology of disease

Huda Y Zoghbi*, Fred H Gage† and Dennis W Choi‡

Advances in technology and basic sciences this past decade have transformed neurobiological research. Practitioners looking prospectively in 1990 could hardly have hoped for the diagnostics and rational therapeutics that have become part of regular practice today. Here, we discuss three areas that have had great impact: genetics, cell death, and stem cell/gene therapy research.

Addresses

*Howard Hughes Medical Institute and Baylor College of Medicine, One Baylor Plaza MS225, Houston, Texas 77030, USA; e-mail: hzoghbi@bcm.tmc.edu

†Laboratory of Genetics, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037, USA; e-mail: gage@salk.edu

‡Department of Neurology, Washington University School of Medicine, Center for the Study of Nervous System Injury, 660 South Euclid Avenue, St Louis, Missouri 63110, USA;

e-mail: wildersp@neuro.wustl.edu

Correspondence: Fred H Gage

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Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
GABA	γ -aminobutyric acid
NMDA	<i>N</i> -methyl-D-aspartate
PD	Parkinson's disease

Introduction

The challenges of summarizing the accomplishments of neurobiology over the past decade are numerous. In the interest of meaningful brevity, we have selected three main areas: neurogenetics, cell injury and death, and repair of the damaged CNS. We have further focused on the unexpected — that is, the discoveries of the past 10 years that could not have been anticipated but that proved relevant to both research and patient care.

Genetics and neurobiology

To paraphrase Carl Jung, the meeting of neurology and genetics in the 1990s has been like the contact of two chemical substances, and both have been transformed by the reaction. Of course, genetics has transformed medicine in general, and its impact on neurology has been similar to its effect on other medical fields: the classification of groups of neurological disease has been overhauled as their distinct genetic etiologies became apparent; a plethora of subtypes of dementia, non-syndromic mental retardation, neuropathies, epilepsies, muscular dystrophies, movement disorders, neuronal migration defects, and motor neuron diseases have come to light (see e.g. Online Mendelian Inheritance in Man at www.ncbi.nlm.nih.gov/OMIM); genetic heterogeneity for specific phenotypes has become

the rule rather than the exception; and molecular testing has lent scientific certainty to the art of clinical assessment.

Neurology is not the only beneficiary of this relationship, however. What could not have been foreseen, even in general terms, are the momentous changes wrought in our understanding of human genetics by research into neurologic diseases. We all knew, for example, that aberrant phenotypes result from sequence alterations within the coding region or regulatory elements of a gene. What we did not know is that single gene dosage can be as important (and liable to disruption) as sequence integrity. Patients with Charcot-Marie Tooth disease type 1A have a tandem duplication of a 1.5 Mbp region on chromosome 17p12; patients with hereditary neuropathy with liability to pressure palsies have a deletion of the same region. Both diseases result from an altered copy number of the dosage-sensitive myelin gene *PMP22*. More importantly, molecular analysis of the mechanism leading to such rearrangements established that the duplications and deletions are caused by recombination between tandem repeated sequences [1]. Such a mechanism accounts for deletions in several other disorders as well (e.g. Williams syndrome, Smith-Magenis syndrome, DiGeorge/velocardiofacial syndrome, Angelman syndrome, Prader-Willi syndrome, and type 1 neurofibromatosis). Studies of these disorders have proven that homologous recombination between region-specific, low-copy repeat gene clusters can cause disease by either loss or duplication of genes sensitive to dosage effects [2].

Until this past decade, it was assumed that genes originating from the maternal and paternal genomes are expressed equally in the offspring. This assumption still holds — unless the genes are imprinted. Genomic imprinting prevents the paternal and maternal alleles of a number of genes on chromosome 15q11-13, for example, from being equally expressed; the gene(s) responsible for Prader-Willi syndrome appears to be expressed from the paternal chromosome, whereas the gene mutated in Angelman syndrome is expressed from the maternal chromosome [3]. Interestingly, the imprinting in Angelman syndrome is tissue specific: only the maternal allele is expressed in Purkinje cells and hippocampal neurons in the brain, whereas bi-allelic expression is present in most other tissues [4]. Imprinted genes are functionally hemizygous; when the mutations are transmitted in families, the inheritance pattern is complex, as it depends on the parent of origin. Imprinting has been found to be involved in additional disorders, such as hereditary paragangliomas and Beckwith-Wiedemann syndrome.

Perhaps the greatest upheaval of Mendelian principles in the 1990s took place with the discovery of dynamic mutations. The assumption that alleles are stably transmitted

(i.e. passed from parent to offspring unchanged) was so deeply embedded that no one thought to question it. But this principle was belied by the observation that disease can be more severe and occurs earlier in successive generations in families with Fragile X syndrome or myotonic dystrophy. Initially ascribed to ascertainment bias, the phenomenon of anticipation provided a striking counter example to normal inheritance patterns. The discovery that some mutations evolve during transmission not only explained the apparent paradox but also opened up a whole new field of research into the so-called triplet repeat diseases. Expanding trinucleotide repeats have been found to cause fourteen neurologic disorders so far, including Fragile X mental retardation, myotonic dystrophy, spinobulbar muscular atrophy, Huntington's disease, Friedreich's ataxia, and several spinocerebellar ataxias [5]. The repeat expansion causes pathology by a variety of mechanisms, depending on the location within the gene. Non-coding repeats may interfere with DNA structure, transcription, or RNA-protein interactions [5]. Translated polyglutamine (CAG) repeats alter protein conformation and interactions. The list of dynamic mutations causing neurologic disease has recently grown beyond the triplet repeats with the discovery that expansion of a dodecamer repeat in the cystatin B gene causes a form of progressive myoclonus epilepsy, and a massive expansion of a pentanucleotide repeat causes dominantly inherited ataxia and seizures [6,7]. It is intriguing that, to date, all known dynamic mutations result in specifically neurologic disorders. Whether this mutational mechanism is to blame for other types of disease remains to be seen, but in the meantime we have learned to scrutinize the human genome for polymorphic and unstable repeat sequences.

Finally, we should mention one development that illuminates the pathophysiology of genetically inherited neurodegeneration without undermining a single genetic principle. In fact, it hints at a single pathogenic mechanism that may apply to more diseases than we currently imagine: protein misfolding. The connection between altered protein conformation and neurodegeneration was first established for prion diseases [8,9], but the suggestion that protein misfolding has greater ramifications comes from more recent research. Over the past several years, polyglutamine researchers have consistently found that mutant proteins (e.g. ataxin-1, huntingtin) have a strong tendency to accumulate in affected neurons along with molecular chaperones, the proteasome, and ubiquitin [10]. Animal models, cell culture, and patient material all yield similar results. Given the role of chaperones in assisting both protein folding and ubiquitin-proteasomal degradation, it was a short step to propose that the expanded polyglutamine tract alters the protein's conformation, and that the resultant misfolded protein is targeted for proteolysis but resists degradation. The glutamine repeat diseases suddenly began to resemble other neurodegenerative conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral

sclerosis (ALS) and, of course, the prion disorders, all of which are characterized by protein aggregates. In turn, research on these disorders turned up more connections between the protein aggregates and protein degradation machinery: the cytoplasmic neurofibrillary tangles of AD contain ubiquitinated tau; beta-amyloid peptides (in AD) have been shown to bind to the proteasome and inhibit its activity; mutations in alpha-synuclein (in PD) accelerate the oligomerization of the protein, and the A53T mutation impedes its degradation; and superoxide dismutase (SOD1) cytoplasmic inclusions in ALS mouse models are ubiquitin-positive [10,11]. Mutations in a deubiquitinating enzyme and in a ubiquitin ligase cause some forms of PD [12,13], and mutations in proteins with domains homologous to chaperones have been implicated in dystonia and spastic ataxia [14,15]. The major implication of this body of work is that enhancing proper protein folding or protein clearance may provide a promising avenue for therapy in these neurodegenerative diseases, with due regard for the distinct pathways that each disorder takes. At the end of the decade, this hope is burning brightly. A recent study has demonstrated that neurons can recover function even after misfolded protein accumulates: in a conditional mouse model of Huntington's disease, in which expression of the mutant gene was blocked after the mice began to display the characteristic phenotype, aggregates disappeared and the behavioral phenotype of the mice improved [16]. That neurons can recover from the onslaught of misfolded proteins is certainly one of the greatest surprises of the decade.

Cell injury and death

The importance of cell death for diseases of the nervous system has long been appreciated, but until the past decade, the topic was widely considered therapeutically intractable, and possibly even dull. On the one hand, premature neuronal death is prominent both after acute insults (e.g. ischemia, trauma, infections, toxins, seizures) and in chronic degenerative conditions stemming from genetic or environmental perturbations. On the other hand, failure of normal death underlies cancer, including nervous system cancer, and suspicions are rising that it may also contribute to the pathogenesis of some mental disorders or neurological disorders of childhood. Thus, the new perspectives on nervous system cell death, especially premature neuronal death, that have arisen in recent years have substantial therapeutic implications.

What has changed? Rather than accepting premature neuronal death as an inevitable consequence of pathogenic injury, we now recognize that it is often a fate reached only after an intricate cascade of events. Two surprising themes have emerged. First, the same cell-cell and intracellular signaling mechanisms that normally serve brain function can, under pathological conditions, mediate cell death. Second, a wide array of different insults, intrinsic and extrinsic to the cell, acute and indolent, can cause neurons to undergo apoptosis.

A primary example of signaling-mediated neuronal death is excitotoxicity, the neuronal death mediated by overactivation of ion-channel-linked glutamate receptors, especially NMDA receptors and Ca²⁺-permeable AMPA receptors, and consequent cellular Ca²⁺ overload [17,18]. Based on the prominent role of excitotoxicity in experimental ischemic brain injury, several anti-excitotoxic treatments have been brought to Phase II clinical trials in the treatment of stroke [19]. Although early results have so far been disappointing, there are reasons to hope that adjustments in trial design and refinements of strategy will recover in humans the powerful neuroprotective effects seen in rodent models of stroke.

There may be many ways to reduce excitotoxicity [20]. The main efforts to date have included blocking NMDA receptors, reducing glutamate release, or reducing Ca²⁺ influx into neurons through voltage-gated Ca²⁺ channels. In the future, NMDA antagonist therapy might be improved by using subtype-selective agents or agents with greater use-dependency that might increase drug effects at overactivated synapses relative to normal synapses. NMDA NR2B receptors are preferentially expressed in forebrain relative to hindbrain, so blocking these receptors may produce greater neuroprotection in forebrain with less interference of motor function than subtype-unselective NMDA antagonists [21]. Other recently identified targets include toxic Zn²⁺ influx through glutamate- or voltage-gated Ca²⁺ channels [22] and intracellular injury mediators, such as MAP kinase pathways (especially p38 and ERK), mitochondrial free-radical production, calpains, nitric oxide synthase, or poly(ADP-ribose) polymerase (PARP) [23–25]. In addition to glutamate, other signaling molecules such as the neurotransmitter dopamine may potentiate neuronal death. Not all signaling is bad, as signaling molecules such as serotonin, GABA, and adenosine may have neuroprotective actions, at least under some circumstances.

Much has been revealed about the molecular basis of programmed cell death over the past decade, creating a powerful wave of information that has enriched all biology. Elegant studies in worms [26] have underscored the highly conserved nature of genes controlling apoptosis and have paved the way for the delineation of mammalian counterparts. We now know a lot about the receptors (e.g. fas, tumor necrosis factor α [TNF α], and the p75 neurotrophin receptor) and injury sensors that trigger apoptosis, the central role of the *bcl-2* family of genes in regulating apoptosis, intracellular signaling pathways (e.g. ceramide, free radicals, c-jun kinase and NF-kappaB), and final downstream steps (e.g. movement of cytochrome *c* from mitochondria to cytosol, and activation of caspases) [27–30]. One could postulate that disease-induced apoptosis only removes cells damaged beyond use, but genetic or pharmacological blockade of apoptosis in animal models of stroke or trauma leads to lesion reduction and functional enhancements [31].

The two themes of signaling-mediated injury and pathological apoptosis are interconnected. Signaling-mediated

injury can trigger pathological apoptosis, but since it may also induce cellular necrosis (excitotoxicity typically may do so), the combined blockade of signaling-mediated injury and apoptosis may yield higher levels of neuroprotection than either strategy alone [32,33]. Such additive neuroprotection has been specifically demonstrated in animal models. However, some therapies may prove to be double-edged swords. For example, reducing glutamate receptor activation and Ca²⁺ influx may be beneficial for neurons at risk for Ca²⁺ overload, but may concurrently promote apoptosis in other neurons in a state of relative Ca²⁺ starvation [34,35]. Neurotrophins such as brain-derived neurotrophic factor (BDNF) or neurotrophin 3 (NT-3) may also be therapeutically risky, as they could enhance neuronal vulnerability to excitotoxic necrosis while attenuating apoptosis [36].

Cellular and molecular approaches to neural repair

Ten years ago we could not have predicted some of the more dramatic approaches to therapy in the CNS that are now being seriously considered. Advances in molecular and developmental biology have paved the way for many of the current approaches to brain repair.

Cellular therapy

While the first wave of human fetal tissue transplants for PD were being evaluated a decade ago [37,38], even the most recent control studies confirm that dissociated fetal tissue implants are not, at present, a reliable therapy for neurodegenerative diseases [39]. However, these pioneering efforts have provided a model for how to design and evaluate these complex approaches for neural repair, and set a benchmark for the extent of acceptable functional recovery. New approaches have emerged from a basic change in the view of adult neuroplasticity.

Even though the pioneering work of Altman [40], Rosenzweig [41], Greenough [42], Nottebohm [43], and colleagues had demonstrated some structural plasticity in the adult brain, these experimental discoveries had not been widely applied to developing therapy for disease. A decade ago, clinical neuroscience was comfortable with the idea that the adult brain was fixed and immutable, with some responding to injury with only limited sprouting. Therapeutic approaches focused primarily on strategies such as neurotransmitter replacement [44]. Studies in the developing nervous system, however, began to reveal the importance of neurotrophic factors [45] and apoptosis [46] in sculpting the developing brain, and, in the past decade, this information has turned into novel approaches to prevent cell death (see section above). Very recently, it has become accepted knowledge that the adult brain has many proliferating cells and that, in some areas of the brain, these cells can give rise to neurons. The recent acceptance of the importance of adult neurogenesis can be attributed to several methodological developments that have confirmed, supplemented and extended the earlier observations of

neurogenesis: in particular, new imaging techniques such as confocal microscopy [47], better and more specific antibodies that can differentiate between neurons and glia [48], and sophisticated quantitative techniques such as stereology [49]. Another important conceptual development emerged out of rapid advances in the general field of stem cell biology, particularly the ability to isolate stem cells from the adult brain. The discovery of the existence of neural stem cells in the adult brain encouraged the view that the new neurons in the brain were not derived from mature neurons dividing, but rather from a residual population of proliferating progenitors. It is now clear that all mammals [50,51], including humans [52], exhibit neurogenesis in at least a limited number of brain regions, including the dentate gyrus of the hippocampus and the olfactory bulb.

Neural stem cells have now been harvested from the hippocampus and the subventricular zone of the lateral ventricular wall of rodent fetal and adult tissue, as well as from human fetal tissue [53–56]. Both types of stem cells can be implanted back to the adult brain where they survive and differentiate into cells appropriate to the area of the brain to which they migrate [57,58]. While the exact origin of adult neural stem cells is still under debate [59,60] — as is the number or variety of cells that the most primitive of these cells can differentiate into [61,62] — there is little doubt about their potential. The general strategy is to isolate primitive cells, expand the population to a number that is sufficient for replacement or repair, then differentiate the cells toward the cell lineage that needs replacement, and then graft the differentiated cells to the location. Studies using strategies for neural transplantation in preclinical models of many diseases from Parkinson's to multiple sclerosis are underway, with mixed experimental results but unflagging optimism. The recognition of the existence of persistent populations of cells, some of which can give rise to neurons, has also raised the possibility that increases or decreases in these cell populations may be relevant to disease. The potential importance of this regulation of adult neurogenesis, specifically in the dentate gyrus of the hippocampus, has been strongly supported by experimental studies that have demonstrated that environmental enrichment [63], learning [64], and exercise [65] can increase the number of new neurons in an adult brain, whereas stress [66] and glucocorticoids [67] can decrease neurogenesis in experimental animals. The clinical importance of the hippocampus has contributed to speculation that structural changes in the brains of patients with mood disorders [68] and epilepsy [69] could, in part, be attributed to changes in the regulation of neurogenesis in the hippocampus. Although the birth of new neurons is limited to restricted areas of the adult brain, the presence of dividing populations throughout the adult brain and even the adult spinal cord [70] has raised the possibility of activating endogenous cells to replace or repair damaged cells in what is being called a 'self-repair' mechanism. The next few years will clearly provide a clearer perspective on these clinical attributions for adult neuroplasticity.

Molecular therapy

The concept of gene therapy was introduced in the 1990s [71]. Early studies focused primarily on the *ex vivo* approach, which involved genetically engineering cells *in vitro* to make a therapeutic gene and then transplanting these cells into specific regions of the brain [72]. The first gene therapy experiment in the CNS was reported as the previous decade closed [73], and, while there was great enthusiasm, there were significant problems surrounding cell survival (choosing the correct cell) and stable gene expression. The 'vectors' or vehicles to deliver genes to cells were inefficient and, in some cases, expression was transient if the gene did not integrate into the genome of the cells and required cell division. In addition, in primary cells the transgene would 'shut off', whereas in immortalized cells the transgene would persist, but the cells would continue to divide. Throughout the past decade, a series of new vector systems has been developed. After some initial failures, there are now several safe and effective methods of delivering genes directly to non-dividing cells in the brain: in particular, lentiviruses [74] and the adeno-associated viruses [75] have been shown to be effective and safe for delivery, and expression from such vectors persists for at least several months. Cell-specific gene delivery [76,77], as well as regulation of the therapeutic gene expression [78,79], will be necessary to make this a routine, safe therapy, but the methods are available. The hard work of preclinical development and clinical testing will be the next steps. A combination of gene transfer and stem cell biology is already being developed and will likely be a hybrid that will be used to address many of the more complicated neurological diseases.

The future

Neuroscience is truly changing the landscape of modern medicine. Progress in neuroscience has made possible, in the past few years, the first drug treatments for several neurological major diseases, including AD, stroke, multiple sclerosis, and ALS. Many patients suffering from poorly controlled seizures are benefiting from the recent deployment of eight new anticonvulsant drugs, the first new anticonvulsants to be introduced in over a decade. Pallidal stimulation, approved by the FDA in 1997, has restored quality of life to some patients previously crippled by PD. The need is still great, as these inaugural treatments are but first steps. Brain and spinal cord disorders account for the majority of our nation's long-term care costs, and the combined costs of hospitalization and prolonged care for neurological, psychiatric, and addictive disorders exceed those of all other diseases combined. What lies ahead?

We can anticipate continued delineation of genetic causes and modifiers of nervous system diseases, enhancements in the power and safety of therapeutic approaches aimed at preventing pathological neuronal or glial cell death, and a harnessing of stem cell biology — both endogenous and transplanted — to repair the injured nervous system. We can anticipate progressive refinements in our neuropharmacological armamentarium, especially sharpening of

selectivity driven by the identification of precise molecular targets such as receptor or channel subtypes, and computer-modeling-assisted drug design. Diseases as disparate as PD, glioma, spinal cord injury, headache, depression, and heroin addiction are likely to benefit from improved drug targeting. We can anticipate that these improved drugs will be joined by other important therapeutic modalities, including gene therapy, alterations in stimulator-evoked or voluntary activity patterns designed to optimize functional recovery after injury, and micro-electronic prostheses that will take over lost functions along the lines of the already successful cochlear implant. The therapeutic power of these treatments will in many cases be fully unleashed only by critical advances in neuroimaging or neurosurgical methodologies. The former will permit identification of brain regions involved in disease processes; the latter, guided by neuroimaging, will permit increasingly precise delivery of drugs (e.g. embedded in slow-release polymers), cells, genes, or devices to needed locations with the least disturbance of normal tissue. Some techniques currently under exploration, such as using magnetic-field-guided beads to move within brain parenchyma, may revolutionize neurosurgical procedures in which direct visualization of tissue is not needed.

Furthermore, we can anticipate continued progress towards understanding how the brain works. Treatments for some of the most severe brain disorders that affect higher cortical functions, such as schizophrenia or addictive disorders, have improved substantially over the past decade, but are still limited and largely empirical. As we gradually develop insights into our most human essence — our consciousness — and understand how we think, feel, hope, and remember, we can look forward to a time when we can cure people with such disorders, and perhaps also enhance the quality of life for everyone.

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