Folding proteins in fatal ways

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Human diseases characterized by insoluble extracellular deposits of proteins have been recognized for almost two centuries. Such amyloidoses were once thought to represent arcane secondary phenomena of questionable pathogenic significance. But it is has now become clear that many different proteins can misfold and form extracellular or intracellular aggregates that initiate profound cellular dysfunction. Particularly challenging examples of such disorders occur in the post-mitotic environment of the neuron and include Alzheimer's and Parkinson's diseases. Understanding some of the principles of protein folding has helped to explain how such diseases arise, with attendant therapeutic insights.

One of the most satisfying moments in scientific investigation occurs when previously disparate phenomena of unclear origin are shown to arise from a common principle. During the past decade, numerous aetiologically distinct diseases have been linked by the likelihood that they result from the progressive misfolding of specific proteins into aggregates that can injure and kill cells. Together, these disorders inflict enormous personal and societal burdens, making it crucial to understand their genesis and to learn how to prevent them.

The amyloidoses have traditionally been defined as diseases in which normally soluble proteins accumulate in the extracellular space of various tissues as insoluble deposits of 10 nm fibrils that are rich in β-sheet structure and have characteristic dye-binding properties[1,2]. There are many examples of secreted, circulating proteins that can, under abnormal circumstances, be converted in part to highly stable extracellular fibrils. These include immunoglobulins in primary systemic amyloidosis and multiple myeloma, amylin in the diabetic pancreas, and small soluble proteins of uncertain function such as the amyloid β-peptide (Aβ) in Alzheimer's disease.

Although the specific polypeptides that comprise the deposits are different for each amyloidosis, the disorders have several key features in common. Perhaps foremost among them is the ability of proteins that are highly soluble in biological fluids to be converted gradually to insoluble filamentous polymers enriched in β-pleated sheet conformation. The common structural motif of virtually all amyloid fibrils consists of cross-β-sheets in which the peptide strands are arranged perpendicular to the long axis of the fibre. Although it was once thought that relatively few proteins have this propensity, recent data suggest that many soluble proteins can, under certain circumstances, undergo this conversion. Of particular significance is the finding that globular proteins with diverse sequences that are not currently associated with a protein-folding disease (for example, muscle myoglobin) can undergo aggregation *in vitro* into fibrils indistinguishable from those found in the amyloidoses[3,4]. This finding supports the concept that aggregation into β-sheet-rich fibrils is a generic property of polypeptide chains regardless of sequence[5].

Another general feature of protein-folding disorders is the prolonged period before clinical manifestations appear. Although the age of onset of symptoms varies widely among the different diseases and even among cases of one disease, most of these disorders become noticeable in middle or late life. There is a prolonged preclinical phase during which proteins misfold, build up and progressively compromise cellular and tissue function. A portion of this long prodrome derives from the energetic barriers to the formation of misfolded species, including the fact that nucleation — the initial development of very small, metastable oligomers of a protein — is a kinetically unfavourable requirement for fibrillogenesis[6,7]. It seems that time, rather than great age, is required, in that some aggressive protein-folding disorders can occur in young and early middle-aged individuals. In such cases, time still has a role but the fibrillogenic process requires less time overall because particular biochemical circumstances promote accelerated nucleation. One striking example is Down's syndrome, in which patients with trisomy 21 develop abundant Aβ aggregates in the brain as early as the age of ten, owing to lifelong overexpression of the β-amyloid precursor protein (APP), which is encoded on chromosome 21. Similarly, inherited missense mutations in amyloid-prone proteins can markedly accelerate their misfolding and fibrillogenesis, producing earlier disease onset than occurs with the wild-type isoform. For example, senile systemic amyloidosis arises late in life from the aggregation of wild-type transthyretin, whereas familial amyloidotic polyneuropathy I generally arises in mid-life from the accelerated aggregation of mutant transthyretin.

In this review, I describe briefly three types of amyloidosis: systemic, organ-limited and intracellular. I then examine how abberant protein folding may occur and how misfolded proteins may disrupt the cell.

Systemic amyloidoses

The list of secreted, circulating proteins that are capable of producing extracellular amyloid deposits in multiple organs is long and growing (Table 1). Amyloidoses can arise when other pathological conditions cause a sharp increase in the concentration of an amyloid-prone polypeptide, as occurs for the serum amyloid A (SAA) protein during the acute-phase response accompanying inflammatory disorders such as rheumatoid arthritis, or chronic granulomatous infections such as tuberculosis. A 76-residue proteolytic fragment of wild-type SAA can then accumulate, misfold, aggregate and be deposited in the connective tissue of multiple organs, including spleen, kidney and liver. Multiple myeloma is associated with the overproduction by plasma cells of monoclonal immunoglobulins that accumulate and form deposits (as holoproteins and/or proteolytic fragments) in the extracellular space of various tissues. Primary systemic amyloidosis also involves progressive multi-tissue deposition of immunoglobulin light chains and their fragments. In some systemic amyloidoses, the basis for an

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Table 1 **Some members of the family of systemic extracellular amyloidoses**

Clinical syndrome	Fibril subunit
Primary systemic amyloidosis	Intact immunoglobulin light chains or fragments thereof
Secondary systemic amyloidosis	Fragments of serum amyloid A protein
Familial Mediterranean fever	Fragments of serum amyloid A protein
Familial amyloidotic polyneuorpathy 1	Mutant transthyretin and fragments thereof
Senile systemic amyloidosis	Wild-type transthyretin and fragments thereof
Familial amyloidotic polyneuropathy II	Fragments of apolipoprotein A-1
Haemodialysis-related amyloidosis	β_2-Microglobulin
Finnish hereditary amyloidosis	Fragments of mutant gelsolin
Lysozyme amyloidosis	Full-length mutant lysozyme
Insulin-related amyloid	Full-length insulin
Fibrinogen α-chain amyloidosis	Fibrinogen α-chain variants

increased concentration of an amyloidogenic protein is a perturbation in its clearance. One striking example is haemodialysis-related amyloidosis, in which chronic haemodialysis can lead to progressive increase in β_2-microglobulin in tissues because this amyloid-prone protein does not pass efficiently through the dialysis membrane and becomes highly concentrated in the post-dialysis serum of the patient.

In these and other systemic amyloid diseases, the precise reasons for selective tissue deposition and the mechanisms of cell dysfunction and organ failure are still under investigation (see below). Because systemic amyloid deposits can sometimes build up in very large amounts, it seems that they can cause injury in part by physical compression and microvascular compromise of adjacent tissue. However, as with the misfolded proteins that define certain neurodegenerative disorders (see below), the systemic extracellular amyloid fibrils and in particular their smaller, more diffusible precursor assemblies (oligomers and protofibrils) might also act in an amphipathic fashion to bind and perturb multiple cell-surface receptors and/or channels rather nonspecifically.

Organ-limited amyloidoses

The other broad class of amyloid disorders is that in which the fibrillogenic protein accumulates locally near its site of cellular production; that is, mostly in one organ (Table 2). Salient examples of this type include calcitonin deposition in medullary carcinoma of the thyroid, deposition of amylin in the pancreas in type II diabetes mellitus, deposition of atrial natriuretic factor in atrial amyloidosis of the heart, and Aβ deposition in Alzheimer's disease. Although these proteins enter the systemic circulation, their high local production in these conditions apparently leads to levels that exceed the critical concentrations for oligomerization and fibrillogenesis solely in that organ. In these disorders, the amyloid fibrils and their precursor aggregates are deposited in the extracellular space of the respective organs and somehow compromise local cell viability. However, there are also a growing number of diseases in which the accumulation of different misfolded proteins occurs intracellularly, particularly in the nervous system, as described in the next section.

Protein misfolding and age-related neurodegeneration

During the past two decades, there has been a marked transformation in our understanding of the causes and pathogenesis of age-related degenerations of the brain. Once fraught with mechanistic ignorance, this class of disorders has become recognized as likely to be mediated by the progressive accumulation of β-sheet-rich protein aggregates. A range of brain disorders of previously unknown cause now falls into this category (Table 3). The most common neurodegenerative disease, Alzheimer's, is the premier example of this. The second most common, Parkinson's, is increasingly considered to involve a protein-folding problem. Two special features of most of these disorders of the central nervous system are that the aggregates accumulate inside cells and that the ultrastructure of the aggregates is not the same as that of extracellular amyloid fibrils. This raises the question of whether such disorders should be classified with the amyloidoses. But because the dye-binding properties (for example, birefringent labelling by Congo red), insolubility and X-ray diffraction pattern (high β-sheet content) of at least some of these deposits seem to be similar to those of classic amyloid fibrils, it is reasonable to consider them a special form of amyloidosis.

A wide variety of proteins of diverse sequence can form intraneuronal filamentous deposits. In Parkinson's disease, α-synuclein, a soluble cytoplasmic protein with a 'natively unfolded' structure, misfolds and accumulates in spherical filamentous masses (Lewy bodies) within selected neuronal cell bodies, particularly the dopaminergic and noradrenergic brainstem neurons whose premature death defines the disease biochemically. A recently recognized group of protein-folding diseases of the central nervous system are the polyglutamine repeat disorders, such as Huntington's disease and several forms of familial spinocerebellar ataxia[8]. Here, unstable DNA mutations in different genes result in the lengthening of glutamine stretches in the cognate proteins, which subsequently misfold and accumulate in the nuclei and cytoplasm of certain neurons. Although the appearance of microscopically detectable filamentous inclusions is variable and subtle compared with the robust formation of neurofibrillary tangles in Alzheimer's disease and Lewy bodies in Parkinson's disease, these highly stable, polyglutamine-rich proteins aggregate and gradually cause the dysfunction and death of their host neurons. It seems that the accumulation of misfolded proteins has particularly dire consequences in the post-mitotic milieu of the adult neuron.

Alzheimer's disease is virtually the only brain disorder that is defined by the accumulation of amyloid-forming proteins both extracellularly (Aβ) and intracellularly (tau). There has been lively debate about which of these, if either, has precedence in the pathogenic mechanism. The issue has been largely resolved by the finding that inherited mutations in APP, or in one of the proteases (called presenilin/γ-secretase) that cleaves APP to release Aβ, cause aggressive, early-onset forms of Alzheimer's disease[9]. In contrast, inherited mutations in the tau protein do not produce Alzheimer's disease but cause the less common but equally devastating disorder, frontotemporal dementia with parkinsonism, in which tau-containing neurofibrillary tangles accumulate in the absence of extracellular amyloid[10].

A remarkable example of protein misfolding causing neurodegeneration is that of the prion disorders. In humans, these include Creutzfeldt–Jacob disease and new-variant Creutzfeldt–Jakob disease, the latter being a human derivative of bovine spongiform encephalopathy ('mad cow disease'). These disorders are very closely related to a range of slowly progressive infectious diseases in certain lower mammals, including the defining disorder, scrapie, in sheep[11]. Very rarely, the normal cellular prion protein (PrP) can undergo a change in its conformation that creates a metastable conformer, which can in turn bind and 'convert' another wild-type prion protein into this same pathological isoform. This misfolding process can apparently occur spontaneously at a very low frequency but occurs more readily if the protein bears certain missense mutations. Prion encephalopathies can thus be inherited, but they also occur in an infectious form: exposure of a healthy subject to small amounts of pathologically conformed but non-mutant prion protein (for example, through a corneal transplant from an individual with

Table 2 **Some of the organ-limited extracellular amyloidoses**

Clinical syndrome	Fibril subunit
Alzheimer's disease	Amyloid β-peptide
Spongiform encephalopathies	Full-length prion protein or fragments thereof
Hereditary cerebral haemorrhage with amyloidosis	Amyloid β-peptide or cystatin C
Type II diabetes	Amylin (islet amyloid polypeptide)
Medullary carcinoma of the thyroid	Procalcitonin
Atrial amyloidoses	Atrial natriuretic factor

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Creutzfeldt–Jacob disease) can lead to progressive and fatal brain degeneration. Exactly how prion conversion and propagation of the disease occur is under study. One theory favours an 'instructive' dimerization of a scrapie-type prion protein conformer with a wild-type molecule that converts the latter to the former form; this can then be magnified geometrically to propagate the process. Alternatively, small oligomers of the scrapie-type conformer might serve as 'seeds' for the progressive addition and conformational conversion of wild-type monomers, in a mechanism akin to those postulated in systemic amyloidoses and Alzheimer's disease. It should be emphasized that, like the prion protein, numerous amyloidogenic proteins can oligomerize and form toxic aggregates in their wild-type state, for example tau in Alzheimer's disease and α-synuclein in idiopathic Parkinson's disease.

Table 3 **Some human brain diseases characterized by progressive misfolding and aggregation of proteins**

Disease	Protein	Locus
Alzheimer's disease	Amyloid β-protein Tau	Extracellular plaques Tangles in neuronal cytoplasm
Frontotemporal dementia with parkinsonism	Tau	Tangles in neuronal cytoplasm
Parkinson's disease; dementia with Lewy bodies	α-Synuclein	Neuronal cytoplasm
Creutzfeldt–Jakob disease; 'mad cow disease'*	Prion protein (PrP^{sc})	Extracellular plaques Oligomers, inside and outside neurons
Polyglutamine expansion diseases (Huntington's disease, spinocerebellar ataxias, and so on)*	Long glutamine stretches within certain proteins	Neuronal nuclei and cytoplasm
Amyotrophic lateral sclerosis*	Superoxide dismutase	Neuronal cytoplasm

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Mechanism of protein misfolding

A principal unanswered question about all of these disorders is the precise manner in which natively soluble proteins of distinct primary structure undergo partial unfolding and aberrant refolding to produce highly stable oligomers and polymers with novel properties. It is clear that supraphysiological concentrations, coupled with prolonged time and certain biochemical conditions, underlie the initiation of the oligomerizaton process. One instructive example that has been studied in some detail is lysozyme, a protein that causes a fatal systemic amyloidosis only when it bears one of two inherited missense mutations, Ile 56→Thr or Asp 67→His. Crystallography of the wild-type and mutant variants revealed subtle but structurally significant changes at the interface between the α- and β-domains that suggest that this region is less constrained in both mutants[12]. In agreement, circular-dichroism studies during conditions of heat denaturation that lead to fibril formation showed that both mutants are less thermostable. Moreover, near the midpoint of the heat-driven unfolding process, the mutants formed partly folded intermediates with considerable helical structure but no persistent tertiary interactions[12]. Wild-type lysozyme unfolds into a partly structured intermediate only when thermal denaturation is performed at very low pH (ref. 13).

It seems that such partly folded forms, which have exposed hydrophobic regions and are therefore prone to self-aggregation, can exist in equilibrium with the native protein. In the case of the mutant lysozymes, the molten-globule-like intermediates have persistent structure in the α-domain but lack stable, native-type structure in the β-domain[14]. It has therefore been proposed that the transient, partly folded forms of the mutant lysozymes associate through their unstable β-domains. The emergence of stable β-structure through such intermolecular self-association might provide a template (seed) for the recruitment of additional peptide chains that ultimately form the hydrogen-bonded, mainly cross-β-sheet core structure of the insoluble amyloid fibril[12] (Fig. 1). In agreement with this model, the NMR structure of a domain of the prion protein (PrP(121–231)) indicates that amino acids that are mutated in inherited prion diseases are involved in the maintenance of the hydrophobic core[15]. Because microscopically visible fibril deposition is not an obligatory feature of the prion diseases, these principles are likely to apply also to pre-fibrillar, oligomeric assemblies of PrP and presumably other amyloidogenic proteins.

How do misfolded proteins cause cell dysfunction?

Perhaps even more complex than the mechanisms by which proteins misfold are the mechanisms by which they inflict cellular injury. This question once focused primarily on the mature, highly stable amyloid fibrils, but now attention has shifted to the apparent cytotoxicity conferred by metastable intermediates of the fibrillogenic process. In diseases in which unmistakeable amyloid formation occurs, deposits of insoluble fibrils are in equilibrium with smaller, more diffusible assemblies that are invisible by light microscopy but can be detected by biochemical methods such as gel electrophoresis and size-exclusion chromatography. In the growing number of diseases in which protein misfolding and oligomerization occur but no definite amyloid fibrils are seen, these smaller diffusible assemblies might be responsible in part for cytotoxicity. Limited studies of such species in human-tissue extracts indicate that they might include dimers, trimers, tetramers and other larger oligomers. But most of the information about the properties of such intermediates has been gleaned from biophysical (*in vitro*) and cell culture studies of synthetic or recombinant versions of the amyloidogenic proteins. The concern here is that perhaps only a small proportion of these artificial assemblies closely resemble the natural oligomers that occur *in vivo*.

Mouse models that transgenically overexpress the polypeptide precursors of amyloid subunits *in vivo* offer another approach. One closely studied example is that of mice overexpressing mutant human APP as a model of the amyloidosis of Alzheimer's disease. In some such mouse lines, presynaptic nerve terminals and neuronal cell bodies are decreased about 30% compared with non-transgenic controls at the age of 2–4 months, when concentrations of soluble Aβ are increasing but well before Aβ deposition (that is, amyloid plaque formation) has begun[16]. Comparisons of transgenic lines with different APP expression levels suggest that these decreases in presynaptic terminals are dependent on Aβ concentrations, not on the Aβ plaque burden[17]. This animal work fits nicely with growing evidence that

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memory and cognitive deficits in Alzheimer's disease and in its harbinger, minimal cognitive impairment, correlate better with cerebral Aβ concentrations than with amyloid plaque numbers[18] and correlate best with the soluble pool of cerebral Aβ, which includes soluble oligomers[19,20]. In the brains of subjects who have died (for other reasons) with only mild clinical impairment, the concentrations of soluble Aβ monomers and oligomers in the cerebral cortex correlate with the degree of synaptic loss[20].

Electrophysiological studies of young mice that are transgenic for human APP bearing Alzheimer's-disease-causing missense mutations reveal significant deficits in basal synaptic transmission and/or long-term potentiation (LTP, an electrophysiological correlate of synaptic plasticity) in the hippocampus, well before the development of microscopically detectable Aβ deposits (reviewed in ref. 21). However, the nature of the 'synaptotoxic' Aβ species in the brain is difficult to define, because these animals accumulate a mixture of Aβ forms (monomers, soluble oligomers, insoluble oligomers, and some insoluble amyloid fibrils) that are likely to exist in dynamic equilibrium. In certain cultured cells expressing mutant human APP, natural oligomers of human Aβ are formed soon after generation of the peptide within intracellular vesicles and are subsequently secreted from the cell at low nanomolar concentrations[22]. Cerebroventricular microinjection of cell medium containing these soluble oligomers and abundant monomers (but no amyloid fibrils) potently inhibits hippocampal LTP in adult rats. Pretreatment of the medium with a protease that selectively degrades Aβ monomers but not oligomers fails to prevent LTP inhibition. In contrast, treatment of the cells with an inhibitor of γ-secretase (one of the two proteases that generate Aβ from APP) markedly decreases oligomer formation at doses that still allow appreciable monomer production, and such medium no longer disrupts LTP[22]. These experiments allow one to attribute an inhibition of synaptic function *in vivo* specifically to soluble oligomers, not monomers or fibrils, of secreted Aβ.

In the polyglutamine expansion disorders, similar phenomena may be occurring, but intraneuronally. For example, in mouse models of spinocerebellar ataxia-1, in which the protein ataxin-1 bears long glutamine repeats and can form microscopically visible intranuclear inclusions in selected neurons, the most vulnerable neurons develop large filamentous inclusions only late in the course of neurodegeneration, whereas neurons showing early inclusion formation are more resistant to cell death[23]. In this sense, inclusions of a misfolded protein might be protective because they sequester the aggregates, at least temporarily. In α-synuclein aggregation in Parkinson's disease, loss of dopaminergic neurons in a *Drosophila* model expressing human α-synuclein was prevented by co-expressing the heat shock protein Hsp70, one of numerous molecular chaperones that guide the correct folding of polypeptides[24]. This work and similar approaches in the polyglutamine disorders[25,26] indicate that such diseases are indeed disorders of protein folding, and they remind us that chaperones and other powerful compensatory mechanisms — such as activation of the ubiquitin–proteasome system — can decrease the accumulation of misfolded proteins or else enhance their clearance.

Whether early-assembly forms or mature polymers or both are important for dysfunction, the question of precisely how such misfolded species perturb cellular homeostasis must also be answered. Many potential mechanisms for the adverse effects of misfolded proteins have been proposed. In the example of Alzheimer's disease, the accumulation of diffusible Aβ oligomers could lead to their non-specific binding to receptors and channel proteins on the synaptic plasma membrane, thus interfering with numerous signal-transduction cascades. One consequence of exposing cultured neurons to synthetic Aβ assemblies is to alter calcium homeostasis and another is to enhance the generation of free radicals, with resultant oxidative damage to membrane lipids and proteins. In the polyglutamine diseases, it could be that small oligomers of the expanded protein adhere to and perturb intracellular membranes and/or sequester vital proteins

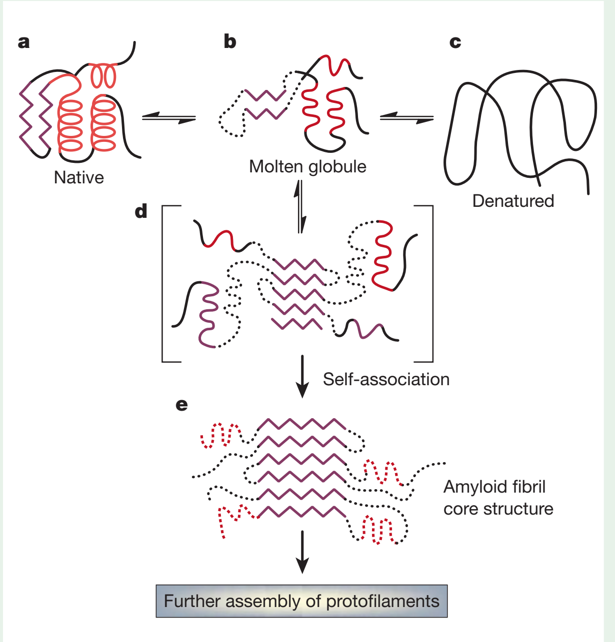

Figure 1 Proposed mechanism for lysozyme amyloid fibril formation. A partly folded, molten globule-like form of the protein (**b**), distinct from the native (**a**) and denatured (**c**) states, self-associates through the β-domain (**d**) to initiate oligomer formation. This provides a template for the further deposition of protein and for the development of the stable, mainly β-sheet, core structure of the protofilaments within an amyloid fibril (**e**). The undefined regions in **e** represent the likelihood that not all of the polypeptide sequence is involved in the cross-β structure. The nature of this residual structure in **e** is not known, and the figure is not intended to represent any defined secondary structural type[12]. Purple, β-sheet structure; red, helical structure; dotted lines, undefined structure. (Modified from ref. 12.)

such as transcription factors. One can assume that the cell biological pathways will differ among the various amyloidogenic proteins, among the targeted cells and tissues, and even among the clinicopathological stages within any one amyloid disease.

Conclusion

The insidious accumulation of misfolded proteins has dire consequences for the organism. Further progress on the two key questions discussed above — how soluble proteins begin to misfold and how the resultant oligomers initiate cell dysfunction — will offer exciting prospects for specific molecular interventions. Some of the treatment modalities that will probably emerge, for example small-molecule inhibitors of monomer–monomer binding, might prove to be useful in treating or preventing more than one amyloidotic disease. We are also likely to see major advances in imaging the extracellular and perhaps even intracellular protein aggregates non-invasively, allowing therapy to be followed dynamically. In addition, the fact that even highly soluble globular proteins not currently implicated in human disease can, under the wrong biochemical circumstances, form filamentous polymers that confer cytotoxicity[4] suggests that numerous other protein-folding disorders await recognition. □

doi:10.1038/nature02264

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements The authors thank S. K. Basu for discussions. P.A. is a Senior Research Fellow of CSIR, India. R.S.G is a Wellcome Trust International Senior Research Fellow in India. This work was also supported by grants to the National Institute of Immunology by DBT, India.

Competing interests statement The authors declare that they have no competing financial interests.

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corrigendum

Delta-promoted filopodia mediate long-range lateral inhibition in *Drosophila*

Cyrille de Joussineau, Jonathan Soulé, Marianne Martin, Christelle Anguille, Philippe Montcourrier & Daniel Alexandre

Nature **426,** 555–559 (2003).

In the authors' published address, 'UMR 5539' should read 'CNRS UMR 5539'. An additional affiliation for the first and last authors (C.d.J. and D.A.) was omitted, who are also at the Laboratoire de Neurogénétique, Unité INSERM 432, Université Montpellier II. □

erratum

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Nature **426,** 900–904 (2003).

In this Insight Review Article, a competing interest was declared by the author that was accidentally omitted. This statement is: "D.J.S. is a founding scientist of Athena Neurosciences, now Elan plc." □

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