The calpain family and human disease

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The number of mammalian calpain protease family members has grown to 14 on last count. Overactivation of calpain 1 and calpain 2 (and their small subunit) has long been tied to acute neurological disorders (e.g. stroke and traumatic brain injury) and recently to Alzheimer's disease. Loss-of-function mutations of the calpain 3 gene have now been identified as the cause of limb-girdle muscular dystrophy 2A. Calpain 10 was recently identified as a susceptibility gene for type 2 diabetes, whereas calpain 9 appears to be a gastric cancer suppressor. This review describes our current understanding of the calpain family members and their mechanistic linkages to the aforementioned diseases as well as other emerging pathological conditions.

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The first documentation of calcium-activated protease, calpain, in mammalian cells was reported over 30 years ago[1]. It was subsequently demonstrated that there are two ubiquitously distributed mammalian calpains: calpain 1 (calpain I, μ-calpain and CAPN1) and calpain 2 (calpain II, m-calpain and CAPN2)[2] (Table 1, Fig. 1). To date, at least 12 additional mammalian calpains have been identified (Table 1, Fig. 1).

The EF-hand subfamily

*The classic calpain 1 and calpain 2 (*capn1*, 2 and* 4*)*

Human calpain 1 and 2 differ in their sensitivity to calcium *in vitro*: they are activated by low and high micromolar free Ca^{2+}, respectively. Yet, both are heterodimeric proteins comprising a large 78–80-kDa catalytic subunit (encoded by *capn1* and *capn2*, respectively)[3,4] and a common 29-kDa regulatory subunit (encoded by *capn4*)[5] (Table 1). Recently, a gene encoding a highly homologous small subunit has been cloned (*capn14*) (Table 1). This subunit has fewer Gly repeats in its N-terminal region. In Capn1 and Capn2 at least four domains (I–IV) have been identified, and the small subunit has at least two regions (V and VI) (Fig. 1). Sequence homology comparison has identified domain II as a cysteine protease[6]. Structural studies of calpains were greatly advanced by the availability of recombinant heterodimeric calpain 1 and 2. Based on the recently available crystal structure of recombinant rat m-calpain[7,8], domain II, like other cysteine proteases such as cathepsin B, can be further divided into subdomain IIa and domain IIb, with a substrate-binding cleft in between (Fig. 2). The catalytic triad residue Cys is on subdomain IIa, whereas His and Asn are part of subdomain IIb. In the absence of calcium, the distance between the catalytic Cys and His is 10 Å, which is too far to form a functional catalytic triad. It is suggested that calcium-induced conformational changes draw subdomain IIa and IIb together. The crystal structure of calpain 2 has revealed that domain I is a short prodomain region of the protease. Domain I is cleaved off during initial activation of calpain 1 or 2 by calcium. Domain III is not homologous to any other known proteins and some suggest that it might serve as a linker region, whereas domain V might be required for interaction with membrane phospholipids. Both domain IV and VI each contain five sets of EF-hand calcium-binding structures. The first four EF-hand structures can bind calcium and are similar to those in calmodulin. The fifth EF-hand does not bind calcium but rather serves as homophilic-association sites between domains IV and VI[9] (Fig. 2). These calcium-binding structures in domain IV and VI have long been viewed as the regions that confer calcium-dependency on the enzyme. However, unlike calmodulin, the 3-D structure of domain VI (and probably domain IV) only undergoes minor conformational changes upon binding Ca^{2+} (Ref. 9). This suggests that additional calcium-binding site(s) might be present to confer further calcium-dependency on the catalytic activity. In fact, both a putative '6th EF-hand' structure and a highly acidic stretch (enriched by Glu and Asp residues) at the N-terminal part of domain III have been proposed to be the elusive calcium-binding site[7,8]. Calpain 1 and 2 have many cellular protein substrates[10]. Although the functions of calpain 1 and 2 have not been completely elucidated, they are likely to be involved in membrane fusion, long term potentiation, platelet activation and cell cycle progression[11,12].

Calpain 3, 8, 9, 11 and 12

The skeletal muscle-specific calpain 3 (nCL-1, p94, CAPN3) is larger than calpain 1 and 2 because of two inserts: IS-1, located in the catalytic domain II, and IS-2, near the end of domain III (Fig. 1)[13]. Interestingly, IS-1 has a nuclear translocation-like sequence (Lys–Lys–Lys–Lys–Xaa–Pro) in its catalytic domain suggesting that it might have nuclear localization functions. Interestingly, calpain 3 protein is highly unstable as a result of rapid autolysis[14]. In the rat, three splice variants exist in the visual system[15–17]. Calpain 3 apparently has an important role in maintaining myofibril homeostasis as the lack of calpain 3 activity leads to formation of muscular dystrophy (see below). Three shorter splice variants of calpain 3 have been detected in rat lens (Lp85, Lp82) and retina (Rt88), respectively[18].

A stomach smooth muscle-specific nCL-2 (proposed name calpain 8) has thus far only been cloned from rat[19], although a homologous human EST sequence has been identified (accession number AA_043093) (Table 1). It can be alternatively spliced to generate a short form (nCL-2′) that lacks two-thirds of domain III and the whole of domain IV (Fig. 1). Calpain 3 and calpain 8 have both been shown to be active calcium-dependent proteases.

 1471-4914/01/$ – see front matter PII: S1471-4914(01)02049-4

Table 1. Mammalian calpain genes

Calpain protein	Calpain gene	Other names	C-term. EF-hand	Tissue distribution	Species	Chromosome number	Genebank accession number
Calpain 1	*capn1*	μ-Calpain, CAPN1	+	Ubiquitous	Human Mouse Rat	11q13 19 NA	X04366 NM_007600 NM_019152
Calpain 2	*capn2*	m-Calpain, CAPN2	+	Ubiquitous	Human Mouse Rat	1 NA NA	NM_001748 NM_009794 NM_017116
Calpain 3	*capn3*	nCL-1, p94, Lp82, Lp85, Rt88	+	Skeletal muscle, lens, retina	Human Mouse Rat	15q15 2 NA	X85030 NM_007601 AF091998 NM_017117 U96367
Small subunit 1	*capn4*		+	Ubiquitous	Human Mouse Rat	19q13 NA NA	NM_001749 NM_009795 RNU10861
Calpain 5	*capn5*	htra3, nCL-3	–	Ubiquitous (high in colon, small intestine and testis)	Human Mouse Rat	11q14 7 NA	NM_004055 NM_007602 NA
Calpain 6	*capn6*	CAPNX, Calpamodulin	–	Placenta	Human Mouse Rat	Xq23 X NA	NM_014289 NM_007603 AF067793
Calpain 7	*capn7*	palBH	–	Ubiquitous	Human Mouse Rat	3p24 NA NA	AB028639 NM_009796 NA
Calpain 8[a]	*ncl-2*, *capn8*	nCL-2	+	Stomach mucosa	Human Mouse Rat	NA NA NA	AA_043093[c] NA D14479-D14480
Calpain 9	*capn9*	nCL-4	+	Digestive track	Human Mouse Rat	1 NA NA	NM_006615 U89513 U89514
Calpain 10	*capn10*, (*capn8*)[b]	CAPN10, CAPN8[b]	–	Ubiquitous	Human Mouse Rat	2q37 NA NA	AF089088, AF089090 -96 NM_011796 AF089089 AF227909
Calpain 11	*capn11*		+	Testis	Human Mouse Rat	6p12 NA NA	AJ242832 NA NA
Calpain 12	*capn12*		+	Ubiquitous (high in hair follicle)	Human Mouse Rat	NA 7 NA	NA AJ289241 NA
Calpain 13[a]	*capn13*[a]	Sol H	–	Ubiquitous	Human Mouse Rat	16p13 17 NA	U85647 NM015830 NA
Small subunit 2	*capn14*[a]		+	NA	Human Mouse Rat	16 NA NA	AC026802 AK009171 NA

[a]Proposed name.
[b]Old name.
[c]Partial sequences only.

The mammalian digestive track contains tissue-specific calpain 9 (also named nCL-4), which is a functional protease[20,21] and appears to play a tumor-suppressing role (discussed below). Testes-specific calpain 11 and the apparently ubiquitous calpain 12 (cloned from mouse) have recently been identified, but little is known about them and their function and substrates[22,23].

Non-EF-hand subfamily (calpain 5, 6, 7, 10 and 13)

Calpain 5 (htra-3) was initially identified as a homologue of the *Caenorhabditis elegans* sex determination gene *tra-3*. Recently, Sokol *et al.* elegantly demonstrated that another sex determinant protein, TRA-2A, is the probable endogenous proteolysis substrate for TRA-3 in *C. elegans*[24]. It is not known whether a human homologue for TRA-2A exists or not. Calpain 5 mRNA

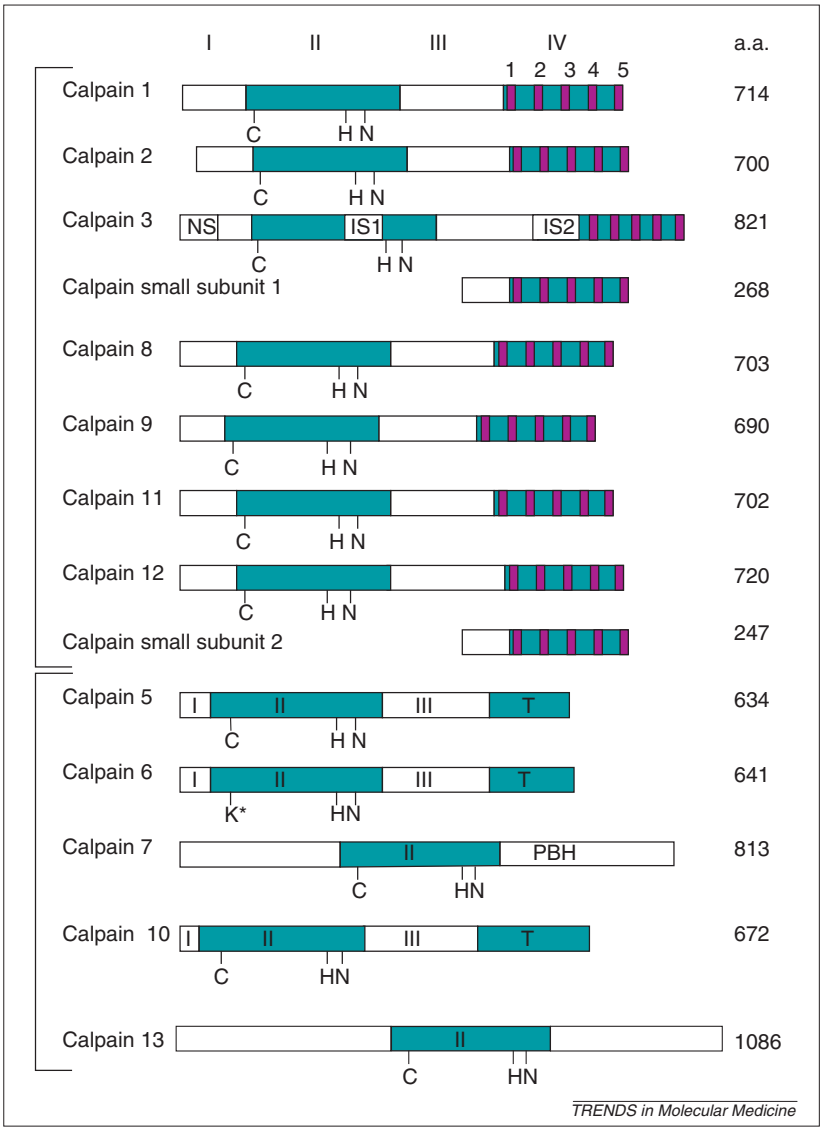


Fig. 1. Mammalian calpain family. Schematic structures of various mammalian calpains and calpastatin. For details on domain definition and abbreviations, see text. C, H, N represent catalytic residues Cys, His and Asn residues. K* represents the unique nonfunctional Lys residue (instead of Cys) in calpain 6. PBH is a domain homologous to a region of the protease PalB.

appears to be present in multiple human tissues, including small intestine, colon, liver and testis[25]. This suggests that calpain 5 might have additional roles in adult tissues. Human calpain 5 (and the rest of the members of this subfamily) lacks the C-terminal cluster of EF-hands. Instead, it contains the loosely defined T-domain, based on homology to *C. elegans* TRA-3 (Ref. 25). Calpain 6 is highly homologous to calpain 5 (47% identity, 56% similarity). Yet it lacks the active-site catalytic Cys and is not a functional protease (Fig. 1)[26]. The calpain 6 gene (*capn6*), which is located on the X-chromosome, might play a role in sex determination[27].

Calpain 7 (PalBH) is the more divergent member with only 26–35% identity to the rest of the calpain members[28] (Fig. 1). In fact, beside the protease domain, it has little homology in domains III and I of calpain 1 and 2. Instead, it is related to the fungal (*Aspergillus nidulans*) protease PalB that is involved in alkaline ambient pH adaptation[28]. A homologue in *C. elegans* (called p92) has also been identified. These proteins are all characterized by a calpain protease domain flanked by a long N-terminal domain N and a so-called PalB homologous (PBH) domain (Fig. 1)[29].

The human calpain 10 gene was recently cloned and identified as a type 2 diabetes susceptibility gene[30] (Table 2). The mouse calpain 10 cDNA and protein sequences have been made available by the same group (accession number AF089089). Interestingly, the mouse calpain 10 cDNA sequence was identical to another mouse calpain gene (accession number NM_011796), which was called *capn8* (Ref. 31). To avoid future confusion, we propose to call this type 2 diabetes-associated gene product calpain 10, whereas nCL-2 will be called calpain 8 (Table 1, Fig. 1). The *capn10* gene is expressed ubiquitously and encodes at least eight alternative splicing variants. Human calpain 10 mRNA levels are highest in the heart, followed by pancreas, brain, liver and kidney. It has domain II and III but its C-terminal domain has no EF-hand motifs and instead is homologous to the T-domain found in calpain 5 (Fig. 1).

Lastly, calpain 13 (Sol H) is a homologue of a *Drosophila* small optic lobe gene product called Sol. It is by far the most divergent member of the calpain family (Fig. 1) (accession number U85647)[57].

Calpains and diseases

Calpain 1 and calpain 2 in acute neurological injuries and Alzheimer's disease

Ischemic strokes and traumatic brain injury. Glutamate doubles as a major excitatory amino acid in the CNS and as a neurotoxin (excitotoxin) when the synaptic glutamate concentration goes beyond the safety threshold. Cerebral ischemia (during strokes and cardiac arrest) and traumatic brain injury (TBI) represent the two most common and well-studied manifestations of *in vivo* excitotoxicity[32] and calpain activation (Box 1). Generally, in or near the core of ischemic or traumatic brain injury, neurons experience large and rapid rises of intracellular calpain activation, which appear to shut down the apoptosis-inducing caspase activation cascade by truncation of caspases-3, -7, -8 and -9 and Apaf-1 (Refs 10,33), ensuring a strict oncotic (necrotic) phenotype. Calpain is also activated in neuronal apoptosis, which is more likely to occur in the penumbra of injury site. First, calpains can compromise lysosomal membrane integrity, leading to cathepsin B and D leakage, which can activate the apoptosis-mediating caspases. In fact, cathepsin B inhibitor CA074 is also neuroprotective[34]. Calpain 1 and 2 can also activate caspase 12 directly[35]. Interestingly, caspase-3 and calpain 1 and 2 can synergistically attack several common or related cytoskeletal, cytosolic and nuclear substrates[10].

Evidence of calpain-mediated αII-spectrin (α-fodrin) breakdown products (SBDPs) are well documented in various excitotoxic or ischemic injuries in both cell culture models and in *in vivo* models for ischemia[32,36] and TBI (Ref. 37). Consistent with this, calpain inhibitors such as MDL28170, PD150606 and SJA6017 have been shown to have significant neuroprotective properties in cell culture models and/or *in vivo* neuro-injury models[10,38,39]. Most impressively, calpain

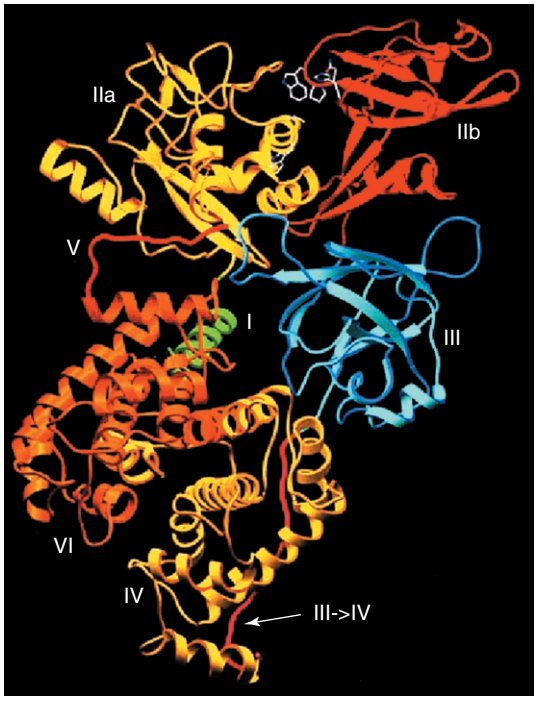


Fig. 2. Three-dimensional structure of non-activated human m-calpain heterodimer (in the absence of Ca^{2+}). Shown are, from the large subunit: the newly redefined short domain I (green), catalytic subdomain IIa (yellow) and subdomain IIb (red), domain III (blue), domain III→IV linker region (magenta) and the '5 EF-hands'-containing domain IV (yellow); from the small subunit: the truncated domain V (magenta) and the '5 EF-hands' domain VI (orange). Side chains of catalytic residues Cys105 (in subdomain IIa), His262 and Asn286 (both in subdomain IIb) are also shown. C- and N-terminals of each polypeptide are also indicated. Adapted from Strobl *et al.* [8].

inhibitors given in a delayed fashion (4 hr) post-ischemic or traumatic injury are still efficacious suggest the presence of the therapeutic window that is both relevant and realistic in paramedic and clinical practices. Cautions should be used however in using calpain inhibitors that are available, as most peptide aldehyde calpain inhibitors (MDL28170, calpain inhibitor I and II and SJA6017 do cross inhibit cathepsin B and L). The use of calpain inhibitor I and II has the further complexity that they also inhibit proteasome[32]. Lastly, the combined use of both calpain and caspase inhibitors provided additive neuroprotection[40], consistent with the synergistic roles of calpain 1 and 2 and caspases in acute neuronal cell death.

Alzheimer's disease. Two major pathological events are consistently identified in Alzheimer's disease (AD) brains. First, the abnormal processing of amyloid precursor protein (APP) to short hydrophobic amyloid β peptides (Aβ1-40, Aβ1-42) leads to extracellular deposit of Aβ aggregate (called amyloid plaques). Second, hyperphosphorylation of tau protein by GSK3b, CDK5 and other kinases lead to its dissociation from microtubules and formation of intracellular tau aggregates in a paired helical filament conformation (called neurofibrillary tangles)[41]. Calpain might be one of the factors that bridge the two pathways (Box 1, Fig. I). Here, amyloid β-peptide and/or glutamate-induced intracellular calcium elevation would set in motion a cascade of calpain-mediated cdk5 activator p35→p25 conversion, cdk5 activation, tau hyperphosphorylation and aggregation, neurofibrillary tangle formation and ultimate neuronal death (Box 1). This is consistent with the reports that both cdk5 inhibition and calpain inhibition can attenuate amyloid β-peptide-induced neuronal death[42,43]. Thus, calpain is an important target for AD treatment.

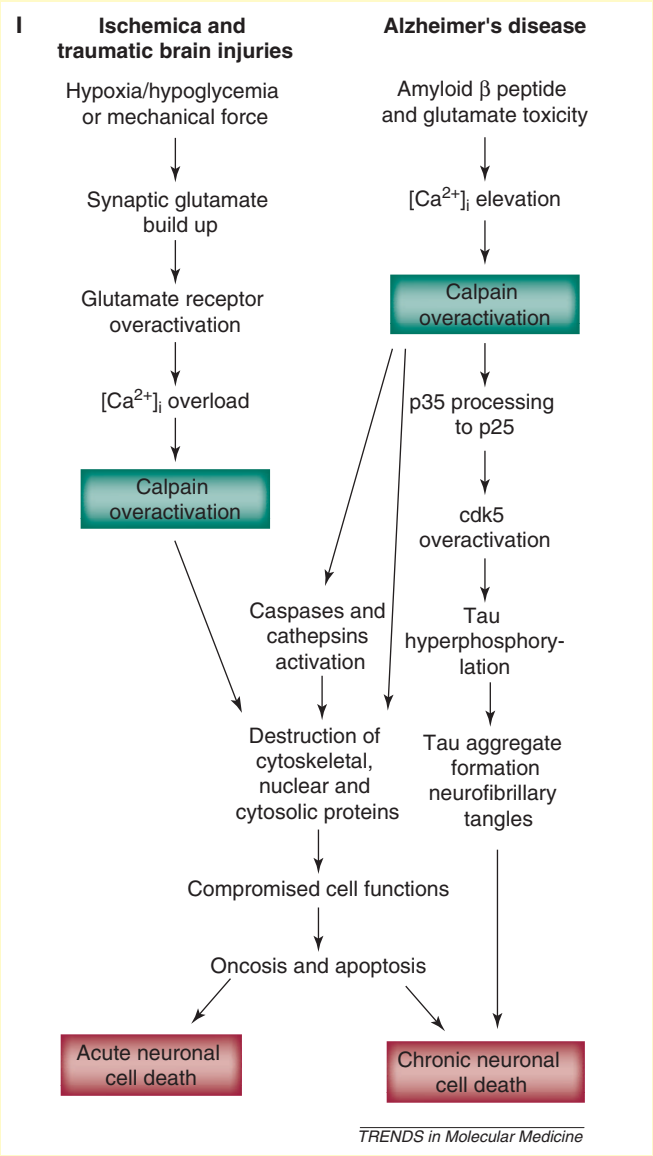
Table 2. Calpain family and various pathological conditions

Calpain	Diseases	Refs
Calpain 1	Stroke, traumatic brain injury, Alzheimer's disease, cataracts	10,32,37–40,61, 63–65,49–51
Calpain 2	Stroke, traumatic brain injury Alzheimer's disease, cataracts	10,32,37–40,62, 63–65,49–51
Calpain 3	Limb-girdle muscular dystrophy 2A, cataracts	44–46,47
Calpain 9	Gastric cancer	21,59
Calpain 8/10	Type 2 diabetes mellitus	60

Calpain 3 and limb-girdle muscular dystrophy 2A

The limb-girdle muscular dystrophies (LGMD) are a group of neuromuscular disorders that are characterized by their genetic heterogeneity and early onset of symptoms, which include progressive symmetrical atrophy and weakness of the proximal limb and trunk muscles[44]. Currently, at least three forms of autosomal-dominant and eight forms of autosomal-recessive can be defined according to the primary gene or protein involved, or to a genetic localization[44]. LGMD2A is a recessive form of the disease in which a product of the *capn3* gene, p94, is the underlying cause[45]. Fifteen different mutations that occurred in exon 2 to exon 22, spanning almost the entire *capn3* gene, were identified in LGMD2A patients. Seven of these mutations are missense mutations that alter the corresponding amino acids. Another seven mutations create premature stop codons or frame shifts that lead to a truncated form of the protein. An acceptor splice site mutation (AG→AA) was found at the junction of exons 6 and 7 causing aberrant splicing and premature termination[45]. Other forms of LGMD primarily resulted from genetic defects of one or more structural proteins. Mutations in any of the α-, β-, γ- and δ-sarcoglycan genes result in the loss of that sarcoglycan and in structural abnormality of myofibril leading to the corresponding form of muscular dystrophy LGMD2C-F (Ref. 44). Mutations in another two structural proteins, caveolin-3 and dysferlin, are found to be responsible for LGMD1C and LGMD2B, respectively. Caveolin-3 and dysferlin both are plasma membrane proteins. Caveolin-3 is believed to play a role in cellular signal transduction, whereas dysferlin is involved in membrane fusion[44]. Unlike these structural proteins, calpain 3 is a proteolytic enzyme. Results of studies have indicated that a functional rather than structural defect of p94 is responsible for the pathogenesis of LGMD2A, namely the loss of calpain 3 proteolytic activity (against a potential substrate, fodrin) is associated with missense *capn3* mutants[44,46]. Others demonstrated that vimentin is an *in vitro* substrate for the LP82 form of calpain 3 (Ref. 47). Understanding the relevant endogenous substrates for p94 could be the key to elucidating the mechanism that causes LGMD2A.

Box 1. Calpain 1 and 2 in cerebral ischemia, traumatic brain injury and Alzheimer's disease



In cerebral ischemia and traumatic brain injury, decreased blood flow to affected brain areas results in increases in presynaptic vesicular glutamate release and inhibition of glutamate re-uptake by adjacent astrocytes. The resultant excessive build up of glutamate overactivates ionotropic glutamate receptors (NMDA, AMPA and kainate receptors) in the postsynaptic membrane and sustained influx of Na^+ and Ca^{2+} through these receptors. Because of membrane depolarization, voltage-gated neuronal Ca^{2+} channels open, enabling Ca^{2+} influx. The net result is a massive calcium overload, which activates several calcium-dependent enzymes, especially calpain 1 and 2 (Ref. a). Overactivated calpain could lead to uncontrolled degradation of cytoskeletal proteins, cytosolic and nuclear enzymes and, ultimately, neuronal death. Importantly, calpain 1 and 2 contribute to both apoptosis and oncotic necrosis in injured neurons[b]. Thus, in both neuronal oncosis and apoptosis, calpain-mediated proteolysis undoubtedly plays a role in disabling the neurons in the signal transduction, membrane and cytoskeleton integrity and nuclear function.

In Alzheimer's disease (AD), chronic toxicity of aggregated amyloid β peptides (Aβ1–40, Aβ1–42) together with build up of glutamate could lead to sustained intracellular calcium elevation in susceptible central neurons and thus calpain 1 and 2 activation[c,d]. Thus, calpain is a probable contributor of both necrotic and apoptotic neuronal cell death directly, as mentioned above. Furthermore, three groups demonstrated that calpain 1 and 2 are the proteases involved in the conversion of cdk5 activator protein p35 to p25 *in vitro* and in cell cultures[e–g]. Taniguchi recently showed that p35 is extremely sensitive to postmortem modification by calpain, so caution should be exercised[g]. Nevertheless, Nath and colleagues also showed increased p35→p25 conversion in ischemia- or malonate-injured brains when compared to controls[e]. Thus, based on this hypothesis, p25-activated cdk5 probably results in hyperphosphorylated tau, which probably leads to NFT formation and neurodegeneration in AD.

References

a Wang, K.K. and Yuen, P.W. (1994) Calpain inhibition: an overview of its therapeutic potential. *Trends Pharmacol. Sci.* 15, 412–419
b Wang, K.K. (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci.* 23, 20–26
c Saito, K. *et al.* (1993) Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 90, 2628–2632
d Grynspan, F. *et al.* (1997) Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease. *Brain Res.* 763, 145–158
e Lee, M.S. *et al.* (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature* 405, 360–364
f Kusakawa, G. *et al.* (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. *J. Biol. Chem.* 275, 17166–17172
g Nath, R. *et al.* (2000) Processing of cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells. *Biochem. Biophys. Res. Commun.* 274, 16–21

Calpain 2 and lens-specific splice variants of calpain 3 in cataracts

In the aging mammalian lens, accumulation of various insults such as those caused by ultra-violet radiation, high sugar levels (diabetes), environmental toxins, free radicals and drugs such as corticosteroids lead to compromised membrane protein and lipid integrity and increased membrane ion permeability in the lens fiber cells[48]. In normal lens fiber cells, crystallin proteins (α, β and γ) are highly concentrated and organized to allow light transmission. Calpain 2 is activated in several experimental rodent cataracts, resulting in fragmentation and precipitation of α- and β-crystallin proteins and hence lens opacity (cataract)[49]. In rodents, lens-specific splice variants of calpain 3, Lp82 (and probably Lp85) are activated in experimental cataracts[18,50]. However, Lp82 and Lp85 are not present in humans, thus are not relevant in contributing to human cataract formation. Cataract can in fact be modeled by subjecting cultured young

Outstanding questions

- How many more calpain genes are there in the human genome?
- What level of redundancy exists for mammalian calpains?
- What is the function of calpain 5 and is the related calpain 6, which lacks protease activity, has dominant-negative function opposing calpain 5?
- What is the key substrate(s) for calpain 9 and what is its relationship to gastric cancer?
- What is the key substrate(s) for calpain 10 and what is its relationship to type 2 diabetes?
- Does calpastatin have additional functions besides inhibiting calpains?

rat lens to chemical oxidants (e.g. sodium selenite and diamide) or calcium-ionophoric agents (e.g. A23187)[51]. The β-crystallin and α-spectrin fragmentation pattern in culture lens subjected to A23187 treatment is similar to that formed *in vitro* by calpain digestion[49]. Experimental cataract can also be chemically induced *in vivo* [e.g. with bolus administration of sodium selenite (20 μmolkg^{-1})] in young rats[52]. In such models, calpain-mediated β-crystallin and α-spectrin fragmentation has been reported. In fact, N-terminal sequencing revealed that the *in vitro* calpain cleavage sites of β-crystallins matched those found *in vivo*[49].

Shearer and colleagues[52] have shown that the calpain inhibitors E64 (5 μM to 1 mM) and MDL28170 (500 μM) reduced opacity and prevented the insolubility of β-crystallin. In a later study, they found that the membrane-permeable E64d was more potent (5–20 μM) than E64 in reducing A23187-induced opacity in rat lens[52]. Sanderson *et al.*[53] also reported that these two compounds could inhibit cytoskeletal protein degradation in A23187-treated lens but not against opacity formation. Most recently, Fukiage *et al.*[54] documented that the novel calpain inhibitor SJA6017 is superior to E64 in both reducing spectrin breakdown and lens opacity in the A23187-treated rat lenses. The effects of E64 on selenite cataract formation have also been investigated *in vivo* in rat. E64 was given as intraperitoneal injection (100 mgkg^{-1}) two hours before selenite was given and the same daily dose was continued for 5 days. In this model, E64 temporarily reduced the frequency of the most severe stage of cataract, namely, nuclear cataract[52]. It should be noted that in the E64-treated group, β-crystallin breakdown continued to occur at a slower rate suggesting that calpain activity was not fully inhibited. Together, these data point to the potential of using calpain inhibitors to slow cataract progression in humans.

Calpain 9 and gastric cancer

Many gene products associated with carcinogenesis are substrates of calpain family enzymes, which include products of oncogenes and tumor suppressor genes, notably c-fos, c-jun, p53, pp60src, the estrogen receptor and the adhesion molecule integrin[36]. Neurofibromatosis type 2 (NF2) protein, a tumor suppressor implicated in schwannomas and meningiomas, is also calpain-sensitive[55]. A growing body of literature has implicated the role of calpain in various aspects involved in carcinogenesis, including cell-cycle progression, cellular differentiation and apoptosis[10,56]. Association between abnormal calpain activity and tumorigenesis has been observed in several studies. For example, *capn1* expression is correlated with increased malignancy in renal cell carcinoma[57]. In breast cancer tissues, activities of calpain were significantly higher compared with those of normal breast tissues, and were higher in the ER-positive tumors than in ER-negative ones[58]. A recent study has reported that expression of calpain 9 was downregulated in gastric cancer tissues and cell lines of both differentiated and poorly differentiated type[21]. Independently, depletion of calpain 9 by antisense RNA strategy resulted in cellular transformation of and tumorigenesis by murine NIH 3T3 fibroblasts[59]. These results suggest that calpain 9 might be a new type of tumor suppressor, probably acting through proteolytic degradation of certain digestive track-specific oncogene products. Thus, identification of endogenous substrate(s) of calpain 9 might help to define underlying mechanisms in the development of gastric cancer.

Calpain 10 and type 2 diabetes

Diabetes mellitus affects a large population of the world with an estimated 4% prevalence for 1995 and 5.4% for 2025. Recent investigation of Mexican American (and Finnish) population revealed an association between genetic variation in the *capn10* gene and type 2 diabetes susceptibility[30]. A G→A transition was found within intron 3 of *capn10* gene. The polymorphism of the *capn10* gene is associated with decreased mRNA expression in muscles and a decreased rate of insulin-mediated glucose turnover[60]. It would thus suggest that loss of function of calpain 10 leads to type 2 diabetes. Future studies should focus on identifying the physiological substrate(s) of calpain 10.

Perspective and future prospective

Calpain research has reached new heights with recent advances in both discovery of new calpain family members and the molecular understanding of the structure–function relationship of calpain 2. Continued refinement of selective and potent calpain 1 and 2 inhibitors might find applications in neurological and neurodegenerative conditions and possibly human cataracts. Identification of relevant physiological substrate protein(s) for calpain 3, 9 and 10 seems to hold the key to understanding and possible intervention of LGMD2A, gastric cancer and type 2 diabetes. Finally, with the completion of the human genome sequence, one expects additional calpain members to be identified and studied at an even faster pace.

Acknowledgements
We are grateful for the contributions from our colleagues R. Nath, S. Dutta, A. Probert, P-W. Yuen, N. Kupina, X. Ren, G. Schielke and E. Hall, Y. Sun and collaborators K. McGinnis, B. Pike, R. Hays, J. Inou and M. Azuma. We also apologize to many researchers whose relevant work we cannot cite owing to space restrictions.

References

1 Guroff, G. (1964) A neutral calcium-activated proteinase from the soluble fraction of rat brain. *J. Biol. Chem.* 239, 149–155

2 Yoshimura, N. *et al.* (1983) Two distinct Ca^{2+} proteases (calpain I and calpain II) purified concurrently by the same method from rat kidney. *J. Biol. Chem.* 258, 8883–8889

3 Aoki, K. *et al.* (1986) Complete amino acid sequence of the large subunit of the low-Ca^{2+}-requiring form of human Ca^{2+}-activated neutral protease (muCANP) deduced from its cDNA sequence. *FEBS Lett.* 205, 313–317

4 Imajoh, S. *et al.* (1988) Molecular cloning of the cDNA for the large subunit of the high-Ca^{2+}-requiring form of human Ca^{2+}-activated neutral protease. *Biochemistry* 27, 8122–8128

5 Ohno, S. *et al.* (1986) Nucleotide sequence of a cDNA coding for the small subunit of human calcium–dependent protease. *Nucleic Acids Res.* 14, 5559

6 Ohno, S. *et al.* (1984) Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein? *Nature* 312, 566–570

7 Hosfield, C.M. *et al.* (1999) Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. *EMBO. J.* 18, 6880–6889

8 Strobl, S. *et al.* (2000) The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. *Proc. Natl. Acad. Sci. U. S. A.* 97, 588–592

9 Lin, G.D. *et al.* (1997) Crystal structure of calcium bound domain VI of calpain at 1.9 Å resolution and its role in enzyme assembly, regulation, and inhibitor binding. *Nat. Struct. Biol.* 4, 539–547

10 Wang, K.K. (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci.* 23, 20–26

11 Carafoli, E. and Molinari, M. (1998) Calpain: a protease in search of a function? [published erratum appears in *Biochem. Biophys. Res. Commun.* 1998 Aug 19; 249(2): 572]. *Biochem. Biophys. Res. Commun.* 247, 193–203

12 Azam, M. *et al.* (2001) Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol. Cell. Biol.* 21, 2213–2220

13 Sorimachi, H. *et al.* (1989) Molecular cloning of a novel mammalian calcium-dependent protease distinct from both m- and mu-types. Specific expression of the mRNA in skeletal muscle. *J. Biol. Chem.* 264, 20106–20111

14 Sorimachi, H. *et al.* (1993) Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in disappearance from muscle. *J. Biol. Chem.* 268, 10593–10605

15 Ma, H. *et al.* (1998) Cloning and expression of mRNA for calpain Lp82 from rat lens: splice variant of p94. *Invest. Ophthalmol. Vis. Sci.* 39, 454–461

16 Ma, H. *et al.* (2000) Lp85 calpain is an enzymatically active rodent-specific isozyme of lens Lp82. *Curr. Eye Res.* 20, 183–189

17 Azuma, M. *et al.* (2000) Identification and characterization of a retina-specific calpain (Rt88) from rat. *Curr. Eye Res.* 21, 710–720

18 Shearer, T.R. *et al.* (2000) Calpains in the lens and cataractogenesis. *Methods Mol. Biol.* 144, 277–285

19 Sorimachi, H. *et al.* (1993) A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+}-binding domain. *J. Biol. Chem.* 268, 19476–19482

20 Lee, H.J. *et al.* (1998) Molecular cloning and characterization of a novel tissue-specific calpain predominantly expressed in the digestive tract. *Biol. Chem.* 379, 175–183

21 Yoshikawa, Y. *et al.* (2000) Isolation of two novel genes, down-regulated in gastric cancer. *Jpn. J. Cancer Res.* 91, 459–463

22 Dear, T.N. *et al.* (1999) CAPN11: A calpain with high mRNA levels in testis and located on chromosome 6. *Genomics* 59, 243–247

23 Dear, T.N. *et al.* (2000) Gene structure, chromosomal localization, and expression pattern of capn12, a new member of the calpain large subunit gene family. *Genomics* 68, 152–160

24 Sokol, S.B. and Kuwabara, P.E. (2000) Proteolysis in *Caenorhabditis elegans* sex determination: cleavage of TRA-2A by TRA-3. *Genes Dev.* 14, 901–906

25 Mugita, N. *et al.* (1997) Identification of a novel, tissue-specific calpain htra-3; a human homologue of the *Caenorhabditis elegans* sex determination gene. *Biochem. Biophys. Res. Commun.* 239, 845–850

26 Dear, N. *et al.* (1997) A new subfamily of vertebrate calpains lacking a calmodulin-like domain: implications for calpain regulation and evolution. *Genomics* 45, 175–184

27 Matena, K. *et al.* (1998) Genomic organization of mouse Capn5 and Capn6 genes confirms that they are a distinct calpain subfamily. *Genomics* 48, 117–120

28 Franz, T. *et al.* (1999) Capn7: a highly divergent vertebrate calpain with a novel C-terminal domain. *Mamm. Genome* 10, 318–321

29 Sorimachi, H. *et al.* (1997) Structure and physiological function of calpains. *Biochem. J.* 328, 721–732

30 Horikawa, Y. *et al.* (2000) Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat. Genet.* 26, 163–175

31 Braun, C. *et al.* (1999) CAPN 8: isolation of a new mouse calpain-isoenzyme. *Biochem. Biophys. Res. Commun.* 260, 671–675

32 Wang, K.K. and Yuen, P.W. (1994) Calpain inhibition: an overview of its therapeutic potential. *Trends Pharmacol. Sci.* 15, 412–419

33 Chua, B.T. *et al.* (2000) Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. *J. Biol. Chem.* 275, 5131–5135

34 Yamashima, T. (2000) Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. *Prog. Neurobiol.* 62, 273–295

35 Nakagawa, T. and Yuan, J. (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J. Cell. Biol.* 150, 887–894

36 Wang, K.K.W. and Yuen, P-W. (1999) *Calpain: Pharmacology and Toxicology of a Calcium-dependent Cellular Protease*, Taylor and Francis

37 Newcomb, J.K. *et al.* (1997) Immunohistochemical study of calpain-mediated breakdown products to alpha-spectrin following controlled cortical impact injury in the rat. *J. Neurotrauma* 14,-369–383

38 Markgraf, C.G. *et al.* (1998) Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. *Stroke* 29, 152–158

39 Kupina, N.C. *et al.* (2000) Calpain-mediated alpha–spectrin degradation in a mouse model of diffuse head injury and the benefit of the calpain inhibitor SJA6017 on functional recovery. *J. Neurotrauma* 17, 964 (E920)

40 Rami, A. *et al.* (2000) mu-Calpain activation, DNA fragmentation, and synergistic effects of caspase and calpain inhibitors in protecting hippocampal neurons from ischemic damage. *Brain Res.* 866, 299–312

41 Buee, L. *et al.* (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res. Brain Res. Rev.* 33, 95–130

42 Alvarez, A. *et al.* (1999) Inhibition of tau phosphorylating protein kinase cdk5 prevents β-amyloid-induced neuronal death. *FEBS Lett.* 459, 421–426

43 Jordan, J. *et al.* (1997) Role of calpain- and interleukin-1 beta converting enzyme-like proteases in the beta-amyloid-induced death of rat hippocampal neurons in culture. *J. Neurochem.* 68, 1612–1621

44 Ono, Y. *et al.* (1999) New aspect of the research on limb-girdle muscular dystrophy 2A: a molecular biologic and biochemical approach to pathology. *Trends Cardiovasc. Med.* 9, 114–118

45 Richard, I. *et al.* (1995) Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell* 81, 27–40

46 Ono, Y. *et al.* (1998) Functional defects of a muscle-specific calpain, p94, caused by mutations associated with limb-girdle muscular dystrophy type 2A. *J. Biol. Chem.* 273, 17073–17078

47 Nakamura, Y. *et al.* (1999) Decreased sensitivity of lens-specific calpain Lp82 to calpastatin inhibitor. *Exp. Eye Res.* 69, 155–162

48 Azuma, M. *et al.* (1997) Activation of calpain in lens: a review and proposed mechanism. *Exp. Eye Res.* 64, 529–538

49 David, L.L. and Shearer, T.R. (1993) β-crystallins insolubilized by calpain II *in vitro* contain cleavage sites similar to beta-crystallins insolubilized during cataract. *FEBS Lett.* 324, 265–270

50 Nakamura, Y. *et al.* (2000) Contribution of calpain Lp82-induced proteolysis to experimental cataractogenesis in mice. *Invest. Ophthalmol. Vis. Sci.* 41, 1460–1466

51 Azuma, M. *et al.* (1992) Superior prevention of calcium ionophore cataract by E64d. *Biochim. Biophys. Acta* 1180, 215–220

52 Azuma, M. *et al.* (1991) Cysteine protease inhibitor E64 reduces the rate of formation of selenite cataract in the whole animal. *Curr. Eye Res.* 10, 657–666

53 Sanderson, J. *et al.* (1996) Calcium ionophore induced proteolysis and cataract: inhibition by cell permeable calpain antagonists. *Biochem. Biophys. Res. Commun.* 218, 893–901

54 Fukiage, C. *et al.* (1997) SJA6017, a newly synthesized peptide aldehyde inhibitor of calpain: amelioration of cataract in cultured rat lenses. *Biochim. Biophys. Acta* 1361, 304–312

55 Kimura, Y. *et al.* (1998) The involvement of calpain-dependent proteolysis of the tumor suppressor NF2 (merlin) in schwannomas and meningiomas. *Nat. Med.* 4, 915–922

56 Cottin, P. *et al.* (1994) Ca^{2+}-dependent proteinases (calpains) and muscle cell differentiation. *Biochim. Biophys. Acta* 1223, 170–178

57 Kamei, M. *et al.* (1998) SOLH, a human homologue of the *Drosophila melanogaster* small optic lobes gene is a member of the calpain and zinc-finger gene families and maps to human chromosome 16p13.3 near CATM (cataract with microphthalmia). *Genomics* 51,197-206

58 Braun, C. *et al.* (1999) Expression of calpain I messenger RNA in human renal cell carcinoma: correlation with lymph node metastasis and histological type. *Int. J. Cancer* 84, 6–9
59 Shiba, E. *et al.* (1996) Ca^{2+}-dependent neutral protease (calpain) activity in breast cancer tissue and estrogen receptor status. *Breast Cancer* 3, 13–17
60 Liu, K. *et al.* (2000) Antisense RNA-mediated deficiency of the calpain protease, nCL-4, in NIH3T3 cells is associated with neoplastic transformation and tumorigenesis. *J. Biol. Chem.* 275, 31093–31098
61 Baier, L.J. *et al.* (2000) A calpain-10 gene polymorphism is associated with reduced muscle mRNA levels and insulin resistance. *J. Clin. Invest.* 106, R69–73
62 Saito, K. *et al.* (1993) Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 90, 2628–2632
63 Grynspan, F. *et al.* (1997) Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease, *Brain Res.* 763, 145–158
64 Lee, M.S. *et al.* (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature* 405, 360–364
65 Kusakawa, G. *et al.* (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. *J. Biol. Chem.* 275, 17166–17172
66 Nath, R. *et al.* (2000) Processing of cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells. *Biochem. Biophys. Res. Commun.* 274, 16–21
67 Taniguchi, S. *et al.* (2001) Calpain-mediated degradation of p35 to p25 in postmortem human and rat brains. *FEBS Lett.* 489, 46–50

Clinical and genetic heterogeneity in nemaline myopathy – a disease of skeletal muscle thin filaments

Despina Sanoudou and Alan H. Beggs

The term nemaline myopathy (NM) encompasses a heterogeneous group of disorders of primary skeletal muscle weakness characterized by the presence of nemaline rods in muscles of affected individuals. Disease severity is variable and unpredictable, with prognosis ranging from neonatal death to almost normal motor function. Recent advances in the identification of NM disease genes demonstrate that NM is a disease of the skeletal muscle sarcomere and, in particular, of the thin filaments. These findings are starting to alter the approach that neurologists and geneticists take to diagnosing and counseling patients with NM, and could lead to insights into specific directed therapies in the future.

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Nemaline myopathy (NM) is a slowly- or non-progressive neuromuscular disorder characterised by muscle weakness and the presence of rod-shaped structures [NEMALINE RODS (see Glossary)] in affected muscle fibers (reviewed in Ref. 1). NM was first described in 1963 by Conen *et al.*[2] and Shy *et al.*[3] and its name reflects the perceived thread-like appearance of the rod bodies (nema being the Greek word for thread). Although a relatively rare disease, it is the most common of the non-dystrophic CONGENITAL MYOPATHIES, occurring worldwide with an estimated incidence of 0.02 per 1000 live births[4]. Many cases are sporadic, but some exhibit either autosomal recessive or dominant patterns of inheritance. As with most other congenital myopathies, NM is pathologically defined on the basis of structural abnormalities of the muscle fibres, which can be visualized after staining of muscle biopsy sections by histochemical or electron microscopic methods. Recent progress in the identification of five different NM genes has shown that, despite a great degree of both clinical and genetic heterogeneity, the common pathological findings are related to the fact that each NM gene encodes a known component of skeletal muscle sarcomeric THIN FILAMENTS.

Clinical description

The nemaline myopathies are defined by primary proximal muscle weakness associated with a myopathic muscle biopsy, characterized by the presence of nemaline rods, and the absence of clinical or pathological findings diagnostic of other disorders. The wide range of clinical presentations represents a continuum from neonatal-lethal forms to late onset slowly progressive weakness[5]. However, to facilitate further study, including potential phenotype–genotype correlation, some categorization has been attempted[6,7] (Table 1).

Typical NM

The typical (i.e. most common) form of NM is usually autosomal recessive and presents with congenital or infantile HYPOTONIA, weakness and often, feeding difficulties[4,8] (Table 1). In cases with profound weakness and hypotonia in the neonatal period, strength often improves with age, leading to delayed attainment of gross motor skills. Patients have a narrow face with a high arched palate, reflecting bulbar weakness, and a lean build, sometimes associated with muscular atrophy. Although fine motor activity is normal, gross motor activity is impaired. Proximal muscles are generally more affected than distal ones, although distal weakness, especially later in life, is not

 1471-4914/01/$ – see front matter PII: S1471-4914(01)02089-5