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Truncating Neurotrypsin Mutation in Autosomal Recessive Nonsyndromic Mental Retardation

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A 4–base pair deletion in the neuronal serine protease neurotrypsin gene was associated with autosomal recessive nonsyndromic mental retardation (MR). In situ hybridization experiments on human fetal brains showed that neurotrypsin was highly expressed in brain structures involved in learning and memory. Immunoelectron microscopy on adult human brain sections revealed that neurotrypsin is located in presynaptic nerve endings, particularly over the presynaptic membrane lining the synaptic cleft. These findings suggest that neurotrypsin-mediated proteolysis is required for normal synaptic function and suggest potential insights into the pathophysiological bases of mental retardation.

Moderate to severe mental retardation (IQ<50) affects 0.3 to 0.8% of the population and its prevalence increases up to 2% if mild MR is included (50<IQ<70) (*1*). The possible causes of MR are diverse and include environmental factors or teratogens, chromosomal anomalies and metabolic diseases impairing neuronal function or brain patterning during development (*2*).

By contrast, MR with apparently normal brain development and no other clinical features (i.e., nonsyndromic MR) represents the most common cognitive dysfunction and remains poorly understood. In fact, the broad genetic heterogeneity of MR and the scarcity of large pedigrees have hitherto hampered linkage analyses in nonsyndromic MR. Indeed, although 10 nonsyndromic X-linked MR genes have been found (*3–12*), none of the genes causing nonsyndromic autosomal recessive MR has been identified so far.

We have investigated a sibship of four mentally retarded (three girls and one boy) and four healthy children born to first cousin Algerian parents (family 1, fig. S1A). Cognitive impairment and a low IQ (below 50) were consistent features in the four affected sibs. A genome-wide screen that used 400 microsatellite markers identified a single region of shared homozygosity on chromosome 4q24–q25 in the four affected sibs. Further marker typing and haplotype analysis reduced the genetic interval to 18 cM (14 Mb) between markers D4S1570 and D4S1575 (Z_{max} = 3.33, at θ = 0 at D4S407 locus) (fig. S1A). This interval encompasses about 29 genes of known function including the *DKK2*, *RPL34*, *CASP6*, *ANK2*, *CAMK2D*, *TRPC3*, and *PRSS12* genes (*13–19*) (fig. S1B).

Neurotrypsin (PRSS12) is a secreted protein of 875 amino acids, belonging to the subfamily of trypsin-like serine proteases. This gene has been identified in a polymerase chain reaction (PCR)–based search for serine proteases that are expressed in the brain (*20*). Neurotrypsin was regarded as a strong candidate gene by both position and function, as expression studies in mouse embryos suggested its likely involvement in synapse maturation and neural plasticity (*21*). The genomic structure of the human neurotrypsin gene was identified by alignment of the cDNA sequence (GenBank accession number: NM_003619) with the sequence of PAC clone 265B3 (GenBank accession number: AC073025). Thirteen exons were identified, and primers were designed for direct sequencing of all coding exons and exon-intron junctions (*22*).

Homozygosity for a 4–base pair (bp) deletion in exon 7 of the *PRSS12* gene was detected in patient II.8 (fig. S2). This deletion disrupted an Aat II restriction site and resulted in a premature stop codon 147 nucleotides downstream of the deletion. Restriction analyses showed cosegregation of the mutation with the disease in all affected individuals, and that both parents were heterozygous (Fig. 1A). Reverse transcription PCR (RT-PCR) analysis of lymphoblastoid cell line RNAs from patient II.8 revealed a slightly reduced amount of mutant neurotrypsin transcript carrying the deletion (*23*). This mutation was not found in 200 unrelated control individuals (100 from Maghrebian and 100 from various ethnic origins).

To estimate the frequency of neurotrypsin mutations, the coding sequence of the gene was screened by DHPLC and direct DNA sequencing in 17 nonsyndromic inbred MR families and 23 mentally retarded children. The same 4-bp deletion was found in another child born to first cousin Algerian parents, apparently unrelated to family 1 but originating from the same area of Eastern Algeria. The course of the disease in this child was very similar to that of affected indi-

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viduals in family 1, as this boy also had normal milestones of psychomotor development in his first 2 years and then became mentally retarded after 2 years of age. The 4-bp neurotrypsin mutation segregated with the disease (Fig. 1B) and was carried on the background of the same haplotype across the *PRSS12* locus (D4S2989 to D4S1575) suggesting either distant consanguinity or a founder effect in the Algerian population.

In order to better understand the function of neurotrypsin, we studied the temporo-spatial expression of the gene by in situ hybridization in fetal human brain. No signal was detected in 32-day-old embryos (*23*). The neurotrypsin gene was expressed in the developing brain from 44 days to 15 weeks of development. At 47 days, prominent expression was seen in the isthmic basal plate, the choroid plexus, the trigeminal nerve, and the facial-statoacustic ganglia (*23*). At 15 weeks, the highest gene expression was detected in the cortical plate, the hippocampal formation, and the tegmental nuclei of the brainstem (Fig. 2). In addition, a weak postero-lateral signal was detected in the thalami. In the hippocampal formation, neurotrypsin was expressed in a spatial gradient. Staining was moderate in the subiculum and reached very high levels in areas CA3 and CA4 of Ammon's horn (cornu ammonis). No staining was detected in the granular layer of the dentate gyrus. In the cortical plate, the gene expression was restricted to the superficial layer (prospective layer II), which contains late migrating neurons. Neurotrypsin expression was also detected in the intermediate zone of the cerebral mantle, a region containing late migrating neurons considered to be at the origin of some interneurons. It is worth remembering that interneurons play a pivotal role in the establishment of neuronal connections and cognitive functions. No expression was detected in the spinal cord. Northern blot analysis of fetal human tissues revealed the strongest hybridization signal with brain mRNA, whereas a faint signal was detected with lung and kidney mRNA (fig. S3). No neurotrypsin transcript was detected in the liver. These results are consistent with those obtained in mouse and suggest that neurotrypsin is mainly expressed in the cerebral cortex and the motor nuclei of the brain stem.

Finally, the expression of neurotrypsin in adult human brain was investigated by immunohistochemistry with both light and electron microscopy. Affinity-purified antibodies raised against its proteolytic domain were used for specific neurotrypsin detection. With light microscopy, we found a high density of neurotrypsin-immunoreactive synaptic boutons in the cortical neuropil (Fig. 3A). A thorough examination by electron-microscopy using peroxidase-conjugated secondary antibodies revealed strong neurotrypsin immunoreactivity in the presynaptic nerve ending of cortical synapses; the most prominent immunoreactivity was found over the presynaptic membrane lining the synaptic cleft (Fig. 3B). With gold-labeled secondary antibodies, the neurotrypsin immunoreactivity was most prominent in a subpopulation of presynaptic vesicles located adjacent to the presynaptic membrane. (Fig. 3, C to F). It appears, therefore, that neurotrypsin is a presynaptic protein in the adult human brain.

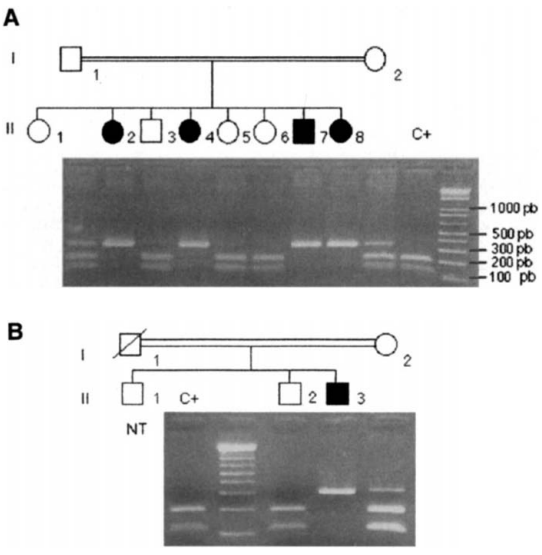

Fig. 1. Mutational analysis of neurotrypsin. (**A**) Simplified pedigree and restriction analysis of the amplified neurotrypsin exon 7. The mutation abolished an Aat II site so that the amplified 370-bp fragment could not be cleaved into the 235-bp and the 135-bp fragments. (**B**) Simplified pedigree and restriction analysis of the amplified neurotrypsin exon 7 in family 2.

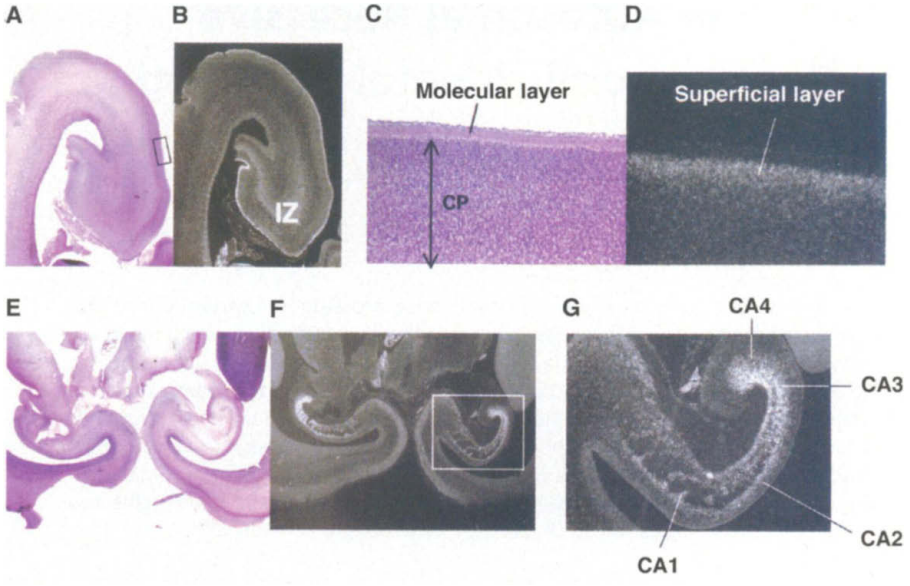

Fig. 2. Neurotrypsin gene expression in the 15-week-old fetal human brain. Sections counterstained with hematoxylin and eosin are shown under bright-field (**A**, **C**, **E**) and dark-field illumination to reveal the localization of the in situ signal (**B**, **D**, **F**, **G**). The expression in the cortex (A to D) and the hippocampus (E to G) is shown. IZ, Intermediate zone; CP, cortical plate.

Impaired proteolysis at the synaptic level has never been considered a possible mechanism in mental retardation. By contrast, an increasing number of studies support a role of extracellular proteases and their inhibitors in the regulation of synaptic plasticity in the developing and adult central nervous system (*24*). Best documented is the role of tissue plasminogen activator (tPA) in adult synaptic function. Qian *et al.* (*25*) reported a marked increase of tPA transcription as an "immediate early gene" concurrent with the induction of long-term potentiation (LTP) in the rat hippocampus. Likewise, an enhanced transcription of tPA mRNA was observed in cerebellar Purkinje neurons when rats were trained to master complex motor tasks (*26*). In line with these results, tPA-deficient mice performed poorly in two-way avoidance tests and exhibited a perturbance in long-lasting LTP (*27*, *28*) An involvement of other serine proteases (thrombin and neuropsin) in adult neural plasticity has recently been demonstrated. Indeed, thrombin has been shown to potentiate the function of the *N*-methyl-D-aspartate receptor (*29*). Abnormalities of synapses and neurons have been observed in neuropsin-deficient mice (*30*). Likewise, the study of serine protease inhibitors supports the role of nexin-1 and neuroserpin in synaptic function. For ex-

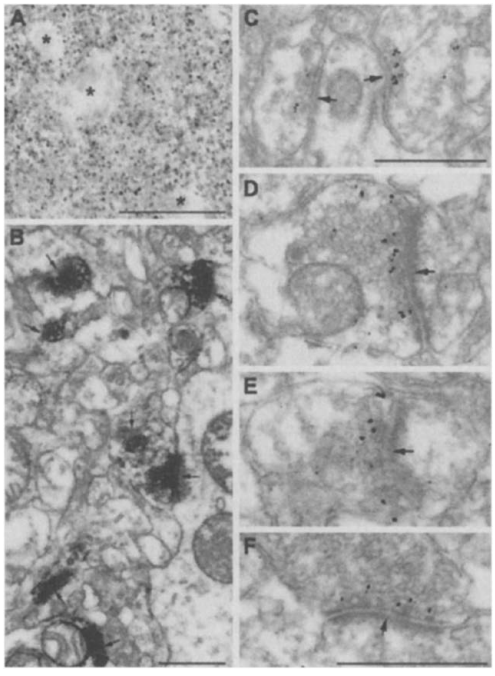

Fig. 3. Immunolocalization of neurotrypsin. Neurotrypsin was visualized in adult human cerebral cortex by using pre-embedding staining with a specific antibody against the proteolytic domain of neurotrypsin. (**A**) Immunohistochemical visualization of neurotrypsin with peroxidase-conjugated second antibody. The strong punctate immunolabeling of the neuropil is typical for a protein with synaptic localization. Neuronal somata (marked by asterisks) were unlabeled. (**B**) Preembedding immuno-EM localization by immunoperoxidase demonstrates the localization of neurotrypsin at presynaptic sites of axospinous and axodendritic asymmetric synapses in the cerebral cortex. The immunoperoxidase reaction product is associated with the presynaptic membrane and the active zone of the presynaptic terminal (arrows). (**C** to **F**) Immunogold localization of neurotrypsin at selected synapses. Note the exclusive labeling of presynaptic terminals in the region lining the synaptic cleft. Scale bars: (A), 100 μm; (B to F), 0.5 μm.

ample, both overexpression and inactivation of protease nexin-1, a serine protease inhibitor of the serpin family, altered hippocampal LTP (*31*). These data suggest that the balance between proteolytic enzymes and their inhibitors is crucial for the regulation of neural plasticity. In this context, neurotrypsin is an excellent candidate as a regulator of synaptic development and/or function, on the basis of its localization in presynaptic terminals; its strong expression in many brain areas during neural development; and its strong expression in adult brain, particularly in areas that are involved in learning and memory.

The results reported here emphasize the crucial role of synaptic proteolysis in higher brain function and open a novel field in the pathophysiology of mental retardation.

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Materials and Methods

Fig. S1 to S3

References

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Neurotoxicity and Neurodegeneration When PrP Accumulates in the Cytosol

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Changes in prion protein (PrP) folding are associated with fatal neurodegenerative disorders, but the neurotoxic species is unknown. Like other proteins that traffic through the endoplasmic reticulum, misfolded PrP is retrograde transported to the cytosol for degradation by proteasomes. Accumulation of even small amounts of cytosolic PrP was strongly neurotoxic in cultured cells and transgenic mice. Mice developed normally but acquired severe ataxia, with cerebellar degeneration and gliosis. This establishes a mechanism for converting wild-type PrP to a highly neurotoxic species that is distinct from the self-propagating PrP^{Sc} isoform and suggests a potential common framework for seemingly diverse PrP neurodegenerative disorders.

Prion diseases are rare and inexorably fatal neurodegenerative disorders that can appear in sporadic, dominantly heritable, and transmissible forms (*1*). These diseases have unusually complex etiologies, and decades of research have failed to elucidate the pathogenic mechanism (*2–5*). Nonetheless, the prion protein PrP plays a pivotal role (*6*, *7*). A rare form of PrP with an altered protease-resistant conformation, PrP^{Sc}, is widely believed to be the infectious agent (or to constitute the major component of it) in transmissible forms of disease (*1*). But increasing evidence suggests that PrP^{Sc} is not itself neurotoxic. PrP^{Sc} is not observed in several inherited and experimentally induced forms of prion disease (*8–11*). And PrP knockout mice

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