Clinical/Scientific Notes

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HYPEREKPLEXIA CAUSED BY DOMINANT-NEGATIVE SUPPRESSION OF GLYRA1 FUNCTION

Hyperekplexia (HE; startle disease; OMIM #149400) is a rare inheritable neurologic disorder characterized by an exaggerated response to sudden stimuli, muscular rigidity, and hyperreflexia, leading to chronic injuries due to unprotected falls. All symptoms are present at birth but gradually decline during the first year of life, although an exaggerated startle response remains during adulthood.[1]

Dysfunctional inhibitory neurotransmission by glycine (Gly) plays a central role in HE pathogenesis. All patients with HE carry mutations in genes encoding either for α_1 (GLYRA1) or β (GLYRB) Gly receptor subunits, presynaptic Gly transporters (SLC6A5), or proteins involved in Gly receptor (GLYR) clustering, such as gephyrin (GPHN) and collybistin (ARHGEF9).[1,2]

GLYRA1 subunits interact in a heteropentameric complex with homologous β subunits ($3\alpha_1 2\beta$). Each subunit comprises an extracellular N-terminus, four α-helical transmembrane segments (TM_1 to TM_4), and an extracellular C-terminus; TM_2 segments are thought to align the Cl^- selective pore.[1]

Different GLYRA1 missense and nonsense mutations have been associated with autosomal dominant, autosomal recessive, and sporadic forms of HE. Missense mutations may show incomplete penetrance and impair channel gating, trafficking, and stability.[3] Nonsense mutations have been reported only in recessive cases, in compound heterozygosity with a missense mutation, or in homozygosity in the offspring of consanguineous nonaffected parents and appear unable to cause the startle phenotype in heterozygosis.[4,5] A recessive form of HE linked to a GLYRA1 null allele has been described,[6] suggesting a nonpathogenic role for haplotype insufficiency.

In this study, we investigated the functional consequences of a de novo GLYRA1 heterozygous nonsense mutation (S296X) in a 1-year-old boy. The patient was born at term after an uneventful pregnancy and delivery; after 1 week, he had high-frequency attacks of generalized stiffness appearing spontaneously or elicited by sensorial stimuli; attacks were not associated with EEG alterations or unconsciousness. Symptoms were successfully treated with valproic acid. Drug treatment was suspended after 6 months; since then, the baby shows normal psychomotor development and is symptom free. Neurologic evaluation and clinical history of the parents were unremarkable.

Molecular analysis of the GLYRA1 gene revealed a heterozygous point mutation in exon 7 (nt c1267a) in the patient, but not in his unaffected parents or in 50 unrelated controls (see methods on the *Neurology* Web site at www.neurology.org for further experimental details). The mutation truncated the protein at codon 296 (S296X), within the TM_3 domain (figure, A).

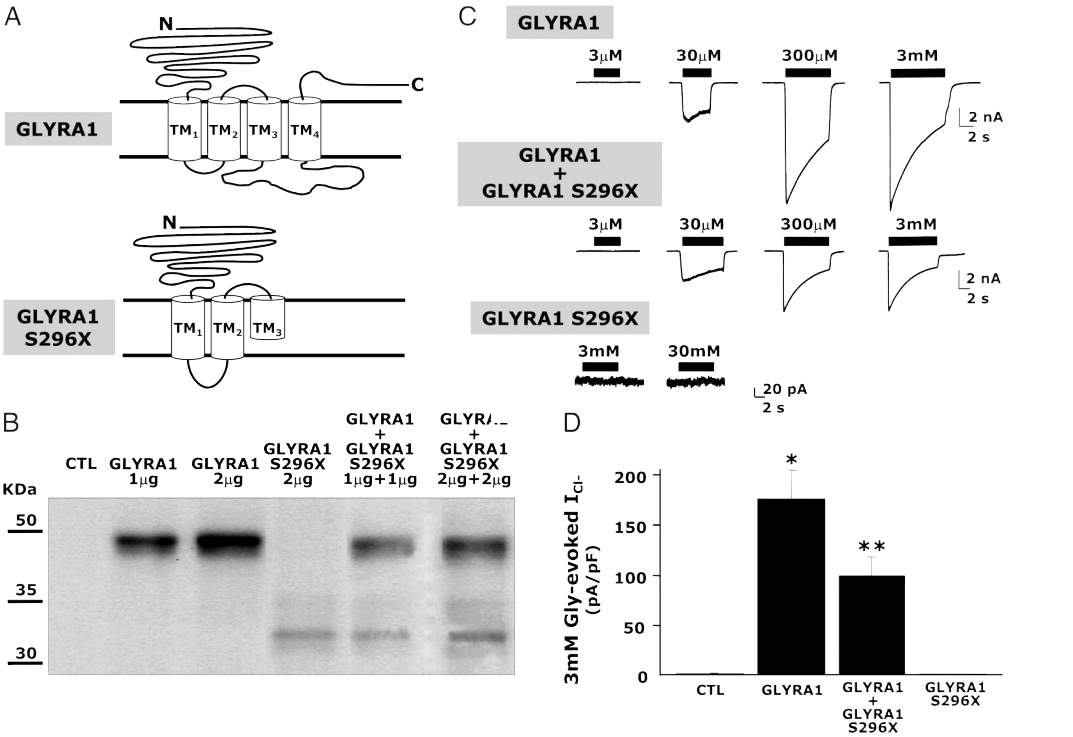
The functional consequences of this mutation were evaluated by biochemical and electrophysiologic techniques upon heterologous expression in HEK-293 cells. In Western blots from total cell lysates and plasma-membrane fractions from GLYRA1-transfected cells, the mAb4a antibody revealed a 48-kd band; by contrast, a 34-kd band, corresponding to truncated subunits, was observed in GLYRA1 S296X–transfected cells. When the two subunits were coexpressed, both total lysates (data not shown) and membrane fractions showed a reduction in the 48-kd band intensity, whereas that of the 34-kd band was unchanged (figure, B).

In whole-cell path-clamp electrophysiologic experiments, cells expressing GLYRA1 subunits exhibited large Gly-gated Cl^- currents; by contrast, in GLYRA1 S296X–transfected cells, Gly failed to elicit Cl^- currents (figure, C). In cells coexpressing GLYRA1 and GLYRA1 S296X subunits, Gly-gated currents were smaller when compared with GLYRA1-expressing cells (figure, C and D), with no significant change in Gly apparent affinity between the two groups of cells.

These results are suggestive of a significant interaction between wild-type and mutant GLYRA1 subunits, leading to a dominant-negative effect. Ligand-gated channels assemble cotranslationally in the endoplasmic reticulum (ER), where they un-

Supplemental data at www.neurology.org

Figure Predicted topology and functional consequences of GLYRA1 subunits carrying the S296X mutation



(A) Presumed topologic arrangement of wild-type and mutant GLYRA1 receptor subunits. (B) Western blot experiment in purified plasma membrane fractions from HEK-293 cells. Bands of 48 kd and 34 kd, corresponding to GLYRA1 and GLYRA1 S296X subunits, were revealed by the mAb4a monoclonal antibody in streptavidin-isolated biotinylated plasma membrane proteins from HEK-293 cells transfected with GLYRA1, GLYRA1 S296X, or both cDNAs, as indicated. The optical density of the 48-kd band in each group, normalized to the values obtained in HEK-293 cells transfected with 2 μg of cDNA, was 0.63 ± 0.23 (GLYRA1, 1 μg), 1.00 ± 0.19 (GLYRA1, 2 μg), 0.22 ± 0.11 (GLYRA1+GLYRA1 S296X, 1 μg + 1 μg), and 0.32 ± 023 (GLYRA1+GLYRA1 S296X, 2 μg + 2 μg). Data are the mean ± SEM from four separate experiments. (C) Representative whole-cell currents evoked by increasing concentration of glycine (Gly) in HEK-293 cells expressing GLYRA1, GLYRA1 S296X, or both subunits. The holding potential was −60 mV. The duration of Gly perfusion is indicated by the length of the bars on top of each current trace. Concentration-response curves for Gly (0.3 μM to 30 mM) were obtained for each group by averaging from different cells the peak inward current values evoked by each Gly concentration, after normalization to the maximal value. The data from the two groups (cells expressing GLYRA1, or GLYRA1+GLYRA1 S296X subunits) were then fitted to the following form of the Hill equation $y = y_{max}([Gly]n_H/[Gly]n_H + EC_{50}n_H)$. EC_{50} and n_H values were 56.8 ± 4.2 μM and 1.2 ± 0.1 for GLYRA1-expressing cells (0.2 μg) and 40.3 ± 1.3 μM and 1.5 ± 0.1 for GLYRA1+GLYRA1 S296X–expressing cells (0.2 μg + 0.2 μg) (n = 12 different cells per group). (D) Quantification of the Cl^- current density induced by 3 mM Gly in HEK-293 cells transfected with GLYRA1 (0.2 μg) or GLYRA1 S296X (0.2 μg) cDNAs, alone or together as indicated. Current densities were calculated in each cell by dividing by membrane capacitance the peak current value evoked by 3 mM Gly. At least 30 cells for each group were recorded from four different transfections. *Value significantly different from controls. **Value significantly different from GLYRA1-transfected cells. CTL = control.

dergo folding and post-translational modifications. S296X GLYRA1 subunits lack the M_3-M_4 loop regulating GlyR membrane concentration,[7] but retain two disulfide bonds (C138-C152, C198-C209), which stabilize the N-terminal region and allow the channel to exit from the ER. Therefore, S296X GLYRA1 subunits likely retain the molecular determinants allowing oligomerization with wild-type GLYRA1 subunits; this interaction suppresses GLYRA1 membrane expression and function.

Two different GLYRA1 nonsense mutations (Y202X[5] and R344X[4]) have been reported in recessive forms of HE, suggesting that both of these mutant alleles failed to exert a clinically significant dominant effect. The Y202X mutation was found in a child of consanguineous parents, whereas the R344X mutation was found in compound heterozygosity with a missense mutation (W96C). Truncated GLYRA1 Y202X subunits lack some of the molecular determinants required to assume a correct topology and are unlikely to assemble into oligomers and exit from the ER. Conversely, the R344X mutation lies in the large positively charged M_3-M_4 intracellular loop; neutralization of all positive charges within this region affects subunit topology.[7]

In conclusion, biochemical and electrophysiologic analyses suggest that GLYRA1 S296X subunits impair GLYRA1 subunit function, thus representing the first description of a GLYRA1 het-

erozygous nonsense mutation causing the startle phenotype by a dominant-negative mechanism.

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SPONTANEOUS TIBIALIS ANTERIOR TENDON RUPTURE: A RARE CAUSE OF FOOT DROP

Tibialis anterior tendon (TAT) rupture is an uncommon musculoskeletal condition that is rarely encountered by neurologists.[1] Most patients present with foot drop and pain and may be misdiagnosed as a peroneal mononeuropathy or L5 radiculopathy. Clinical recognition and distinction of this entity from other causes of foot drop are important for neurologists. We describe two patients with spontaneous TAT ruptures who presented for neurologic consultation for suspected peroneal neuropathies.

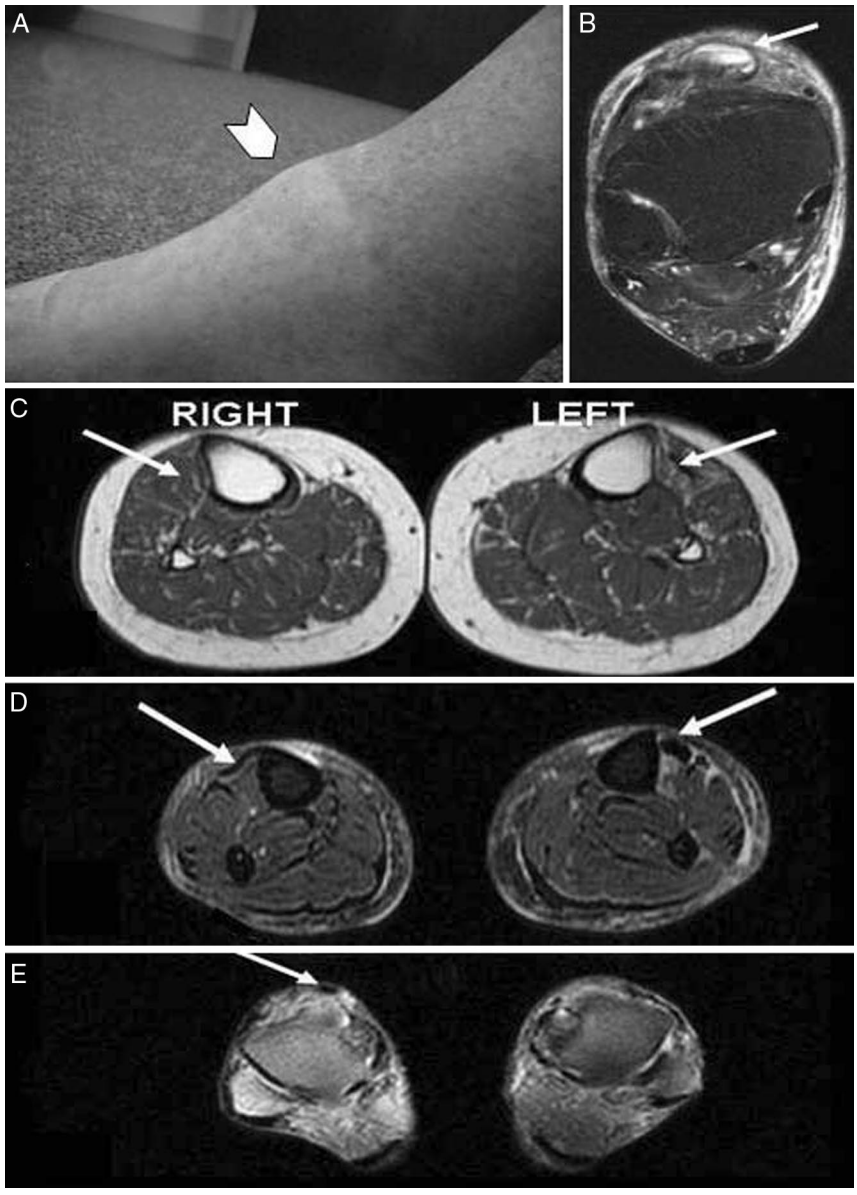
Case 1. A 73-year-old man with a history of atrial fibrillation and hypertension was walking on his treadmill when he noted a painless "pop" in his right anterior ankle followed by "flopping" of his foot. A few days later, he noted swelling on the anterior aspect of his ankle and difficulty dorsiflexing his right foot. He denied back pain and bladder dysfunction. Neurologic examination demonstrated mild weakness in right ankle dorsiflexion with normal strength in all other muscles including toe extension and ankle eversion and inversion. There was swelling on the anterior surface of the right ankle (figure, A). The extensor hallucis longus and digitorum longus tendons were intact, but the TAT was not palpable at its insertion site.

Nerve conduction studies, including right peroneal motor, were normal for age. Needle EMG demonstrated residual of an old right L5 radiculopathy, but examination of the TA muscle demonstrated a normal recruitment pattern of motor unit potentials. MRI of the right ankle demonstrated a complete tear of the TAT at the level of the extensor retinaculum. A small fluid collection was seen in the region of the tendon sheath. (figure, B) The patient elected conservative therapy.

Case 2. A 79-year-old woman with hypertension, hyperlipidemia, and coronary artery disease was evaluated for an 8-week history of a left foot drop. The precise onset of the foot drop was not known. She denied precipitating trauma, sudden foot or back pain, progressive weakness, and numbness. Examination demonstrated weakness of the left TA muscle with normal strength in all other lower extremity muscles and a steppage gait. Reflexes and sensory examination were normal. Orthopedic consultation noted retraction of the left TAT proximal to the ankle.

Nerve conduction studies were normal. Needle EMG revealed poor activation but an otherwise

Figure Cases 1 and 2



(A) Swelling on the dorsum of the ankle (arrowhead) due to a retracted tibialis anterior tendon 3 weeks following rupture in Patient 1. (B) MRI of right ankle demonstrating fluid collection (arrow) in the region of the anterior tibialis tendon sheath. (C) Axial T1-weighted MRI across the proximal third of the leg showing the fatty replacement of the left tibialis anterior muscle as compared with the normal appearing tibialis anterior on the right in Patient 2. (D) Axial T2-weighted fat saturation MRI sequences across the distal third of the leg showing the thickened retracted tibialis anterior tendon on the left as compared with the normal tibialis anterior tendon on the right. (E) Axial T2-weighted fat saturation MRI sequences across the ankle showing the normal tibialis anterior tendon on the right as compared with absence of the tendon on the left.

normal recruitment pattern in the left TA muscle. Other muscles examined were normal. MRI of the leg revealed retraction and thickening of the left TAT at the level of the tibiotalar joint beyond which it was noted to terminate abruptly (figure, C through E). Due to the chronicity of the rupture, she was treated conservatively.

Discussion. Rupture of the TAT is an uncommon musculoskeletal condition that presents as foot drop but is rarely encountered by neurologists.[1] Most cases are caused by direct or indirect foot trauma. Diabetes, systemic lupus erythematosus, hyperparathyroidism, psoriasis, and gout may produce tenosynovitis and are other associated etiologies.[1-4] Spontaneous TAT rupture is rare and may be the most difficult form to distinguish from a neurogenic etiology of foot drop.[4,5] The most prominent clinical feature of TAT rupture is weakness of foot dorsiflexion. However, increased compensatory action of the extensor hallucis longus and extensor digitorum longus may minimize the degree of weakness and make identification more difficult.[5,6] The absence of toe extensor, foot eversion, and hip abduction weakness helps to distinguish TAT rupture from other neuromuscular causes of foot drop, such as peroneal neuropathy, lumbosacral plexopathy, or L5 radiculopathy. The pathognomonic sign of TAT rupture is the presence of the retracted tendon, which produces a palpable defect or swelling along the dorsum of the ankle (figure). Normal findings on needle EMG of the TA muscle is useful to distinguish TAT rupture from neuromuscular causes of foot drop

where reduction in the recruitment of motor units would be seen. MRI of the ankle is the imaging modality of choice to confirm TAT rupture by identifying partial or complete ruptures of the tendon.[7]

No consensus on conservative management vs surgical repair of TAT rupture has been established. In cases of acute ruptures or in young, active individuals, surgical repair may provide better functional outcome. Potential long-term complications if the diagnosis is missed include pain from the retracted stump, neuroma, and extensor substitution with clawing, flat foot, weakness, and heel cord contractures.[6]

In conclusion, TAT rupture should be considered in the differential diagnosis of foot drop. The identification of a swelling on the ankle and normal electrodiagnostic testing in the context of clinical weakness are clues to help in suspecting the diagnosis, and MRI of the lower leg is necessary for confirmation.

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MUTATIONS IN *VAPB* ARE NOT ASSOCIATED WITH SPORADIC ALS

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ALS is the third most common adult-onset neurodegenerative disorder. The majority of cases are sporadic (SALS), whereas 5 to 10% are familial (FALS). There are 13 known loci, with causative genes identified at seven, including superoxide dismutase 1 (*SOD1*) and angiogenin (*ANG*).[1,2] Recently, mutations in vesicle-associated membrane protein (VAMP) *a*ssociated *p*rotein *B* (*VAPB*) have been associated with autosomal dominant, adult-onset ALS8.[3]

VAPB is ubiquitously expressed, and evidence suggests that it plays a role in vesicle trafficking.[4] As *VAPB* was the only gene, other than *SOD1*, known to cause adult-onset, autosomal dominant ALS, we undertook a mutation screening strategy to establish the contribution of *VAPB* mutations in a large cohort of ALS patients. Although it is appreciated that mutations would be most likely to be found in familial cases, mutations in *SOD1* and more recently *ANG* have been reported in SALS. Therefore, both FALS and SALS cases were screened for mutations in *VAPB*.

Methods. DNA was extracted from 301 cases of ALS (23 FALS in whom *SOD1* mutations had been excluded and 278 SALS cases), originating from 86 postmortem CNS samples in the Sheffield Brain Tissue Bank and 215 blood samples in our DNA database; all were United Kingdom (UK) cases of Caucasian ethnicity. All patients had definite or probable ALS by revised El Escorial criteria.[5] Controls samples were obtained from either our local DNA database or from healthy donors at the Sheffield Blood Transfusion Service after appropriate ethics approval and donor consent. All individuals were neurologically normal and older than 50 years of age; ethnicity for all cases was UK Caucasian.

All six exons of the *VAPB* gene were amplified using intronic primers to ensure all coding regions and splice sites were sequenced (table). PCR products were purified using ExoSAP-IT (USB/Amersham Biosciences) and then bidirectionally sequenced using ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (ABI). Samples were run on an ABI 3730 capillary analyzer (Applied Biosystems).

To determine whether the deletion of Ser159 was present in the normal population, restriction enzyme digestion using *Mbo*II was used to screen controls. The frequency of the C to T polymorphism 35 bp downstream of exon 3 in intron 3 was screened by digestion with *Mae*III (Roche). Direct sequencing was used to screen controls for the G to A nucleotide substitution 4 bp upstream of exon 6.

Results. Mutation screening of the entire coding region and intron/exon boundaries in 301 ALS cases

Table VAPB primers and annealing temperatures used for mutation screening

Primer	Sequence 5′–3′	Annealing temperature	Product size
VAPB1F	CCG ACC GGT CCC CGC CTT TTT G		
VAPB1R	CGG CCA CTC CGG GCA GTT CTG	58 °C	353 bp
VAPB2F	TAC AGC TCT CTT TTC CAC AAA CCT T		
VAPB2R	GCC CAC CCT CTA GGC CTC	50 °C	261 bp
VAPB3F	ACA ACC AAA GTA CAA GTA TTA GCA TAA TAA ATG		
VAPB3R	GCA TGC ACC CAC AAT TCC A	50 °C	266 bp
VAPB4F	AAA TTG TCA GTT ATA GGA AGA AAG		
VAPB4R	TCA GAC ATC AGA TAA AGG AAA AT	54 °C	277 bp
VAPB5F	TTT GCT GAG AAC TAC TTT ACT TT		
VAPB5R	TAT ATT CAC TGG CTC ATC AAC TTC	52 °C	350 bp
VAPB6F	GCT GTA CAG TTG ACT CCC CTT TCT		
VAPB6R	GTC ATC ATA CAT TAA TTT CTT TTT	54 °C	327 bp

identified a 3-bp deletion (del 746-8 TCT) in exon 5, resulting in the loss of Ser159 in two SALS cases. Screening of 120 controls using *Mbo*II restriction enzyme digests identified a further two individuals with this deletion, suggesting this change is a polymorphism found in the general population.

Three nucleotide substitutions were also found. The first, C763T, was novel but did not alter the amino acid. The second, a G to A 4 bp upstream of exon 6, was only found in one SALS case and not in 111 controls. This change was not predicted to alter the splice site (www.fruitfly.org/seq_tools/splice). Finally, a C to T polymorphism was found 35 bp downstream of exon 3. There were no significant differences between the polymorphic frequencies in ALS and controls (n = 107) (ALS: C = 60.0%, T = 40.0%; controls: C = 64.5%, T = 35.5%; χ^2: $p = 0.24$).

Discussion. Screening of *VAPB* in 278 SALS cases has identified no clear pathogenic mutations associated with the disease. This study provides robust evidence that mutations in *VAPB* are not associated with SALS in the UK, although it is not possible to rule out that the mutation upstream of exon 6 may produce an abnormal transcript. *VAPB* mutations were not found in any of the non-SOD1 FALS cases screened, although due to the small sample size and apparent rarity of *VAPB* mutations, it cannot be ruled out that *VAPB* is a rare cause of FALS in the UK.

The initial P56S mutation was identified in seven Brazilian families of either African or Caucasian descent with varying clinical phenotypes.[3] However, further studies have shown that this mutation is due to a common founder 23 generations previously, when Brazil was colonized by the Portuguese.[6] More recently, a study concluded that *VAPB* mutations were not a common cause of SALS in Italy.[7] Taking these studies into consideration, it is suggested that *VAPB* mutations do not significantly contribute to the genetic causes of SALS in the UK and Northern Europe. However, further studies of the Portuguese ALS population may identify ancestors of the common founder, and it is possible that *VAPB* mutations may contribute to SALS in other populations.

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