# Altered fast and slow inactivation of the N440K Na<sub>v</sub>1.4 mutant in a periodic paralysis syndrome

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#### ABSTRACT

**Objective:** To electrophysiologically characterize the Na<sub>v</sub>1.4 mutant N440K found in a Korean family with a syndrome combining symptoms of paramyotonia congenita, hyperkalemic periodic paralysis, and potassium-aggravated myotonia.

**Methods:** We characterized transiently expressed wild-type and mutant  $Na_v1.4$  using whole-cell voltage-clamp analysis.

**Results:** N440K produced a significant depolarizing shift in the voltage dependence of fast inactivation and increased persistent current and acceleration in fast inactivation recovery, which gave rise to a 2-fold elevation in the dynamic availability of the mutant channels. In addition, the mutant channels required substantially longer and stronger depolarization to enter the slow-inactivated state.

**Conclusions:** N440K causes a gain of function consistent with skeletal muscle hyperexcitability as observed in individuals with the mutation. How the same mutation results in distinct phenotypes in the 2 kindreds remains to be determined. *Neurology*<sup>®</sup> **2012;79:1033-1040** 

#### GLOSSARY

**ADM** = abductor digiti minimi; **CMAP** = compound muscle action potential; **LET** = long exercise test; **PEMP** = postexercise myotonic potential; **PMC** = paramyotonia congenita; **SET** = short exercise test.

Mutations in genes encoding voltage-gated sodium (Nav) channel isoforms that result in altered channel behavior lead to inheritable diseases affecting tissues in which the isoforms are expressed. For example, mutations in SCN4A, the gene coding for the skeletal muscle sodium channel Na, 1.4, cause paroxysmal muscle dysfunction known as paramyotonia congenita (PMC), an autosomal dominant disorder with cold- and exercise-induced stiffness as well as weakness.<sup>1-7</sup> Not all SCN4A mutations, however, lead to this phenotype. Of the 66 mutations identified so far (see appendix e-1 on the Neurology® Web site at www.neurology.org), only half are associated with PMC. The other half give rise to sodium channel myotonia and hyperkalemic or hypokalemic periodic paralysis. The diagnosis of these SCN4A channelopathies can be difficult, because a reliable genotype-phenotype correlation does not exist. The situation is especially complicated, because a single mutation can have diverse phenotypic presentations. In a recent study, a group in Germany reported a novel Na, 1.4 variation, N440K, which produced cold-insensitive myotonia with warm-up phenomenon, muscular hypertrophy, and flaccid weakness as well as potassium-aggravated weakness.<sup>8</sup> We found the same mutation in a Korean family (figure 1, A and B) with cold-sensitive paradoxical myotonia; however, warm-up phenomenon, muscular hypertrophy, and potassium sensitivity were absent, which is consistent with the universally accepted phenotype of PMC. To gain insight into the basis for the observed muscle dysfunction, we examined its electromyographic charac-

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Figure 1



(A) Pedigree with affected individuals shown as solid symbols. Family members IV-3, IV-8, IV-13, and IV-15 were not available for DNA analysis. Individuals I-1, II-1, and II-2 had died from natural cause, stroke, or hepatocellular carcinoma, respectively. Symbols and nomenclature follow established guidelines.<sup>28</sup> (B) The heterozygous substitution c.1320T>A/ p.(Asn440Lys or N440K) is present in all patients, but not in healthy family members or 200 control subjects. Top: the proband's electropherogram showing the exchange (arrow). The reading frame is indicated by black bars under the sequence. Bottom: single-letter amino acid alignment of various Na<sub>v</sub> sequences shows the affected residue to be highly conserved even in evolutionary distant orthologs. GenBank accession numbers are as follows: human skeletal muscle Na<sub>v</sub>1.4, P35499.4; human heart Na<sub>v</sub>1.5, Q14524.2; rat brain isoform II rNa<sub>v</sub>1.2, NP\_036779.1; rat sensory neuron rNa<sub>v</sub>1.8, NP\_058943.1; *Electrophorus electricus* sodium channel, AAA79960.1; *Drosophila melanogaster* sodium channel  $\alpha$  subunit, AAB59191.1; zebrafish Na<sub>v</sub>1.4a, Q2XVR3.2; zebrafish Na<sub>v</sub>1.4b, Q20JQ7.1; and jellyfish SCN1, AAC09306.1. (C) Na<sub>v</sub>1.4 channel topology with 4 homologous domains (D1–D4) of 6 transmembrane regions each (S1–S6).  $\bigcirc$ , previously published PMC mutations;  $\blacklozenge$ , N440K. The numbering refers to additional material in appendix e-1.

teristics and functionally analyzed the N440K substitution in a heterologously expressed Na<sub>v</sub>1.4 construct using whole-cell voltage clamping.

**METHODS Standard protocol approvals, registrations, and patient consents.** Informed written consent was obtained from all participants or their guardians, and the project was approved by the institutional review board.

**Diagnosis.** Author I.-N.S. made all diagnoses, finding the following presentation in all participants: (1) paradoxical myotonia, (2) absence of a warm-up phenomenon, (3) insensitivity to oral potassium, (4) normal muscle build, and (5) childhood onset (with the exception of the proband, who first presented with symptoms at 15 months of age). Of the patients, 60% (9 of 15) of the patients were male.

EMG. A routine nerve conduction study was performed using standard techniques to rule out underlying neuropathy. Needle EMG was performed on at least 2 different muscles in each extremity. The short exercise test (SET) was performed on the right abductor digiti minimi (ADM) at 32°C skin temperature or after cooling the left ADM to 20°C skin temperature. The SET consisted of a 10-second isometric contraction and measurement of the compound muscle action potential (CMAP) immediately thereafter, as well as every 10 seconds for a total of 1 minute.9,10 The long exercise test (LET) was done similarly, using a 5-minute contraction of the right ADM that was released for 5 seconds every 50 seconds; CMAPs were recorded 2 seconds after exercise and every 5 minutes for 40 additional minutes. The baseline CMAP amplitude, as evoked by supramaximal stimulation of the ulnar nerve, was determined immediately before each exercise test.

**DNA sequencing.** Peripherally extracted genomic DNA served as a template for PCRs amplifying all *SCN4A* exons. The resulting amplicons were bidirectionally sequenced using standard BigDye Terminator technology, and the electropherograms inspected with Chromas 2.13 (Technelysium Pty. Ltd., Queensland, Australia). The variant nomenclature adheres to specifications set by the Human Genome Variation Society (http://www.hgvs. org). Additional information is provided in appendix e-1.

**Mutagenesis, tissue culture, and electrophysiology.** Wild-type human Na<sub>v</sub>1.4 cDNA in the pRc-CMV vector (gift of Dr. Alfred L. George, Vanderbilt University) was subjected to site-directed mutagenesis using a QuikChange Kit (Agilent Technologies, Santa Clara, CA). Technical details, including the

Table 1	Clinical data							
Subject	Sex	Age, y	Onset	Paramyotonia	Percussion myotonia	Aggravating factor		
III-1	М	55	Childhood	+	-	Cold, exercise		
111-4	F	46	Childhood	+	+/-	Cold, exercise		
III-6	М	40	Childhood	+	-	Cold, exercise		
III-7	М	39	Childhood	+	-	Cold, exercise		
III-8	F	45	Childhood	+	+	Cold, exercise, pregnancy		
IV-2	F	26	Childhood	+	-	Cold, exercise		
IV-9	М	20	Childhood	+	-	Cold, exercise		
IV-16	М	15	15 mo	+	+	Cold, exercise		

primer sequences, tissue culture, and transfection methods, as well as a thorough description of the electrophysiology methods are provided in appendix e-1.

**Data analysis and statistical evaluation.** Electrophysiology data were analyzed off-line using Clampex 9.2 software (Axon Instruments), OriginPro 7.5 (OriginLab, Northampton, MA), and Excel (Microsoft, Seattle, WA) and plotted as mean values  $\pm$  SE. Student's *t* tests yielding p < 0.05 were deemed statistically significant.

**RESULTS Clinical features.** The proband (IV-16 in figure 1A and table 1) came to our attention at 8 years of age. During infancy, he had difficulty raising his head and standing after strenuous crying. At age 5 years, he developed muscle stiffness affecting the eyelids, jaw, hands, and legs with prolonged exercise or after cold exposure. Postexercise weakness of the affected muscles lasted up to 3 days. There was no myalgia. On examination, exercise-induced paramyotonia was readily apparent in the hands, as was percussion myotonia, which was also present in the tongue. Strength and external muscle appearance as well as deep tendon reflexes were normal. The blood potassium level during an acute attack of muscle weakness was normal. Sustained immersion in ice-cold water readily reproduced the patient's symptoms.

The proband's mother reported subtle but frequent spasms in the eyelids and hands since preschool, which worsened with pregnancy at age 29, when she had difficulty chewing and unfolding her fists after exposure to cold. The attacks were exacerbated with continued activity and were followed by prolonged, incapacitating weakness. With age, the condition improved, rendering her intermittently asymptomatic. Clinical examination revealed percussion myotonia in the hands and the tongue. Maternal uncles, aunts, cousins, and sister were similarly affected since preschool, with improvement over age. The maternal grandfathers and great-grandmother were deemed to be affected postmortem based on family reports. There was no evidence of renal or endocrine system failure that could have triggered the symptoms secondarily.

**EMG.** Electrodiagnostic studies (figure 2, table 2) of the proband produced the following findings: (1) normal motor and sensory nerve conduction between paralysis attacks; (2) abundant myotonic discharges in all muscles studied; (3) appearance of a postexercise myotonic potential (PEMP) in the first postexercise CMAP (figure 2A) and disappearance with repeated exercise; (4) drastic reduction in the CMAP amplitude after room temperature SET (figure 2B); (5) further CMAP reduction after SET with cold exposure (figure 2B); and (6) mild reduction of CMAP amplitude after room temperature LET (fig-

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(A) The proband's first compound muscle action potential (CMAP) after short exercise with a postexercise myotonic potential (arrow, bottom trace) that was absent in the pre-exercise control (top trace). (B, C) Short exercise test and CMAP amplitude in the proband (B) and other affected family members (C) (n = 7-8). Note the distinct reduction in CMAP amplitude compared with pre-exercise values at room temperature (**II**), which is enhanced by lowering of the temperature (**O**). (D, E) Long exercise test in the proband (D) and affected family members (E) (n = 3) at room temperature produced a mild reduction in CMAP amplitudes. All CMAP amplitude values are expressed as a percentage of pre-exercise values and are plotted against the time elapsed after cessation of the exercise trial.

ure 2D). Affected family members produced similar results: CMAP amplitudes after SET were smaller than those collected before the trial  $(-15.7 \pm 1.3\%)$ , n = 7); cooling enhanced this effect  $(-37.6 \pm 2.1\%)$ , n = 6) (figure 2D). During and after the room temperature LET, the CMAP amplitudes were reduced  $(-27.4 \pm 2.3\%)$ , n = 3) (figure 2E).

**DNA analysis.** Direct sequence analysis of the *SCN4A* coding region revealed a c.1320T>A exchange in all affected individuals (figure 1B) that was not present in healthy relatives or in 200 normal control subjects. The predicted exchange, p.Asn440Lys (N440K), replaces a highly conserved and electrostatically neutral asparagine with a positively charged lysine in D1/S6 of Na, 1.4 (figure 1C). The same substitution was

previously reported by Lehmann-Horn et al.<sup>8</sup>, albeit with a different nucleotide (c.1320T>G).

N440K electrophysiology. Heterologously expressed wild-type Na, 1.4 produced a current-voltage relationship, voltage dependence of activation, and inactivation kinetics that were identical between wild-type and mutant constructs (figure 3, A, B, and E). Clearly different, on the other hand, was the voltage dependence of inactivation (figure 3C), which showed a depolarizing shift that yielded an approximate 40% increase in mutant channel availability at the wild-type half-maximal inactivation level. A similar gain-of-function effect was seen in fast inactivation recovery (figure 3D), for which the mutant reprimed noticeably faster, allowing accelerated reentry into action. Persistent current was increased from 0.06% in the wild-type to 0.48% in the mutant. The combined effect of these alterations became obvious when we stimulated the channels at high frequency (figure 3F), which produced mutant peak currents that were 60%-100% larger than the wild-type controls. Protocols looking at entry into slow inactivation showed a prominent shift toward larger time constants, consistent with a need for longer depolarization (figure 3G). This was paired with changes in the voltage dependence of slow inactivation: not only was there a pronounced decrease in the voltage sensitivity as expressed by a larger slope factor, but there was also a sizable depolarizing shift in half-maximal inactivation, showing that N440K channels require stimuli approximately 25 mV more depolarized than what is necessary in the wild-type channels to achieve the same level of slow inactivation (figure 3H). Slow inactivation recovery was unchanged (figure 3I).

**DISCUSSION** The impetus for this study was the identification of a Korean family with PMC in which the N440K mutation is present in affected family members but not in healthy relatives. Because the sequence variation also does not occur in normal control subjects, it is very likely to be related to PMC in this family, although conclusive linkage analysis data are not available. Interestingly, the same SCN4A gene variation was previously associated with a distinct phenotype in which there is potassiumaggravated myotonia and hyperkalemic periodic paralysis.8 To confirm that the disorder in our family is clinically different from the previously described syndrome, we conducted extensive patient interviews and EMG tests. Each affected subject exhibited the hallmark symptoms for PMC, including paradoxical myotonia that worsened with cold temperature. In addition, each subject exhibited several distinctly abnormal EMG features: (1) PEMPs after 10 seconds of exercise; (2) a reduction in CMAP amplitude after

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Table 2	Needle EMG: electrodiagnostic presentation								
		Room tempera							
Subject	Myotonic discharge	SET: PEMP?	SET: CMAP change, %	LET: CMAP change, %	After cooling: SET: CMAP change, %				
III-1	Abundant	Yes	$-16.2\pm1.8$	ND	$-34.2\pm1.5$				
III-6	Abundant	Yes	$-6.2\pm0.6$	ND	$-14.8\pm0.5$				
III-7	Abundant	Yes	$-6.6\pm1.2$	ND	ND				
III-8	Abundant	Yes	$-6.2\pm0.6$	$-10.9\pm1.1$	$-21.4\pm0.5$				
IV-2	Abundant	Yes	$-21.2\pm1.2$	ND	$-39.1\pm2.2$				
IV-9	Abundant	Yes	$-23.1\pm0.8$	$-43.8\pm1.7$	$-50.8\pm1.2$				
IV-15	Abundant	Yes	$-10.9\pm0.9$	ND	$-47.9\pm1.8$				
IV-16	Abundant	Yes	$-35.1\pm0.9$	$-26.6\pm2.3$	$-54.8\pm2.2$				

Abbreviations: CMAP = compound muscle action potential; LET = long exercse test; ND = not determined; PEMP = postexercise myotonic potential; SET = short exercise test.

the SET and the LET; and (3) enhancement of the latter effect with cooling. These findings are in keeping with the field-accepted diagnostic criteria for PMC.9,11 The presence of PEMPs after the first stimulation indicates that several muscle fibers fire repeatedly in a synchronous fashion and is suggestive of paramyotonia or classic myotonia.9,12 The SET after cold exposure invariably resulted in a significant reduction of CMAP amplitude in all subjects studied. Together, the clinical features in conjunction with the unique electromyographic findings are consistent with the diagnosis of PMC. Moreover, because there were no reports of potassium- or exercise-induced paralytic attacks, the syndrome is distinct from that described previously in a multigenerational Turkish family with the same mutation.

The underlying basis for the difference in the phenotypes in the 2 families is unknown, but modification through secondary, yet to be identified, factors is a possibility. Linkage analysis data that could confirm the suggested phenotypic heterogeneity are not available for either family.

There is little doubt that the net outcome for N440K is a gain of function. Nav1.4 channels with the mutation require stronger depolarization to become inactivated and recover from inactivation more quickly (figure 3, C and D, respectively). Thus, the channels are active under conditions in which the wild-type channel is closed, and it is channel closing, not opening, that is responsible for the observed pathology. Wild-type channels exhibit a refractory period determined by the time for recovery from inactivation. The mutant channels have a reduced refractory period, which is expected to permit higher frequency firing. This, in conjunction with the requirement for stronger depolarization to inactivate, results in enhanced dynamic availability that generates approximately twice the current of wild-type

channels at all frequencies examined (figure 3F). An interesting parallel to our findings exists in a report describing the PMC mutation p.(Arg1448Cys), which produced a similar alteration in the dynamic availability of  $Na_v 1.4$ .<sup>13</sup> Physiologically, this translates into muscle stiffness, one of the main symptoms in our proband.

The observed defects in fast inactivation would probably suffice to create abnormal excitation patterns, but N440K also interferes with Nav1.4 slow inactivation in that the mutant channels require dramatically longer depolarization to enter the slowinactivated state (figure 3G). Thus, to the extent that slow inactivation plays a role in limiting excessive muscle excitation, the defect in slow inactivation probably contributes to the myotonic phenotype, because it permits prolonged high-frequency firing and with it persistent contraction, the hallmark of myotonia. At the voltages examined, the N440K channels never reached the level of slow inactivation seen in control subjects but retained an availability that was minimally twice that of wild-type channels (figure 3H). Our patients' sustained weakness probably arises from elevated persistent current and, with it, subtle depolarization that is sufficient to cause fast inactivation of Na<sup>+</sup> channels, resulting in membrane inexcitability.14,15 A critical component of this model, as Ruff pointed out, is a defect in slow inactivation, because normal slow inactivation would ultimately terminate the abnormal conduction of the mutant channels.16

Previous biophysical characterizations of PMC mutants have revealed diverse defects including shifts in the voltage dependence of activation and inactivation, slowing of the rate of inactivation, accelerated recovery from fast inactivation, slowing in the rate of deactivation, shifts in the voltage dependence of slow inactivation, and alterations in the recovery from slow inactivation.<sup>2,4,13,17-21</sup> Most of these defects have been interpreted as a gain of function, but some findings were consistent with loss of function. However, a consensus has emerged that PMC is caused by excess Na<sub>v</sub>1.4 current, rather than by too little. The outcome of our analyses is fully compatible with this notion, although the individual findings are not necessarily identical to earlier studies. There was an obligatory, but subtle, enlargement of the window current because of the depolarizing shift in steadystate availability and, along with it, persistent current as reported by others, but we found no evidence that the inactivation kinetics in N440K were altered, whereas in other PMC mutants, the inactivation rate is frequently slowed.<sup>2,4,13,16-21</sup> Another unique feature of N440K is its slow inactivation deficiency. Although frequently perturbed in Na<sup>+</sup> channelopa-



All experiments used a holding potential of -120 mV; step protocols are indicated in each panel. Additional technical details are provided online (appendix e-1). (A) Current-voltage (*IV*) relationship: the peak current amplitude (*I*) at each potential (*V*) was normalized to the cell's capacitance to obtain the current density. (B) Voltage dependence of activation. Maximum normalized peak conductances based on *IV* data were recalculated with  $G(V) = I(V)/(V - E_{Na})$  and Boltzmann fitted, where  $E_{Na}$  is the experimentally determined Na<sup>+</sup> reversal potential,  $V_{1/2}$  is the half-maximal activation potential, and *k* is the slope factor. (C) Steady-state availability. Maximum-normalized P2 peak current responses, Boltzmann fitted. (D) Recovery from inactivation. A two-pulse protocol using a conditioning pulse (P1, -10 mV) was followed by a variable recovery period ( $\Delta t$ ) and a P2 test pulse. The P2 response was normalized to P1 and double-exponentially fitted. (E) Fast inactivation kinetics. The *IV* current decay data were exponentially fitted from approximately 90% of the peak to baseline for the indicated voltages. (F) Use dependence. The cells were pulsed to -10 mV for 5 msec at the indicated frequencies for a total of 500 msec. Solid symbols, wild-type; open symbols, N440K. The peak current amplitude of each pulse was normalized to the first pulse response and exponentially fitted. (G) Onset of slow inactivation. The P2/P1 peak current ratio was exponentially fitted. (H) Steady-state slow inactivation. P2 maximum-normalized peak currents were Boltzmann fitted. (I) Recovery from slow inactivation. All results are summarized in tables e-1 and e-2.

thies,<sup>13,14</sup> the extent of the slow inactivation alteration in N440K is unusual, which suggests a vital role for asparagine 440 in slow inactivation. All these differences may be the result of the position of N440K within the channel topology. PMC mutations commonly cluster in and around the voltage-sensing D4/S4 region.<sup>22,23</sup> In contrast, N440K lies in D1/S6, which lines the channel pore. Only one other PMC mutation, p.(Gln270Lys), localizes to D1. This mutation is situated in the S5–S6 linker, and it produces a slowing of the fast inactivation rate.<sup>6,13</sup> PMC, potassium-aggravated myotonia, and hyperkalemic periodic paralysis are distinct clinical syndromes caused by compromise of the skeletal muscle sodium channel Na<sub>v</sub>1.4. The diversity in the phenotypic expression of different Na<sub>v</sub>1.4 mutants is generally attributed to their distinct biophysical properties. This notion clearly does not explain how the N440K mutation causes classic PMC in one family and a complex syndrome of myotonia and hyperkalemic periodic paralysis in another. In recent years, a variety of examples of specific ion channel muta-

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tions that cause distinct clinical syndromes in different members of the same pedigree, and in some cases the 2 conditions present in the same affected individual. For example, various mutations in Na<sub>v</sub>1.4 have been found to cause PMC in some family members and hyperkalemic periodic paralysis in others.<sup>24</sup> In the present situation we have described a single mutation that is associated with distinct clinical presentations in 2 different families. It is possible that such phenotypic heterogeneity is the result of genetic modification through secondary genes or phenotype modulation. The factors involved, whether genetic, epigenetic, hormonal, or external, remain to be determined.

As a final note, we comment on the implications of the present work for therapy development. There is emerging evidence that certain drugs including lacosamide,<sup>25</sup> ranolazine,<sup>26,27</sup> and rufinamide can enhance slow inactivation. Agents such as these could be of benefit for the treatment of hyperexcitability muscle disorders in general, but specifically in patients in whom there is defective slow inactivation, as with the N440K Na<sub>v</sub>1.4 mutation described here. Clinical trials of the therapeutic potential of these compounds in the myotonias are warranted.

#### AUTHOR CONTRIBUTIONS

Christoph Lossin: performed all tissue culture and transfections, designed and executed biophysical analyses of wild-type and mutant  $Na_V 1.4$ , analyzed electrophysiologic data including statistical analyses, drafted, revised, and submitted the manuscript, and contributed to funding support. Tai-Seung Nam: contributed to drafting/revising of the manuscript for intellectual content and data acquisition/analysis. Shahab Sean Shahangian: conducted electrophysiologic experiments and contributed to tissue culture as well as analysis and helped revise the manuscript. Michael A. Rogawski: contributed to drafting/revising of the manuscript and funding support. Seok-Yong Choi: contributed to study conception, data acquisition/interpretation/analysis, study supervision, and drafting/revising of the manuscript and obtaining funding. Myeong-Kyu Kim: contributed to study conception, data acquisition/interpretation/analysis, study supervision, drafting/revising of the manuscript, and obtaining funding. Il-Nam Sunwoo: contributed to data acquisition/analysis and patient diagnosis.

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#### DISCLOSURE

The authors report no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

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### Thank you, Dr. John F. Kurtzke!

The Neurology online archive has recently been updated to include the following seminal articles related to early research in MS:

**Rose AS, Kuzma JW, Kurtzke, JF, et al.** Cooperative study in the evaluation of therapy in multiple sclerosis; ACTH vs placebo in acute exacerbations. Neurology 1968 (June); 18 (6 Part 2): 1–10.

**Rose AS, Kuzma JW.** A protocol for a cooperative study to evaluate the therapeutic effectiveness of ACTH on multiple sclerosis in acute exacerbations. Neurology 1968 (June); 18 (6 Part 2): 1–20 + study forms.

**Rose AS, Kuzma JW, Kurtzke, JF, et al.** Cooperative study in the evaluation of therapy in multiple sclerosis: ACTH vs. placebo – final report. Neurology 1970 (May); 20 (5 Part 2) 1–59.

Kurtzke, JF. Multiple Sclerosis: What's in a name? Neurology 1988;38:309-316.

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