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Rescuable folding defective $Na_V1.1$ (*SCN1A*) mutants in epilepsy: Properties, occurrence, and novel rescuing strategy with peptides targeted to the endoplasmic reticulum

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ABSTRACT

Mutations of the voltage gated Na^+ channel $Na_V1.1$ (*SCN1A*) are important causes of different genetic epilepsies and can also cause familial hemiplegic migraine (FHM-III). In previous studies, some rescuable epileptogenic folding defective mutants located in domain IV of $Na_V1.1$ have been identified, showing partial loss of function also with maximal rescue. Variable rescue may be one of the causes of phenotypic variability, and rescue might be exploited for therapeutic approaches. Recently, we have identified a folding defective FHM-III $Na_V1.1$ mutant that showed overall gain of function when rescued, consistent with a differential pathomechanism.
Here, we have evaluated functional properties and cell surface expression of six $Na_V1.1$ epileptogenic missense mutations in different rescuing conditions, including a novel one that we have developed expressing a selective sodium channel toxin (CsEI) targeted to the endoplasmic reticulum (ER). All the mutants showed loss of function and reduced cell surface expression, consistently with possibility of rescue. Four of them were rescuable by incubation at low temperature and interactions with different co-expressed proteins or a pharmacological chaperone (phenytoin). Notably, CsEI was able to rescue four mutants. Thus, $Na_V1.1$ folding defective mutants can be relatively common and mutations inducing rescuable folding defects are spread in all $Na_V1.1$ domains. Importantly, epileptogenic mutants showed overall loss of function even upon rescue, differently than FHM-III ones. The effectiveness of CsEI demonstrates that interactions in the ER are sufficient for inducing rescue, and provides a proof of concept for developing possible therapeutic approaches that may overcome some limitations of pharmacological chaperones.

Introduction

Voltage gated Na^+ channels (Na_V) are essential for the generation of cellular excitability and their mutations are important causes of genetic diseases (Mantegazza et al., 2010; Catterall, 2012). Na_V are composed of a principal pore-forming α subunit (nine isoforms: $Na_V1.1$–$Na_V1.9$ for the proteins, *SCN1A*–*SCN11A* for the genes), and by auxiliary β subunits (four isoforms: β1–β4 for the proteins, *SCN1B*–*SCN4B* for the genes). The primary sequence of α subunits contains four homologous domains (DI–DIV), each comprising six predicted transmembrane segments (S1–S6) that form voltage-sensing modules (S1–S4; S4 is the voltage sensor) and pore modules (S5–S6) in each domain. The β subunits contain a single transmembrane segment.

SCN1A/$Na_V1.1$ (MIM# 182389) is one of the most clinically relevant epilepsy genes (Helbig et al., 2008; Guerrini et al., 2014), with hundreds of mutations reported thus far in different epilepsy syndromes characterized by variable phenotypes, and is also the target of some familial hemiplegic migraine (FHM-type III; MIM# 609634) mutations (see www.molgen.ua.ac.be/SCN1AMutations and http://www.scn1a.info/ for *SCN1A* variant databases). The most severe epileptic phenotype associated with $Na_V1.1$ mutations is Dravet syndrome (DS; MIM# 607208), also known as Severe Myoclonic Epilepsy of Infancy (SMEI), an extremely severe epileptic encephalopathy for which it is important to develop treatments, characterized by onset in the first year of life as prolonged seizures triggered by fever and later appearance of severe

Abbreviations: AaHII, α toxin from the *venom* of the scorpion *Androctonus australis Hector*; CFP, cyan fluorescent protein; CsEI, β toxin from the venom of the scorpion *Centruroides sculpturatus* Ewing; DS, Dravet syndrome; ER, endoplasmic reticulum; FS, febrile seizures; GEFS+, Generalized (Genetic) Epilepsy with Febrile Seizures Plus; INa_P, persistent Na^+ current; INa_t, transient Na^+ current; MTLE&HS, mesial temporal lobe epilepsy with hippocampal sclerosis; Na_V, voltage gated Na^+ channels; YFP, yellow fluorescent protein.

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afebrile seizures, drug resistance, ataxia, delayed psychomotor development and cognitive impairment (Dravet et al., 2005). In general, it is caused by de novo deletions or missense mutations (Claes et al., 2001; Depienne et al., 2009), which lead to haploinsufficiency (Bechi et al., 2012). Generalized (Genetic) Epilepsy with Febrile Seizures Plus (GEFS+; MIM# 604233) patients carry missense $Na_V1.1$ mutations and present with febrile and afebrile seizures; this syndrome is characterized by highly heterogeneous intra-familial phenotypes which vary from asymptomatic to very severe, in some cases DS-like (Scheffer et al., 2009). Moreover, mutations have also been identified in some patients presenting with different epileptic encephalopathies, ranging from Lennox–Gastaut syndrome to epilepsy aphasia syndrome (Depienne et al., 2009; Marini and Mantegazza, 2010; Carvill et al., 2013; Guerrini et al., 2014). One of the mildest epileptic phenotypes associated with missense $Na_V1.1$ mutations is benign simple febrile seizures (FS; MIM# 604403) (Mantegazza et al., 2005b), although some patients of this family developed also mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE&HS). Interestingly, genome-wide association studies have linked common *SCN1A* single nucleotide polymorphisms (rs7587026 and rs11692675) to development of MTLE&HS upon a history of febrile seizures (Kasperaviciute et al., 2013), and a further *SCN1A* polymorphism has been identified as a risk factor for idiopathic/genetic generalized epilepsies (rs11890028) (Steffens et al., 2012). Notably, also FHM-III families carry missense $Na_V1.1$ mutations, in some cases without any signs of epileptic phenotypes (Cestele et al., 2013a).

Several evidences point to a loss of function as the common effect of $Na_V1.1$ epileptogenic mutations, with mutations that cause complete loss of function (e.g. truncating mutations) leading in general to more severe phenotypes (Tang et al., 2009; Catterall et al., 2010; Marini and Mantegazza, 2010; Mantegazza, 2011). This finding has been confirmed by results obtained studying gene-targeted mice carrying DS or GEFS+ $Na_V1.1$ mutations (Yu et al., 2006; Ogiwara et al., 2007; Martin et al., 2010). We have recently shown that the GEFS+ missense mutation R1648H causes interneuron-specific hypoexcitability by a generalized defect in action potential initiation, inducing multisystem disinhibition and network hyperexcitability in different brain regions (Hedrich et al., 2014).

However, the phenotypic variability caused by $Na_V1.1$ mutations is striking (Guerrini et al., 2014). In particular, it is not possible to predict the phenotype caused by a $Na_V1.1$ missense mutation, complicating early diagnosis and genetic counselling, and the cause of this high phenotypic variability is not completely clear yet. Notably, mosaicism has been excluded in several GEFS+ families presenting with severe phenotypes (Depienne et al., 2010). Thus, other factors can modulate the severity of the effect of the mutations, and it is not possible to evaluate the risk of appearance of severe phenotypes in these families. Missense mutations listed in *SCN1A* variant databases are more than 300 (more than 40% of the total) and, although their effect on channel's properties cannot be inferred without functional studies, only few tens of mutants have been evaluated functionally (Escayg and Goldin, 2010; Mantegazza, 2011), and functional rescue has been thus far evaluated for just 12 mutants.

We have previously studied two $Na_V1.1$ GEFS+ mutations (M1841T and R1916G) located in the C-terminus (DIV), which induce loss of function because of folding defects (Rusconi et al., 2007, 2009). Folding defective mutants are recognized as incorrectly folded proteins by the quality control system of the endoplasmic reticulum and degraded (Bernier et al., 2004). We have shown that interacting proteins and pharmacological chaperones can rescue these $Na_V1.1$ mutants attenuating the loss of function and acting as modulators of the effect of the mutation; thus, possibly of the phenotype. We hypothesized that this could be one of the causes of the phenotypic variability observed in GEFS+ families, and that defective rescue can generate severe phenotypes. Recently published observations have confirmed these results. One study showed that pharmacological chaperones can rescue function and cell surface expression of two DS mutants: the missense mutations R1648C, localized in DIV-S4 segment, and G1674R, localized in DIV-S5 segment (Thompson et al., 2012). Another study showed that two mutations of the same residue in DIV-S5 segment can cause either DS (A1685D) or mild GEFS+ (A1685V), and that only the GEFS+ mutant could be rescued, consistent with a milder phenotype (Sugiura et al., 2012).

Thus, interacting proteins and their genetic variants may modulate the effect of mutations, and other rescuing strategies may ameliorate severe phenotypes caused by mutations that are not rescued by ordinary in vivo interactions. However, thus far rescuable mutants have been identified only for few mutations located in DIV, and mutations in other domains may be less amenable to rescue (e.g. because the C-terminal domain is particularly rich in binding sites). Furthermore, rescue can transform a non-functional $Na_V1.1$ mutant into a gain of function one, as we have recently reported for the L1649Q FHM-III mutation located in the S4 segment of DIV (Cestele et al., 2013a). This finding is consistent with our hypothesis of $Na_V1.1$ gain of function as the pathomechanism of FHM-III (Cestele et al., 2008, 2013a), but the same finding for an epileptogenic mutant would contrast with this hypothesis and it is important to find out the effect of rescue on different epileptogenic mutants.

Therefore, rescuable folding defects have been implicated in the pathomechanism of $Na_V1.1$ mutations, but several important issues still need to be clarified. We have undertaken the functional study and evaluated cell surface expression of six $Na_V1.1$ missense mutations found in families with variable phenotypes, including severe ones, in order to: 1) evaluate the occurrence of folding defects in this group of mutants, 2) better characterize molecular interactions and mechanisms that can rescue the mutants, 3) verify if rescued epileptogenic mutants still show loss of function, differently than the L1649Q FHM-III mutant that we have recently studied (Cestele et al., 2013a), 4) develop improved rescuing strategies, and 5) find out whether folding defective mutations are located in other domains than DIV.

Material and methods

Plasmids and mutagenesis

The cDNA of the human $Na_V1.1$ Na^+ channel α subunit ($hNa_V1.1$) was provided by Dr. Jeff Clare (GlaxoSmithKline, Stevenage, Herts, UK) and encodes the shorter splice variant isoform of 1998 amino acids (deletion of 11 amino acids in the cytoplasmic loop between domains I and II in comparison with the longer splice variant), which is the predominant $Na_V1.1$ variant expressed in the brain (Schaller et al., 1992; Lossin, 2009). $hNa_V1.1$ cDNA was subcloned into the pCDM8 vector because in our experience this plasmid was able to stabilize the cDNA of several Na^+ channel α subunits (Mantegazza et al., 2001, 2005a). We propagated it in TOP10/P3 or MC1061/P3 *Escherichia coli* (Invitrogen) grown at 28 °C for >48 h in order to minimize the rearrangement rate and the entire coding sequence was sequenced after each propagation. We introduced the mutations by means of the Quick Change XL kit (Stratagene) using pCDM8-$hNa_V1.1$ as template and the following primers: 5′CATGGAGCACTGTCCAATGACGGACC (forward) and 5′GTCCGTCATTGGACAGTGCTCCATG (reverse) for Y790C; 5′GATTATCTGTTCTCTGTTCATTTCG (forward) and 5′CGAAATGAACAGAGAACAGATAATC (reverse) for R859C; 5′ATAGAGACCACCGTGGGACTGTATG (forward) and 5′ACAGTCCCACGTGGTCTCTATCC (reverse) for M956T; 5′GGAAAACAACGGTGGAACCTGAGAAG (forward) and 5′AGGTTCCACCGTTGTTTTCCTCTGCC (reverse) for W1204R; 5′GCATCATGGGCATAAATTTGTTTGC (forward) and 5′GCAAACAAATTTATGCCCATGATGC (reverse) for V1366I; 5′TCTTTGCTTTGAAGATGTCCCTTC (forward) and 5′GGGACATCTTCAAAGCAAAGAGCAG (reverse) for M1664K. The mutations Y790C and M1664K were introduced also in the chimeric construct pCDM8-YFP$hNa_V1.1$ (N-terminal tagging of $hNa_V1.1$ with the yellow fluorescent protein, YFP) (Rusconi et al., 2007). The cDNAs of human voltage gated Na^+ channel β1 and β2 subunits were provided