Original Research

Novel missense alleles of SIGMAR1 as tools to understand emerin-dependent gene silencing in response to cocaine

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Impact statement

The Sigma-1 Receptor (Sigma1R; *SIGMAR1*) binds neuroactive drugs—both therapeutic and addictive—and associates with the nuclear membrane protein emerin and its partners lamin A/C and BANF1 in response to cocaine, through unknown mechanisms. We identified two novel *SIGMAR1* missense variants of special interest due to their prevalence in human populations and their potential to perturb Sigma1R function at the nuclear envelope. Despite its importance in physiology and pharmacology, many aspects of Sigma1R including its membrane topology are unclear. Our findings lay the foundation for future molecular studies to understand how Sigma1R associates with emerin, lamin A/C, and BANF1 and manipulates their activity in response to agonist.

Abstract

Sigma-1 receptor (Sigma1R; *SIGMAR1*), an integral membrane protein of the endoplasmic reticulum and nuclear envelope, has a hydrophobic drug-binding pocket that binds with high affinity to addictive drugs (cocaine, methamphetamine) and therapeutics used to treat a wide spectrum of neurological disorders. Cocaine enhances Sigma1R association with three nuclear lamina proteins (emerin, lamin A/C, BANF1), causing Sigma1R-dependent and emerin-dependent recruitment and transcriptional repression of a gene, *MAOB1*, involved in dopamine removal from neural synapses. The mechanism of Sigma1R association with emerin and the molecular impact of cocaine on their association are unknown. Mutations in Sigma1R, as a proposed regulator or mis-regulator of the nuclear lamina, have the potential to alter nuclear lamina function in brain or other tissues. We examined the frequency of *SIGMAR1* missense alleles among 60,706 unrelated individuals in the ExAC database. We identified two novel *SIGMAR1* missense variants of particular interest due to their frequency and potential to impact molecular association with emerin or other nuclear lamina proteins. Variant p.Q2P was widespread in ExAC (overall allele frequency 18.4%) with broad ethnic distribution among non-Finnish Europeans, Africans, South Asians, Latinx (allele frequencies ~15% to 23%), and East Asians (~38%). The p.R208W allele was identified in ~0.78% of individuals overall with enrichment in Africans, Latinx, and East Asians (~1.9–2.9%). These and other novel Sigma1R variants provide tools for future studies to determine the molecular basis of Sigma1R association with emerin and the mechanism of nuclear lamina misregulation by cocaine and potentially other Sigma1R agonists.

Keywords: Emerin, cocaine, nuclear lamina, LMNA, BANF1, Sigma-1 receptor

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Introduction

The human Sigma-1 receptor (Sigma1R; *SIGMAR1*) is a 223-residue integral membrane protein localized in endoplasmic reticulum (ER) and nuclear envelope (NE) membranes, including the inner nuclear membrane (INM).[1–3] Sigma1R protein is expressed in all mammalian tissues, highly conserved (>90%), and enriches at sites where the ER closely contacts mitochondria or the plasma membrane.[4] *SIGMAR1* missense variant p.E102Q is linked to disease (juvenile-onset amyotrophic lateral sclerosis)[5] and leads to Sigma1R protein aggregation and decreased cell viability.[6,7] Sigma1R also has a hydrophobic drug-binding site. This site can bind cocaine and methamphetamine with nanomolar to micromolar affinity, and binds with nanomolar affinity to drugs used therapeutically to treat depression, psychosis, anxiety, pain, dementia, and Alzheimer's disease.[8–14]

While studying dopaminergic neurons, Tsai *et al.*[15] discovered that cocaine enhanced Sigma1R association with three NE proteins: emerin (an integral INM protein), lamin A or lamin C (nuclear intermediate filament

proteins), and barrier to autointegration factor 1 (BANF1; an essential chromatin protein).[16] Cocaine increased Sigma1R co-immunoprecipitation with emerin, lamin A/C, BANF1, and HDAC3 (an emerin-stimulated transcriptional silencer).[15,17] Furthermore, cocaine stimulated the Sigma1R-dependent and emerin-dependent recruitment and transcriptional repression of *MAOB1* (monoamine oxidase B), which facilitates the removal of dopamine and monoamine neurotransmitters from synapses.[15,18] These discoveries[15] provided the first evidence that cocaine influences emerin and other nuclear lamina proteins with dynamic roles in 3D genome organization and tissue-specific gene silencing at the NE.[16,19,20]

We hypothesize that Sigma1R functions as an agonist-sensitive regulator or mis-regulator of emerin and its partners in brain and possibly other tissues. Mutations in emerin (*EMD*), lamins A/C (*LMNA*), *BANF1*, or other nuclear lamina proteins cause a spectrum of tissue-specific disorders. These disorders ('laminopathies') include muscular dystrophy, cardiomyopathy, lipodystrophy, type 2 diabetes, progeria syndromes, and a neurological disorder (Charcot–Marie–Tooth)[21,22]; demyelinating disorders are linked to *LMNB1* (encoding lamin B1).[23]

Human genetic variation in *SIGMAR1* has not to our knowledge been examined in large populations. This information is critical because Sigma1R variants might influence individual responses to therapeutic agonists or an individual's risk of addiction. At the molecular level, we hypothesized that genetic variants in Sigma1R might affect its interactions with emerin or other NE partners, with unknown consequences for disease or responses to Sigma1R agonists.

To begin testing our hypothesis we analyzed *SIGMAR1* missense alleles among 60,706 unrelated individuals in the ExAC database.[24] This database is a collection of cohorts in which about half of the individuals present a known condition (either type 2 diabetes, heart disease, or psychiatric disorders comprising schizophrenia, bipolar disorder, or Tourette syndrome) and half are unaffected controls for that specific condition. Our recent analyses of *LMNA*, *EMD*, and *BANF1* variants in ExAC revealed novel variants and unexpectedly high frequencies of disease-causing dominant variants in *LMNA*, as well as novel association with psychiatric disease (*LMNA* variant p.K108E) or type 2 diabetes (*LMNA* variant p.G602S)[25] and novel association with healthy metabolic traits (*EMD* variant p.D149H).[26] Here we report the identification of novel *SIGMAR1* missense variants in ExAC, two of which were widespread and common with allele frequencies of ~2–3% or >18% in specific ethnic groups. These and other variants of interest are discussed in relation to the atomic structure of the full-length Sigma1R trimer[27] and still-unresolved questions about its membrane topology and orientation at the INM.[3,28]

Materials and methods

Manipulation and analysis of ExAC data

We queried DNA sequence data for *SIGMAR1* in ExAC (http://exac.broadinstitute.org/), focused on the most biologically relevant splice variant ENST00000277010 (encodes the canonical 223-residue protein), and downloaded the raw data for all possible mutation categories (Supplemental Table 1). We then focused on missense alleles. The data were filtered based on overall allele count and variants identified at least twice are shown in Figure 1. All data processing and analysis was done using Python version 3.7. We developed a Python script that neatly visualizes and summarizes raw ExAC data in table format; this script and instructions are available at https://github.com/aditharun/ExAC-Viewer.

Visualizing Sigma1R protein

The atomic structure of Sigma1R was accessed via the Protein Data Bank[27] and visualized using UCSF ChimeraX software (http://www.cgl.ucsf.edu/chimera). Different protein views and other manipulations were generated using ChimeraX software.

Results

We identified 49 total missense variants in the *SIGMAR1* gene, including 26 unique variants (identified in a single individual; Supplemental Table 1) and 23 variants each identified in two or more individuals (Figure 1, Supplemental Table 1). We did not find any alleles that partitioned with

Variant	Total Alleles	# HOM ind.*	Total ind.*	Allele Freq. Overall (%)
p.Gln2Pro	1985	184	1801	18.4
p.Gln24His	8	0	8	0.04661
p.Gln38Arg	2	0	2	0.006321
p.Ile42Val	2	0	2	0.005594
p.Leu65Gln	4	0	4	0.004085
p.Pro70Ser	2	0	2	0.002166
p.Phe83Leu	2	0	2	0.002936
p.Asn85Ser	7	0	7	0.01107
p.Gly87Ser	2	0	2	0.003489
p.Met93Val	2	0	2	0.004665
p.Gly155Arg	8	1	7	0.006614
p.Gly155Trp	3	0	3	0.00248
p.Gly174Ser	2	0	2	0.001649
p.Arg175Trp	2	0	2	0.001649
p.Val177Ile	2	0	2	0.001649
p.Ala183Thr	2	0	2	0.001649
p.Ala185Val	4	0	4	0.003299
p.Ala187Thr	4	0	4	0.003299
p.Gln194His	2	0	2	0.00165
p.Arg204Cys	8	0	8	0.006611
p.Arg204His	2	0	2	0.001653
p.Arg208Trp	941	19	922	0.7782
p.Arg211Gln	12	0	12	0.009934
*ind. = individuals				
*HOM = homozygote				

Figure 1. Summary of *SIGMAR1* missense variants identified two or more times in ExAC.

psychiatric diseases represented in the ExAC database. Most variants were considered rare (<1%) in ExAC, with three exceptions. One synonymous allele (p.G51G, in a splice region) was identified 602 times in ExAC (Supplemental Table 1); whether this allele affects mRNA splicing is a question for future work. Missense variant p.R208W was identified 941 times and had an allele frequency approaching 1% (specifically, 0.78%); missense variant p.Q2P was identified 1985 times for an overall allele frequency of 18.4% (Figure 1). Both missense variants were also identified in homozygosity: 2% of individuals carrying the p.R208W allele were homozygous for this allele, as were 10% of individuals with the p.Q2P allele (Figure 1). These and other missense alleles showed further enrichment in specific ethnic groups.

Variants-of-interest enriched in South Asians, East Asians, or non-Finnish Europeans

Five variants identified in 7–12 individuals each (Figure 1) were concentrated in a specific ethnic group (Figure 2). All eight individuals with p.Q24H were South Asian (allele frequency 0.1%). All seven individuals with p.N85S were non-Finnish European (allele frequency 0.02%). Eight individuals with p.G155R were non-Finnish European or Latinx (allele frequencies 0.009% and 0.017%, respectively). All eight individuals with p.R204C were South Asian (allele frequency 0.048%), and 10 of 12 individuals with p.R211Q were East Asian (allele frequency 0.11%). Although still rare, several of these variants were interesting as non-conservative substitutions in functionally important regions of the Sigma1R protein, described below.

Novel p.R208W allele identified at frequencies of ~1.9–2.9% in three ethnic groups

Variant p.R208W was widely distributed in human populations. This allele was identified at frequencies of ~0.25–0.77% in four groups (non-Finnish Europeans, Finnish, South Asian, 'Other') and at higher frequencies in Africans and Latinx (~1.9% each) and East Asians (~2.9%; Figure 2). As discussed below, variant p.R208W affects an Arg residue that is both membrane-associated and solvent-exposed, and is therefore positioned to interact with emerin or other integral membrane partners (see 'Discussion' section).

Novel p.Q2P allele identified at frequencies of ~16–38% in five ethnic groups

Variant p.Q2P was identified in nearly all ancestries represented in ExAC with high allele frequencies in Africans (~21%), East Asians (~38%), non-Finnish Europeans (~16%), Latinx (~23%), and South Asians (~19%; Figure 2). This allele was also identified in one (of two) Finnish individuals in whom this region was sequenced, and in ~16% of 'Others' (Figure 2). We conclude that variant p.Q2P is widespread and common in human populations. Based on its molecular location, discussed below, p.Q2P also has the potential to perturb Sigma1R binding to emerin or other nuclear lamina proteins.

	Allele Frequency Expressed as Percentage (%)							
Variant	Overall	African	E. Asian	Euro (NF)	Finnish	Latino	S. Asian	Other
p.Gln2Pro	18.4	20.75	38.51	15.7	50	22.95	18.84	16.1
p.Gln24His	0.04661	0	0	0	0	0	0.102	0
p.Gln38Arg	0.00632	0	0.0576	0	0	0.046	0	0
p.Ile42Val	0.00559	0	0	0	0	0	0.0218	0
p.Leu65Gln	0.00409	0	0	0.00729	0	0	0	0
p.Pro70Ser	0.00216	0	0	0.00387	0	0	0	0
p.Phe83Leu	0.00293	0	0	0.00534	0	0	0	0
p.Asn85Ser	0.01107	0	0	0.0202	0	0	0	0
p.Gly87Ser	0.00345	0	0	0	0	0.0465	0	0
p.Met93Val	0.00466	0	0	0	0	0	0.0203	0
p.Gly155Arg	0.00661	0	0	0.00902	0	0.017	0	0
p.Gly155Trp	0.00248	0	0	0	0	0.02549	0	0
p.Gly174Ser	0.00164	0	0	0	0.0151	0	0.00605	0
p.Arg175Trp	0.00164	0	0	0	0	0.00864	0.00605	0
p.Val177Ile	0.00164	0	0	0.003	0	0	0	0
p.Ala183Thr	0.00164	0	0	0.003	0	0	0	0
p.Ala185Val	0.0033	0.00962	0	0	0.0045	0	0	0
p.Ala187Thr	0.0033	0	0	0	0	0	0.0242	0
p.Gln194His	0.00165	0	0	0.003	0	0	0	0
p.Arg204Cys	0.00661	0	0	0	0	0	0.0484	0
p.Arg204His	0.00165	0.00965	0	0.0015	0	0	0	0
p.Arg208Trp	0.7782	1.89	2.89	0.263	0.247	1.939	0.442	0.779
p.Arg211Gln	0.00993	0	0.11	0.0015	0	0.00866	0	0

Figure 2. Ethnic distribution of *SIGMAR1* alleles identified two or more times in ExAC.

Locations of affected residues in Sigma1R trimers and subunits

The structure of the Sigma1R trimer was determined by X-ray crystallography.[27] Each subunit has a short N-terminal domain (residues 1–9), one transmembrane domain (TMD) (residues 10–30), and a large globular domain. One surface of the globular domain is intimately membrane-associated, two other surfaces mediate subunit–subunit association, and the remaining surface is solvent-exposed in the trimer.[3,27] Many ExAC variants affect residues that are solvent-exposed on the globular domain of the trimer, including p.Q38R, p.P70S, p.F83L, p.G155R, p.G155W, p.R175W, p.V177I, p.Q194H, p.R204C, p.R204H, p.R208W, and p.R211Q (Figure 3(a) and (b)). These variants have the potential to perturb Sigma1R association with partners located on the same side of the lipid bilayer (see 'Discussion' section). We next considered each molecular region of Sigma1R in detail.

Short N-terminal domain: The most frequent Sigma1R variant, p.Q2P, affects the short N-terminal domain (Figure 3(b)). Although proline substitutions can be disruptive, this residue varies between species[3] from Gln (humans, guinea pig, ermine, brushtail possum) to Pro (rat, mouse), Ser (zebrafish), Ala (Xenopus, roughskin newt), Gly (red junglefowl), or Cys (cow). Given this variability and the null hypothesis (genetic variants are often phenotypically silent), future testing in the context of human cell lines and nuclear lamina partners may be essential to determine whether human Sigma1R is functionally perturbed by p.Q2P.

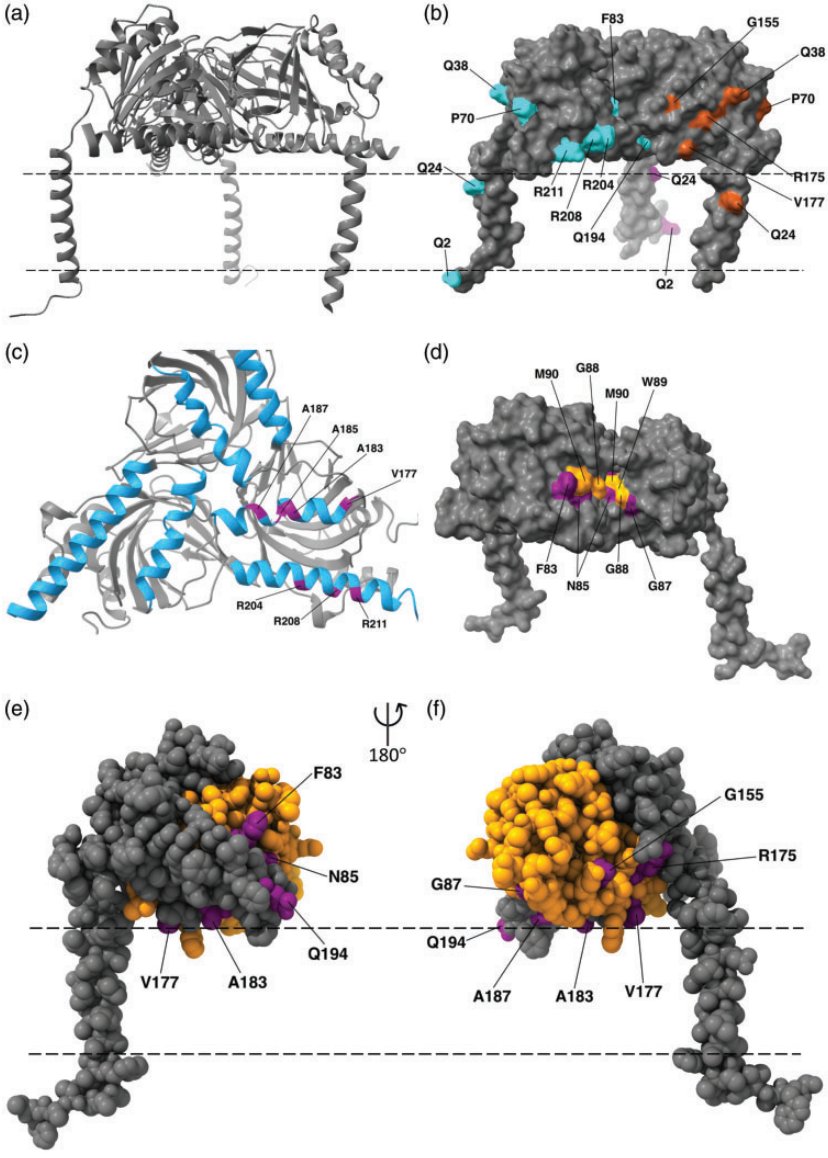

Figure 3. ExAC-affected residues in the Sigma1R trimer. (a,b) Ribbon and surface views of the Sigma1R trimer.[27] Wildtype surface-exposed residues affected by ExAC variants in Figure 1 are colored (blue, orange, or purple) and labeled where visible on each subunit. Dashed line indicates the lipid bilayer. (c) Ribbon view of the membrane-proximal surface of the globular domain of the Sigma1R trimer. Each subunit has two membrane-associated α-helices formed by residues 177–192 and residues 195–220, colored blue; residues affected by ExAC variants are purple. (d) Side surface view of two subunits of the Sigma1R trimer. Subunit–subunit contact involves residues 87–91 (residues 88-90 colored orange); ExAC-affected residues in (G87) or near (F83, N85) this region are purple. (e,f) Surface views of a single Sigma1R subunit, rotated 180°. Residues that form the ligand-binding pocket (β-barrel residues 81–176) are orange. ExAC-affected residues in or near this pocket are purple. Dashed lines indicate the lipid bilayer. (A color version of this figure is available in the online journal.)

TMD: The TMD of Sigma1R was affected by one variant: p.Q24H (Figure 3(b)). Remarkably, residue Q24 is the *only* TMD residue that did not vary among 12 vertebrate species.[3] The conservation of this residue and its position in the lipid bilayer suggest it may be required for an interaction within the bilayer, for example with the TMD of an integral membrane partner such as emerin or a hypothetical INM protein that 'bridges' Sigma1R association with emerin (see 'Discussion' section).

Residues that interact with the membrane surface: In the structure of the Sigma1R trimer, the globular domain of each subunit includes two hydrophobic α-helices that parallel the membrane surface (Figure 3(c)). The first such 'pontoon' formed by residues 177–192 (also known as Sterol Element Binding Protein-Like 2) was previously considered a potential TMD.[29–31] The other 'pontoon' is formed by residues 195–220 (Figure 3(c)). We identified several ExAC variants in or near these α-helices; some were relatively conservative substitutions (p.V177I, p.A185V, p.V190L, p.Q194H, p.L199F, p.R211Q, p.L218F) whereas others introduced a polar residue (p.A183T, p.A185T, p.A187T) or potentially disruptive residues (p.R175W, p.L182P, p.D188N, p.R204C/H, p.R208W, p.R211W, p.L212R; Figure 4(b) and (c); Supplemental Table 1). Four putative 'disruptors' including p.R208W (identified in 1.9–2.9% of three ancestries; Figure 2) affect Arg residues that

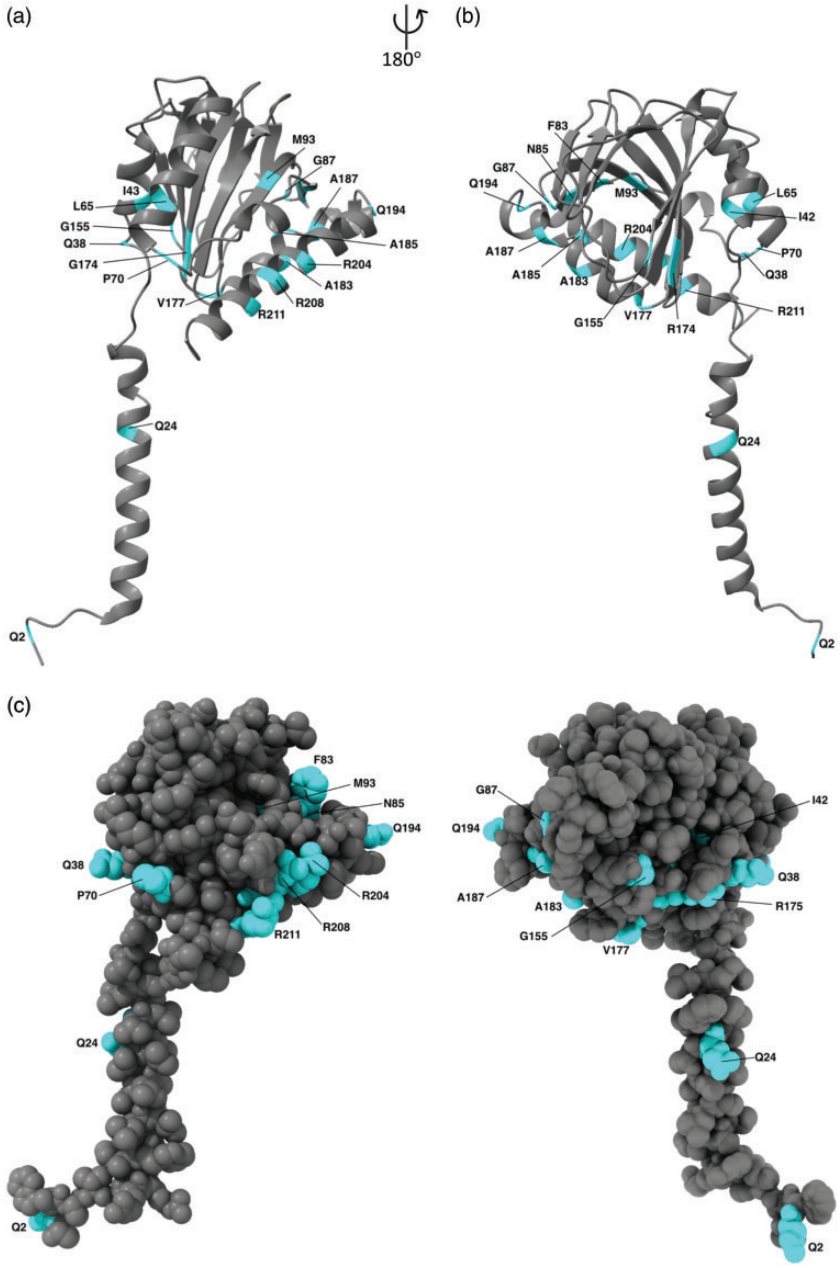

Figure 4. Alternative views of one Sigma1R subunit showing ExAC-affected residues. (a, b) Ribbon and (c) corresponding surface views of a single Sigma1R subunit (from the trimer structure), rotated by 180° in panel B. Wildtype residues affected by the ExAC variants listed in Figure 1 are blue and labeled. These views show ExAC-affected residues that are surface-exposed in the subunit but buried (p.F83L, p.N85S, p.G87S, p.M93V) or partially buried (p.Q194H) in the trimer. (A color version of this figure is available in the online journal.)

are membrane-associated but also solvent-exposed in the Sigma1R trimer (Figures 3(b) and 4(a)). This region might plausibly interact with other integral membrane proteins (see 'Discussion' section).

Subunit–subunit interactions (trimerization): Subunit–subunit association in the Sigma1R trimer is mediated in part by wildtype residues H116, R119, D195, and T198.[3,27] Oligomerization also requires a tight hairpin turn involving 'GxxxG' motif residues 87–91 at the three-fold symmetry axis (Figure 3(d)).[27] Substitutions here and elsewhere can perturb both oligomerization and ligand binding, as suggested by mutagenesis studies.[3,8] For example, hydrophobic substitutions at residue G87 (G87I, G87L) reduced both ligand binding and the stability of the oligomer.[3,8] ExAC variants in or near this region were rare but included p.F83L, p.N85S and p.G87S (purple in Figure 3(d)).

Ligand-binding pocket: Residues that form the hydrophobic ligand-binding 'pocket' within each subunit are highly conserved in vertebrates.[27] This pocket is located within a cone-shaped β-barrel (residues 81–176; orange in Figure 3(e)).[6,27] Studies using photoreactive analogs of cocaine or fenpropimorph showed ligand is enveloped by two α-helices comprising residues 91–109 (also known as SBDL1) and residues 176–194 (inner surface of the SBDL2 'pontoon' in Figure 3(c)).[29,32,33] This conserved-yet-flexible structure explains how Sigma1R can bind with high affinity to chemically diverse hydrophobic ligands.[3,27] Four variants (p.V177I, p.A183T, p.Q194H, p.A187T) affect residues in SBDL2 that interact with the membrane surface (Figure 3(c)); three (p.G155R, p.G155W, p.R175W) affect solvent-exposed residues (Figure 3(b)); and three (p.F83L, p.N85S, p.G87S) affect 'trimerization' residues near the ligand-binding pocket (Figure 3(d)), any of which might influence ligand selectivity or affinity. Variants in this region were rare (Figure 2).

Additional ExAC variants in 'buried' regions

Certain variants listed in Figure 1 affected residues that were not visible in Figure 3. These and other residues are shown in two ribbon views (Figure 4(a) and (b)) and corresponding surface views of a single Sigma1R subunit (Figure 4(c)). Most ExAC variants in the globular domain were either membrane-associated, buried, or solvent-exposed in the context of the trimer, as discussed above. However, the structure of the Sigma1R monomer alone is unsolved. Sigma1R monomers and oligomers are thought to exist in equilibrium, with ligands such as cocaine driving oligomerization through unknown mechanisms.[3] Five ExAC-affected residues were solvent-exposed in single subunits and were either buried (p.N85S, p.G87S, p.M93V) or partially buried (p.F83L, p.Q194H) in the trimer (compare Figure 4 with Figure 3(b)). Residues that are solvent-accessible only in the monomer might provide binding sites for hypothetical partners that regulate oligomerization.

Discussion

We identified two novel Sigma1R variants of special interest due to their prevalence in human populations and their potential to disrupt Sigma1R function. Variant p.Q2P was the most common variant in ExAC (identified in 18.4% of the entire cohort) with allele frequencies of ∼15–23% in non-Finnish Europeans, Africans, South Asians, and Latinx, and ∼38% in East Asians. The second-most frequent variant, p.R208W, was identified in ∼0.78% of the cohort and enriched among Africans, Latinx, and East Asians (∼1.9–2.9%). These and other Sigma1R variants identified in this study may be phenotypically silent. However, future functional studies are warranted since these variants have the potential to alter individual patient responses to a broad range of drugs (therapeutic or addictive), potentially in association with emerin or emerin's partners at the nuclear lamina.

Sigma1R associates with emerin, lamin A/C, and BANF1 directly or indirectly, and somehow manipulates their activity in response to cocaine.[15] The molecular basis of this control is completely unknown. Despite its importance in physiology, pharmacology, and addiction, the Sigma1R protein remains a multifunctional 'mystery' with fundamental aspects of its structure still uncertain. Residues ∼135–165 were predicted to be intrinsically disordered in the monomer,[3] a property that might enable conformational changes in response to ligand binding. Two ExAC variants (p.G155W and p.G155R) are likely to restrict conformational freedom in this region. The structure of the Sigma1R trimer was entirely unexpected since previous studies predicted two TMDs and a readily accessible drug-binding site.[3,6,27] Even now the topology of the Sigma1R trimer with respect to the NE/ER lumen is uncertain: the globular domain could be located in the lumen as modeled in Figure 5(a), or the nucleoplasm as modeled in Figure 5(b).

By contrast the membrane topology of emerin is known (Figure 5) and its partners lamin A/C and BANF1 are strictly nucleoplasmic. Future investigation of their interactions with Sigma1R might therefore yield insight into the topology of Sigma1R itself. Emerin has an N-terminal LEM-domain and large intrinsically disordered region, both of which are nucleoplasmic, followed by a TMD and short lumenal domain.[26,34] Thus, emerin has the potential to bind Sigma1R in either orientation, and to be affected by specific variant(s), if emerin is the direct partner as modeled in Figure 5. If emerin is not the direct partner, the same logic will apply to other candidates not depicted in Figure 5 (e.g. lamin A/C, BANF1). The Sigma1R variants identified in this study warrant future testing to determine if they alter the Sigma1R response to agonists, regardless of their potential impact on nuclear lamina proteins. They also richly warrant further investigation as tools to dissect how Sigma1R interacts with emerin and the nuclear lamina. Does emerin bind Sigma1R directly, or is their association bridged by an unidentified (potentially neuron-specific) INM protein? Does emerin associate with Sigma1R monomers or trimers (or both), and is this balance affected by cocaine? Does cocaine trigger Sigma1R-and-emerin-dependent silencing of just one gene (*MAOB1*), or genome-wide changes in 3D chromosome organization[16,19,20] that favor addiction? Might Sigma1R agonists influence lamina-dependent activities relevant to

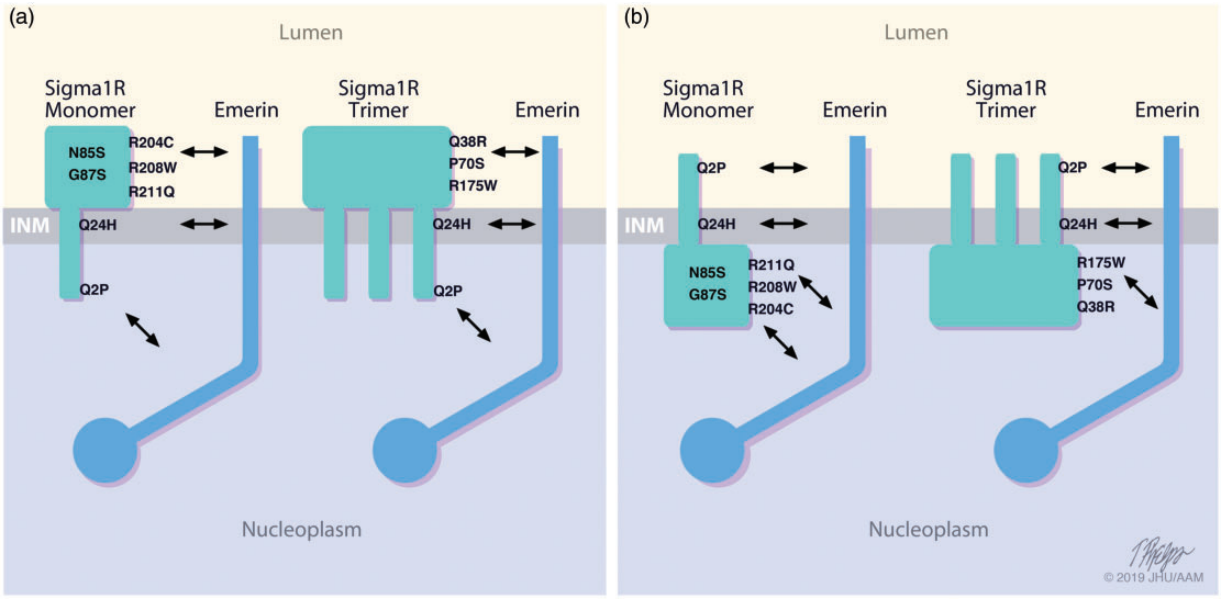

Figure 5. Too many degrees of freedom. Schematic diagrams of emerin alongside a Sigma1R monomer (here represented by one subunit of the trimer) and a Sigma1R trimer. Emerin has an N-terminal LEM-domain and large intrinsically disordered region (both located in the nucleoplasm), one TMD and a short lumenal domain. Because the membrane topology of the Sigma1R trimer is uncertain, we show two potential orientations at the INM with the globular domain located either in the lumen (a) or nucleoplasm (b). These models provide a roadmap for future studies to determine which (if any) region(s) of Sigma1R bind emerin and whether Sigma1R variants (examples shown) affect binding. If emerin is not the direct partner, the same strategy can be applied to emerin-and-Sigma1R-associated proteins (not depicted) such as lamin A/C, BANF1, HDAC3, or potentially novel INM proteins. (A color version of this figure is available in the online journal.)

neuromuscular or metabolic disease, progeria, or other laminopathies? These and other questions raised by this work suggest fascinating new directions for molecular research on Sigma1R, emerin, and the nuclear lamina.

Authors' contributions: ASA and CRE contributed equally to this paper. Conception and first draft: KLW and CRE. Preparation of figures and tables: ASA. Data analysis: KLW, ASA, CRE. Intellectual input, manuscript preparation and editing: CRE, ASA, KLW.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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