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INVESTIGATION

Novel Mutations in Synaptic Transmission Genes Suppress Neuronal Hyperexcitation in *Caenorhabditis elegans*

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ABSTRACT Acetylcholine (ACh) receptors (AChR) regulate neural circuit activity in multiple contexts. In humans, mutations in ionotropic acetylcholine receptor (iAChR) genes can cause neurological disorders, including myasthenia gravis and epilepsy. In *Caenorhabditis elegans*, iAChRs play multiple roles in the locomotor circuit. The cholinergic motor neurons express an ACR-2-containing pentameric AChR (ACR-2R) comprised of ACR-2, ACR-3, ACR-12, UNC-38, and UNC-63 subunits. A gain-of-function mutation in the non-α subunit gene *acr-2* [*acr-2(gf)*] causes defective locomotion as well as spontaneous convulsions. Previous studies of genetic suppressors of *acr-2(gf)* have provided insights into ACR-2R composition and assembly. Here, to further understand how the ACR-2R regulates neuronal activity, we expanded the suppressor screen for *acr-2(gf)*-induced convulsions. The majority of these suppressor mutations affect genes that play critical roles in synaptic transmission, including two novel mutations in the vesicular ACh transporter *unc-17*. In addition, we identified a role for a conserved major facilitator superfamily domain (MFSD) protein, *mfsd-6*, in regulating neural circuit activity. We further defined a role for the sphingosine (SPH) kinase (Sphk) *sphk-1* in cholinergic neuron activity, independent of previously known signaling pathways. Overall, the genes identified in our study suggest that optimal modulation of synaptic activity is balanced by the differential activities of multiple pathways, and the novel alleles provide valuable reagents to further dissect neuronal mechanisms regulating the locomotor circuit.

KEYWORDS
acetylcholine receptor
sphingosine kinase/*sphk-1*
major facilitator superfamily domain (MFSD) proteins
acetylcholine transporter
unc-17
lipid
seizure
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locomotion

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Cholinergic transmission underlies a variety of processes including learning, memory, and movement. iAChRs are evolutionarily conserved pentameric channels that regulate neuronal activity in the central nervous system and at the neuromuscular junction (Albuquerque *et al.* 2009). Multiple mutations in the human iAChR subunits encoded by CHRNA2 (α2), CHRNA4 (α4), and CHRNB2 (β4), have been linked to autosomal dominant forms of epilepsy (Boillot and Baulac 2016). Most disease-associated mutations in α2, α4, or β4 cluster in the second or third transmembrane (TM) domain and generally elicit gain-of-function phenotypes (Bertrand *et al.* 2002, 2005; Leniger *et al.* 2003; Hoda *et al.* 2008).

The *Caenorhabditis elegans* genome encodes over 30 AChR subunits (Hobert 2013). Decades of study have revealed the subunit composition of heteromeric and homomeric channels that act in different tissues or cells and that display differences in channel physiology and pharmacology. We previously characterized the ACR-2R pentameric ion channel that is expressed in cholinergic motorneurons (Jospin *et al.*

2009). A V309M gain-of-function mutation in the second TM domain of the ACR-2 subunit causes elevated cholinergic activity. Additionally, *acr-2(gf)* results in a cell nonautonomous decrease in the activity of inhibitory GABAergic neurons (Jospin *et al.* 2009; Stawicki *et al.* 2011). The concurrent increase in cholinergic excitation and decrease in GABA inhibition results in overexcitation of the motor circuit. This activity imbalance causes defective locomotion accompanied by spontaneous contractions of the body wall muscles, referred to as convulsions. Previous studies of genetic mutations that restored wild-type locomotion to *acr-2(gf)* animals identified UNC-38, UNC-63, and ACR-12 as the other subunits that form functional receptors with ACR-2 (Jospin *et al.* 2009). Additional suppressors of the *acr-2(gf)* convulsion phenotype defined a divalent cation transient receptor potential channel subfamily M (TRPM) that modulates locomotor circuit via ion homeostasis, and a novel mutation in *unc-13* that affects synaptic transmission through positional docking of synaptic vesicles (Stawicki *et al.* 2011; Zhou *et al.* 2013).

To further characterize the molecular pathways that mediate the effects of the overactive ACR-2R(gf), we expanded the genetic suppressor screen of *acr-2(gf)*. Here, we report the identification of novel mutations in multiple genes that regulate synaptic transmission. Many mutations are partial loss-of-function alleles in genes required for synaptic function. We identified multiple mutations affecting a conserved MFSD protein, *mfsd-6*. In addition, our analysis of *sphk-1*, the *C. elegans* homolog of human Sphk, suggests a neuronal subtype specific role for this kinase in promoting cholinergic activity in *acr-2(gf)* animals. This screen expands our understanding of the function of AChRs and provides a useful resource to dissect how synaptic transmission is modulated in the context of an *in vivo* neural circuit.

MATERIALS AND METHODS

C. elegans genetics and mutagenesis screen

Strains were maintained at room temperature or 20° as described (Brenner 1974). Genetic crosses were performed using standard methods. The genotypes of strains are listed in Supplemental Material, Table S1. The previous suppressor mutations of *acr-2(gf)* were selected based on faster movement than *acr-2(gf)* (Jospin *et al.* 2009). Here, we performed a semiclonal screen, focusing on mutations that primarily reduced the convulsion frequency. Briefly, *acr-2(n2420gf)* L4 animals (CZ10402) were subjected to 50 mM ethyl methanesulfonate following standard protocols (Kutscher and Shaham 2014). Forty P0 animals were placed on individual plates to allow egg-laying for 24 hr, averaging 30–50 F1 per P0. The P0 animals were transferred to fresh plates every day for 3 d, giving rise to ~20,000 mutagenized haploid genomes. The F2 progeny were screened for a reduction in convulsion frequency. Only one line derived from an individual P0 was kept for subsequent analysis, resulting in a total of 31 lines. Since levamisole-resistant mutations were overrepresented in the previous screen (Jospin *et al.* 2009), we first tested the suppressor mutations for levamisole sensitivity. Eight lines were found to be resistant to 1 mM levamisole and were not pursued in further analysis.

Mutation identification by outcrossing and whole-genome sequencing

Twenty-three suppressor lines that showed normal sensitivity to levamisole were outcrossed to N2. Three suppressor mutations were linked to the X-chromosome and were identified to be one intragenic loss-of-function mutation of *acr-2(gf)* and two loss-of-function mutations in *acr-12*. These suppressor lines were not subjected to further analysis. We obtained whole-genome sequencing data on 20 outcrossed suppressor strains. The Galaxy platform (Afgan *et al.* 2016) was used to analyze raw sequence files with a custom-designed workflow. We used custom-designed software to identify SNPs affecting restriction enzyme sites in the mutagenized suppressor strains compared to the reference N2 sequences. These SNPs were then used to follow chromosome linkage after further outcrossing of the suppressors to N2 (Table S2). For example, for mapping *sphk-1(ju831)*, all of the outcrossed strains contained a SNP on chromosome II but not SNPs on chromosome III or IV. For mapping the *unc-63(ju815)* allele, CZ24017 (Table S2) was outcrossed to N2, and linkage of the *ju815* mutation to chromosome I was determined based on cosegregation of the suppression effect with the *unc-63* SNP and a SNP in *anc-1(I)*, ~1.36 map units from the *unc-63* (*I*) locus (Table S1). Multiple outcrossings of *unc-63(ju815)* were then conducted to eliminate other SNPs nearby to generate CZ25251.

Among the remaining 20 independent lines, only *unc-63(ju815)* behaved as a completely dominant suppressor of *acr-2(gf)*. To outcross the *unc-63(ju815)* mutation, wild-type males heterozygous for an integrated fluorescent transgene, either (*juIs76*) or (*juIs14*), were crossed into the *ju815*; *acr-2(gf)* strain. The fluorescent transgenes were used to verify isolation of suppressed cross progeny. Nonconvulsing F2s were isolated from heterozygous F1s carrying either *juIs14* or *juIs76* transgenes and verified as homozygous for the *acr-2(gf)* mutation by Sanger sequencing. Using the combination of whole-genome sequencing and SNP mapping analyses, we identified the causative mutations in all but two of the 20 levamisole-sensitive suppressor lines. Both *ju807* and *ju863* showed linkage to chromosome II; however, the causative mutations have not been determined (Table S2).

Convulsion behavioral observation and pharmacological analysis

All behavioral observations were made on mutations that were outcrossed with N2 at least four times. Convulsions were defined as simultaneous contraction of the body wall muscles producing a concerted shortening in body length. The convulsion frequency for d1 adult animals was calculated during a 90 sec period of visual observation.

For levamisole sensitivity, 10 d1 adult animals were transferred to fresh plates containing 1 mM levamisole and were monitored every 15 min for paralysis. For aldicarb sensitivity, 0.5 mM aldicarb was used for strains containing *acr-2(gf)* or *mfsd-6* alleles, and 1.5 mM aldicarb was used on all other strains. Aldicarb sensitivity was assessed by transferring 10 d1 adults to fresh aldicarb plates, and by monitoring worms for paralysis every 30 min by gently touching the animal with a platinum wire. Aldicarb sensitivity was quantified for at least three independent experiments.

Fluorescent microscopy and image analysis

SPHK-1::GFP (*nuIs197*) was analyzed by confocal microscopy (LSM710, Zeiss) in wild-type and *acr-2(gf)* animals. The dorsal cord of L4 animals was imaged under identical settings for all samples, as previously described (Cherra and Jin 2016). Fluorescence intensity and area of each punctum was measured from a 0.5 μm Z-plane using the Analyze Particles function in NIH ImageJ.

Data availability

All reagents including strains and the diagnostic SNP analysis program are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS AND DISCUSSION

To identify additional genes contributing to the *acr-2(gf)* convulsion phenotype, we performed a semiclonal genetic suppressor screen for worms that showed a reduction in convulsion frequency. Following pharmacological tests using levamisole, an agonist of muscle iAChRs,

and aldicarb, an acetylcholinesterase inhibitor, as well as outcrossing and genetic mapping, we identified a total of 20 independent suppressor lines that showed normal sensitivity to levamisole. Two loss-of-function mutations affected the TRPM channel *gtl-2* (Stawicki *et al.* 2011; Takayanagi-Kiya *et al.* 2016). One mutation, *ju825*, was characterized as a gain-of-function mutation in the ACC family of ligand-gated channel *lgc-46* (Takayanagi-Kiya *et al.* 2016). Two suppressors were loss-of-function mutations in the neuronal calcium sensor protein *ncs-2* (Zhou *et al.* 2017). Two suppressors were mapped to chromosome II, but the causative mutations have yet to be identified (Table 1). The 13 suppressors described here in detail can be organized into two major categories: novel mutations in ACR-2R subunits, and mutations that affect synaptic vesicle loading, exocytosis, or recycling (Table 1).

Table 1 *acr-2(gf)* suppressors

Gene	Allele	Nucleotide Change[a]	Amino Acid Change[b]
AChR subunits			
unc-38	*ju852*	cCc/cAc	P494H
unc-38	*ju857*	Gga/Aga	G321R
unc-63	*ju860*	atG/atA	M150I
unc-63	*ju815*	tGt/tAt	C294Y
Synaptic genes			
unc-31	*ju818*	Cga/Tga	R1180*
sphk-1	*ju831*	Cca/Tca	P177S
unc-17	*ju840*	tCc/tTc	S398F
unc-17	*ju854*	Ccc/Tcc	P415S
unc-41	*ju873*	ctG/ctA	Q91*
unc-13	*ju874*	atttcaGcttccttg/ atttcaActtccttg	Splice site: intron 26/exon 27
mfsd-6	*ju833*	Gga/Aga	G524R
mfsd-6	*ju866*	gGa/gAa	G421E
mfsd-6	*ju870*	ttG/ttA	Q76*
Unidentified			
	ju807(II)		
	ju863(II)		

AChR, acetylcholine receptor.
[a] Capital letters indicate mutated nucleotide. The left is the reference sequence and on the right, is the mutated sequence. For the *ju874* mutation, underlined sequence is intronic sequence prior to splice site.
[b] Amino acid position is based on that for protein isoform UNC-31B, UNC-41A, UNC-13A, respectively.
*indicates stop codon.

Novel mutations in iAChR subunits suppress acr-2(gf)

UNC-63 and UNC-38 are both ACh-binding α subunits of the ACR-2R. We have previously identified multiple recessive alleles of *unc-63* and *unc-38* that suppress the *acr-2(gf)* convulsion frequency and also show strong resistance to levamisole (Jospin *et al.* 2009). Here, we found several levamisole-sensitive alleles of *unc-63* and *unc-38* that behaved as recessive suppressors of *acr-2(gf)* (Table 1). The M150I mutation in UNC-63 (*ju860*) and the G321R or P494H mutation in UNC-38 (*ju852*, *ju857*) showed disparate distribution throughout the receptors (Figure 1, A and B). These mutations may either alter the binding of ACh but not levamisole, or may alter the function of neuronal iAChRs but only mildly affect the muscle iAChRs. Of particular note, for UNC-63, animals harboring the C151Y mutation are resistant to levamisole (Lewis *et al.* 1980) but the M150I mutants remain sensitive to levamisole, highlighting the importance of obtaining a deeper understanding of the structure–function relationship of iAChR subunits.

While all other mutations in *unc-63* or *unc-38* were recessive for suppression of *acr-2(gf)*, the *ju815* mutation in *unc-63*, which affects TM2, acted in a dominant manner to suppress *acr-2(gf)* convulsion frequency (Figure 1, A–C). We verified the dominant suppression of *ju815* following extensive outcrossing and reisolation (Figure 1C, Table S1, and Table S2). *unc-63(ju815)* completely suppressed *acr-2(gf)* convulsions but does not cause a noticeable defect in locomotion as compared to *unc-63* null alleles (Figure 1C, File S1, File S2, and File S7). To more quantitatively compare *unc-63(ju815)* to an *unc-63* null mutation, *unc-63(x37)* (Lewis *et al.* 1980), we assayed these mutants for sensitivity to levamisole. *unc-63(ju815)* mutants were not as resistant to levamisole as *unc-63(x37)* animals, but *unc-63(ju815)* animals showed mild resistant to levamisole as compared to wild-type (Figure 1D).

The highly conserved TM2 domain lines the receptor pore and is critical for regulating the activity of iAChRs (Unwin 2005). The heteromeric nature of iAChRs presents a difficulty for understanding the functional interactions of the subunits without disrupting the entire receptor complex. The observation that *unc-63(ju815)* dominantly reduces *acr-2(gf)*-induced behavior yet does not show resistance to levamisole suggests that the requirement for the UNC-63 α subunit in neuronal ACR-2R and muscle Lev-R may differ significantly to provide different gating properties or ion flux. Together with the missense mutations that were reported previously to be resistant to levamisole (Jospin *et al.* 2009), these alleles provide useful information to further tease apart how heteromeric iAChRs with similar subunit compositions can have separate functions to regulate neural circuit and muscle activity.

Novel mutations in presynaptic proteins suppress acr-2(gf)

Several suppressors affected a set of genes that are required to maintain the efficient transmission of neurotransmitters or neuropeptides. We found *ju874* to be an allele of *unc-13*, which is a phorbol ester/diacylglycerol-binding protein with multiple C2 domains (Maruyama and Brenner 1991). UNC-13 and its Munc13 homologs are presynaptic active zone proteins required for synaptic vesicle priming, and loss of *unc-13* greatly reduces synaptic transmission (Aravamudan *et al.* 1999; Augustin *et al.* 1999; Richmond *et al.* 1999). We previously reported that both strong loss-of-function mutations of *unc-13* and a unique mutation in the C2A domain of UNC-13 suppress convulsions of *acr-2(gf)* (Zhou *et al.* 2013). The *ju874* allele disrupts the splice site between intron 26 and exon 27 and would be predicted to affect the extreme C-terminus of the protein (Table 1). *unc-13(ju874)* behaved as partial loss-of-function, as the animals show normal locomotion.

The *ju873* mutation affects *unc-41*, the *C. elegans* Stoned B homolog, which is generally agreed to function in synaptic vesicle recycling (Walther *et al.* 2004; Diril *et al.* 2006; Mullen *et al.* 2012). The *unc-41* gene produces two isoforms: the A isoform is broadly expressed in the nervous system, while the B isoform is exclusively expressed in GABA motor neurons (Mullen *et al.* 2012). The *unc-41(ju873)* allele results in a premature stop codon in the first exon of the A isoform (Figure 2A and Table 1). While *unc-41* null mutations are not lethal, they cause multiple defects in locomotion and egg-laying (Mullen *et al.* 2012). We confirmed that *ju873* is a new allele of *unc-41* through complementation tests with a null allele, *unc-41(e268)*. Consistent with *unc-41(ju873)* being partial loss-of-function, the locomotion defects of *unc-41(ju873)* are less severe than for *unc-41(e268)* (File S3 and File S4). Interestingly, *unc-41(e268); acr-2(gf)* and *unc-41(ju873); acr-2(gf)* animals displayed strongly reduced convulsion frequencies (Figure 2B), suggesting that the function of the UNC-41A isoform is rate-limiting for synaptic transmission in *acr-2(gf)*.

Consistent with our previous studies that revealed neuropeptide modulation of *acr-2(gf)* (Stawicki *et al.* 2013; Zhou *et al.* 2013), we identified *ju818* to be a new allele of *unc-31*, the calcium-dependent secretion activator (Table 1). *unc-31(ju818)* causes a premature termination in all

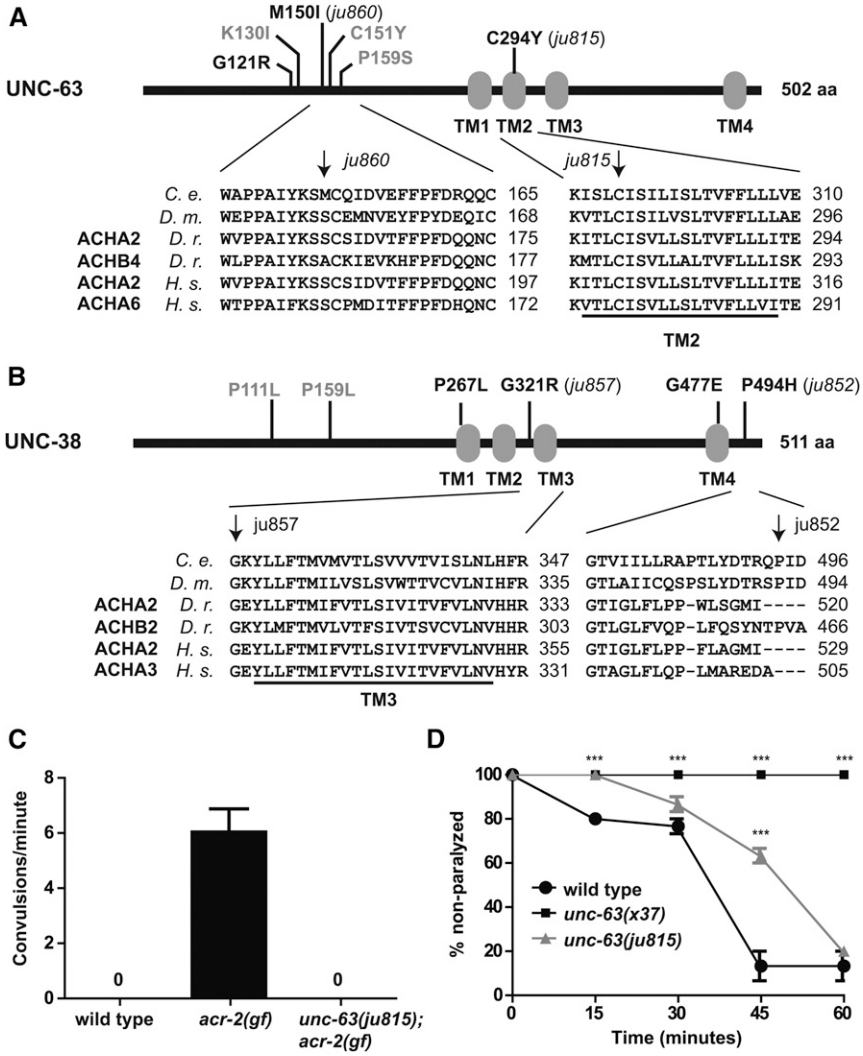

Figure 1 Levamisole-sensitive *unc-63* and *unc-38* mutants suppress *acr-2(gf)*. (A) Protein diagrams illustrating known and new alleles of *unc-63*. New alleles isolated in the *acr-2(gf)* suppressor screen are indicated with a *ju* allele number. G121R, K130I, and P159S were previously isolated through an *acr-2(gf)* screen (Jospin *et al.* 2009), and C151Y was isolated through a levamisole resistance screen (Lewis *et al.* 1980). Levamisole-sensitive alleles are labeled in black and levamisole-resistant alleles are labeled in gray. *unc-63(ju815)* affects the TM2 domain at a residue conserved from *C. elegans* to mammals. Protein alignments labeled as: *C. e.* = *C. elegans* UNC-63, *D. m.* = *Drosophila melanogaster* ACH4, *D. r.* = *Danio rerio* ACHA2 and ACHB4, and *H. s.* = *Homo sapiens* ACHA2 and ACHA6. (B) Protein diagrams illustrating known and new alleles of *unc-38*. P159L was previously isolated in a levamisole resistance screen (Garcia *et al.* 2001), and P111L, P267L, and G477E were previously isolated in an *acr-2(gf)* suppressor screen (Jospin *et al.* 2009). Black and gray alleles indicate levamisole sensitivity and resistance, respectively. Protein alignments labeled as *C. e.* = *C. elegans* UNC-38, *D. m.* = *Drosophila melanogaster* ACH4, *D. r.* = *Danio rerio* ACHA2 and ACHB2, and *H. s.* = *Homo sapiens* ACHA2 and ACHA3. (C) *unc-63(ju815)* completely suppresses *acr-2(gf)* convulsions. $N \geq 17$. (D) *unc-63(ju815)* does not cause strong resistance to 1 mM levamisole. *unc-63(x37)* results in a premature stop and is a null allele. $N = 10$ animals each trial, average of three trials in shown. *** $P < 0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test. aa, amino acid; TM, transmembrane.

but one predicted isoform, and behaves as a null allele of *unc-31* based on the suppression of *acr-2(gf)*.

In addition to genes that directly regulate synaptic vesicle release and recycling, we have also identified two mutations affecting *unc-17* (Figure 2, C and D and Table 1), the *C. elegans* vesicular ACh transporter (VAChT). Null alleles of *unc-17* are lethal, and most strong loss-of-function mutants are extremely defective in locomotion and growth. The *unc-17* mutants isolated from our screen behave as hypomorphs and show essentially normal locomotion (File S5 and File S6). The new alleles of *unc-17* map to the region around TM11, which contains one previously reported partial loss-of-function mutation, Y400N, isolated in aldicarb resistance screens (Figure 2C) (Zhu *et al.* 2001). A previous study of *unc-17* mutations affecting TM domains has used pharyngeal pumping or animal thrashing assays to rank mutation severity: TM9 mutations > TM6 mutations > TM5 mutations ≥ TM10 mutations (Zhu *et al.* 2001). The new alleles, *ju840* and *ju854*, reside in TM11, and are similar to or slightly weaker than mutations in TM5 or TM10 based on pharyngeal pumping (data not shown), thus placing these alleles at the lower end of the severity spectrum. Previous transport activity analysis of *unc-17* mutations has led to the identification of the binding site for vesamicol, a VAChT antagonist (Zhu *et al.* 2001). The alleles isolated here will provide additional coverage to further understand how VAChT functions to transport ACh and how this transporter is modulated. Overall, reduction-of-function mutations in genes involved in either SV loading, release, or endocytosis are strong suppressors of *acr-2(gf)* phenotypes, likely through reducing the efficiency of neurotransmission.

Loss-of-function in a novel conserved MFSD gene, mfsd-6, suppresses acr-2(gf)

The MFSD proteins are generally characterized by 10–12 TMs and play broad roles as transporters in vesicular or plasma membranes (Yan 2013). Members of this family include GluT, VAChT, and VGAT. We mapped three *acr-2(gf)* suppressor mutations to the R13A5.9 open reading frame (Figure 3A). R13A5.9 displays > 23% identity to the vertebrate protein known as MFSD6; therefore, this gene is renamed *mfsd-6*. An independent mutation, *tm3356*, deletes 227 bases in exon six, removing TM4, TM5, and part of TM6, and is therefore likely a null mutation (Figure 3A). The *tm3356* allele suppressed *acr-2(gf)* convulsion frequency to a similar degree as the point mutations isolated in *mfsd-6*, which suggests that the suppression is due to loss-of-function in *mfsd-6* (Figure 3B). Animals harboring null mutations in *mfsd-6* were homozygous viable with no obvious locomotion defects; however, these mutants were resistant to aldicarb, an acetylcholinesterase inhibitor that causes eventual paralysis in wild-type animals (Figure 3C). Similar results have been observed for a different deletion allele of *mfsd-6* (Ogurusu *et al.* 2015). Mutations in *mfsd-6*

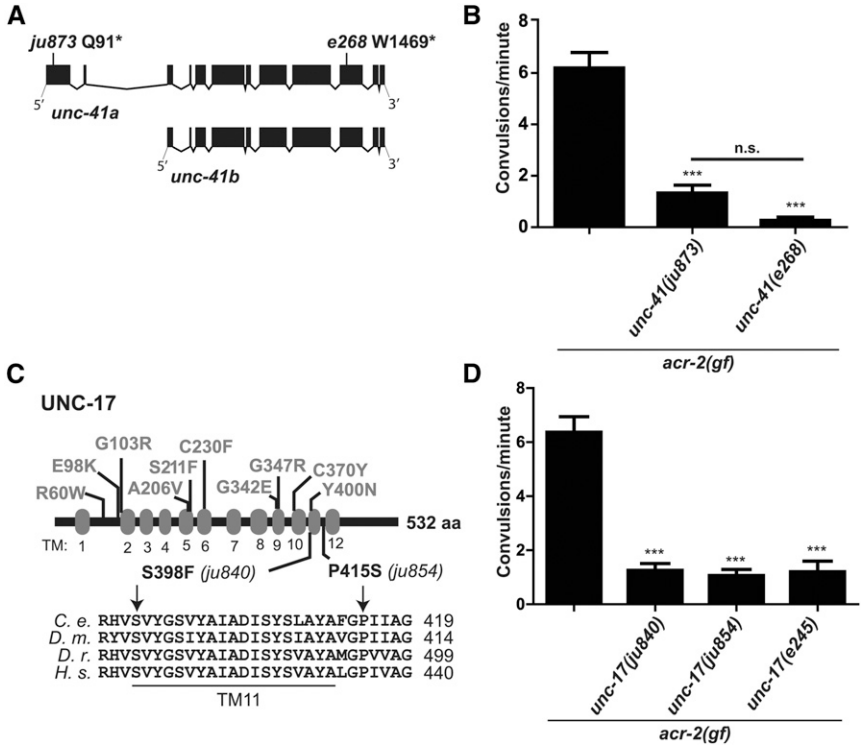

Figure 2 Novel mutations that alter synaptic vesicle function suppress *acr-2(gf)*. (A) Gene structures of *unc-41a* and *unc-41b* isoforms are depicted as described in (Mullen *et al.* 2012). *unc-41(ju873)* causes a premature stop in the first exon of *unc-41a*, which is broadly expressed in the nervous system. *unc-41(e268)* is a premature stop and is a null for both *unc-41* isoforms, producing no functional protein (Mullen *et al.* 2012). (B) *unc-41* loss-of-function suppresses *acr-2(gf)* convulsions. $N \geq 19$ each genotype. *** $P < 0.001$, one-way ANOVA on ranks followed by Dunn's *post hoc* test. n.s., not significant. (C) Diagram of UNC-17 protein with previously studied aa changes in the UNC-17 protein labeled in gray (Zhu *et al.* 2001). The mutations isolated in our screen, both of which alter evolutionarily conserved residues, are labeled in black. Protein alignments labeled as: *C. e.* = *C. elegans* UNC-17, *D. m.* = *Drosophila melanogaster* VAChT, *D. r.* = *Danio rerio* VAChT-B, and *H. s.* = *Homo sapiens* VAChT. (D) *unc-17(lf)* mutations suppress *acr-2(gf)* convulsion frequency. $N \geq 10$ *** $P < 0.001$, One-way ANOVA on ranks followed by Dunn's *post hoc* test. aa, amino acid; VAChT, vesicular acetylcholine transporter.

were not resistant to levamisole, suggesting that *mfsd-6* mutants do not display defects in muscle response to ACh. Since mutations in *mfsd-6* prevent the paralysis caused by aldicarb, which causes a build-up of ACh, leading to prolonged muscle contraction, we hypothesize that mutations in *mfsd-6* suppress the *acr-2(gf)* convulsions by disrupting presynaptic release of ACh. MFSD-6 localizes to presynaptic terminals in or near synaptic vesicles (Ogurusu *et al.* 2015). Therefore, we speculate that MFSD-6 may regulate synaptic vesicle trafficking or exocytosis to enable efficient synaptic transmission.

The TMs of MFSD family transporters have been grouped into three functional classes: substrate coordination, TM1, 4, 7, and 10; interdomain interactions, TM2, 5, 8, and 11; and structural integrity, TM3, 6, 9, and 12 (Yan 2013). As found with UNC-17, the most severe *unc-17* mutants fall in the structural TMs TM9 or TM6. Interestingly, our current screen has identified mutations in both *unc-17* and *mfsd-6* that affect the interdomain interactions modulated by TM8 or TM11. There are currently multiple hypotheses regarding how the MFSD family may transport solutes (Quistgaard *et al.* 2016), and these novel mutations may provide

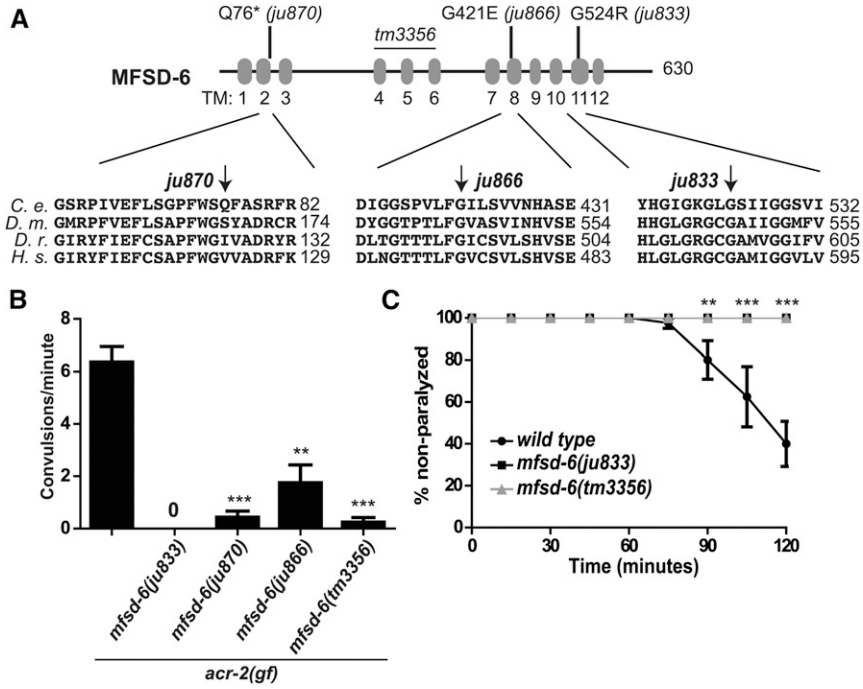

Figure 3 Loss-of-function in a novel conserved major facilitator superfamily domain (MFSD) gene, *mfsd-6*, suppresses *acr-2(gf)*. (A) Diagram of MFSD-6 protein with transmembrane (TM) domains labeled in gray. Two of the mutations identified in this screen alter evolutionarily conserved residues. Protein alignments labeled as: *C. e.* = *C. elegans* MFSD-6, *D. m.* = *Drosophila melanogaster* uncharacterized encoded by *jef*, *D. r.* = *Danio rerio* MFSD6-A, and *H. s.* = *Homo sapiens* MFSD6. (B) Loss-of-function in *mfsd-6* suppresses *acr-2(gf)* convulsions. $N \geq 8$ worms per genotype *** $P < 0.001$, ** $P < 0.01$, one-way ANOVA on ranks followed by Dunn's *post hoc* test. (C) *mfsd-6* mutations cause aldicarb resistance. Percent of animals not paralyzed over time on plates with 0.5 mM drug are shown. $N = 10$ animals in each trial, average of four trials shown, *** $P < 0.001$, ** $P < 0.01$, two-way ANOVA followed by Bonferroni's *post hoc* test.

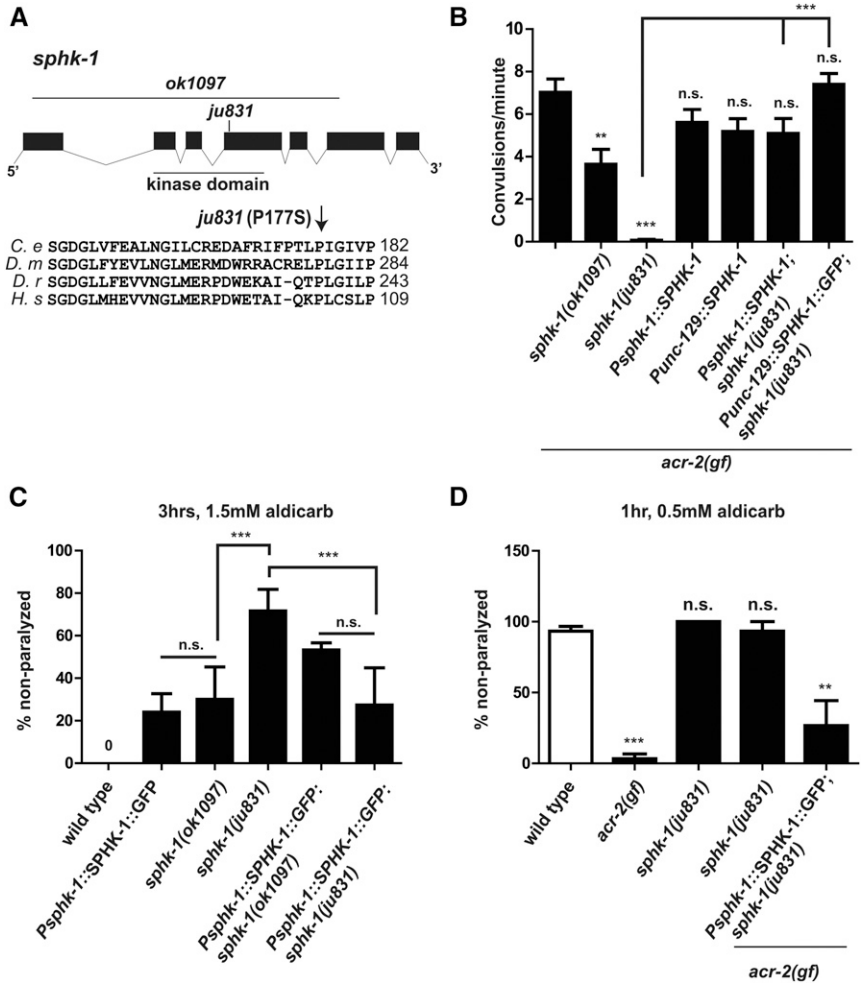

Figure 4 Loss of *sphk-1* function suppresses *acr-2(gf)* phenotypes. (A) *ju831* affects the kinase domain of *sphk-1*. Shown is the *sphk-1* genomic locus and protein alignment of part of the kinase domain. *sphk-1(ju831)* is G/A transition in the fourth exon of the gene, causing a P177S mutation. *ok1097* is a large deletion and null allele. Protein alignments labeled as: *C. e.* = *C. elegans* SPHK-1, *D. m.* = *Drosophila melanogaster* Sphk1, *D. r.* = *Danio rerio* Sphk1, and *H. s.* = *Homo sapiens* Sphk1. (B) *sphk-1(ju831)* and *sphk-1(ok1097)* both suppress *acr-2(gf)* convulsions. Transgenic expression of SPHK-1 under its endogenous promoter or the *unc-129* promoter rescues *sphk-1(ju831)* suppression of *acr-2(gf)*. $N \geq 18$ each genotype *** $P < 0.001$, n.s., not significant. One-way ANOVA on ranks followed by Dunn's *post hoc* test. (C) *sphk-1* mutations cause aldicarb resistance. Shown are percentages of animals not paralyzed after 3 hr on 1.5 mM aldicarb. (D) *sphk-1(ju831)* suppresses *acr-2(gf)* aldicarb hypersensitivity. Shown are the percentages of animals that were not paralyzed after 1 hr on 0.5 mM aldicarb. Pharmacological data are averaged from at least three trials, $N \geq 9$ animals per strain per trial. *** $P < 0.001$, ** $P < 0.01$, n.s., not significant, one-way ANOVA followed by Bonferroni's *post hoc* test. SPHK, sphingosine kinase.

further insight into how these proteins function, for example by illuminating the underlying structural changes that occur during solute transport. Mammalian MFSD6 shows expression in many areas of the brain, including the cortex, hippocampus, and midbrain [Allen Mouse Brain Atlas, Lein *et al.* (2007)], and its *in vivo* function remains unknown. Overall, the mutations in *mfsd-6* provide a valuable entry point to investigate the function of this conserved protein family.

Loss-of-function mutations in sphk-1 suppress acr-2(gf) hyperactivity

We identified the *ju831* mutation as affecting *sphk-1*, the sole *C. elegans* homolog of the conserved Sphk, which phosphorylates the lipid SPH to generate SPH-1-phosphate (S1P) (Spiegel and Milstien 2003). Previous studies have shown that SPHK-1 is localized near presynaptic terminals and that *sphk-1* loss-of-function mutants exhibit a reduced evoked release from excitatory motor neurons, possibly by modulating synaptic vesicle recycling (Chan *et al.* 2012; Chan and Sieburth 2012; Shen *et al.* 2014). *sphk-1(ju831)* causes a conserved P177S mutation in the kinase domain, close to the ATP-binding site (Figure 4A). *sphk-1(ju831)* strongly suppresses *acr-2(gf)* convulsion frequency, which was rescued by expressing an SPHK-1 cDNA transgene driven by the endogenous *sphk-1* promoter (Figure 4B). The null allele, *sphk-1(ok1097)*, suppressed *acr-2(gf)* locomotion defects, but reduced convulsion frequency to a lesser degree as compared to *ju831* (Figure 4B), suggesting that *sphk-1(ju831)* might act as a dominant-negative mutation.

Previous studies have found that *sphk-1(ok1097)* mutants are resistant to 1.5 mM aldicarb as compared to wild-type (Chan *et al.* 2012). *sphk-1(ju831)* animals also showed resistance to paralysis after 3 hr incubation on 1.5 mM aldicarb, relative to wild-type animals (Figure 4C and Table S3). Consistent with being a dominant-negative mutation, *sphk-1(ju831)* mutants are more resistant to aldicarb than *sphk-1(ok1097)*. Interestingly, we observed that overexpression of *sphk-1* also induced aldicarb resistance to a similar level as *sphk-1(ok1097)*, suggesting that in these conditions, excessive levels of *sphk-1* also inhibit neurotransmission. Overexpression of wild-type *sphk-1* reduced the aldicarb resistance of *sphk-1(ju831)* animals to that of the overexpression line alone (Figure 4C). In contrast to *sphk-1* mutants, *acr-2(gf)* animals are hypersensitive to aldicarb (Jospin *et al.* 2009), becoming paralyzed after just 1 hr on a lower concentration of drug, 0.5 mM (Figure 4D and Table S4). *sphk-1(ju831)* suppressed the aldicarb hypersensitivity of *acr-2(gf)* animals back to wild-type levels (Figure 4D). Transgenic overexpression of wild-type *sphk-1* restored aldicarb hypersensitivity to *sphk-1(ju831)*; *acr-2(gf)* double mutants (Figure 4D), indicating that *sphk-1* is critical for regulating cholinergic synaptic activity.

We have previously shown that the *acr-2(gf)* mutation is capable of driving the convulsion phenotype when expressed under the *unc-129* promoter in the cholinergic motorneurons that form synapses in the dorsal cord (Qi *et al.* 2013). Interestingly, we found that expression of *sphk-1* was also required only in this subset of cholinergic

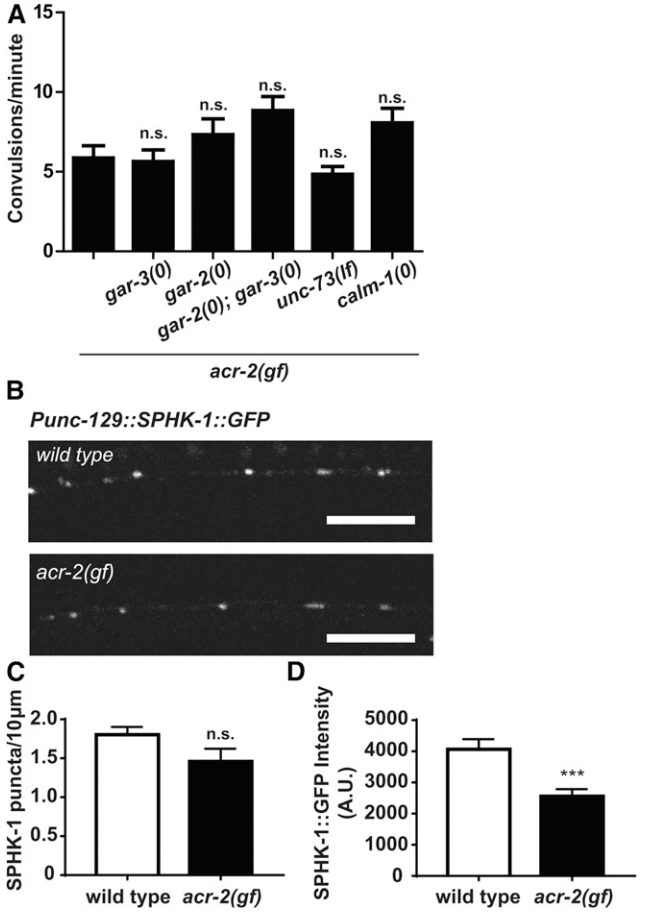

Figure 5 SPHK-1 regulates *acr-2(gf)* convulsions independent of known regulatory mechanisms. (A) Loss-of-function mutations in *gar-2*, *gar-3*, *unc-73*, and *calm-1* genes do not suppress *acr-2(gf)* convulsion rate. $N \geq 19$, n.s., not significant, one-way ANOVA on ranks followed by Dunn's *post hoc* test. (B) Expression of *Punc-129*::SPHK-1::GFP in the dorsal cord of wild-type and *acr-2(gf)* animals. Bar, 10 µm. (C) Quantification of SPHK-1::GFP punctal density. (D) Quantification of SPHK-1::GFP intensity. $N \geq 19$ each genotype. n.s., not significant ($P > 0.05$), *** $P < 0.001$, Student's *t*-test. SPHK, sphingosine kinase.

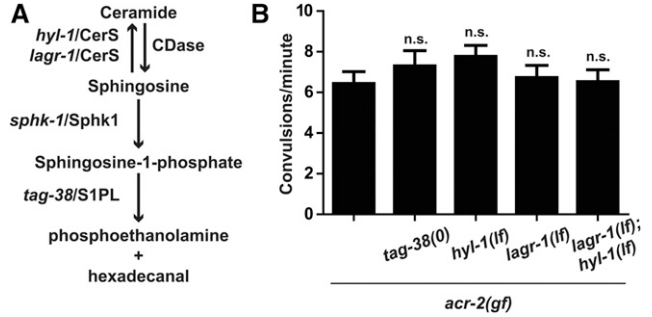

Figure 6 Mutations in sphingolipid metabolism genes do not modulate *acr-2(gf)* convulsions. (A) Diagram of the sphingosine metabolism pathway with *C. elegans* homologs noted. (B) Loss-of-function mutations in the sphingosine metabolism pathway do not suppress *acr-2(gf)* convulsion rate. The *hyl-1* and *lagr-1* mutations are small deletions that disrupt the catalytic domains of these proteins (Deng *et al.* 2008), while the *tag-38* mutation is a large deletion resulting in a molecular null. $N \geq 19$, n.s., not significant, one-way ANOVA on ranks followed by Dunn's *post hoc* test. CDase, ceramidase.

motorneurons to rescue the suppression effect of *sphk-1(ju831)* on convulsion frequency of *acr-2(gf)* (Figure 4B). These data suggest that SPHK-1 acts directly in the cholinergic neurons to mediate the convulsion behavior of *acr-2(gf)* animals.

sphk-1 contributes to acr-2(gf) convulsions independently of its regulatory Gqα pathway

In *C. elegans* motor neurons, activation of Gqα signaling, through either treatment with arecoline, a muscarinic agonist, or by a gain-of-function mutation in *egl-30*/Gqα, caused increased punctal expression of SPHK-1::GFP in axons and increased sensitivity to aldicarb (Chan *et al.* 2012, 2013; Chan and Sieburth 2012). The activity-induced SPHK-1::GFP expression required a G-protein signaling pathway involving the muscarinic receptor gene *gar-3*, the guanine exchanger factor Trio *unc-73*, and a calmodulin-like calcium binding protein *calm-1* (Chan *et al.* 2012, 2013; Chan and Sieburth 2012). We wanted to address whether the increased cholinergic activity caused by *acr-2(gf)* might promote *sphk-1* expression through the GAR-3 G-protein signaling pathway. Therefore, we used genetic analyses to test whether this pathway was required for *acr-2(gf)* convulsions, similar to *sphk-1*.

Double mutant combinations were made between *gar-3* or *calm-1* null alleles, or *unc-73(ce362)*, a partial loss-of-function allele, and *acr-2(gf)*. None of these double mutants showed detectable suppression of convulsions (Figure 5A), although *calm-1(0)*; *acr-2(gf)* animals showed a slight, but not statistically significant, increase in convulsion rate. We next investigated whether functional redundancy from similar receptors could mask a role for GAR-3 in the cholinergic neuron response to *acr-2(gf)*. To test this, we examined *gar-2*, which is also expressed in the cholinergic motorneurons and is thought to inhibit circuit activity through Goα signaling (Dittman and Kaplan 2008). We found that both *gar-2(0)*; *acr-2(gf)* and *gar-2(0)*; *gar-3(0)*; *acr-2(gf)* mutants were not significantly different from *acr-2(gf)* alone (Figure 5A). Altogether, these genetic data suggest that, while the activity of *calm-1* or the G-protein signaling pathway regulates SPHK-1 localization and function in a wild-type background, they are not necessary for *sphk-1* function when cholinergic motor neurons are hyperactivated in *acr-2(gf)* mutants.

Although the G-protein signaling pathway that regulates SPHK-1 localization did not affect *acr-2(gf)* convulsions, it was possible that hyperactive ACR-2R caused by the *acr-2(gf)* mutation alters SPHK-1 localization through a parallel pathway to cause convulsions. Therefore, we investigated the localization and fluorescence intensity of SPHK-1::GFP in cholinergic motor neurons. Compared to wild-type, *acr-2(gf)* caused a small but statistically significant decrease in SPHK-1::GFP fluorescence intensity without altering synapse number (Figure 5, A, B and C). Therefore, unlike activation of Gqα signaling, which increased the expression of SPHK-1 in cholinergic motor neurons, hyperactivation of the ACR-2R does not cause increased synaptic SPHK-1 levels.

Loss-of-function in SPH metabolism genes does not affect acr-2(gf) convulsions

SPH can be converted to ceramide by ceramide synthase enzymes or to S1P through the activity of Sphk (Figure 6A) (Spiegel and Milstien 2003; Maceyka *et al.* 2012). Furthermore, S1P is irreversibly degraded by the enzyme S1P lyase. *sphk-1* is the sole Sphk homolog in *C. elegans*, and *sphk-1(lf)* mutants should lack S1P. We next tested whether other enzymes in the SPH metabolism pathway both upstream and downstream of SPHK-1, were involved in mediating *acr-2(gf)* behaviors.

The *C. elegans* homologs of ceramide synthase include the genes *hyl-1* and *lagr-1*, while *tag-38* encodes the worm S1P lyase homolog. Lipid profiling has found that both *hyl-1(lf)* and *sphk-1(ok1097)* mutants accumulate SPH, while *hyl-1(lf)* was also shown to cause decreased levels of long-chain ceramides (Menuz *et al.* 2009). Both *tag-38* and *hyl-1* are expressed in the *C. elegans* cholinergic motor neurons, and TAG-38 strongly colocalizes with SPHK-1 in axons (Chan and Sieburth 2012). Null or strong loss-of-function mutants of *tag-38* and *hyl-1* are superficially wild-type, with normal locomotion, although *hyl-1(lf)* animals are hypersensitive to aldicarb, possibly due to increased SPH and S1P levels (Chan and Sieburth 2012). Given the increased aldicarb sensitivity of *hyl-1(lf)* mutants, one prediction would be that increased SPH and/or S1P levels resulting from *hyl-1(lf)* or *tag-38(0)* might enhance *acr-2(gf)* phenotypes. However, we found that neither *tag-38(0)* nor *hyl-1(lf)* had any effect on *acr-2(gf)* convulsion rate (Figure 6B). In addition to *hyl-1*, the *C. elegans* genome contains another putative ceramide synthase gene, *lagr-1*. Although no locomotion phenotype has been reported for *lagr-1(lf)*, this mutation conferred resistance to radiation-induced apoptosis in the germline (Deng *et al.* 2008). In contrast, *sphk-1(ok1097)* results in increased radiation-induced cell death in the germline. *lagr-1(lf)* was epistatic to *sphk-1(ok1097)* in germline apoptosis due to lack of a ceramide, a proapoptotic lipid. However, we found that neither *lagr-1(lf)* nor *lagr-1(lf); hyl-1(lf)* had any significant effect on *acr-2(gf)* convulsions (Figure 6B). Therefore, increased SPH and S1P levels do not seem to affect *acr-2(gf)* convulsion rate. These results are consistent with the observation that overexpression of SPHK-1 also does not enhance convulsions (Figure 4B). Taken together, these genetic data highlight a novel, specific function of *sphk-1* in the motor circuit in the context of *acr-2(gf)* that may be independent of the SPH metabolism pathway.

S1P has been shown to function primarily as a signaling molecule that regulates multiple processes, particularly apoptosis, and has been well-studied for its role in cancer (Maceyka *et al.* 2012). Some reports from mouse models and cell culture studies also support a conserved function for Sphk1 in promoting excitatory neurotransmission. Work in murine models found a role for Sphk1 in excitatory transmission in the hippocampus to promote learning and memory (Kanno *et al.* 2010). In cell culture experiments, Sphk1 localized to sites of endocytosis, and knockdown of Sphk1 resulted in decreased rates of endocytic uptake (Shen *et al.* 2014). These studies suggest that Sphk1 may function to mediate activity-dependent effects on endocytic recycling in the nervous system. Coincidentally, our screen has revealed a selective role of *unc-41/stoned* and *sphk-1*, both implicated in promoting endocytosis, in modulating the effects of a hyperactive neuronal AChR in the locomotion circuit. Future studies will explore possible links between these pathways.

Conclusions

Neurological disorders such as epilepsy often result from hyperactivity of cholinergic receptors, which can lead to disruptions in a diverse set of genes and pathways. We have used *C. elegans* to understand how the activity of neural circuits can be modulated in the context of circuit hyperactivity. The primary effect of *acr-2(gf)* is increased cholinergic release, and disruption of the ACR-2R itself (Jospin *et al.* 2009) or components of presynaptic release machinery are key points to modulate *acr-2(gf)* phenotypes (Zhou *et al.* 2013). This is further supported by the identification of novel hypomorphic alleles of genes, known to function in presynaptic release in the cholinergic system, that strongly suppress *acr-2(gf)*. Our recent studies of another suppressor mutation *lgc-46(ju825)* also revealed an ACC family of ligand-gated anion channels that localize to the presynaptic terminal and may provide rapid feedback inhibition on synaptic vesicle release (Takayanagi-Kiya *et al.* 2016). Altogether, our findings demonstrate the power of genetic pathway dissection using the suppression of *acr-2(gf)* as a functional readout.

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