Title
Novel Mutations in Synaptic Transmission Genes Suppress Neuronal Hyperexcitation in Caenorhabditis elegans.

Permalink
https://escholarship.org/uc/item/76p466r1

Journal
G3 (Bethesda, Md.), 7(7)

ISSN
2160-1836

Authors
McCulloch, Katherine A
Qi, Yingchuan B
Takayanagi-Kiya, Seika
et al.

Publication Date
2017-07-05

DOI
10.1534/g3.117.042598

Peer reviewed
Novel Mutations in Synaptic Transmission Genes Suppress Neuronal Hyperexcitation in Caenorhabditis elegans

Katherine A. McCulloch,*,1 Yingchuan B. Qi,*,1,2 Seika Takayanagi-Kiya,*,3 Yishi Jin,*,†,‡,4 and Salvatore J. Cherra III,*,4

*Section of Neurobiology, Division of Biological Sciences, †Department of Cellular and Molecular Medicine, and ‡Howard Hughes Medical Institute, University of California, San Diego, La Jolla, California 92093

ORCID ID: 0000-0002-9371-9860 (Y.J.)

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 epilepsy locomotion

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 CHRNA2/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.
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; Stavicki 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 divergent cation 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 (Stavicki 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 spkh-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°C 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 selected based on cosegregation of the suppression effect with acr-2(n2420gf) and were crossed with N2 at least four times. Convulsions were determined based on cosegregation of the suppression effect with acr-2(gf), 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 (nuls197) 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 gil-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 Ig–46 (Takayanagi-Kiya et al. 2016). Two suppressors were loss-of-function mutations in the neuronal calcium sensor protein nes-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).

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, but not levamisole, or may alter the function of neuronal iAChRs (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 ju874 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

### Table 1 acr-2(gf) suppressors

| Gene   | Allele   | Nucleotide Change* | Amino Acid Change* |
|--------|----------|--------------------|--------------------|
| AChR subunits |
| unc-38 | ju852    | C/Cc/CcAc          | P494H              |
| unc-38 | ju857    | G/Ga/Ga            | G321R              |
| unc-63 | ju860    | aG/aA             | M150I              |
| unc-63 | ju815    | tG/tAt            | C294Y              |
| Synaptic genes |
| unc-31 | ju818    | G/ga/Tga          | R1180*             |
| spk-1  | ju831    | C/Ca/Tca           | P177S              |
| unc-17 | ju840    | tC/tCtC            | S398F              |
| unc-17 | ju854    | C/Ct/CtC           | P415S              |
| unc-41 | ju873    | Cc/CcA            | Q91*               |
| unc-13 | ju874    | atttcaGttccttg/atttcaAgtccttg | Splice site: intron 26/exon 27 |

| mfsd-6 | ju833    | G/ga/Ga            | G524R              |
| mfsd-6 | ju866    | g/Ga/gAa           | G421E              |
| mfsd-6 | ju870    | ttG/tAt            | Q76*               |

**AChR**, acetylcholine receptor.

*Capitals 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 intrinsic sequence prior to splice site.

**Amino acid position is based on that for protein isoform UNC-31B, UNC-41A, UNC-13A, respectively.**

*Indicates stop codon.
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-38, P159L was previously isolated in a levamisole resistance screen (Zhu 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-63, D. m. = Drosophila melanogaster ACH4, D. r. = Danio rerio ACH2 and ACH4, and H. s. = Homo sapiens ACHA2 and ACHA6.

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). The three alleles isolated (ju815, 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

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.
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](https://example.com/figure3.png)

**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 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 = 19; One-way ANOVA on ranks followed by Dunn’s post hoc test. aa, amino acid; VACHT, vesicular acetylcholine transporter.
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 mfds-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 motor neurons 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
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, as confirmed by genetic analyses of double mutants (Figure 5A). These data suggest that SPHK-1 acts directly in the cholinergic neurons to mediate the convulsion behavior of acr-2(gf) animals.

Limitations of the G-protein signaling pathway for SPHK-1 might promote 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::GFP 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(cc362), 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 Goxα 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 function of acr-2(gf) in a wild-type background, they are not necessary for sphk-1 function when cholinergic motor neurons are hyperactivated in acr-2(gf) mutants.

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(0) 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 lgr-1, while tag-38 encodes the worm S1P lyase homolog. Lipid profiling has found that both hyl-1(rlf) and spkh-1(ok1097) mutants accumulate SPH, while hyl-1(rlf) was also shown to cause decreased levels of long-chain ceramides (Menuez 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(rlf) 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(rlf) mutants, one prediction would be that increased SPH and/or S1P levels resulting from hyl-1(rlf) or tag-38(0) might enhance acr-2(gf) phenotypes. However, we found that neither tag-38(0) nor hyl-1(rlf) 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, lgr-1. Although no locomotor phenotype has been reported for lgr-1(rlf), this mutation conferred resistance to radiation-induced apoptosis in the germline (Deng et al. 2008). In contrast, spkh-1(ok1097) results in increased radiation-induced cell death in the germline. lgr-1(rlf) was epistatic to spkh-1(ok1097) in germline apoptosis due to lack of a ceramide, a proapoptotic lipid. However, we found that neither lgr-1(rlf) nor lgr-1(rlf); hyl-1(rlf) 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 spkh-1 in the motor circuit in the context of acr-2(gf) that may be independent of the S1P metabolism pathway.

SIP 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 spkh-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 *lgs-46*(null25) 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.

**ACKNOWLEDGMENTS**

We thank Bhavika Anandpura for assistance in mapping the *ju815* allele. We thank Derek Sieburth for OJ802 and KP4010 strains and Shohei Mitani at National BioResource Project in Japan for deletion alleles. Some strains were provided by the National Institutes of Health (NIH) Office of Research Infrastructure Programs (P40 OD010440). This work was supported in part by NIH institutional training grants (T32 NS07220 to K.A.M. and S.J.C. and T32 AG000216 to K.A.M.), and grants to S.J.C. (F33 NS081945 and K99 NS097638) and Y.J. (R01 NS035546). Y.J. is an investigator of the Howard Hughes Medical Institute.

**LITERATURE CITED**

Afgan, E., D. Baker, M. van den Beek, D. Blankenberg, D. Bouvier et al., 2016 The galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2016 update. Nucleic Acids Res. 44: W3–W10.

Albuquerque, E. X., E. F. Pereira, M. Alkondon, and S. W. Rogers, 2009 Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol. Rev. 89: 73–120.

Aravamudan, B., T. Fergestad, W. S. Davis, C. K. Rodesch, and K. Broadie, 1999 *Drosophila* UNC-13 is essential for synaptic transmission. Nat. Neurosci. 2: 965–971.

Augustin, L., C. Rosenmund, T. C. Sudhof, and N. Brose, 1999 Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. Nature 400: 457–461.

Bertrand, D., F. Picard, S. Le Hellard, S. Weiland, I. Favre et al., 2002 How mutations in the nAChRs can cause ADNFLE epilepsy. Epilepsia 43 (Suppl. 5): 112–122.

Bertrand, D., F. Elsmie, E. Hughes, J. Trounce, T. Sander et al., 2005 The CHRN82 mutation 1312M is associated with epilepsy and distinct memory deficits. Neurobiol. Dis. 20: 799–804.

Boillot, M., and S. Baulac, 2016 Genetic models of focal epilepsies. J. Neurosci. Methods 260: 132–143.

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics 77: 71–94.

Chan, J. P., and D. Sieburth, 2012 Localized sphingolipid signaling at pre-synaptic terminals is regulated by calcium influx and promotes recruitment of priming factors. J. Neurosci. 32: 17909–17920.

Chan, J. P., Z. Hu, and D. Sieburth, 2012 Recruitment of sphingosine kinase to pre-synaptic terminals by a conserved muscarinic signaling pathway promotes neurotransmitter release. Genes Dev. 26: 1070–1085.

Chan, J. P., T. A. Staab, H. Wang, C. Mazzarette, Z. Butte et al., 2013 Extra-synaptic muscarinic acetylcholine receptors on neuronal cell bodies regulate presynaptic function in *Caenorhabditis elegans*. J. Neurosci. 33: 14146–14159.

Cherra, S. T., III, and Y. Jin, 2016 A two-immunoglobulin-transmembrane protein mediates an epidermal-neuronal interaction to maintain synapse density. Neuron 89: 325–336.

Deng, X., Y. Yin, R. Allan, D. D. Lu, C. W. Maurer et al., 2008 Ceramide biogenesis is required for radiation-induced apoptosis in the germ line of *C. elegans*. Science 322: 110–115.

Diril, M. K., M. Wiensisch, N. Jung, J. Klingauff, and V. Haucke, 2006 Stonin 2 is an AP-2-dependent endocytic sorting adaptor for synaptotagmin internalization and recycling. Dev. Cell 10: 233–244.

Dittman, J. S., and J. M. Kaplan, 2008 Behavioral impact of neurotransmitter-activated G-protein-coupled receptors: muscarinic and GABAB receptors regulate *Caenorhabditis elegans* locomotion. J. Neurosci. 28: 7104–7112.

Garcia, L. R., P. Mehta, and P. W. Sternberg, 2001 Regulation of distinct muscle behaviors controls the *C. elegans* male’s copulatory spicules during mating. Cell 107: 777–788.
