Gain-of-function mutations in the UNC-2/CaV2α channel lead to hyperactivity and excitation-dominant synaptic transmission in *Caenorhabditis elegans*

Yung-Chi Huang¹, Jennifer K. Pirri¹, Diego Rayes¹,², Shangbang Gao³,⁴, Ben Mulcahy³, Jeff Grant¹, Yasunori Saheki⁵, Michael M. Francis¹, Mei Zhen³ and Mark J. Alkema¹

¹ Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA 01655, USA

² Present address: Instituto de Investigaciones Bioquímicas de Bahía Blanca (CONICET), Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, Bahía Blanca, Argentina.

³ Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada Department of Molecular Genetics, Department of Physiology, Department of Cell and Systems Biology, University of Toronto, Toronto, Ontario, Canada

⁴ Present address: College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei 430074, China

⁵ Lulu and Anthony Wang Laboratory of Neural Circuits and Behavior, The Rockefeller University, New York, NY 10065, USA Present address: Lee Kong Chian School of Medicine, Nanyang Technological University, 308232, Singapore

*Correspondence: Mark.Alkema@umassmed.edu*
Abstract

Mutations in pre-synaptic voltage gated calcium channels can lead to familial hemiplegic migraine type 1 (FHM1). While mammalian studies indicate that the migraine brain is hyperexcitable due to enhanced excitation or reduced inhibition, the molecular and cellular mechanisms underlying this excitatory/inhibitory (E/I) imbalance are poorly understood. We identified a gain-of-function (gf) mutation in the Caenorhabditis elegans CaV2 channel α1 subunit, UNC-2, which leads to increased calcium currents. unc-2(gf) mutants exhibit hyperactivity and seizure-like motor behaviors. Expression of the unc-2 gene with FHM1 substitutions R192Q and S218L leads to hyperactivity similar to that of unc-2(gf) mutants. unc-2(gf) mutants display increased cholinergic- and decreased GABAergic-transmission. Moreover, we reveal that increased cholinergic transmission in unc-2(gf) mutants leads to reduction of GABA synapses in a TAX-6/calcineurin dependent manner. Our studies provide mechanistic insight into how CaV2 gain-of-function mutations disrupt excitation-inhibition balance in the nervous system.

Introduction

Maintenance of proper brain function requires the balance of excitatory and inhibitory synaptic transmission. There is an increasing amount of evidence that the disruption of E/I balance in neural circuits is associated with neurological disorders, including autism, epilepsy and migraine (Nelson and Valakh, 2015; Vecchia and Pietrobon, 2012). Several studies have proposed that impaired inhibitory function may drive a shift in E/I balance towards excitation, and underlie the phenotypic changes observed in these disorders (Selten et al., 2018; Mainero and Louapre, 2014). While animal model studies provide support for this hypothesis, our understanding of the molecular and cellular mechanisms that lead to E/I imbalance remains limited.

Mutations in the CACNA1A gene, which encodes the pore-forming α subunit of the CaV2.1 (P/Q type) voltage-gated calcium channel (VGCC), are associated with a broad spectrum of autosomal dominant neurological disorders. CaV2 VGCCs are the predominant channels in presynaptic nerve terminals, where they mediate the Ca\(^{2+}\) influx that triggers the fusion of synaptic vesicles with the presynaptic membrane (Catterall, 2000; Bidaud et al., 2006). CACNA1A mutations can cause episodic ataxia type 2 (EA2), epileptic seizures and familial hemiplegic migraine type 1 (FHM1) (Pietrobon, 2010). Episodic ataxia type 2 (EA2), whose clinical features include the lack of voluntary coordination of muscle movements and epileptic seizures, is associated with a range of missense, nonsense-, and...
splice site mutations throughout the CACNA1A gene. Familial hemiplegic migraine type 1 (FHM1), a severe variant of migraine that can co-occur with tonic-clonic seizures, has been found to be associated with missense mutations near the voltage sensors of the α1 subunit (Adams and Snutch, 2007). Electrophysiological analyses suggest that EA2 mutations lead to diminished channel functions, whereas both gain- and loss-of-channel function phenotypes have been reported for FHM1-associated mutations (Cao et al., 2004; Tottene et al., 2002). Although these disorders have been conventionally distinguished, they exhibit considerable overlap in clinical presentations, leaving a precise correlation between genotype and phenotype unresolved.

Animal model studies can provide mechanistic insights into the pathology of CACNA1A mutations. Mice carrying FHM1 missense mutations R192Q or S218L in the cacna1a gene display gain-of-function CaV2 phenotypes with increased Ca^{2+} current density at lower voltages (Tottene et al., 2009b; van den Maagdenberg et al., 2004a; van den Maagdenberg et al., 2010). In FHM1 knock-in mice, glutamatergic neurotransmission in cortical pyramidal cells is enhanced, while GABAergic neurotransmission is unaltered. These findings suggest that FHM1 mutations cause a dysregulation of cortical excitatory-inhibitory balance (Vecchia and Pietrobon, 2012) but the mechanisms that underlie this excitatory/inhibitory (E/I) imbalance in the nervous system remain poorly understood.

Animal model studies can provide mechanistic insights into the pathology of CACNA1A mutations. Mice carrying FHM1 missense mutations R192Q or S218L in the cacna1a gene display gain-of-function CaV2 phenotypes with increased Ca^{2+} current density at lower voltages (Tottene et al., 2009b; van den Maagdenberg et al., 2004a; van den Maagdenberg et al., 2010). In FHM1 knock-in mice, glutamatergic neurotransmission in cortical pyramidal cells is enhanced, while GABAergic neurotransmission is unaltered. These findings suggest that FHM1 mutations cause a dysregulation of cortical excitatory-inhibitory balance (Vecchia and Pietrobon, 2012) but the mechanisms that underlie this excitatory/inhibitory (E/I) imbalance in the nervous system remain poorly understood.

The genome of the nematode Caenorhabditis elegans encodes a single CaV2α subunit gene: unc-2 (Schafer and Kenyon, 1995). UNC-2/CaV2α is exclusively expressed in the nervous system (Mathews et al., 2003) and localizes to presynaptic zones, at synaptic vesicle release sites (Saheki and Bargmann, 2009), as well as at the plasma membrane of neural somas (Gao et al., 2018). Behaviorally, unc-2 loss-of-function (lf) mutants are sluggish and uncoordinated (Mathews et al., 2003). Furthermore, unc-2( lf) mutants have a reduced frequency of spontaneous excitatory postsynaptic currents (EPSCs) (Richmond et al., 2001), and reduced intrinsic neuronal calcium oscillations (Gao et al., 2018).

In this study, we characterize a novel unc-2/CaV2α gain-of-function (gf) mutant, which, in sharp contrast to the loss-of-function mutant, exhibits hyperactive- as well as seizure-like motor behaviors. We show that the expression of an unc-2 gene carrying FHM1 mutations results in a similar hyperactive behavioral phenotype, while the intragenic suppressor alleles of unc-2(gf) resemble EA2 mutations and are lethargic. We reveal that the unc-2(gf) mutation shifts the E/I balance towards excitatory transmission, and an increased excitatory signaling leads to the destabilization of GABAergic synapses in a TAX-6/calcineurin-dependent manner.

Results

zf35 mutants are hyperactive
*C. elegans* locomotion is biased towards sustained forward runs, interrupted by periodic brief reversals. From a forward genetic screen for animals with locomotion defects, we isolated a mutant, *zf35*, which failed to execute sustained forward or backward runs and continually switched the direction of locomotion in a jerky manner (reversal frequency: *zf35*: 43.1 ± 2.0/3 min, n = 59; wild type: 6.8 ± 0.4/3 min, n = 59) (Fig. 1, Supplementary Movie 1). This clonic seizure-like phenotype of *zf35* mutants was accompanied by an increased locomotion rate during bouts of forward or backward locomotion. On average, *zf35* mutants moved approximately 1.5 fold faster than wild-type animals in both directions (Fig. 1a, b). Animals heterozygous for the *zf35* mutation also displayed increased velocity and reversal frequency (Fig. 1a-c), albeit to a lesser extent when compared to homozygous mutants. This indicates that the *zf35* mutation is semi-dominant.

*zf35* mutant animals were slightly smaller than wild-type animals (0.82 ± 0.03 mm, n = 75 vs 1.00 ± 0.04 mm, n = 88) (Fig 1d) and had a reduced brood size (wild type: 207 ± 11, n = 5, *zf35*: 150 ± 16, n = 5). Furthermore, *zf35* adults retained a reduced number of eggs in the uterus (*zf35*: 3.6 ± 0.2, n = 86; wild type: 14.1 ± 0.6, n = 80) (Fig. 1e). *zf35* mutants laid eggs that are at an earlier developmental stage than wild-type animals, indicating that the time between fertilization and egg-laying was reduced (Fig. 1f). Therefore *zf35* mutants are hyperactive in both locomotion and egg-laying behaviors.

**zf35 mutant's hyperactivity is caused by a missense mutation in the unc-2/CaV2α gene**

We mapped the *zf35* mutation to the left end of chromosome X between genetic markers lon-2 and dpy-3. This region contains a gene, *unc-2*, which encodes the α1 subunit of the *C. elegans* CaV2 voltage-gated calcium channel. Sequencing analysis of the *zf35* allele revealed a single-base transition (GGA -> AGA) in the 17th exon of *unc-2* (Fig. 2a). UNC-2/CaV2α consists of four homologous domains (TMI-IV) each containing six hydrophobic membrane-spanning segments (S1–S6). The *zf35* mutation results in a glycine to arginine substitution (G1132R) in the highly conserved intracellular linker between TM III-S6 and TM IV-S1 (Fig. 2b,c). To determine if UNC-2(G1132R) in *zf35* mutant animals is sufficient to confer the hyperactive phenotype, we generated an *unc-2(zf35)* cDNA clone, which encodes the UNC-2/CaV2α(G1132R) protein. Pan-neuronal expression of the *unc-2(zf35)* transgene, in both wild-type and *unc-2* loss-of-function mutant (lf) backgrounds, induced hyperactive behavior similar to that of the *zf35* mutant. Transgenic overexpression of the wild-type *unc-2* cDNA rescued the uncoordinated and lethargic phenotype of *unc-2(lf)* mutants, but did not induce hyperactive behavior (Fig. 2e).

*unc-2(zf35)* mutants did not display obvious defects in neural morphology (data not shown). To determine if the *zf35* mutation affected UNC-2 localization, we generated transgenic animals carrying C-terminus GFP tagged *unc-2(zf35)* cDNA. UNC-2(G1132R)::GFP was observed in the cell soma, and in puncta along the neuronal processes (Fig. 2d). The fluorescence expression pattern of UNC-
2(G1132R)::GFP animals displayed no obvious difference with that of a UNC-2(WT)::GFP transgene (Fig. 2d) (Saheki and Bargmann, 2009). This indicates that UNC-2(G1132R) is properly processed and trafficked to presynaptic sites.

**Intragenic mutations suppress the unc-2(zf35) hyperactivity phenotype**

*unc-2* loss-of-function (lf) mutants are sluggish and exhibit reduced motor activities (Mathews et al., 2003). *unc-2(lf)* mutants are also slightly longer than wild type animals, most likely due to reduced muscle contraction. The contrasting phenotypes between *unc-2(zf35)* and *unc-2(lf)* mutants suggested that the *zf35* G1132R mutation is a rare gain-of-function mutation. If so, secondary, loss-of-function mutations in the *unc-2* locus should function as intragenic suppressors of the hyperactivity phenotype of *unc-2(zf35)*. From a screen of mutagenized *unc-2(zf35)* mutants, we identified 7 intragenic suppressor alleles that harbor missense or non-sense secondary mutations in the *unc-2* gene (Fig. 2b).

Four suppressors, *zf109*, *zf113*, *zf115* and *zf124* reverted the *zf35* hyperactivity phenotype to sluggish locomotion, similar to the canonical loss-of-function *unc-2(e55)* allele (Fig 2f). The *zf113*(W551stop) and *zf124*(M1371stop) alleles result in premature stop codons and therefore likely represent null alleles of *unc-2*. The *zf115*(C324Y) and *zf109*(L1357F) missense mutations result in substitutions of conserved amino acids in the S5-S6 loop of domain I and IV, respectively. Two suppressors, *zf134* and *zf130*, caused moderate locomotion defects. The *zf134*(I970T) mutation affects an amino acid in the conserved voltage sensor, and the *zf130*(G1457D) mutation affects the C-terminal region, between a conserved EF-hand and the IQ-like motif. One suppressor, *zf114*(D892N), which changes an amino acid in the domain III S2, restored locomotion behavior of *unc-2(zf35)* to approximately wild-type levels. These intragenic suppressors represent an allelic series of hypomorphic *unc-2* mutations. Their ability to revert the hyperactive phenotype of *zf35* mutants to that of the wild-type or *unc-2(lf)* mutants strongly suggest that the *zf35* mutation is a gain-of-function allele of *unc-2*. Therefore, from here on the *unc-2(zf35)* allele will be referred to as *unc-2(gf)*.

**unc-2(gf) mutation leads to increased CaV2 channel activity**

To investigate the functional consequences of the UNCF2/CaV2α G1132R gain-of-function mutation, we introduced the corresponding change (G1518R) into the human P/Q type CaV2.1 channel α1 subunit, CACNA1A (Fig. 2b). CaV2.1α expression constructs were transfected into a HEK 293 cell line that stably expresses the auxiliary β1c and α2δ subunits (Piedras-Renteria et al., 2001). Whole-cell patch clamp experiments (Fig. 3a) showed that the CACNA1A(G1518R) CaV2.1α channel exhibited a -10 mV shift in activation potential when compared to the wild-type CaV2.1α channel (Fig.
The maximal current density was 1.7-fold larger for G1518R channels (80.6 ± 5.7 pA/pF, n = 11) compared to wild type (47.5 ± 4.3 pA/pF, n = 13) (Fig. 3b).

The slope of the activation curve was not significantly affected in the CACNA1A(G1518R) channel (K<sub>a</sub> WT = 3.8 ± 0.2 mV; K<sub>a</sub> G1518R = 4.1 ± 0.1 mV, Fig 3c). Both wild-type and G1518R CaV2.1 channels decayed with similar mono-exponential time courses (T<sub>inac</sub> CACNA1A(wt) = 177 ± 45 ms and T<sub>inac</sub> CACNA1A(G1518R) = 196 ± 32 ms at a 0 mV pulse). This suggests that the transition from the open to the inactive states was not affected by the G1518R mutation. To determine if inactivation following closed states was altered, we compared steady-state inactivation properties of wild-type and G1518R channels (Fig. 3d). The membrane potential at which half of the current was inactivated in the G1518R channels exhibited a 7.7 mV shift to more positive potentials compared to wild type (V<sub>0.5inact</sub> = -55.0 ± 1.0 mV and -47.3 ± 1.0 mV for the wild-type and G1518R channels, respectively). This displacement indicates that the proportion of activatable channels is increased for CACNA1A(G1518R) channels at a given membrane potential. Thus, the G1518R mutation leads to channels that are activated at lower membrane potentials, and inactivated at higher membrane potentials. Together, these properties lead to increased current density by CACNA1A(G1518R). The conservation in the linker between TM III and TM IV between C. elegans and mammals strongly suggests that UNC-2(G1132R) exhibits similar gain-of-function effects in activation and inactivation kinetics of CaV2α channel.

**FHM1-analogous mutations in UNC-2/CaV2α lead to behavioral hyperactivity**

Several missense mutations in the human CACNA1A gene result in familial hemiplegic migraine type 1 (FHM1) (Pietrobon, 2010). Electrophysiological analysis of the effects on CaV2.1 channel kinetics of FHM1 mutations in heterologous expression systems vary considerably and can even be contradictory. For instance, while some reports find that the R192Q mutation decreases CaV2.1 calcium transients (Cao et al., 2004; Tottene et al., 2002), others find that the same mutation results in an increased calcium influx at lower membrane potentials (Hans et al., 1999; Van Den Maagdenberg et al., 2004b). In knock-in mouse models, the R192Q and S218L, FHM1 mutations increased Ca<sup>2+</sup> current density indicating a gain-of-function effect (Van Den Maagdenberg et al., 2004b; Tottene et al., 2009a; van den Maagdenberg et al., 2010). To determine the effects of FHM1 mutations in C. elegans, we introduced analogous R192Q and S218L mutations into unc-2 (Fig. 4a). Pan-neuronal expression of the unc-2(R192Q) or unc-2(S218L) transgene in C. elegans resulted in phenotypes similar to unc-2(gf) mutants. Specifically, both unc-2(R192Q) and unc-2(S218L) animals exhibited increased reversal frequencies (25.5/min ± 0.9, n = 34 and 16.5/min ± 0.9, n = 33, respectively) when compared to wild type animals (4.2/min ± 0.5, n = 29) (Fig. 4b). They also displayed hyperactive egg-
laying behavior (Fig. 4c). *unc-2*(FHM1) transgenic animals laid eggs that are at an earlier developmental stage and retained fewer eggs in the uterus (*unc-2*(R192Q): 5.7 ± 0.4, n = 37; *unc-2*(S218L): 8.4 ± 0.6, n = 32, respectively), when compared to wild-type animals (16.5 ± 0.8, n = 23). These experiments provide strong genetic evidence that, similar to *unc-2*(zf35), the FHM1 mutations are gain-of-function mutations that lead to increased CaV2 activity.

**unc-2/CaV2α gain-of-function mutations increase sensitivity to aldicarb**

Our electrophysiological recordings suggested the UNC-2/CaV2α(GF) channel may increase Ca$^{2+}$ influx, resulting in elevated neurotransmitter release. To assess if *unc-2*(gf) mutants have altered synaptic transmission, we analyzed their sensitivity to the acetylcholinesterase inhibitor, aldicarb. *C. elegans* body wall muscles receive input from excitatory cholinergic motor neurons (White et al., 1986; Richmond and Jorgensen, 1999). Aldicarb treatment causes the accumulation of acetylcholine (ACh), inducing muscle hypercontraction and acute paralysis (Miller et al., 1996). Approximately 50% of wild type animals exposed to 1 mM aldicarb became paralyzed within 1 hour, consistent with previous findings (Miller et al., 1996; Mathews et al., 2003), while less than 15% *unc-2*(lf) mutants were paralyzed within one hour (Fig. 4d). In sharp contrast, almost 100% of *unc-2*(gf) mutants became paralyzed within 30 minutes (Fig. 4d). Heterozygous *unc-2*(gf)/+ mutants also paralyzed more rapidly than the wild-type, confirming that the *unc-2*(gf) mutation is semi-dominant.

Pan-neuronal expression of *unc-2*(R192Q) or *unc-2*(S218L) also induced hypersensitivity to aldicarb (Fig. 4e). This hypersensitivity is not due to overexpression of the *unc-2* transgene because expression of a wild-type *unc-2* transgene, which restored the locomotion defects in *unc-2*(lf) mutants, led to wild-type sensitivity to aldicarb (Fig 4e). Therefore, animals with gain-of-function mutations in *unc-2/CaV2α* are hypersensitive to aldicarb, which may reflect increased ACh release at the neuromuscular junction (NMJ).

**unc-2/CaV2α(gf) mutants exhibit increased cholinergic and decreased GABAergic spontaneous postsynaptic currents (sPSCs) at the neuromuscular junction**

To directly assay the effect of the *unc-2*(gf) mutation on synaptic function, we measured the frequency of spontaneous neurotransmitter release events in recordings of postsynaptic currents (PSCs) from *C. elegans* body wall muscles. *C. elegans* body wall muscles are innervated by both excitatory (cholinergic) and inhibitory (GABAergic) motor neurons (White et al., 1986; McIntire et al., 1993; Lewis et al., 1980). To examine the total spontaneous PSC events, we performed recordings under conditions where both cholinergic and GABAergic PSCs appear as inward currents (-60 mV holding potential, see Material and Methods). *unc-2*(gf) mutants showed an over two-fold increase in
the overall frequency of spontaneous PSCs when compared to wild-type animals (Fig. 5a,b), with no significant changes in the mean amplitude (Fig. 5a,c).

Since excitatory and inhibitory neurotransmitter systems appear to be differentially affected in FHM1 mouse models (Tottene et al., 2009b; Vecchia et al., 2014; Vecchia et al., 2015), we analyzed the effect of the unc-2(gf) mutation on cholinergic and GABAergic transmission. To isolate cholinergic currents, we performed recordings at a holding potential of -60mV in the GABA receptor/unc-49 mutant background. The frequency of spontaneous excitatory postsynaptic currents (EPSCs) was increased by approximately 1.5-fold in unc-2(gf); unc-49 double mutants compared with control unc-49 single mutants (Fig. 5d-f). To isolate spontaneous GABAergic inhibitory postsynaptic currents (IPSCs), we performed recordings in the presence of 0.5 mM d-tubocurarine at a holding potential of -10 mV, a condition that specifically eliminates EPSCs (Maro et al., 2015). The frequency of spontaneous IPSC was reduced by half, without significant changes in the amplitude (Fig. 5g-i).

Our data show that, despite being expressed by both cholinergic and GABAergic motor neurons, the unc-2(gf) mutation leads to increased cholinergic and decreased GABAergic transmission to body wall muscles. Thus, instead of causing a uniform increase of neural signaling, the UNC-2/CaV2α(GF) mutation differentially affects excitatory and inhibitory signaling, shifting the E/I balance towards excitatory transmission.

**unc-2/CaV2α(gf) differentially affects excitatory and inhibitory synapses**

How does an increase of UNC-2/CaV2α activity lead to an E/I imbalance? Since changes in neuronal activity can modulate synaptic protein distribution (Frank, 2014; Turrigiano, 2012), we examined the morphology of pre- and post-synaptic markers at cholinergic and GABAergic NMJs (Fig. 6). We labeled cholinergic NMJs with the presynaptic vesicle marker RAB-3::mCherry (Pacr-2::RAB-3::mCherry) and the postsynaptic nicotinic ACh receptor (AChR) UNC-29::GFP (Punc-29::UNC-29::GFP) (Fig. 6a-d). RAB-3::mCherry puncta were larger in unc-2(gf) mutants (Fig. 6a,b), consistent with the notion that increased calcium influx can recruit more synaptic vesicles to release sites (Thanawala and Regehr, 2013). Importantly, we also observed a marked increase in the size of UNC-29::GFP clusters, indicating a concomitant increase in the postsynaptic receptors (Fig. 6c,d). To pharmacologically test if the increase in UNC-29::GFP fluorescence reflects an increase in the expression of functional AChRs at the cell surface, we examined the response of unc-2(gf) mutants to an AChR agonist, levamisole. Levamisole induces hyper-contraction and paralysis through the activation of a class of UNC-29-containing AChRs in body wall muscles (Lewis et al., 1980). unc-2(gf) mutants were hypersensitive to levamisole, consistent with an increased AChR expression on the muscle cell membrane (Supplementary Fig. 1a). These pre- and postsynaptic morphological changes
and pharmacological responses are consistent with the notion that the UNC-2/CaV2α(GF) mutation increases excitatory signaling to body wall muscle cells.

We observed a different effect on GABAergic NMJ morphology. We visualized GABAergic NMJs with the same presynaptic vesicle marker RAB-3 (Punc-25::RAB-3::mCherry) and the GABA<sub>A</sub> receptor UNC-49 (Punc-49::UNC-49::GFP). In unc-2(gf) mutants, RAB-3::mCherry puncta were enlarged, to a level comparable to that observed for cholinergic NMJs (Fig. 6e,f). However, UNC-49::GFP puncta were severely reduced in both size and number (Fig. 6g,h). At some NMJs, we noted the presence of RAB-3::mCherry puncta without punctate UNC-49::GFP apposition (Supplementary Fig. 2), suggesting post-synaptic silencing of GABA synapses.

The reduced UNC-49::GFP fluorescence in unc-2(gf) mutants is in sharp contrast to the increased fluorescence of the UNC-29::GFP cholinergic receptor. To determine if the morphological changes in UNC-49::GFP fluorescence signals reflect reduced levels of functional UNC-49 on the muscle cell surface, we analyzed unc-2(gf) mutants’ response to the GABA receptor agonist muscimol. Muscimol induces hyperpolarization of body wall muscles through UNC-49/GABA<sub>A</sub>-mediated inward Cl<sup>-</sup> currents (Richmond and Jorgensen, 1999). Muscimol sensitivity is assessed by the animal’s ability to respond to head touch. Wild-type animals typically initiate backward locomotion when touched to their heads. After treatment with 1mM muscimol, severely affected wild-type animals become flaccid, unable to respond to head touch. Moderately affected animals respond with a rubber band phenotype, in which the body wall muscles initially contract but then fully relax, failing to generate backward locomotion (de la Cruz et al., 2003). unc-2(gf) mutants exhibited reduced sensitivity to muscimol: most unc-2(gf) mutants were able to generate backward locomotion upon the head touch (Supplementary Fig.1b). The partial resistance of unc-2(gf) mutants to muscimol-induced muscle relaxation is consistent with reduction of UNC-49/GABA<sub>A</sub> expression at the muscle cell surface.

Thus, consistent with the electrophysiological analyses, our pharmacological studies demonstrate that a gain-of-function mutation in UNC-2/CaV2α has distinct effects on cholinergic and GABAergic synapses. However, both spontaneous EPSC and IPSC amplitudes are not significantly different between unc-2(gf) mutants and the wild type. This suggests that the density of functional cholinergic and GABAergic receptors at individual synapses is unchanged in unc-2(gf) mutants. As individual synapses can be difficult to resolve with confocal microscopy, single fluorescent puncta often represent multiple synapses. Given this, the synaptic fluorescence changes we observe most likely indicate increases or decreases in the number of excitatory and inhibitory synaptic connections. An increased number of cholinergic synapses would account for the increased sEPSC frequency, the levamisole hypersensitivity, and increased UNC-29::GFP fluorescence intensity in the of nerve cord. Similarly, a reduced number of GABAergic synapses is consistent with a reduced spontaneous IPSC
frequency, reduced sensitivity to muscimol, and reduced UNC-49::GFP fluorescence intensity in the nerve cord.

**unc-2(gf) expression in cholinergic neurons impairs GABA synapse formation**

The striking difference in excitatory and inhibitory neuromuscular signaling in *unc-2/CaV2a(gf)* mutants is surprising since both cholinergic and GABAergic neurons express *unc-2/CaV2a*. Why does a gain-function-mutation in the presynaptic CaV2 channel lead to a reduction in the number of GABAergic synapses?

The simplest explanation is that cholinergic and GABAergic synapses respond differently to increased presynaptic activity. For instance, while increased ACh release may result in the increase of cholinergic synapses, increased GABA release may result in a homeostatic reduction of GABAergic synapses. To test this possibility, we analyzed the GABAergic synaptic markers in animals that specifically express the *unc-2(gf)* transgene in GABAergic motor neurons (*Punc-47::UNC-2(GF)) in a wild-type background. Expression in GABAergic motor neurons alone resulted in an increase in both presynaptic RAB-3::mCherry and post-synaptic UNC-49::GFP fluorescence (Fig. 7a,b and Supplementary Fig. 3). Thus, the reduction of GABAergic synapses in *unc-2(gf)* mutants is not a direct consequence of elevated GABAergic neuron activity. Instead, our results indicate that increased GABAergic motor neuron activity in principle leads to increases in both presynaptic and postsynaptic termini similar to that observed for cholinergic synapses.

Previous studies showed that cholinergic signaling affects the development and transmission of GABAergic neurons (Jospin et al., 2009; Barbagallo et al., 2017). Therefore, increased cholinergic transmission in *unc-2(gf)* mutants may negatively affect the formation of GABAergic synapses. Indeed, when we expressed *unc-2(gf)* only in cholinergic neurons (*Pacr-2::UNC-2(GF)), UNC-49::GFP fluorescence was reduced to a similar degree as in the *unc-2(gf)* mutants (Fig. 7a,b). Presynaptic RAB-3::mCherry fluorescence in GABAergic neurons was slightly increased in *Pacr-2::UNC-2(GF)* animals (Supplementary Fig. 3), which may reflect increased stimulation of GABAergic motor neurons by cholinergic motor neurons. Together, these results suggest that increased activity of cholinergic motor neurons in *unc-2(gf)* mutants is not only required, but causes the decrease in GABAergic synapses.

**Increased excitatory signaling leads to calcineurin-dependent reduction of inhibitory synapses**

How might increased cholinergic input lead to a reduction in GABAergic synapses? First, we examined whether reducing cholinergic synaptic transmission was sufficient to restore UNC-49::GFP expression in *unc-2(gf)* mutants. ACR-12, expressed by cholinergic motor neurons, and UNC-29,
expressed by body wall muscles, are subunits of ionotropic AChRs. The loss of ACR-12 reduces excitability of cholinergic motor neurons (Jospin et al., 2009; Petrash et al., 2013). Loss of UNC-29, a subunit of the levamisole-sensitive AChR, reduces cholinergic depolarization of body wall muscles (Fleming et al., 1997; Richmond and Jorgensen, 1999). In both unc-2(gf); acr-12 and unc-2(gf); unc-29 mutants, UNC-49::GFP fluorescence was restored to wild-type levels (Fig. 7a,b). This finding indicates that increased cholinergic input to body wall muscles is the primary signal for decreasing the number of GABA synapses. Cholinergic motor neurons simultaneously innervate body wall muscles and GABAergic motor neurons (White et al., 1986). Both acr-12 and unc-29 are also expressed by GABAergic motor neurons, and play a role in cholinergic activation of not only body wall muscles, but also GABA motor neurons (Petrash et al., 2013; Philbrook et al., 2018). However, GABA signaling was not required for UNC-49/GABA<sub>A</sub>R expression or localization in body wall muscles (Gally and Bessereau, 2003). Together, these results suggest that increased cholinergic input to body wall muscles negatively regulates GABAergic postsynapse formation or stability.

To directly test this possibility, we examined UNC-49::GFP expression in animals where we specifically increased cholinergic input to body wall muscles. Muscle-specific expression of the hyperactive levamisole-sensitive AChR (L-AChR(GF)) containing gain-of-function mutations in L-AChR subunits UNC-29 and UNC-38, leads to increased excitation of body wall muscles, but no obvious defects in muscle structure or cholinergic synapses (Bhattacharya et al., 2014). L-AChR(gf) transgenic animals exhibited normal presynaptic marker expression at GABAergic NMJs (Supplementary Fig. 3). However, similar to unc-2(gf) mutants, postsynaptic UNC-49::GFP fluorescence was markedly reduced in L-AChR(gf) animals (Fig. 7c,d). Transgenic expression of the wild-type L-AChR (Pmyo-3::L-AChR(wt)) did not affect UNC-49::GFP fluorescence. This indicates that increased cholinergic signaling onto muscles in unc-2(gf) mutants negatively regulates GABAergic postsynapse formation.

Several studies with cultured hippocampal neurons suggest GABAergic receptors are modulated by excitatory neuronal activity. In particular, sustained high Ca<sup>2+</sup> levels reduce inhibitory synaptic strength through a calcineurin-dependent lateral diffusion of GABA<sub>A</sub> receptor from synapses (Bannai et al., 2009; Bannai et al., 2015; Muir et al., 2010). We examined whether <i>C. elegans</i> calcineurin, TAX-6, is required for the decrease in GABAergic postsynapses in unc-2(gf) mutants. UNC-49::GFP expression was not significantly different in a tax-6(lf) mutants (Fig. 7c,d). However, a tax-6(lf) mutation restored UNC-49::GFP fluorescence in unc-2(gf) mutants. Together, these results indicate that increased cholinergic input to body wall muscles reduces the number of GABAergic postsynapses in a calcineurin-dependent manner.
Discussion

Gain- and loss-of-function mutations in unc-2/CaV2α result in opposing phenotypes

Presynaptic voltage-gated calcium channels (CaV2) are crucial regulators of neuronal excitability and synaptic transmission. Here we report the isolation of a gain-of-function mutation in the unc-2 gene. unc-2(gf) mutants are hyperactive and exhibit seizure-like motor behaviors, in contrast to the lethargus of unc-2(lf) mutants (Schafer and Kenyon, 1995; Mathews et al., 2003). The unc-2(gf) mutation results in a G-to-R substitution in a highly conserved region in the intracellular linker between TMIII and TMIV. Our electrophysiological analyses indicate that this G-to-R substitution causes a shift to lower voltages of activation and reduced inactivation of the channel to increase Ca\(^{2+}\) influx. A similar G-to-R mutation in an intracellular linker of the human CaV1.2 channel results in similar defects in channel inactivation that underlies Timothy syndrome (Splawski et al., 2004). The negative shift in the activation potential of UNC-2/CaV2α(GF) channel is reminiscent of similar observations for several mutant human CaV2.1α channels that have been identified in patients with familial hemiplegic migraine type 1 (FHM1) (Hans et al., 1999; Tottene et al., 2005; Müllner et al., 2004). While both loss- and gain-of-function phenotypes in CaV2.1 channels with FHM1 mutations have been reported in various expression systems, most FHM1 mutations appear to lead to channel activation at lower voltages and/or increased channel open probability. The gain-of-function effect of FHM1 mutations is supported by knock-in mouse models of the FHM1 R192Q and S218L channel, which activate at lower membrane potentials and have an increase in open probability (Tottene et al., 2009b; van den Maagdenberg et al., 2010).

Intragenic suppressor mutations of the unc-2(gf) allele include both premature stop codons and missense mutations. Most intragenic suppressor mutations result in uncoordinated and lethargic phenotypes, indicating that they are hypomorphic alleles. Interestingly, some intragenic suppressor mutations resemble those found in CACNA1A in EA2 patients, including the C324Y mutation in the domain I S5-S6 loop (analogous to the CACNA1A(C287Y) mutation) (Wan et al., 2005), and the L1357F mutation in the domain IV S5-S6 loop analogous to CACNA1A (L1749P) mutation (Maksemous et al., 2016). These and other CACNA1A(EA2) missense mutations are partial or total loss-of-function mutations that lead to defects in channel trafficking or positive shifts in the voltage threshold for activation (Jeng et al., 2008; Mezghrani et al., 2008).

We found that expression of an unc-2 transgene carrying FHM1 mutations R192Q and S218L in C. elegans recapitulated the behavioral hyperactivity of unc-2(gf) mutants, whereas EA2-like CACNA1A(lf) mutations led to decreased motor activity. These studies provide strong genetic evidence that EA2 mutations are reduction-of-function mutations, while FHM1 mutations are gain-of-function
mutations. *C. elegans*, which has a single CaV2α gene, thus provides an efficient *in vivo* system to determine the genetic nature of VGCC mutations associated with neurological disorders.

**An unc-2 gain-of-function mutation results in E/I imbalance**

Presynaptic Ca\(^{2+}\) influx through CaV2 channels is tightly coupled to neurotransmitter release. In accordance, *unc-2* loss-of-function mutants are resistant to the acetylcholinesterase inhibitor aldicarb (Miller et al., 1996), and have a reduction in spontaneous EPSC frequency (Richmond et al., 2001; Tong et al., 2015; Liu et al., 2018). The *unc-2(gf)* mutation increases Ca\(^{2+}\) influx, which would lead to an increase in neurotransmitter release probability. In accordance, *unc-2(gf)* mutants are hypersensitive to aldicarb, and show a two-fold increase in spontaneous EPSC frequency. In contrast, spontaneous IPSC frequency is significantly reduced in *unc-2(gf)* mutants. Therefore, even though UNC-2 is expressed by both cholinergic and GABAergic motor neurons, the UNC-2/CaV2α(GF) mutation differentially affects excitatory and inhibitory signaling, shifting the E/I balance towards excitatory transmission.

Human studies indicate that cortical hyperexcitability in migraine patients (Aurora and Wilkinson, 2007; Pietrobon and Striessnig, 2003), could result from enhanced excitation and/or reduced inhibition. This has led to the hypothesis that migraine is a disorder of brain E/I imbalance (Vecchia and Pietrobon, 2012; Mainero and Louapre, 2014). Our data strongly support this hypothesis. The differential effect on excitatory and inhibitory signaling was also observed in FHM1 mouse models (Tottene et al., 2009b). The R192Q FHM1 knock-in mice exhibit increased excitatory glutamatergic signaling, while inhibitory GABAergic transmission appears unaffected. In the R192Q FHM1 mice, an increase in glutamate release is thought to play a key role in initiation of cortical spreading depression, but the molecular and cellular mechanisms that underlie this E/I imbalance in mammals remain unclear. Our results provide new insights into how CaV2 gain-of-function mutations may lead to the E/I imbalances.

**Increased excitatory transmission leads to destabilization of GABAergic synapses**

The *C. elegans* neuromuscular system, where both excitatory (cholinergic) and inhibitory (GABAergic) motor neurons regulate muscle activity, provides a suitable and complementary model for mechanistic studies of E/I imbalance (Stawicki et al., 2011; Safdie et al., 2016; Zhou et al., 2017). In our system, *unc-2(gf)* mutations led to a modest increase in RAB-3 expression in the neurites of both excitatory and inhibitory motor neurons, consistent with the notion that increased Ca\(^{2+}\) influx may potentiate the recruitment of synaptic vesicles (Gracheva et al., 2008; Han et al., 2011). However, the *unc-2(gf)* mutation led to pronounced and opposite effects on the density of cholinergic and GABAergic receptors in the ventral nerve cord: an increase of AChR, but a marked decrease of GABA\(_A\)R, which parallel the increased sEPSC frequency and reduced sIPSC frequency. Like the wild-type UNC-
2/CaV2α, UNC-2/CaV2α(GF) channel proteins localize to presynapses to mediate Ca\textsuperscript{2+} influx and exocytosis of neurotransmitters so these effects are not attributable to channel mislocalization. Unchanged amplitudes of spontaneous EPSCs and IPSCs suggest the density of receptors at individual cholinergic and inhibitory synapse is not affected by unc-2(gf). Therefore, the differential density of cholinergic and GABAergic receptors and the frequency of spontaneous EPSCs and IPSCs reflects the number of functional synapses from excitatory and inhibitory motor neurons to the body wall muscles.

Our results show that the reduced GABAergic neuromuscular signaling in unc-2(gf) mutants is a consequence of increased cholinergic signaling onto the same muscle target. A possible explanation of this observation is a differential response of cholinergic and GABAergic synapses to increased stimulation: strengthening of excitatory and homeostatic compensation of inhibitory synapses (Malenka and Bear, 2004; Glanzman, 2010; Gaiarsa et al., 2002). However, our results argue against this possibility: GABAergic-specific expression of the gain-of-function UNC-2/CaV2α channel leads to increased density of GABA\textsubscript{A} receptors in the nerve cord, hence the CaV2(GF) channel in principle should increase synaptic strength in both synapse types. Our results instead reveal that the reduction of GABAergic neuromuscular signaling is a consequence of increased cholinergic input to the muscle cells.

In the mammalian brain, excessive neuronal excitation can induce long-term depression of GABAergic transmission (Gaiarsa et al., 2002). Long-term depression of GABAergic transmission is associated with decreased GABA\textsubscript{A} receptor clustering (Bannai et al., 2009). NMDA receptor mediated Ca\textsuperscript{2+} influx can induce LTD at GABAergic synapses by activating calcineurin (Lu et al., 2000; Wang et al., 2003). Sustained activity-dependent Ca\textsuperscript{2+} influx reduces inhibitory synaptic strength through a calcineurin-dependent increase in the lateral mobility of synaptic GABA\textsubscript{A} receptors (Bannai et al., 2009; Muir et al., 2010). GABA\textsubscript{A} receptor clustering is regulated in part by lateral diffusion on the cell surface (Triller and Choquet, 2008), utilizing several evolutionarily conserved molecular mechanisms (Maro et al., 2015; Tong et al., 2015; Tu et al., 2015). In C. elegans, increased AChR-mediated Na\textsuperscript{+}/Ca\textsuperscript{2+} influx in unc-2(gf) mutants may similarly increase GABA\textsubscript{A} receptor diffusion to disassemble or prevent the assembly of GABAergic postsynapses. UNC-49/GABA\textsubscript{A} receptor localization to postsynaptic sites is restored by removing TAX-6/calcineurin, implicating a conserved activity-dependent mechanism to modulate of synaptic inhibition.

We propose that UNC-2/CaV2 gain-of-function mutations change the E/I balance of the C. elegans neuromuscular system: increased excitatory signaling strengthens excitatory synapses, resulting in the destabilization of inhibitory synapses in a calcineurin-dependent manner. A decrease in synaptic inhibition has been implicated in epilepsy, schizophrenia and autism (Eichler and Meier, 2008;
Nelson and Valakh, 2015; Vecchia and Pietrobon, 2012). Since the role of CaV2 channels in excitatory and inhibitory signaling is conserved, the processes we describe provide valuable insights into molecular and neural mechanisms of E/I imbalance that underlie neurological disorders.

**Materials and Methods**

**Strains**

All strains were cultured at room temperature (22-24˚C) on nematode growth media (NGM) agar plates with the *E. coli* strain OP50 as a food source. Experiments were performed on young adult animals (24 hr post-L4 larva) at room temperature (22-24˚C). The wild-type strain was Bristol N2. Transgenic strains were obtained by microinjection of plasmid DNA into the germline with coinjection marker *lin-15* rescuing plasmid pL15EK both at 80 ng/μl into *unc-2(e55); lin-15(n765ts)* or *lin-15(n765ts)* animals. At least three independent transgenic lines were obtained. The data presented are from a single representative line. The following strains were utilized in this study:

- CB55 *unc-2(e55)*
- QW37 *unc-2(zf35gf)*
- QW355 *unc-2(zf109)*
- QW359 *unc-2(zf113)*
- QW360 *unc-2(zf114)*
- QW441 *unc-2(zf115)*
- QW720 *unc-2(zf124)*
- QW726 *unc-2(zf130)*
- QW849 *unc-2(zf134)*
- QW388 *unc-2(e55); zfEx51[Ptag-168::unc-2(zf35gf)]*
- QW383 *zfEx51[Ptag-168::unc-2(zf35gf)]; lin-15(n765ts)*
- QW392 *unc-2(e55); zfEx52[Ptag-168::unc-2]*
- QW863 *unc-2(e55); zfEx327[Ptag168::unc-2(FHM1 S218L); lin-15(n765ts)]*
- QW864 *unc-2(lj1); zfEx328[Ptag168::unc-2(FHM1 R192Q); lin-15(n765ts)]*
- QW1317 *unc-2(e55); zfEx575[Ptag168::unc-2::gfp]; lin-15(n765ts)*
- QW1362 *unc-2(e55); zfEx601[Ptag168::unc-2(zf35gf)::GFP]; lin-15(n765ts)*
- lZ930 *ufIs58[Punc-47::RAB-3::mCherry]; oxls19[Punc-49::UNC-49::GFP]*
- lZ106 *unc-29(x29); ufIs7[UNC-29::GFP]*
- QW1970 *unc-2(zf35gf); ufIs58[Punc-47::RAB-3::mCherry]; oxls19[Punc-49::UNC-49::GFP]*
- QW1703 *unc-2(zf35gf); acr-12; ufIs58[Punc-47::RAB-3::mCherry]; oxls19[Punc-49::UNC-49::GFP]*
QW1726 unc-2(zf35gf); unc-29; ufIs58[Punc-47::RAB-3::mCherry]; oxIs19[Punc-49::UNC-49::GFP]
QW1367 ufIs58[Punc-47::RAB-3::mCherry]; oxIs19[Punc-49::UNC-49::GFP]; zfEx609[Punc-47::unc-2(zf35gf);+rol-6(+)];
QW1375 ufIs58[Punc-47::RAB-3::mCherry]; oxIs19[Punc-49::UNC-49::GFP]; zfEx613[Pacr-2::unc-2(zf35gf); rol-6(+)]
QW1849 tax-6(p675); oxIs19[Punc-49::UNC-49::GFP]; ufIs58[Punc-47::RAB-3::mCherry];
QW1841 unc-2(zf35gf); tax-6(p675); oxIs19[Punc-49::UNC-49::GFP]; ufIs58[Punc-47::RAB-3::mCherry]
IZ539 akIs26[Pmyo-3::lev-1(gf);Pmyo-3::unc-29(gf); lin-15(+)(L-AChR(gf)); ufIs58[Punc-47::RAB-3::mCherry]; oxIs19[Punc-49::UNC-49::GFP]
IZ818 ufIs47[Pmyo-3::UNC-38; Pmyo-3::UNC-29; Pmyo-3::LEV-1; lin-15(+)(L-AChR(wt)); ufIs58[Punc-47::RAB-3::mCherry]; oxIs19[Punc-49::UNC-49::GFP]

**Molecular Biology and plasmids**

The unc-2(zf35gf) mutation was introduced in the *Ptag-168::unc-2(wt)* clone (Saheki and Bargmann, 2009) using site-directed mutagenesis. For cell-specific unc-2(zf35gf) transgene expression, cell specific promoters for GABAergic (*Punc*-47) and cholinergic (*Pacr*-2) (Barbagallo et al., 2010) motor neurons were amplified by PCR with FseI restriction site at the 5' end and an AscI site at the 3' end. The *Ptag-168::unc-2(zf35gf)* construct was digested with FseI and AscI to remove the *Ptag-168* promoter and replace with cell specific promoters of interest. For unc-2 transgene carrying human FHM1 mutations, unc-2(R192Q) and unc-2(S218L). UNC-2 and human CACNA1A amino acid sequences were aligned to locate the corresponding amino acid substitutions in UNC-2. The mutations were then introduced in the *Ptag-168::unc-2(wt)* construct by site-directed mutagenesis. The wild-type human CaV2.1 cDNA used in the HEK cell recording was obtained from Y. Cao and R. Tsien (Cao and Tsien, 2010). To generate the human CaV2.1 G1518R cDNA, UNC-2 and human CACNA1A amino acid sequences were aligned to locate the corresponding unc-2(zf35gf) glycine(G) to arginine(R) substitution in CACNA1A. The mutation was then introduced in the CACNA1A cDNA by site-directed mutagenesis.

**Isolation of unc-2(zf35) mutants, mapping and cloning**

The unc-2(zf35) allele was isolated in a screen for animals that were resistant to the immobilizing effects exogenous tyramine as previously described (Pirri et al., 2009). We mapped unc-2(zf35) to LG X based on its hyperactive locomotion phenotype using SNP mapping (Wicks et al., 2001; Davis et al., 2005). Three-factor mapping placed unc-2(zf35) to the left of lon-2 and close to dpy-3.
Isolation and identification of intragenic unc-2(zf35gf) suppressors

unc-2(zf35gf) L4 animals (P0) were mutagenized with 0.5 mM N-ethyl-N-nitrosourea (ENU) for 4 hr. Approximately 10,000 F1 animals were bleached to obtain F2 eggs. F2 eggs were plated on NGM plates containing 0.25 mM aldicarb and examined for viable progeny after 7 and 14 days. Aldicarb resistant animals were individually transferred to fresh NGM plates, and their progeny were retested for aldicarb resistance. All suppressors isolated from the screen backcrossed with the wild-type N2. Suppressors that showed linkage to the X-chromosome were tested for complementation with unc-2(lf) mutants. Molecular changes of unc-2(zf35) intragenic suppressors were identified by DNA sequencing of the unc-2 gene.

Behavioral and pharmacological assays

Spontaneous reversal frequency was scored on NGM plates with freshly seeded OP50. The animals were transferred from their culture plate to a new plate, and allowed to recover for 1 min. After the recovery period the number of reversals was counted for 3 min.

To quantify the instantaneous velocity and average forward velocity, animals were transferred from their culture plate to a new NGM plate seeded with a thin bacterial lawn and allowed to recover for 1 min. After the recovery period the animals were tracked for 90 sec using a single worm tracker (Yemini et al., 2013). Videos were recorded at 30 frames per second and each frame was analyzed with wormtracking software (Leifer et al., 2011) to measure instantaneous velocity of single animals. Reversals, as well as 10 frames before and following each reversal, were discarded from the average forward velocity.

To examine defects in movement, individual young adult worms were transferred into 96-well plates containing 50 μl M9 buffer in each well. After a 30 sec recovery period, body bends were counted for 30 s. A body bend was defined as a change in direction of bending at the mid-body. Egg-laying assays were performed as described (Koelle and Horvitz, 1996). Rates of egg-laying behaviors were measured by two different assays: the numbers of unlaid fertilized eggs accumulated inside of adult animals, and the developmental stages of freshly laid eggs. Briefly, in both assays, L4 larvae were isolated and allowed to develop for 40 hr. In the first method, the adults were then incubated in 96-well plates containing 1% sodium hypochlorite until the bodies were dissolved. In the second method, the adults were transferred to a fresh plate. After 30 min, the developmental stage of each freshly laid egg was determined by viewing under a high-magnification dissecting microscope.

To quantify aldicarb resistance, young adult animals were transferred to NGM plates supplemented with 1 mM aldicarb. The percentage of paralyzed animals was scored at 15 min intervals. Animals were scored as paralyzed when they did not move when prodded with a platinum wire.
Electrophysiology with HEK 293 cells

A stable HEK 293 cell line expressing the calcium channel auxiliary subunits $\beta_{1c}$ and $\alpha_{2\delta}$ (Cao and Tsien, 2010) was used to transiently transfect 5 $\mu$g of the wild-type or G1518R CaV2.1 $\alpha_1$ subunit using the calcium phosphate method. A plasmid encoding the green fluorescent protein (pGreen lantern) was also transfected to allow identification of transfected cells. Cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum and 1000 U/ml penicillin–streptomycin. Whole-cell inward currents were recorded 24–36 hr after transfection with a HEKA EPC-9 patch clamp amplifier. Recordings were filtered at 2 kHz and acquired using Patchmaster software (HEKA). The extracellular recording solution contained 5 mM BaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 40 mM TEACl, 10 mM glucose, and 87.5 mM CsCl, pH 7.4. Typically the pipettes exhibited resistances ranging from 2 to 4 M$\Omega$ and were filled with internal solution containing: 105 mM CsCl, 25 mM TEACl, 1 mM CaCl$_2$, 11 mM EGTA, and 10 mM HEPES, pH 7.2.

Cell capacitance (16.7 ± 6.7 pF; $n = 24$) and series resistance (9.7 ± 4.6 M$\Omega$ before compensation; $n = 24$) were measured from the current transient after a voltage pulse from 80 to 90 mV. Series resistance was typically compensated by 80–90%. Cells with large currents in which errors in voltage control might appear were discarded. I-V curves were generated by measuring the peak currents obtained after stepping the membrane potential from a holding potential of -120 mV to voltages between -55 and 40 mV in 5 mV increments for 200 msec. I-V curves were fitted with Equation 1: $I = G(G - E_{rev}) (1+\exp{(V_{0.5} - V)/k_a})^{-1}$ where $G$ is membrane conductance, $E_{rev}$ is the reversal potential, $V_{0.5}$ is the midpoint, and $k_a$ the slope of the voltage dependence. Current densities were obtained by dividing the current peak amplitude to the cell capacitance for each experiment.

To measure steady-state inactivation profiles, conditioning pre-pulses (10 sec) from -90 to 20 mV in 10mV steps were applied, and the membrane was then stepped to the peak of the I–V curve. Currents were normalized to the maximal value obtained at the test pulse and plotted as a function of the prepulse potential. Data were fitted with Boltzmann equations: $I/I_{\text{max}} = (1 + \exp[(V-V_{0.5})/k_i]-1)$

Data analysis was performed using the IgorPro software (WaveMetrics Inc., Lake Oswego, OR); figures, fitting and statistical analysis were done using the SigmaPlot software (version 11.0; Systat Software Inc.). Data are presented as mean ± SD. Significant differences were determined using Student’s t test with the significance value set at $p<0.01$.

Electrophysiology with C. elegans neuromuscular preparations

Total spontaneous postsynaptic currents were recorded from body wall muscles as previously described (Gao and Zhen, 2011). Intracellular solution: K-gluconate, 115 mM; KCl, 25 mM; CaCl$_2$, 0.1
mM; MgCl₂, 5 mM; BAPTS, 1 mM; HEPES, 10 mM; Na₂ATP, 5 mM; Na₂GTP, 0.5 mM cAMP, 0.5 mM; cGMP, 0.5mM. pH 7.2 with KOH, ~320mOsm. Extracellular solution: NaCl, 150 mM; KCl, 5 mM; CaCl₂, 5 mM; MgCl₂, 1 mM; glucose, 10 mM; sucrose, 5mM; HEPES, 15mM. pH 7.3 with NaOH, ~330 mOsm, and the membrane potential was held at -60 mV. To isolate spontaneous excitatory postsynaptic currents, total spontaneous postsynaptic currents were recorded in unc-49/GABA receptor mutant background. To isolate spontaneous inhibitory postsynaptic currents, 0.5mM d-tubocurarine (dTBC) was added to the extracellular solution to block acetylcholine receptors, and the membrane potential was held at -10mV so IPSCs appeared as outward currents (Maro et al., 2015). All electrophysiology experiments were carried out at room temperature (20-22˚C).

**Synaptic marker imaging**

L4-stage transgenic animals expressing synaptic markers were picked a day before imaging. Young adults were mounted on 2% agarose pads containing 60 mM sodium azide for 5 minutes and immediately examined for fluorescent protein expression and localization patterns. Images were captured under consistent detector settings with a Hamamatsu Photonics C2400 CCD camera on a Zeiss Axioplan2 Imaging System. Images were projected into a single plane using NIH ImageJ software.

**Acknowledgments.** We thank Yu-Qin Cao for CACNA1A constructs and cell lines, the *Caenorhabditis* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440) for nematode strains, Andrew Leifer for Worm tracking software, Jan Czerminski and Micah Belew for experimental assistance and Vivian Budnik for comments on the manuscript. This work was supported by NIH grant GM084491 to M.J.A, CIHR FDS 154274 and NSERC RGPIN-2017-06738 to M.Z. and NSFC 31671052 to S.G.

**References:**

Adams, P. J., and Snutch, T. P. (2007). Calcium channelopathies: voltage-gated calcium channels. Sub-cellular biochemistry 45, 215-251.

Aurora, S. K., and Wilkinson, F. (2007). The brain is hyperexcitable in migraine. Cephalalgia 27, 1442-1453.

Bannai, H., Lévi, S., Schweizer, C., Inoue, T., Launey, T., Racine, V., Sibarita, J. B., Mikoshiba, K., and Triller, A. (2009). Activity-Dependent Tuning of Inhibitory Neurotransmission Based on GABAAR Diffusion Dynamics. Neuron 62, 670-682.
Bannai, H., Niwa, F., Sherwood, M. W., Shrivastava, A. N., Arizono, M., Miyamoto, A., Sugiura, K., Lévi, S., Triller, A., and Mikoshiba, K. (2015). Bidirectional Control of Synaptic GABAAR Clustering by Glutamate and Calcium. Cell Reports 13, 2768-2780.

Barbagallo, B., Philbrook, A., Touroutine, D., Banerjee, N., Oliver, D., Lambert, C. M., and Francis, M. M. (2017). Excitatory neurons sculpt GABAergic neuronal connectivity in the C. elegans motor circuit. Development (Cambridge, England) 1807-1819.

Barbagallo, B., Prescott, H. A., Boyle, P., Climer, J., and Francis, M. M. (2010). A Dominant Mutation in a Neuronal Acetylcholine Receptor Subunit Leads to Motor Neuron Degeneration in Caenorhabditis elegans. Journal of Neuroscience 30, 13932-13942.

Bhattacharya, R., Touroutine, D., Barbagallo, B., Climer, J., Lambert, C. M., Clark, C. M., Alkema, M. J., and Francis, M. M. (2014). A conserved dopamine-cholecystokinin signaling pathway shapes context-dependent Caenorhabditis elegans behavior. PLoS Genet 10, e1004584.

Bidaud, I., Mezghrani, A., Swayne, L. A., Monteil, A., and Lory, P. (2006). Voltage-gated calcium channels in genetic diseases. Biochimica et biophysica acta 1763, 1169-1174.

Cao, Y. Q., and Tsien, R. W. (2010). Different relationship of N- and P/Q-type Ca2+ channels to channel-interacting slots in controlling neurotransmission at cultured hippocampal synapses. J Neurosci 30, 4536-4546.

Cao, Y. Q., Piedras-Renteria, E. S., Smith, G. B., Chen, G., Harata, N. C., and Tsien, R. W. (2004). Presynaptic Ca2+ channels compete for channel type-preferring slots in altered neurotransmission arising from Ca2+ channelopathy. Neuron 43, 387-400.

Catterall, W. A. (2000). Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 16, 521-555.

Davis, M. W., Hammarlund, M., Harrach, T., Hullett, P., Olsen, S., and Jorgensen, E. M. (2005). Rapid single nucleotide polymorphism mapping in C. elegans. BMC genomics 6, 118.

de la Cruz, I. P., Levin, J. Z., Cummins, C., Anderson, P., and Horvitz, H. R. (2003). sup-9, sup-10, and unc-93 may encode components of a two-pore K+ channel that coordinates muscle contraction in Caenorhabditis elegans. The Journal of neuroscience : the official journal of the Society for Neuroscience 23, 9133-9145.

Eichler, S. A., and Meier, J. C. (2008). E-I balance and human diseases - from molecules to networking. Front Mol Neurosci 1, 2.

Fleming, J. T., Squire, M. D., Barnes, T. M., Tornoe, C., Matsuda, K., Ahnn, J., Fire, A., Sulston, J. E., Barnard, E. A., Sattelle, D. B., and Lewis, J. A. (1997). Caenorhabditis elegans levamisole resistance genes lev-1, unc-29, and unc-38 encode functional nicotinic acetylcholine receptor subunits. J Neurosci 17, 5843-5857.
Frank, C. A. (2014). How voltage-gated calcium channels gate forms of homeostatic synaptic plasticity. Front Cell Neurosci 8, 40.

Gaiarsa, J. L., Caillard, O., and Ben-Ari, Y. (2002). Long-term plasticity at GABAergic and glycinergic synapses: mechanisms and functional significance. Trends Neurosci 25, 564-570.

Gally, C., and Bessereau, J.-L. (2003). GABA is dispensable for the formation of junctional GABA receptor clusters in Caenorhabditis elegans. The Journal of neuroscience : the official journal of the Society for Neuroscience 23, 2591-2599.

Gao, S., Guan, S. A., Fouad, A. D., Meng, J., Kawano, T., Huang, Y. C., Li, Y., Alcaire, S., Hung, W., Lu, Y., Qi, Y. B., Jin, Y., Alkema, M., Fang-Yen, C., and Zhen, M. (2018). Excitatory motor neurons are local oscillators for backward locomotion. Elife 7,

Gao, S., and Zhen, M. (2011). Action potentials drive body wall muscle contractions in Caenorhabditis elegans. Proceedings of the National Academy of Sciences of the United States of America 108, 2557-2562.

Glanzman, D. L. (2010). Common mechanisms of synaptic plasticity in vertebrates and invertebrates. Curr Biol 20, R31-6.

Gracheva, E. O., Hadwiger, G., Nonet, M. L., and Richmond, J. E. (2008). Direct interactions between C. elegans RAB-3 and Rim provide a mechanism to target vesicles to the presynaptic density. Neuroscience Letters 444, 137-142.

Han, Y., Kaeser, P. S., Südhof, T. C., and Schneggenburger, R. (2011). RIM determines Ca²⁺ channel density and vesicle docking at the presynaptic active zone. Neuron 69, 304-316.

Hans, M., Luvisetto, S., Williams, M. E., Spagnolo, M., Urrutia, A., Tottene, A., Brust, P. F., Johnson, E. C., Harpold, M. M., Stauderman, K. A., and Pietrobon, D. (1999). Functional consequences of mutations in the human alpha1A calcium channel subunit linked to familial hemiplegic migraine. The Journal of neuroscience : the official journal of the Society for Neuroscience 19, 1610-1619.

Jeng, C. J., Sun, M. C., Chen, Y. W., and Tang, C. Y. (2008). Dominant-negative effects of episodic ataxia type 2 mutations involve disruption of membrane trafficking of human P/Q-type Ca²⁺ channels. Journal of Cellular Physiology 214, 422-433.

Jospin, M., Qi, Y. B., Stawicki, T. M., Boulin, T., Schuske, K. R., Horvitz, H. R., Bessereau, J. L., Jorgensen, E. M., and Jin, Y. (2009). A neuronal acetylcholine receptor regulates the balance of muscle excitation and inhibition in Caenorhabditis elegans. PLoS Biology 7,

Koelle, M. R., and Horvitz, H. R. (1996). EGL-10 regulates G protein signaling in the C. elegans nervous system and shares a conserved domain with many mammalian proteins. Cell 84, 115-125.
Leifer, A. M., Fang-Yen, C., Gershow, M., Alkema, M. J., and Samuel, A. D. T. (2011). Optogenetic manipulation of neural activity in freely moving Caenorhabditis elegans. Nature Methods 8, 147-U71.

Lewis, J. A., Wu, C. H., Levine, J. H., and Berg, H. (1980). Levamisole-resistant mutants of the nematode Caenorhabditis elegans appear to lack pharmacological acetylcholine receptors. Neuroscience 5, 967-989.

Liu, H., Li, L., Wang, W., Gong, J., Yang, X., and Hu, Z. (2018). Spontaneous Vesicle Fusion Is Differentially Regulated at Cholinergic and GABAergic Synapses. Cell Rep 22, 2334-2345.

Lu, Y. M., Mansuy, I. M., Kandel, E. R., and Roder, J. (2000). Calcineurin-mediated LTD of GABAergic inhibition underlies the increased excitability of CA1 neurons associated with LTP. Neuron 26, 197-205.

Mainero, C., and Louapre, C. (2014). Migraine and inhibitory system - I can’t hold it. Curr Pain Headache Rep 18, 426.

Makseious, N., Roy, B., Smith, R. A., and Griffiths, L. R. (2016). Next-generation sequencing identifies novel CACNA1A gene mutations in episodic ataxia type 2. Mol Genet Genomic Med 4, 211-222.

Malenka, R. C., and Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. Neuron 44, 5-21.

Maro, G. S., Gao, S., Olechwier, A. M., Hung, W. L., Liu, M., Özkan, E., Zhen, M., and Shen, K. (2015). MADD-4/Punctin and Neurexin Organize C. elegans GABAergic Postsynapses through Neuroligin. Neuron 86, 1420-1432.

Mathews, E. A., García, E., Santi, C. M., Mullen, G. P., Thacker, C., Moerman, D. G., and Snutch, T. P. (2003). Critical Residues of the Caenorhabditis elegans unc-2 Voltage-Gated Calcium Channel That Affect Behavioral and Physiological Properties. 23, 6537-6545.

McIntire, S. L., Jorgensen, E., Kaplan, J., and Horvitz, H. R. (1993). The GABAergic nervous system of Caenorhabditis elegans. Nature 364, 337-341.

Mezghrani, A., Monteil, A., Watschinger, K., Sinnegger-Brauns, M. J., Barrère, C., Bourinet, E., Nargeot, J., Striessnig, J., and Lory, P. (2008). A destructive interaction mechanism accounts for dominant-negative effects of misfolded mutants of voltage-gated calcium channels. The Journal of neuroscience : the official journal of the Society for Neuroscience 28, 4501-4511.

Miller, K. G., Alfonso, A., Nguyen, M., Crowell, J. A., Johnson, C. D., and Rand, J. B. (1996). A genetic selection for Caenorhabditis elegans synaptic transmission mutants. Proceedings of the National Academy of Sciences of the United States of America 93, 12593-12598.

Muir, J., Arancibia-Carcamo, I. L., MacAskill, A. F., Smith, K. R., Griffin, L. D., and Kittler, J. T. (2010). NMDA receptors regulate GABAA receptor lateral mobility and clustering at inhibitory synapses through serine 327 on the γ2 subunit. Proc Natl Acad Sci U S A 107, 16679-16684.
Müllner, C., Broos, L. A. M., Van Den Maagdenberg, A. M. J. M., and Striessnig, J. (2004). Familial hemiplegic migraine type 1 mutations K1336E, W1684R, and V1696I alter Cav2.1 Ca2+ channel gating: Evidence for β-subunit isoform-specific effects. Journal of Biological Chemistry 279, 51844-51850.

Nelson, S. B., and Valakh, V. (2015). Excitatory/Inhibitory Balance and Circuit Homeostasis in Autism Spectrum Disorders. Neuron 87, 684-698.

Petrash, H. A., Philbrook, A., Haburcak, M., Barbagallo, B., and Francis, M. M. (2013). ACR-12 ionotropic acetylcholine receptor complexes regulate inhibitory motor neuron activity in Caenorhabditis elegans. The Journal of neuroscience 33, 5524-5532.

Philbrook, A., Ramachandran, S., Lambert, C. M., Oliver, D., Florman, J., Alkema, M. J., Lemons, M., and Francis, M. M. (2018). Neurexin directs partner-specific synaptic connectivity in. Elife 7.

Piedras-Renteria, E. S., Watase, K., Harata, N., Zhuchenko, O., Zoghbi, H. Y., Lee, C. C., and Tsien, R. W. (2001). Increased expression of alpha 1A Ca2+ channel currents arising from expanded trinucleotide repeats in spinocerebellar ataxia type 6. The Journal of neuroscience : the official journal of the Society for Neuroscience 21, 9185-9193.

Pietrobon, D., and Striessnig, J. (2003). Neurobiology of migraine. Nat Rev Neurosci 4, 386-398.

Pietrobon, D. (2010). CAv2.1 channelopathies. Pflugers Archiv European Journal of Physiology 460, 374-393.

Pirri, J. K., McPherson, A. D., Donnelly, J. L., Francis, M. M., and Alkema, M. J. (2009). A Tyramine-Gated Chloride Channel Coordinates Distinct Motor Programs of a Caenorhabditis elegans Escape Response. Neuron 62, 526-538.

Richmond, J. E., and Jorgensen, E. M. (1999). One GABA and two acetylcholine receptors function at the C. elegans neuromuscular junction. Nature neuroscience 2, 791-797.

Richmond, J. E., Weimer, R. M., and Jorgensen, E. M. (2001). An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature 412, 338-341.

Safdie, G., Liewald, J. F., Kagan, S., Battat, E., Gottschalk, A., and Treinin, M. (2016). RIC-3 phosphorylation enables dual regulation of excitation and inhibition of Caenorhabditis elegans muscle. Mol Biol Cell 27, 2994-3003.

Saheki, Y., and Bargmann, C. I. (2009). Presynaptic CaV2 calcium channel traffic requires CALF-1 and the alpha(2)delta subunit UNC-36. Nature neuroscience 12, 1257-1265.

Schafer, W. R., and Kenyon, C. J. (1995). A calcium-channel homologue required for adaptation to dopamine and serotonin in Caenorhabditis elegans. Nature 375, 73-78.

Selten, M., van Bokhoven, H., and Nadif Kasri, N. (2018). Inhibitory control of the excitatory/inhibitory balance in psychiatric disorders. F1000Res 7, 23.
Splawski, I., Timothy, K. W., Sharpe, L. M., Decher, N., Kumar, P., Bloise, R., Napolitano, C., Schwartz, P. J., Joseph, R. M., Condouris, K., Tager-Flusberg, H., Priori, S. G., Sanguinetti, M. C., and Keating, M. T. (2004). CaV1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. Cell 119, 19-31.

Stawicki, T. M., Zhou, K., Yochem, J., Chen, L., and Jin, Y. (2011). TRPM channels modulate epileptic-like convulsions via systemic ion homeostasis. Curr Biol 21, 883-888.

Thanawala, M. S., and Regehr, W. G. (2013). Presynaptic calcium influx controls neurotransmitter release in part by regulating the effective size of the readily releasable pool. J Neurosci 33, 4625-4633.

Tong, X. J., Hu, Z., Liu, Y., Anderson, D., and Kaplan, J. M. (2015). A network of autism linked genes stabilizes two pools of synaptic GABAA receptors. eLife 4, 1-19.

Tottene, A., Conti, R., Fabbro, A., Vecchia, D., Shapovalova, M., Santello, M., van den Maagdenberg, A. M., Ferrari, M. D., and Pietrobon, D. (2009a). Enhanced excitatory transmission at cortical synapses as the basis for facilitated spreading depression in Ca(v)2.1 knockin migraine mice. Neuron 61, 762-773.

Tottene, A., Conti, R., Fabbro, A., Vecchia, D., Shapovalova, M., Santello, M., van den Maagdenberg, A. M. J. M., Ferrari, M. D., and Pietrobon, D. (2009b). Enhanced Excitatory Transmission at Cortical Synapses as the Basis for Facilitated Spreading Depression in CaV2.1 Knockin Migraine Mice. Neuron 61, 762-773.

Tottene, A., Fellin, T., Pagnutti, S., Luvisetto, S., Striessnig, J., Fletcher, C., and Pietrobon, D. (2002). Familial hemiplegic migraine mutations increase Ca(2+) influx through single human CaV2.1 channels and decrease maximal CaV2.1 current density in neurons. Proceedings of the National Academy of Sciences of the United States of America 99, 13284-13289.

Tottene, A., Pivotto, F., Fellin, T., Cesetti, T., Van Den Maagdenberg, A. M. J. M., and Pietrobon, D. (2005). Specific kinetic alterations of human Cav2.1 calcium channels produced by mutation S218L causing familial hemiplegic migraine and delayed cerebral edema and coma after minor head trauma. Journal of Biological Chemistry 280, 17678-17686.

Triller, A., and Choquet, D. (2008). New concepts in synaptic biology derived from single-molecule imaging. Neuron 59, 359-374.

Tu, H., Pinan-Lucarré, B., Ji, T., Jospin, M., and Bessereau, J. L. (2015). C. elegans Punctin Clusters GABA(A) Receptors via Neuroligin Binding and UNC-40/DCC Recruitment. Neuron 86, 1407-1419.

Turrigiano, G. (2012). Homeostatic synaptic plasticity: Local and global mechanisms for stabilizing neuronal function. Cold Spring Harbor Perspectives in Biology 4,
van den Maagdenberg, A. M., Pietrobon, D., Pizzorusso, T., Kaja, S., Broos, L. A., Cesetti, T., van de Ven, R. C., Tottene, A., van der Kaa, J., Plomp, J. J., Frants, R. R., and Ferrari, M. D. (2004a). A Cacna1a knockin migraine mouse model with increased susceptibility to cortical spreading depression. Neuron 41, 701-710.

van den Maagdenberg, A. M., Pizzorusso, T., Kaja, S., Terpolilli, N., Shapovalova, M., Hoebeek, F. E., Barrett, C. F., Gherardini, L., van de Ven, R. C., Todorov, B., Broos, L. A., Tottene, A., Gao, Z., Fodor, M., De Zeeuw, C. I., Frants, R. R., Plesnila, N., Plomp, J. J., Pietrobon, D., and Ferrari, M. D. (2010). High cortical spreading depression susceptibility and migraine-associated symptoms in Ca(v)2.1 S218L mice. Ann Neurol 67, 85-98.

Van Den Maagdenberg, A. M. J. M., Pietrobon, D., Pizzorusso, T., Kaja, S., Broos, L. A. M., Cesetti, T., Van De Ven, R. C. G., Tottene, A., Van Der Kaa, J., Van Der Kaa, J., Plomp, J. J., Frants, R. R., and Ferrari, M. D. (2004b). A Cacna1a knockin migraine mouse model with increased susceptibility to cortical spreading depression. Neuron 41, 701-710.

Vecchia, D., Tottene, A., van den Maagdenberg, A. M., and Pietrobon, D. (2014). Mechanism underlying unaltered cortical inhibitory synaptic transmission in contrast with enhanced excitatory transmission in CaV2.1 knockin migraine mice. Neurobiol Dis 69, 225-234.

Vecchia, D., Tottene, A., van den Maagdenberg, A. M., and Pietrobon, D. (2015). Abnormal cortical synaptic transmission in CaV2.1 knockin mice with the S218L missense mutation which causes a severe familial hemiplegic migraine syndrome in humans. Front Cell Neurosci 9, 8.

Vecchia, D., and Pietrobon, D. (2012). Migraine: A disorder of brain excitatory-inhibitory balance? Trends in Neurosciences 35, 507-520.

Wan, J., Khanna, R., Sandusky, M., Papazian, D. M., Jen, J. C., and Baloh, R. W. (2005). CACANA1A mutations causing episodic and progressive ataxia alter channel trafficking and kinetics. Neurology 64, 2090-2097.

Wang, J., Liu, S., Haditsch, U., Tu, W., Cochrane, K., Ahmadian, G., Tran, L., Paw, J., Wang, Y., Mansuy, I., Salter, M. M., and Lu, Y. M. (2003). Interaction of calcineurin and type-A GABA receptor gamma 2 subunits produces long-term depression at CA1 inhibitory synapses. J Neurosci 23, 826-836.

White, J. G., Southgate, E., Thomson, J. N., and Brenner, S. (1986). The structure of the nervous system of the nematode Caenorhabditis elegans. Philos Trans R Soc Lond B Biol Sci 314, 1-340.

Wicks, S. R., Yeh, R. T., Gish, W. R., Waterston, R. H., and Plasterk, R. H. (2001). Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. Nature genetics 28, 160-164.
Yemini, E., Jucikas, T., Grundy, L. J., Brown, A. E., and Schafer, W. R. (2013). A database of Caenorhabditis elegans behavioral phenotypes. Nat Methods 10, 877-879.

Zhou, K., Cherra, S. J., Goncharov, A., and Jin, Y. (2017). Asynchronous Cholinergic Drive Correlates with Excitation-Inhibition Imbalance via a Neuronal Ca. Cell Rep 19, 1117-1129.
**Figure Legends**

**Figure 1. zf35 animals are hyperactive in both locomotion and egg-laying behaviors.**
(a) Representative traces from single worm tracking showing instantaneous velocity of indicated genotypes on OP50 thin lawn plates (see Methods). Positive and negative values indicate forward and backward locomotion, respectively. Transition from positive to negative values indicates reversal events. (b) Shown is the average velocity for the wild-type (0.118 ± 0.01 worm lengths/sec, n = 9), zf35 (0.156 ± 0.01 worm lengths/sec, n = 10), zf35/+ (0.155 ± 0.01 worm lengths/sec, n = 10) animals (c) Quantification of the reversal frequency in 3 minutes on regular OP50 plates: average reversal numbers made by wild type (6.8 ± 0.4 reversals, n = 59), zf35 (43.1 ± 2.0 reversals, n = 59) and zf35/+ (33.2 ± 1.6 reversals, n = 23). Error bars represent SEM for at least three trials. Statistical difference from wild type *p<0.05, ****p<0.0001, One way ANOVA with Dunnett’s multiple comparisons test. Statistical difference between zf35 and zf35/+ **p<0.01, unpaired t-test. (d) Representative images of wild type and zf35 animals. Average of midline lengths of the wild type: 0.82 ± 0.03 mm, n = 75 and zf35: 1.00 ± 0.04 mm, n = 88. Scale bar is 200 µm. (e) Representative Nomarski images of unlaid eggs in adult wild-type and zf35 animals. Arrowheads indicate eggs; asterisk denotes the position of the vulva. The average numbers of eggs in the uterus: wild type (14.1 ± 0.6 eggs, n = 80), zf35 (3.6 ± 0.2 egg, n = 86) animals. Scale bar, 50 µm. (f) Embryonic stages of freshly laid eggs of the wild type and zf35 mutants. 43% of the laid eggs from zf35 animals are at 1-16 cell stage, while only 5% from the wild type laid eggs are at 1-16 cell stage. Five independent trials with 75 animals for each genotype. Statistical difference from wild type ****p<0.0001, Chi-squared test.
**Figure 2. zf35 is a novel allele of the CaV2α subunit gene unc-2**

(a) The genetic map and gene structure of unc-2. Coding sequences are represented as black boxes. The zf35 allele is a single nucleotide transition (GGA -> AGA) resulting in a glycine to arginine (G -> R) amino acid substitution at position 1132. (b) Diagram of the secondary structure of UNC-2/CaV2α. UNC-2/CaV2α consists of four transmembrane (TM) domains (TMI – TMIV) each containing six alpha-helix segments (S1 – S6). The UNC-2 (G1132R) mutation localizes in the intracellular loop between TM domain III and IV, indicated by the blue circle. Purple circles indicate positions of intragenic unc-2(zf35) suppressors, red circles indicate the location of human FHM1 mutations. (c) The G1132R mutation occurs in a highly conserved region of the CaV2α subunit. Amino acid alignment of C-terminus region of the transmembrane III alpha-helix segment 6 (TM III S6) and the beginning of the third intracellular loop of CaV2α subunits from human (*H. sapiens*, CACNA1A), rainbow fish (*P. reticulata*, cacna1a), fly (*D. melanogaster*, Cacophony) and nematode (*C. elegans*, UNC-2). Identities are shaded in dark grey, similarities in light grey. Location of the G1132R mutation is indicated. (d) Representative images of GFP tagged UNC-2(wt) and UNC-2(zf35) in the ventral nerve cord. Asterisks point the cell bodies of the motor neurons and arrows indicate the presynaptic sites. Both constructs are expressed under pan-neuronal promoter tag-168. Scale bar, 10 μm. (e) Quantification of the reversal frequency: wild type (6.6 ± 0.4, n = 70), unc-2(zf35) (43.3± 1.9, n = 65), unc-2(lf) (2.4 ± 0.2, n = 59), wild-type animals expressing unc-2(wt) transgene (7.5 ± 0.6, n = 10) and unc-2(zf35) transgene (33 ± 2.1, n = 22), and unc-2(lf) rescued with unc-2(wt) transgene (3.8 ± 0.7, n = 12) and unc-2(zf35) transgene (41.3 ± 3.6, n = 21). Error bars represent SEM for at least three trials with indicated totaling animals number. Statistical difference from wild type ****p<0.0001, One way ANOVA with Dunnett’s multiple comparisons test. (f) Intragenic unc-2(lf) suppressors suppress unc-2(zf35) hyperactive locomotion. Shown are numbers of thrashes in 30 seconds in M9 for the wild type (107.0 ± 14.0, n = 60), unc-2(zf35) (128.1 ± 13.5, n = 60), unc-2(lf) (4.8 ± 2.1, n = 57), unc-2(zf35,zf109) (6.9 ± 4.3, n = 53); unc-2(zf35,zf113) (5.6 ± 3.7 thrashes, n = 57); unc-2(zf35,zf114) (80.2 ± 9.9, n = 60); unc-2(zf35,zf115) (6.9 ± 3.8, n = 56); unc-2(zf35,zf124) (5.3 ± 3.1, n = 57); unc-2(zf35,zf130) (67.1 ± 22.5, n = 58); unc-2(zf35,zf134) (31.2±17.9, n = 50). Error bars represent SEM. Statistical difference from unc-2(zf35) mutants unless otherwise indicated, ****p<0.0001, One way ANOVA with Tukey’s multiple comparisons test.
Figure 3. The UNC-2 G1132R corresponding mutation in human CaV2α1 subunit results in increased channel activity.

(a) Representative macro-currents of wild type and G1518R CaV2 channels. Currents were generated by stepping membrane potential to voltages between -55 and 40 mV in 5mV increments for 200 ms from a holding potential of -120 mV. (b) Voltage dependence of whole-cell current density for wild type and G1518R CaV2 channels. Current density values were obtained by dividing current amplitudes and cell capacitance. (Wild type, n = 9; G1518R, n = 11). (c) Voltage dependence of Ba\(^{2+}\) current activation. The activation curve of G1518R exhibits a significant shift of the \(V_{0.5}\) value towards more negative membrane potentials. (d) Steady-State inactivation curves. The G1518R mutation causes a slight positive shift in the midpoint voltage in the steady-state inactivation curves (\(V_{0.5\text{inact}}\) = -55.0 ± 1.0 and -47.3 ± 1.0 for wild type and G1518R, respectively). Currents were normalized to the maximal value obtained at the test pulse and plotted as a function of the prepulse potential. Data were fitted with the Boltzmann equation: \(I\text{max} = (1+\exp[(V-V_{0.5})/k])^{-1}\). All recordings were carried out in Ba\(^{2+}\) solution to exclude the effects from calcium-dependent inactivation.
Figure 4. FHM1 mutations in *unc-2/CaV2α* result in a hyperactive phenotype.

(a) The amino acid alignment of the conserved region of transmembrane domain I membrane-spanning segments 4 (TM I S4) and the following linker region from human (CACNA1A) and worm (UNC-2) CaV2α subunits. Identities are dark grey and similarities are light grey. Indicated are the known human FHM1 mutations: R192Q and S218L. (b) Shown is the average number of reversals in 90 seconds on thin lawn OP50 plates: wild type (4.2 ± 0.5, n = 29), *unc-2(zf35gf)* (30.3 ± 1.2, n = 20), *Ptag-168::unc-2(R192Q)* (25.5 ± 0.9, n = 34), and *Ptag-168::unc-2(R192Q)* (16.5 ± 0.9, n = 33). (c) Average numbers of eggs in the adult uterus: wild type (16.5 ± 0.8 eggs, n = 23), *unc-2(zf35gf)* (2.7 ± 0.2, n = 35), *Ptag-168::unc-2(R192Q)* (5.7 ± 0.4, n = 37), and *Ptag-168::unc-2(S218L)* (8.4 ± 0.6, n = 32). Each bar represents the mean ± SEM for at least three trials with indicated totaling animals number. Statistical difference from wild-type, ****p<0.0001, one way ANOVA with Dunnett’s multiple comparisons test. (d) Quantification of movement on 1 mM aldicarb. Each data point represents the mean ± SEM of the percentage of animals paralyzed every 15 minutes. 50% of the wild type animals were paralyzed at 60 minutes. *unc-2(lf)* animals were resistant to the effects of aldicarb and reached 50% paralysis at 90 minute. Homozygous *unc-2(gf)* mutants were sensitive to aldicarb; 50% of the *unc-2(gf)* mutants were paralyzed at 20 minute. Heterozygous *unc-2(gf)* mutants have 50% paralysis at 40 minutes. Three independent trials with totaling at least 50 animals for each genotype. **p<0.01, ****p<0.0001, two way ANOVA with Tukey’s multiple comparisons test. (e) Quantification of paralysis percentage on 1 mM aldicarb at the 60-minute time point: 55.5% ± 4.5 of wild type, 56.7% ± 3.3 of *Ptag-168::unc-2(wt)* and 98.3% ± 3.3 of *Ptag-168::unc-2(gf)* expressed in wild-type animals, 27.1% ± 7.3 of *unc-2(lf)* animals, 54.8% ± 2.9 of *Ptag-168::unc-2(wt)*, 100% of *Ptag-168::unc-2(gf)*, and 100% of *Ptag-168::unc-2(R192Q) and *Ex unc-2(S218L)* in *unc-2(lf)* background. **p<0.01, ***p<0.001, one way ANOVA with Dunnett’s multiple comparisons test.
Figure 5. *unc-2(gf)* mutants lead to increased spontaneous EPSCs and decreased spontaneous IPSCs.

(a) Representative traces of total spontaneous postsynaptic currents (sPSCs) from ventral body wall muscles in wild-type and *unc-2(gf)* mutants. (b and c) Mean spontaneous PSC frequency and amplitude of wild-type and *unc-2(gf)* mutants. (d) Representative traces of spontaneous cholinergic EPSCs in *unc-49* and *unc-49; unc-2(gf)* mutants. (e and f) Mean spontaneous EPSC frequency and amplitude *unc-49* and *unc-49; unc-2(gf)* mutants. (g) Representative traces of spontaneous GABAergic IPSCs in wild-type and *unc-2(gf)* mutants. (h and i) Mean IPSC frequency and amplitude of wild-type animals and *unc-2(gf)* mutants. Error bars depict SEM. *p<0.05, **p<0.01, two-tailed Student’s t test.*
Figure 6. *unc-2*(gf) mutants have decreased GABA<sub>A</sub> receptor expression at the NMJ.

(a and c) Representative images of cholinergic synapses in wild type and *unc-2*(gf) mutants. Presynaptic sites are labeled with synaptic vesicle marker RAB-3::mCherry while postsynaptic nicotinic acetylcholine receptors are labeled by UNC-29::GFP. Scale bar represents 10 μm. (b and d) Quantification of the fluorescence intensity of RAB-3::mCherry and UNC-29::GFP along the ventral nerve cord at cholinergic synapses in wild type and *unc-2*(gf) mutants. Arbitrary fluorescence units of individual animals are normalized to the mean value of wild type. Normalized fluorescence of cholinergic RAB-3::mCherry: 99.5 ± 7.3%, n = 19 in wild type and 128 ± 12.3%, n = 12 in *unc-2*(gf) mutants. UNC-29::GFP: 99.9 ± 8%, n = 17 in wild type and 128 ± 10.1%, n = 22 in *unc-2*(gf) mutants. (e and g) Representative images of GABAergic synapses in wild type and *unc-2*(gf) mutants. Presynaptic sites are labeled with synaptic vesicle marker RAB-3::mCherry while postsynaptic GABA receptors are labeled by UNC-49::GFP. Scale bar represents 10 μm. (f and h) Quantification of the fluorescence intensity of RAB-3::mCherry and UNC-49::GFP along the ventral nerve cord at GABAergic synapses in wild type and *unc-2*(gf) mutants. Arbitrary fluorescence units of individual animals are normalized to the mean value of wild type. Normalized fluorescence of GABAergic RAB-3::mCherry: 100 ± 4.2%, n = 18 in wild type and 130.4 ± 10.2%, n = 20 in *unc-2*(gf) mutants. UNC-49::GFP: 100 ± 9.1%, n = 18 in wild type and 66.5 ± 7.4%, n = 20 in *unc-2*(gf) mutants. For all the quantification above, error bars depict SEM. *p<0.05, ****p<0.0001, two-tailed Student’s t test.
Figure 7. Increased excitatory signaling leads to the reduction of GABA\textsubscript{A} receptor in \textit{unc-2(gf)} mutants.

(a and c) Representative images of GABAergic post-synaptic sites labeled with UNC-49::GFP of indicated genotypes. Scale bar represents 10 μm (b) Quantification of the fluorescence intensity of UNC-49::GFP along the nerve cord of indicated genotypes. Arbitrary fluorescence units of individual animals are normalized to the mean value of wild type. Normalized UNC-49::GFP fluorescence: wild type (1 ± 0.06, n = 62), \textit{unc-2(gf)} (0.5 ± 0.07, n = 24), \textit{Punc-47::unc-2(gf)} (1.5 ± 0.12, n = 13), \textit{Pacr-2::unc-2(gf)} (0.7 ± 0.07, n = 16), \textit{acr-12; unc-2(gf)} (1 ± 0.11, n = 15) and \textit{unc-29; unc-2(gf)} (0.9 ± 0.07, n = 14). (d) Quantification of the fluorescence intensity of UNC-49::GFP along the nerve cord of indicated genotypes. Arbitrary fluorescence units of individual animals are normalized to the mean value of wild type. Normalized UNC-49::GFP fluorescence: wild type (1 ± 0.04, n = 82), \textit{L-AChR(wt)} (1 ± 0.05, n = 22), \textit{L-AChR(gf)} (0.8 ± 0.05, n = 23), \textit{unc-2(gf)} (0.6 ± 0.07, n = 27), \textit{tax-6} (1.1 ± 0.06, n = 54), \textit{tax-6;unc-2(gf)} (1 ± 0.05, n = 29). For all the quantification above, error bars depict SEM. *p<0.05, ****p<0.0001, one way ANOVA with Dunnett’s multiple comparisons. (e) Model: The UNC-2/CaV2\textalpha gain-of-function mutation shifts the E/I balance to an excitation-dominant transmission through the destabilization of GABA synapses in a TAX-6/calcineurin dependent manner (See text for explanation).
Supplementary figure 1

(a) Quantification of movement on 0.5 mM levamisole. Each data point represents the mean ± SEM of the percentage of animals paralyzed by levamisole every 15 minutes for at least three trials, totaling a minimum of 50 animals. (b) Percentage of animals that displayed the muscimol-induced rubberband phenotype on 1 mM muscimol plates at 60-minute time point. Wild type animals: category 0: 0%, category 1: 3 ± 2.9%, category 2: 17 ± 5.8%, category 3: 62 ± 2.7% and category 4: 32 ± 13.7%. unc-2(gf): category 0: 12 ± 3.3%, category 1: 33 ± 2.2%, category 2: 26 ± 2.4%, category 3: 25 ± 4.5% and category 4 and 3 ± 1%. Severity of muscimol-induced phenotype is increased from 0 (normal locomotion) to 4 (complete flaccid category), see Materials and Methods for scoring details. ****p<0.001, Chi-squared test.
Supplementary figure 2
Representative images of GABAergic synapses pre- and post-synaptic apposition in wild-type and unc-2(gf) animals. Presynaptic sites are labeled with synaptic vesicle marker RAB-3::mCherry while postsynaptic GABA receptors are labeled by UNC-49::GFP. Scale bar represents 10 μm.
Supplementary figure 3

(a) Representative images of GABAergic presynaptic sites labeled with RAB-3::mCherry of indicated genotypes. Scale bar represents 10 μm. (b) Quantification of the fluorescence intensity of RAB-3::mCherry along the ventral nerve cord of indicated genotypes. Arbitrary fluorescence units of individual animals are normalized to the mean value of wild type. Normalized RAB-3::mCherry fluorescence: wild type (1 ± 0.05, n = 51), unc-2(gf) (1.4 ± 0.07, n = 20), Punc-47::unc-2(gf) (1.3 ± 0.08, n = 14), Pacr-2::unc-2(gf) (1.2 ± 0.08, n = 16), acr-12; unc-2(gf) (1.2 ± 0.06, n = 15) and unc-29; unc-2(gf) (1.2 ± 0.08, n = 14). Punc-47::unc-2(gf) and Pacr-2::unc-2(gf) are strains with unc-2(gf) transgene specifically expressed in GABAergic and cholinergic motor neurons respectively. For all the quantification above, error bars depict SEM. *p<0.05, **p<0.01, ****p<0.0001, one way ANOVA with Dunnett’s multiple comparisons.
Figure 1
Figure 2
Figure 3
CACNA1A  Hs  188  
UNC-2  Ce  210  

FHM R192Q  
FHM S218L  

Eggs in the Uterus  
****  ****  ****  ****  
unc-2(gf) Ex unc-2  
unc-2(FHM R192Q)  
unc-2(FHM S218L)  

Reversals in 90 secs  
unc-2(lf)  ****  ****  ****  ****  
unc-2(gf) Ex unc-2  
unc-2(FHM R192Q)  
unc-2(FHM S218L)  

Eggs in the Uterus  
unc-2(lf)  ****  ****  ****  ****  
unc-2(gf) Ex unc-2  
unc-2(FHM R192Q)  
unc-2(FHM S218L)  

Paralyzed (%)  
unc-2(lf)  ***  ***  ***  ***  
unc-2(gf) Ex unc-2  
unc-2(FHM R192Q)  
unc-2(FHM S218L)  

Paralyzed (%)  
unc-2(lf)  ***  ***  ***  ***  
unc-2(gf) Ex unc-2  
unc-2(FHM R192Q)  
unc-2(FHM S218L)  

Time (min)  
Paralyzed (%)  
unc-2(lf)  ***  ***  ***  ***  
unc-2(gf) Ex unc-2  
unc-2(FHM R192Q)  
unc-2(FHM S218L)  

Figure 4
Figure 5
Figure 6
Figure 7
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 3