Caenorhabditis elegans provides a convenient drug screening platform for *GNAO1*-related disorders and highlights the potential role of caffeine in controlling dyskinesia

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Abstract

De novo mutations in the *GNAO1* gene cause an emerging group of childhood-onset neurological disorders characterized by developmental delay, intellectual disability, movement disorders, drug-resistant seizures, and neurological deterioration. *GNAO1* encodes the α-subunit of a heterotrimeric GTP/GDP-binding protein, which controls inhibitory signaling from G-protein coupled receptors, regulating ion channel activity and neurotransmitter release. The pathogenic mechanisms underlying *GNAO1*-related disorders remain largely elusive and there are no effective therapies.

Here, we report on the functional impact of the c.139A>G (p.S47G) mutation identified in a child with a severe clinical presentation. An equivalent change at the orthologous position of the *Caenorhabditis elegans* gene (*goa-1*) was generated using CRISPR/Cas9. Like null mutants, *goa-1* [S47G] animals showed increased egg laying and were hypersensitive to aldicarb, an inhibitor of acetylcholinesterase, suggesting excessive neurotransmitter release by different classes of motor neurons. Using an automated tracking system, *goa-1*[S47G] mutants were shown to move faster than control animals, with more frequent body bends and a higher reversal rate, and appeared uncoordinated. The hyperactive behavior was tested in both homozygous and heterozygous animals, revealing a severe loss-of-function rather than a dominant-negative effect of the mutation on $G\alpha_o$ signaling. Finally, we proved the efficacy of caffeine to improve the aberrant motor function of *goa-1* mutants.

Overall, our findings establish a suitable platform for drug discovery, which may assist in accelerating the development of new therapies for this devastating condition, and highlight the potential role of caffeine in controlling *GNAO1*-related dyskinesia.

Keywords: Pediatric movement disorders, GNAO1, caffeine, C. *elegans*, neurotransmitter release.

goa-1, the *C. elegans* orthologue of *GNAO1*, shows 90% homology with the human gene, with 82% identity in their amino acid sequence. We introduced the c.139_141TCG>GGA (p.S47G) nucleotide change at the orthologous position of the *C. elegans* gene by CRISPR/Cas9 to generate *goa-1*(*pan5*[S47G]) animals (hereafter *goa-1*[S47G]). The resulting nematodes, homozygous for the desired variant, showed slow growth and a penetrant protruding vulva (Pvl) phenotype, resembling those observed in knock-out (KO) worms carrying the *goa-1*(*sa734*) allele (p.Q52*) (Robatzek and Thomas, 2000; PMID: 11063685). $G\alpha_o$ signaling was previously shown to inhibit serotonin release from HSN motor neurons, which innervate vulval muscles stimulating egg-laying, and acetylcholine (ACh) release from ventral cord motor neurons onto body wall muscles (Koelle, 2018; PMID: 26937633). Thus, to explore the effect of the p.S47G on $G\alpha_o$ function, we counted the number of eggs retained in the uterus of gravid hermaphrodites (Fig. 1) and assessed sensitivity to aldicarb (an acetylcholinesterase inhibitor) (Fig. 2), levamisole (a cholinergic agonist) (Fig. 3), and pentylenetetrazole (PTZ) (an inhibitor of GABA signaling) (Fig. 4). Our findings demonstrated defective $G\alpha_o$-mediated inhibition of neurotransmitter release by HSN and ventral cord motor neurons in both *goa-1*[S47G] and KO animals, with a more severe phenotype generally observed in null mutants pointing to a strong hypomorphic rather than a complete LOF effect of the disease-associated change on $G\alpha_o$-mediated signaling.

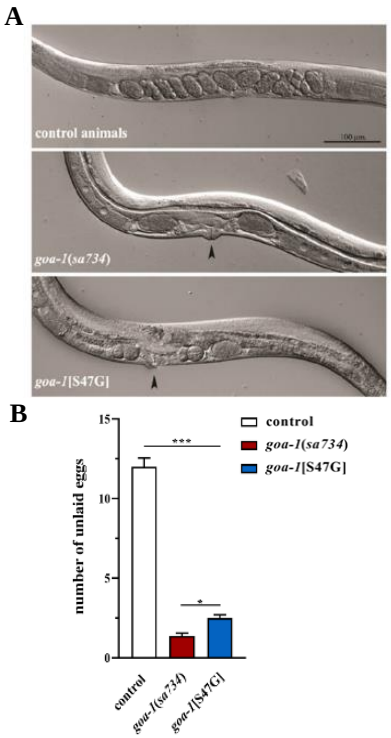

Figure 1. *goa-1*[S47G] and KO animals display increased egg laying activity. (**A**) Representative images of adult mid-body regions of wild-type *C. elegans* (upper panel) and *goa-1* mutants (lower panels). Unlaid eggs are evident as oval objects inside the body. A protruding vulva phenotype is also visible in mutant animals (black arrows). (**B**) Control animals display 12-16 unlaid eggs on average, whereas homozygous null mutants (*sa734*) and *goa-1*[S47G] animals laid eggs as soon as they are generated, suggesting defective $G\alpha_o$ function.

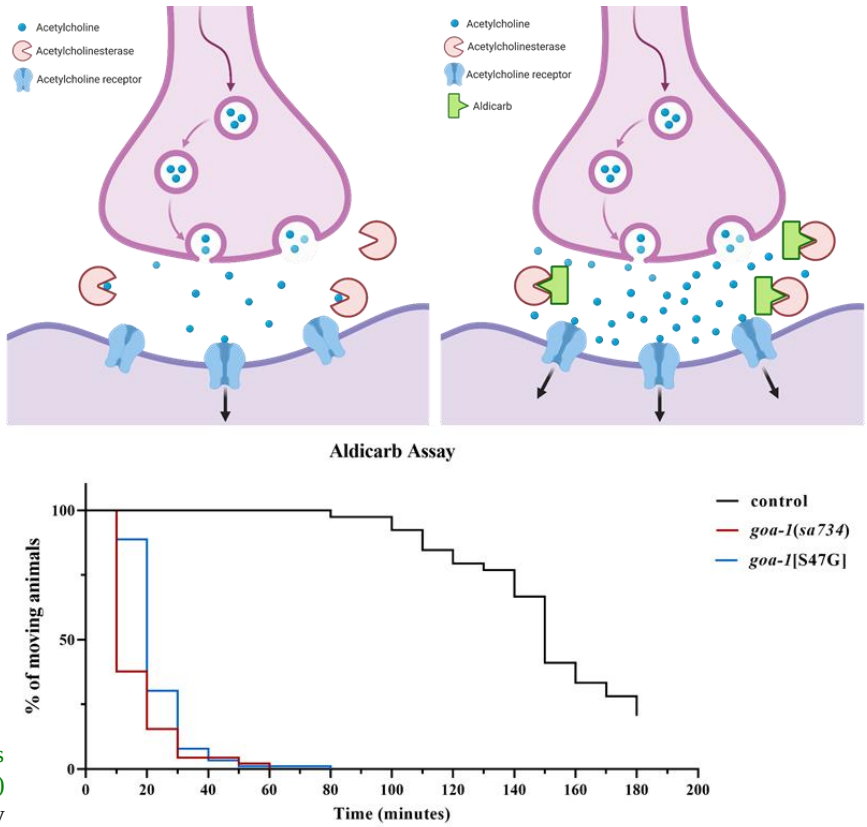

Figure 2. Mutant animals exhibit aldicarb hypersensivity. Schematic structure of the *C. elegans* neuromuscular junction (NMJ) (upper panels). ACh is released in the synaptic cleft and binds to its receptor on body wall muscles, inducing muscle contraction (left). Acetylcholinesterase catabolizes ACh, thereby terminating its synaptic function. In the presence of aldicarb, an acetylcholinesterase inhibitor, ACh accumulates in the synaptic cleft, causing persistent muscle contraction and paralysis (right). Aldicarb assay (lower panel) showed that *goa-1*(*sa734*) and homozygous *goa-1*[S47G] animals display hypersensitivity to aldicarb (1 mM)-induced paralysis ($p<0.0001$ in both comparisons; log-rank test).

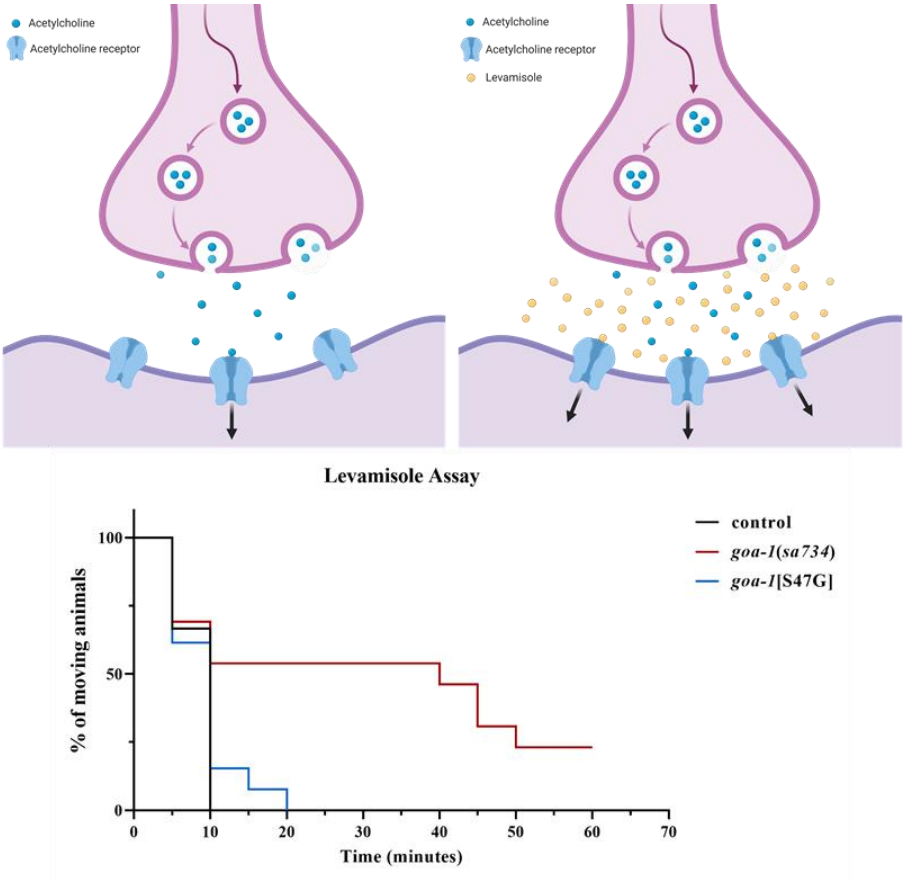

Figure 3. The cholinergic defects in knock-in animals is presynaptic. Levamisole is a strong nicotinic agonist acting on postsynaptic muscles, inducing persistent contraction and paralysis (upper panel). Worms carrying the p.S47G substitution show normal sensitivity to levamisole (1mM) (lower panel), further supporting the presynaptic origin of the cholinergic defect. It is worth noting, however, that *goa-1* null mutants display a slight resistance to this drug, suggesting a possible compensatory post-synaptic effect that needs to be further investigated ($p<0.05$; log-rank test).

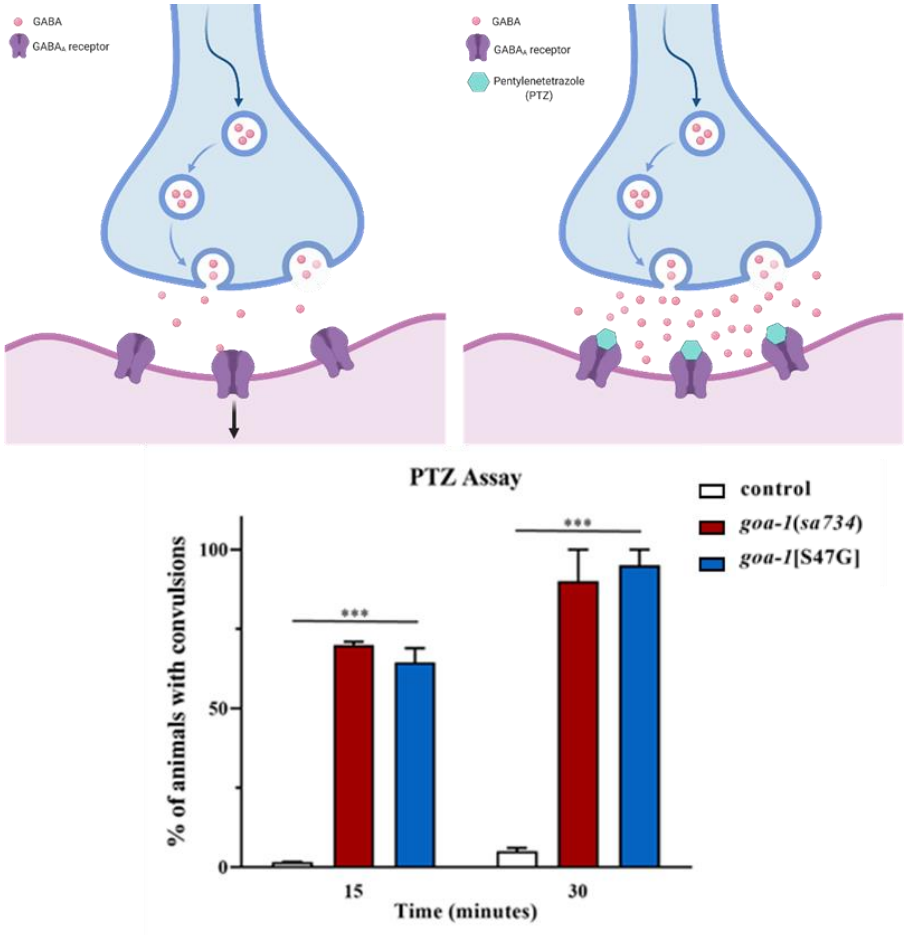

Figure 4. Knock-in and KO animals display PTZ hypersensitivity. PTZ is a competitive inhibitor of GABA, preventing its binding to $GABA_A$ receptors on body-wall muscles (upper panel). Exposure to PTZ leads to a shift in the equilibrium between excitatory and inhibitory inputs towards the former, resulting in a convulsion phenotype. All mutants display hypersensitivity to PTZ, indicating defective GABAergic signaling, which is likely due to augmented release of acetylcholine at the *C. elegans* NMJ (lower panel) (***$p<0.0001$ in all comparisons; Fisher's exact test with Bonferroni correction for multiple comparisons).

Characterization of the hyperactive behaviour in mutant animals by using an automated tracking system

Automated analysis of *C. elegans* locomotion indicated that both *goa-1*[S47G] and KO animals are characterized by hyperactive crawling (Fig. 5A) and move faster than control animals (Fig. 5B). They also showed more frequent and deeper body bends (Fig. 5C) and a higher reversal rate (change of direction) (Fig. 5D), and display uncoordinated locomotion, assuming a tightly coiled position and tending to remain for long time in this position (Fig. 5E). Again, a more severe phenotype was observed in *goa-1*(*sa734*) null mutants, further supporting the hypomorphic effect of the p.S47G pathogenic variant. Phenotypic profiling of heterozygous animals indicated that p.S47G has no dominant negative (DN) activity (Figure 6).

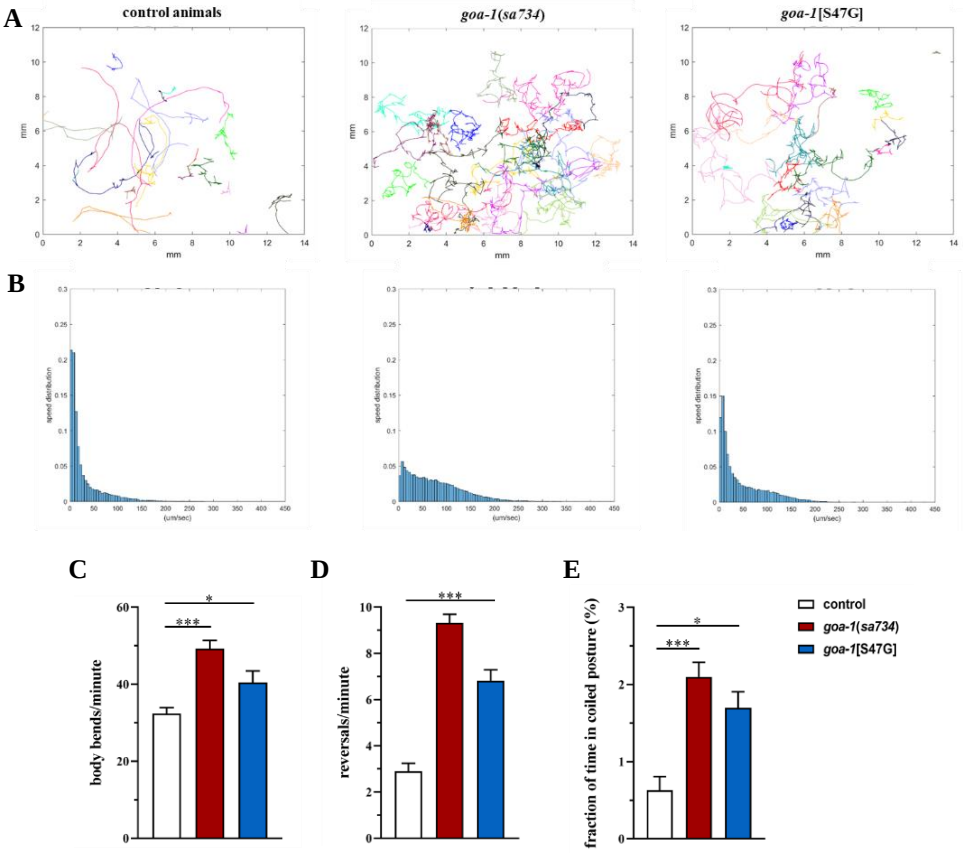

Figure 5. Mutant animals exhibit aberrant locomotion. (**A**) Trajectories of multiple *C. elegans* (n=20) on 35 mm plates seeded with a thin lawn of *E.coli* OP50 bacteria. Animals were recorded for 10 minutes. Different colors refer to different nematodes. All mutants show hyperactive crawling, with a higher frequency of change in direction, compared to controls. (**B**) Speed histograms of the same tracks indicate a clear difference between wild type and mutant animals, the latter being faster on average ($p<10^{-6}$, two-sample t-test). The aberrant locomotor activity of *goa-1* mutants is also revealed by the higher number of body bends per minute (**C**), reversals per minute (**D**), and the average fraction of time spent in a coiled position (**E**) compared to control worms (*p<0.01 and ***p<0.0001; one-way ANOVA with Bonferroni correction).

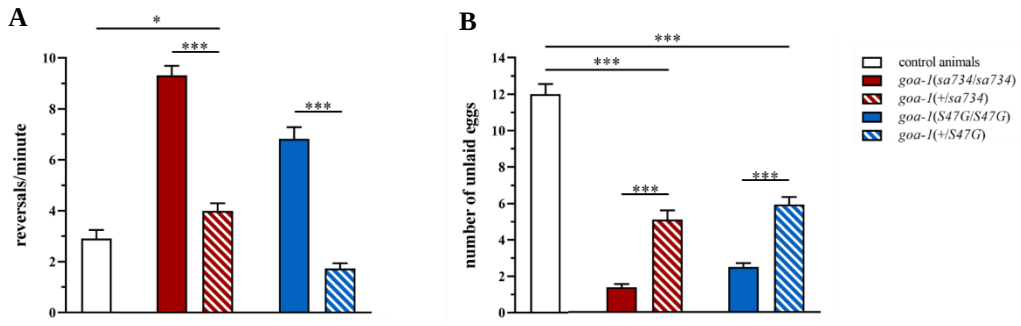

Figure 6. Phenotypic analysis of heterozygous animals reveals that p.S47G does not behave as a DN allele. Number of reversals per minute (**A**) and number of unlaid eggs (**B**) were counted in F1 progeny after crossing homozygous mutant hermaphrodites to control males carrying wild-type *goa-1*. Results show that *goa-1(+/sa734)* null heterozygotes display a clear phenotype in terms of both number of unlaid eggs and increased reversal rate, although less severe than that observed in homozygous null mutants, indicating that *goa-1* is a haploinsufficient gene in *C. elegans* for these particular phenotypes. Analysis of heterozygous knock-in animals show a residual phenotype only in terms of unlaid eggs which is however milder compared to that of their homozygous counterparts, indicating that p.S47G has no DN activity (*p<0.05 and ***p<0.0001; two-way ANOVA with Bonferroni correction).

Caffeine ameliorates aberrant locomotor behavior of *goa-1* mutants

Exposure of *goa-1*[S47G] to caffeine (2 hours) was shown to rescue aberrant locomotion in terms of number of reversals per minute in a dose-dependent manner (Fig. 7A). Of note, longer or "chronic" exposure to the drug appeared to be less efficient in improving the reversal frequency compared to shorter, "acute" exposures (Fig. 7B). Automated recordings of *C. elegans* trajectories highlighted the beneficial effect of caffeine (10 mM) on the hyperactive crawling of *goa-1* mutant animals. Indeed, caffeine is able to reduce the number of reversals per minute (Fig. 7C) and the time spent in a coiled position (Fig. 7D) in both knock-in and KO worms. Our findings suggests that caffeine is able to improve aberrant motor function of mutant animals by blocking a putative adenosine receptor in the nematode, since its known role as a psychotropic agent acting as an antagonist of adenosine GPCRs in mammals (Rivera-Oliver and Díaz-Ríos, 2014; PMID: 24530739). Since caffeine is able to suppress aberrant motor function even in null mutants, it acts as a bypass suppressor, thus bypassing the requirement for the *goa-1* gene. Overall our findings demonstrate that *C. elegans* is an excellent model to functionally characterize *GNAO1* pathogenic variants and establish the nematode as an efficient experimental platform for drug discovery. Our data also highlight the potential role of caffeine in controlling *GNAO1*-related dyskinesia.

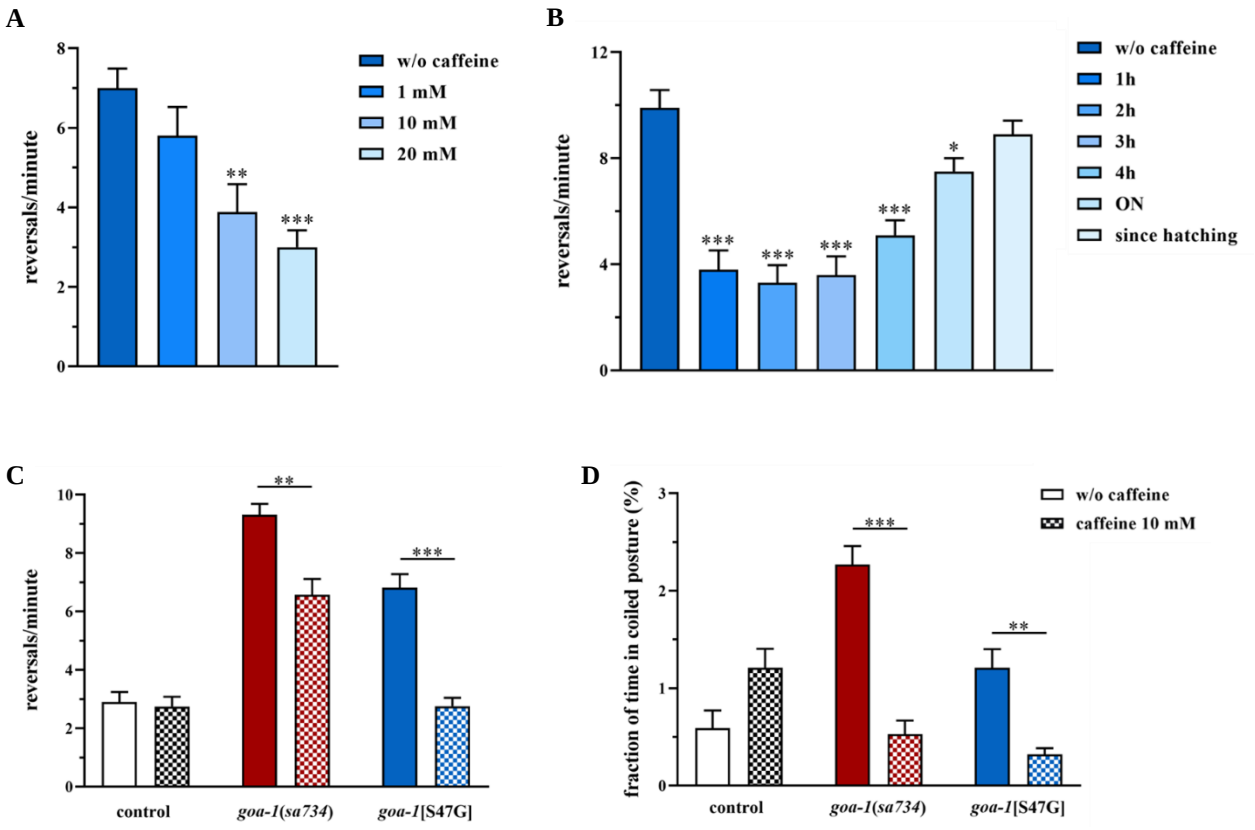

Figure 5. Caffeine suppresses increased reversals and uncoordinated locomotion in *goa-1* mutants. (**A**) Exposure to caffeine (2h) rescues the increased reversal rate of *goa-1*[S47G] animals in a dose-dependent manner (***p*=0.0025 and ****p*<0.0001; one-way ANOVA with Bonferroni correction). (**B**) Longer exposures to caffeine (10 mM) is less efficient in ameliorating the reversal phenotype of *goa-1*[S47G] worms compared to acute exposures (**p*<0.05 and ****p*<0.0001). Computational analysis reveals that caffeine (10 mM, 2h) is able to diminish the number of reversals per minute (C) (***p*<0.001 and ****p*<0.0001) and the time spent in a coiled position (D) (***p*<0.002 and ****p*<0.0001) of *goa-1* mutants.