# A microbial metabolite synergizes with endogenous serotonin to trigger *C. elegans* reproductive behavior

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**Natural products are a major source of small-molecule therapeutics, including those that target the nervous system. We have used a simple serotonin-dependent behavior of the roundworm *Caenorhabditis elegans*, egg laying, to perform a behavior-based screen for natural products that affect serotonin signaling. Our screen yielded agonists of G protein-coupled serotonin receptors, protein kinase C agonists, and a microbial metabolite not previously known to interact with serotonin signaling pathways: the disulfide-bridged 2,5-diketopiperazine gliotoxin. Effects of gliotoxin on egg-laying behavior required the G protein-coupled serotonin receptors SER-1 and SER-7, and the $G_q$ ortholog EGL-30. Furthermore, mutants lacking serotonergic neurons and mutants that cannot synthesize serotonin were profoundly resistant to gliotoxin. Exogenous serotonin restored their sensitivity to gliotoxin, indicating that this compound synergizes with endogenous serotonin to elicit behavior. These data show that a microbial metabolite with no structural similarity to known serotonergic agonists potentiates an endogenous serotonin signal to affect behavior. Based on this study, we suggest that microbial metabolites are a rich source of functionally novel neuroactive molecules.**

*C. elegans* | serotonin | animal–microbe interaction

In the vertebrate brain, the neurotransmitter serotonin is produced by a small number of neurons that project widely to regulate diverse neural circuits. Accordingly, a large number of psychiatric diseases are treated with small-molecule therapeutics that agonize or antagonize serotonin signaling (1, 2). Small molecules that boost endogenous serotonin signaling, such as inhibitors of serotonin catabolism or reuptake and serotonin receptor agonists, are used to treat major depression, anxiety disorders, and alcohol and nicotine addiction (3–5). Compounds that inhibit serotonin receptors are also clinically important; many antipsychotics are potent antagonists of a subset of G protein-coupled serotonin receptors. Although the links between serotonin and psychiatric disease have been firmly established and several classes of small-molecule therapeutics that target serotonin signaling systems are available in the brain, there is still a need for new small-molecule agonists and antagonists of serotonin signaling. Many patients do not respond to available therapeutics, which can also cause undesired side effects (6, 7).

The tiny roundworm *Caenorhabditis elegans* offers the opportunity to use high-throughput behavior-based screens to discover small molecules that target serotonin signaling. The *C. elegans* nervous system uses serotonin to generate simple and stereotyped behaviors. One such behavior is egg laying. A pair of serotonergic neurons—the hermaphrodite specific motor neurons (HSNs)—innervate egg-laying muscles (ELMs) (8). HSNs are necessary for normal egg laying, and activation of HSNs is sufficient to trigger egg-laying behavior. This behavior is particularly well suited for high-throughput screening because it leaves a visible trace that obviates the need to observe the behavior in real time: eggs that have been released into the environment. Also, the egg-laying circuit is readily accessible to pharmacological agents. Exogenous serotonin stimulates egg laying (9, 10) as do many canonical regulators of serotonin signaling that are in clinical use: e.g., serotonin-reuptake inhibitors and receptor agonists (10–13). Importantly, the *C. elegans* egg-laying system uses molecular mechanisms of serotonin signaling that have counterparts in the vertebrate brain. HSNs use conserved pathways for the synthesis, storage, and release of serotonin (14–18). G protein-coupled serotonin receptors that are homologous to mammalian HTR2 and HTR7 mediate activation of ELMs (19, 20), and these nematode serotonin receptors signal via conserved $G_q$ and $G_s$ subunits to activate highly conserved second-messenger signaling cascades. Mutants exist for many of the key components of the serotonin signaling pathway that promote egg laying. This permits genetic analysis of mechanisms of drug action, which can accelerate the process of matching novel compounds to their biological targets.

Here, we report the identification of a microbial metabolite, the 2,5-diketopiperazine (DKP) gliotoxin, as a potent activator of *C. elegans* egg-laying behavior. Genetic studies of gliotoxin sensitivity and physiological measurements of the effects of gliotoxin on serotonin neurons and their targets indicate that gliotoxin potentiates signaling via $G_q$-coupled serotonin receptors in a manner that strictly depends on the presence of serotonin. Although this is reminiscent of serotonin-reuptake inhibitors such as imipramine and fluoxetine, which work by amplifying endogenous serotonin signals, gliotoxin does not require serotonin-reuptake transporters for its effects on behavior, indicating that it acts via a novel mechanism. Our data suggest

## Significance

Neurochemical signaling pathways are important therapeutic targets for the treatment of brain disorders, including complex psychiatric disorders that affect poorly understood cognitive and affective mechanisms. Despite a pressing need for improved psychopharmacology, it has proved difficult to discover new types of small molecules that target neurochemical signaling. We have used a behavior-based screen to identify natural products that strongly and specifically interact with serotonin signaling in the nervous system of the nematode *Caenorhabditis elegans*. This approach identified a microbial metabolite that is structurally unrelated to known activators of serotonin signaling. Our study suggests that microbe-derived small molecules can be a rich source of novel neuroactive compounds.

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The authors declare no competing interest.

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that gliotoxin-like compounds might constitute a useful class of neuroactive small molecules with therapeutic potential. We further suggest that microbial metabolites are an unexplored resource for neuroactive small molecules with new and useful properties, and we discuss the possibility that these molecules arise during coevolution of microbes and soil-dwelling nematodes such as *C. elegans*.

## Results

**The DKP Gliotoxin Is a Potent Activator of Egg-Laying Behavior.** We assembled a library of structurally and functionally diverse natural products, comprising commercially available compounds and recently isolated molecules, and screened this library for agonist of *C. elegans* behavior (*Materials and Methods*). For the screen, five gravid *C. elegans* hermaphrodites were placed in each well of a 96-well microtiter plate with 100 μL of S basal buffer and exposed to compounds from the library at a final concentration of 100 μM for 2 h. Under these conditions, undosed animals or animals exposed only to the dimethyl sulfoxide (DMSO) vehicle did not lay eggs. Wells were then inspected for eggs, and compounds that elicited the release of two or more eggs per animal were retested for activity. Of 576 compounds tested, we focused on 7 that reliably elicited a behavioral response (Fig. 1*A*). Two of these compounds—lysergol and methylergonovine—have structural similarities to lysergic acid diethylamide (LSD) and are known agonists of G protein-coupled serotonin receptors (Fig. 1*B*) (21). Three compounds—ingenol 3,20-dibenzoate (IDB), 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA), and phorbol 12-myristate 13-acetate (PMA)—are known agonists of protein kinase C (PKC) (Fig. 1*B*) (22, 23), which is an effector of $G_q$-coupled serotonin receptors (24). Our behavior-based screen, therefore, was able to recover known agonists of serotonin signaling pathways from a library.

Our screen also identified the disulfide-bridged DKP gliotoxin (Fig. 1*B*). Like the PKC agonists and serotonin receptor agonists, gliotoxin elicited behavior when present in the environment at micromolar concentrations (Fig. 1 *C*–*E*). The kinetics of gliotoxin action were also similar to those of serotonin receptor agonists and PKC agonists; animals exposed to these compounds began laying eggs within 40 to 60 min and continued to lay eggs for the remainder of the assay (*SI Appendix*, Fig. S1). DKPs are a diverse class of natural products that have been used to generate clinically important drugs (25). We therefore selected gliotoxin for further characterization as a potential serotonergic agonist.

DKPs are doubly condensed dipeptides, and gliotoxin is a member of a class of DKPs that possess an additional six-membered ring formed by a disulfide bridge (Fig. 2*A*). We tested whether this disulfide bridge was required for effects of gliotoxin on behavior in two ways. First, we exposed gliotoxin to the reducing agent dithiothreitol (DTT) and then tested whether reduced gliotoxin could elicit a behavioral response. Reduced gliotoxin did not trigger egg laying, suggesting that the disulfide bridge in gliotoxin and the associated three-dimensional structure are required for its biological activity (Fig. 2*B*). Second, we tested the gliotoxin analog bis(methylthio)gliotoxin, in which the sulfurs that span the core DKP ring are methylated and therefore cannot engage in a disulfide bond. (Fig. 2*A*). Bis(methylthio) gliotoxin, like reduced gliotoxin, had no detectable ability to stimulate egg laying (Fig. 2*B*). These data indicate that the disulfide bridge, which is a distinguishing feature of gliotoxin, is required for its biological activity.

**Resistance to Gliotoxin Is Widespread among Wild Isolates of *C. elegans*.** Gliotoxin is produced by many species of fungi that are found in temperate soil ecosystems around the globe. Some gliotoxin-producing fungi (e.g., fungi of the genus *Myrothecium*) are symbionts that associate with plant roots; others are saprophytic and found in decaying plant matter (e.g., fungi of the genus *Penicillium* and the genus *Aspergillus*) (25–27). Like gliotoxin-producing fungi, *C. elegans* is widely distributed in temperate soil ecosystems and associates with decaying vegetable matter and plants (28, 29). We hypothesized that gliotoxin-producing fungi might interact with *C. elegans* in the wild, and some wild populations of *C. elegans* might, therefore, have acquired resistance to the effects of gliotoxin on behavior. To test this, we used a panel of wild *C. elegans* strains—the *C. elegans* Natural Diversity Resource (CeNDR) (30)—to screen for naturally occurring gliotoxin resistance. We tested 154 strains for resistance to 100 μM gliotoxin, a concentration at which gliotoxin's effects on egg laying by the domesticated N2 laboratory strain are saturated. Seventy-four strains released significantly fewer eggs in response to gliotoxin treatment than did the N2 control (Fig. 3*A* and *SI Appendix*, Table S1), indicating that these strains are resistant to the effects of gliotoxin on egg-laying behavior. We also found four wild strains that responded more strongly to gliotoxin than the N2 strain (Fig. 3*A* and *SI Appendix*, Table S1). Wild strains with altered gliotoxin sensitivity laid comparable amounts of eggs to N2 control when treated with serotonin and, like the N2 strain, did not lay eggs when exposed only to vehicle (Fig. 3 *B* and *C*). These data indicated that resistance to gliotoxin is not the result of a nonspecific defect in the control of egg laying. We noted that strains with resistance and sensitivity to gliotoxin came from diverse geographical regions (Fig. 3*D*), suggesting that these traits arose independently in different wild populations of *C. elegans*.

**Effects of Gliotoxin on Behavior Require Signaling via Metabotropic and Ionotropic Receptors.** To determine how gliotoxin triggers egg laying, we performed a genetic study of gliotoxin resistance. First, we determined the sensitivity of mutants lacking serotonergic HSNs or G protein-coupled serotonin receptors to gliotoxin and to other compounds identified by our screen (Fig. 4*A*). The *egl-1(gf)* mutants carry a mutation that selectively eliminates the HSNs by programmed cell death (10). These mutants are sensitive to the serotonin receptor agonist lysergol (Fig. 4*B*) and to the PKC agonist IDB (Fig. 4*C*). By contrast, *egl-1(gf)* mutants were profoundly resistant to gliotoxin (Fig. 4*D*). Deletion of serotonin receptors expressed by ELMs—the metabotropic receptors SER-1 and SER-7—conferred strong resistance to lysergol as expected (Fig. 4*B*). SER-1/SER-7 receptor mutants were sensitive to the PKC agonist IDB (Fig. 4*C*), which was also expected because PKC functions downstream of G protein-coupled receptors (GPCRs) in many contexts (24). We tested mutants for different PKC isozymes to determine whether PKC agonists identified from our screen target specific isoforms of PKC on egg laying. The *pkc-1*/PKCε mutants and *pkc-2*/PKCα mutants (31–33) exhibited resistance to these PKC agonists (*SI Appendix*, Fig. S2). By contrast, these PKC agonists did not target *tpa-1*/PKCθ (34) on egg laying (*SI Appendix*, Fig. S2). Furthermore, SER-1/SER-7 receptor mutants, like mutants that lack HSNs, were resistant to gliotoxin (Fig. 4*D*). These data indicate that the effects of gliotoxin on egg-laying behavior strictly require serotonergic HSNs and G protein-coupled serotonin receptors expressed by HSN targets.

Serotonin also regulates other *C. elegans* behaviors, including pharyngeal pumping and locomotion. We therefore tested whether gliotoxin mimics the effects of serotonin on these behaviors as it does with respect to egg-laying behavior. To measure effects on pharyngeal pumping, we exposed immobilized animals either to serotonin, gliotoxin, or vehicle only while recording pharyngeal movement. In the absence of an agonist, we observed no pumping whereas 10 mM serotonin robustly stimulated pumping as previously described (35) (*SI Appendix*, Fig. S3 *A* and *B*). By contrast, we observed no activation of pumping in the presence of concentrations of gliotoxin that potently activate egg laying (*SI Appendix*, Fig. S3 *A* and *B*. These data suggest that gliotoxin does not affect serotonin signaling in the *C. elegans* pharynx. We also tested whether gliotoxin affects the *C.*

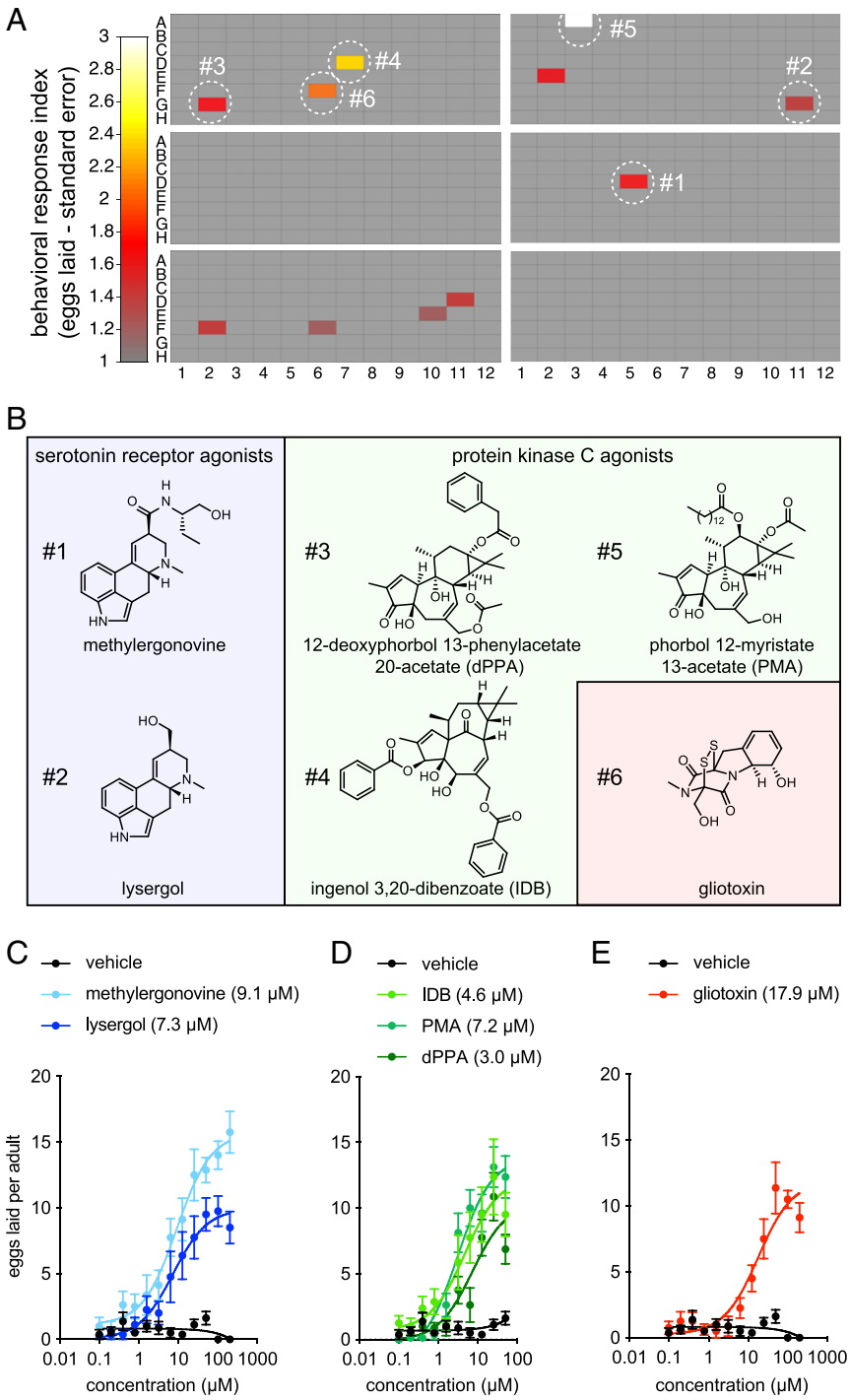


**Fig. 1.** A screen for natural products that elicit a serotonin-dependent behavior of *C. elegans*. (*A*) A summary of a screen for small-molecule agonists of serotonin-dependent behavior showing a heat map of behavioral response to compounds corrected for the variance of the response; 576 chemicals were arranged in six 96-well plates. Chemicals that elicited release of two or more eggs per animal were retested at least twice. For these compounds, a behavioral response index was calculated by subtracting the SEM from the mean number of eggs laid to identify compounds that reproducibly stimulate egg laying. Six compounds that reproducibly stimulate egg laying are annotated in #1 to #6. (*B*) The structure of six agonists recovered by the screen. Lead compounds include known serotonin receptor agonists (blue), known PKC agonists (green), and gliotoxin, a compound not previously known to interact with serotonin signaling pathways (red). (*C–E*) Dose–response curves of compounds that stimulate egg-laying behavior. Data are presented as mean ± SEM. Each point represents the mean number of eggs laid per animal in eight trials of cohorts of five animals. Numbers in parentheses are computed $EC_{50}$s for tested compounds.

*elegans* motor system, which is inhibited by serotonin via the metabotropic receptor SER-4 and the ionotropic serotonin receptor MOD-1 (36). We treated animals with vehicle, serotonin, or gliotoxin and assayed their thrashing behavior over time. Gliotoxin, like serotonin, caused transient paralysis (*SI Appendix*, Fig. S3 *C–E*). Importantly, *ser-4; mod-1* mutants, which lack the receptors that mediate the paralytic effects of serotonin, were resistant to paralysis by gliotoxin (*SI Appendix*, Fig. S3 *C–E*). Together, these data indicate that the effects of gliotoxin are not limited to serotonin signaling in the egg-laying system, but that

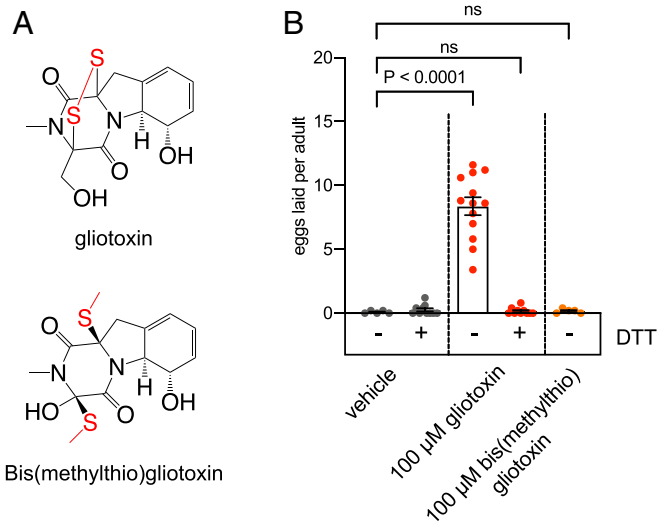


**Fig. 2.** The disulfide bridge of gliotoxin is essential for its activity. (*A*) Structure of gliotoxin and its analog, bis(methylthio)gliotoxin. Sulfur atoms are shown in red. (*B*) Behavioral response of animals exposed to 100 μM gliotoxin, 100 μM DTT-treated gliotoxin, and 100 μM bis(methylthio)gliotoxin. Data are presented as mean ± SEM. Each point represents the mean number of eggs laid per animal in 10 to 13 trials of cohorts of five animals. Data were analyzed by one-way ANOVA. ns, not significant.

not all serotonin signaling pathways are equally sensitive to this compound.

We next tested the gliotoxin sensitivity of mutants defective for specific aspects of HSN function and GPCR signaling (Fig. 5*A*). In addition to serotonin, HSNs release neuropeptides that are important activators of egg-laying behavior (37–39). Mutants are available which are selectively defective either for serotonin synthesis or for neuropeptide synthesis, allowing us to determine whether these neurochemical signals are required for the behavioral effects of gliotoxin. We found that *unc-13* mutants, which are broadly defective in neurotransmitter and neuromodulator release (40), did not lay eggs in response to gliotoxin (*SI Appendix*, Fig. S4). The *tph-1* mutants lack a key enzyme for serotonin synthesis and therefore lack endogenous serotonin (17). We found that *tph-1* mutants were resistant to high concentrations of gliotoxin (Fig. 5*B*). Other than serotonin, HSNs are also thought to release acetylcholine (41, 42). We found that gliotoxin was able to elicit egg laying in *unc-17* mutants, which are defective for cholinergic transmission (*SI Appendix*, Fig. S4) (43). Moreover, *egl-3* mutants, which lack a prohormone convertase required for the synthesis of many neuropeptides (37), were sensitive to gliotoxin (Fig. 5*B*). We also tested *unc-31* mutants, which are defective for the regulated release of neuropeptides stored in dense-core vesicles (44). Unlike serotonin-deficient mutants, *unc-31* mutants laid eggs in response to gliotoxin (Fig. 5*B*).These data indicated that serotonin signals are required for the effects of gliotoxin on behavior but that neuropeptide signals are dispensable.

The genetics of gliotoxin resistance are similar to the genetics of resistance to drugs that interfere with serotonin reuptake: i.e., like serotonin-reuptake inhibitors, gliotoxin requires endogenous serotonin to stimulate egg laying (11, 45). Serotonin-reuptake inhibitors act on a defined molecular target, the serotonin-reuptake transporter, which, in *C. elegans*, is encoded by the gene *mod-5* (13). We found that gliotoxin was able to elicit egg laying in *mod-5* mutants (Fig. 5*B*), indicating that, although gliotoxin shares with serotonin-reuptake inhibitors a requirement for endogenous serotonin, it acts via a distinct molecular mechanism.

We used another set of mutants to further define the pathway downstream of serotonin receptors that is required for gliotoxin's action. EGL-30 is the sole $G_q$ homolog in *C. elegans* (46), and EGL-8 is a phospholipase C β homolog that is regulated by $G_q$ (47). A strong loss-of-function mutation in *egl-30* and a null allele of *egl-8* each conferred strong resistance to gliotoxin (Fig. 5*C*). Together, our analysis of the genetics of gliotoxin resistance indicates that gliotoxin requires endogenous serotonin and signaling via metabotropic serotonin receptors to exert its effects on *C. elegans* egg-laying behavior.

**Gliotoxin Activates Downstream Targets of Serotonin Neurons in a Serotonin-Dependent Manner.** The genetics of gliotoxin resistance are consistent with two models of gliotoxin action: 1) Gliotoxin might stimulate HSN neurons to trigger a serotonin-dependent behavior, or 2) gliotoxin might act in parallel to serotonin signals from the HSNs to trigger behavior. To discriminate between these possibilities, we determined the effects of gliotoxin on HSN physiology and on the physiology of ELMs, which are HSN targets. We first used in vivo calcium imaging to monitor the physiology of HSN neurons and determine whether gliotoxin affected their activity (Fig. 6*A*). Under control conditions, HSNs display spontaneous calcium transients (48–50). Mutations that increase or decrease the frequency and amplitude of these transients have corresponding effects on egg-laying behavior (48, 50), indicating that these transients reflect a functionally important aspect of HSN physiology. We treated animals either with vehicle alone or with gliotoxin and measured HSN calcium transients. We observed no gross differences between the activity of HSNs in these two cohorts (Fig. 6*B*), and, when we compared the cumulative calcium signaling in HSNs from controls and gliotoxin-dosed animals, we found no evidence for an effect of gliotoxin (Fig. 6 *C* and *D*).

These data suggested that gliotoxin does not act by stimulating HSN activity. To further test this idea, we asked whether sensitivity to gliotoxin could be restored to animals that lack HSNs by simply supplying exogenous serotonin. We dosed *egl-1(gf)* mutants with serotonin in the presence of varying concentrations of gliotoxin. We found that, across a range of serotonin concentrations, gliotoxin increased egg laying by *egl-1(gf)* mutants (Fig. 6*E*), indicating that sensitivity to gliotoxin had been restored. Low concentrations of gliotoxin potentiated serotonin-induced egg laying, reducing the half-maximal effective concentration, 50% ($EC_{50}$) of serotonin nearly fourfold (Fig. 6*E*). By plotting the $EC_{50}$ of serotonin as a function of the concentration of added gliotoxin, we estimated that gliotoxin has a half-maximal effect on serotonin-sensitivity at 3.8 μM (Fig. 6*F*). Because gliotoxin had no measurable effect on HSN physiology and because gliotoxin sensitivity could be restored to mutants lacking HSNs, we concluded that serotonergic HSNs were not the cellular targets of gliotoxin.

We next tested whether gliotoxin affected the physiology of ELMs, which receive synaptic inputs from HSNs. Like HSNs, ELMs display spontaneous calcium transients (Fig. 7 *A* and *B*). Gliotoxin had a marked effect on calcium transients in ELMs and increased the frequency of calcium spikes (Fig. 7*B*). Accordingly, gliotoxin increased the cumulative calcium signaling in ELMs (Fig. 7 *C* and *D*). Like its effect on egg-laying behavior, the effect of gliotoxin on ELMs required HSNs; gliotoxin had no effect on calcium signaling in the ELMs of *egl-1(gf)* mutants (Fig. 7 *E* and *F* and *SI Appendix*, Fig. S5). Strikingly, the sensitivity of ELMs to gliotoxin could be restored to *egl-1* mutants with exogenous serotonin (Fig. 7 *G* and *H* and *SI Appendix*, Fig. S5). We concluded that the synergistic effects of gliotoxin and serotonin that we observed in behavioral studies could be explained by their combined actions on the synaptic targets of serotonin neurons in the egg-laying system.

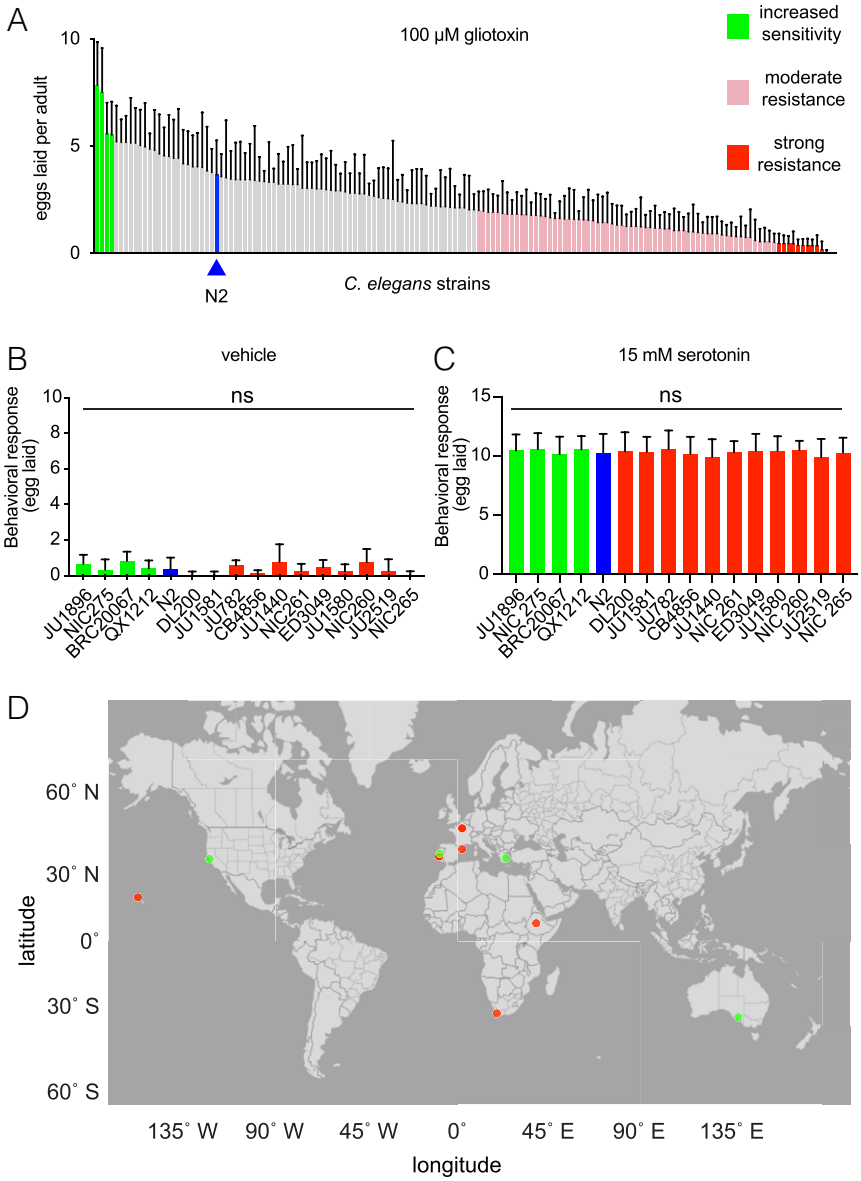


**Fig. 3.** Gliotoxin resistance occurs in wild populations of *C. elegans*. (*A*) Egg-laying responses of 154 wild isolates after 2 h of exposure to 100 μM gliotoxin in microtiter wells are shown. Data are presented as mean ± SD. The laboratory reference strain, N2, is indicated in blue. Strains whose mean response was more than one SD above the N2 mean are colored green. Strains whose responses were less than one SD below the N2 mean are colored pink. Those with responses less than two SD below the N2 mean are colored red. Each set comprises 7 to 20 trials of cohorts of five animals. (*B* and *C*) Egg-laying responses of selected strains during 2 h of exposure to vehicle or 15 mM serotonin. Data are presented as mean ± SD, $n = 20$ trials of cohorts of five animals. Data were analyzed by two-way ANOVA. ns, not significant. (*D*) The geographic distribution of strains with increased sensitivity (green) and strong resistance (red) plotted on a world map.

## Discussion

Our screen of natural products for compounds that elicit a serotonin-dependent behavior of *C. elegans* identified the DKP gliotoxin as a potent behavioral agonist. Gliotoxin has not previously been linked to the function of the nervous system. As its name indicates, gliotoxin was initially identified as a cytotoxin produced by multiple types of fungal pathogen (51–53). Subsequently, high concentrations of gliotoxin were shown to inhibit farnesyltransferases (54) that lipidate small GTPases, interfere with NFκB signaling (55), and inhibit the proteosome (56). It has been suggested that gliotoxin impacts so many cellular processes because its ring-spanning disulfide bridge can react with diverse cysteine-containing proteins to disrupt their function (57). The inability of bis(methylthio)gliotoxin to trigger egg laying suggests that the disulfide is essential for interfering with pathways that underlie this process. Our data indicate that gliotoxin has previously unappreciated effects on serotonin signaling in an animal nervous system and that these effects are likely mediated by high-affinity interactions with cellular targets. First, we observed that micromolar concentrations of gliotoxin in the environment trigger egg-laying behavior. This is comparable to the concentrations of well-characterized high-affinity serotonin receptor agonists that elicit this behavior. Second, gliotoxin, serotonin receptor agonists, and serotonin itself showed similar pharmacokinetics and elicited egg-laying behavior over similar timescales. These data are consistent with a model in which gliotoxin has a high-affinity interaction with a cellular target and is limited by its ability to cross diffusion barriers of cuticle and skin and permeate the nematode.

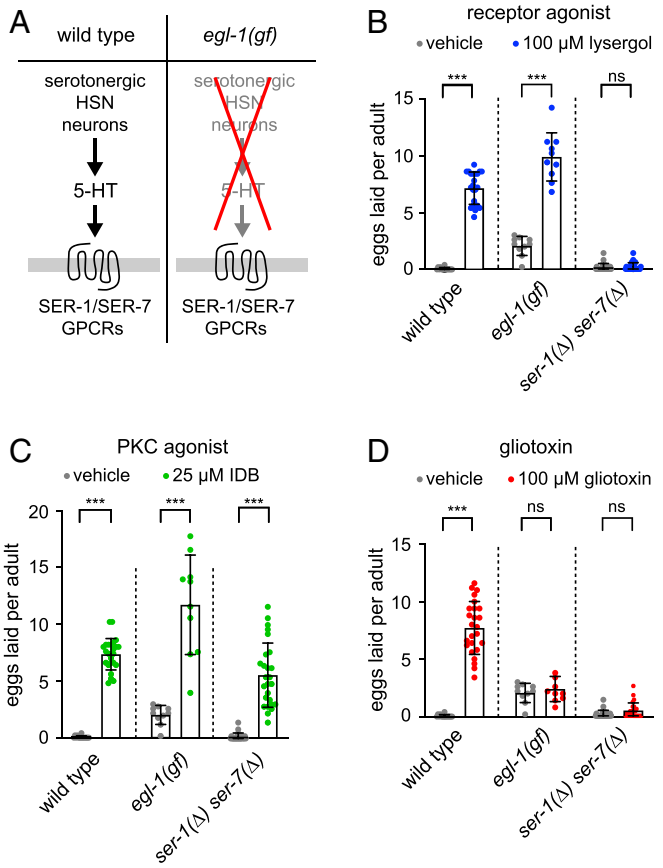


**Fig. 4.** Gliotoxin action requires serotonin neurons and metabotropic serotonin receptors. (*A*) Schematic of the egg-laying system showing serotonergic HSNs, which are eliminated in *egl-1(gf)* mutants, and SER-1/SER-7 GPCRs, which are expressed by postsynaptic ELMs. (*B–D*) Egg-laying responses of wild type, *egl-1*, and *ser-1 ser-7* mutants to a serotonin receptor agonist, a PKC agonist, and gliotoxin. Data are presented as mean ± SEM. Each point represents the mean of 20 to 30 trials of cohorts of five animals. Data were analyzed by two-way ANOVA. ***$P \leq 0.0001$. ns, not significant.

A remarkable aspect of gliotoxin's action is that it strongly requires endogenous serotonin to affect behavior. Mutants that lack serotonin neurons in the egg-laying system or that are defective for serotonin synthesis are profoundly resistant to gliotoxin. One known class of psychopharmaceuticals that acts in this manner comprises inhibitors of serotonin reuptake via the plasma membrane serotonin-selective transporter (10, 58). In *C. elegans*, only one gene encodes this transporter: *mod-5* (13). We found that mutants carrying a null allele of *mod-5* remain sensitive to gliotoxin, suggesting that gliotoxin boosts endogenous serotonin signaling through a mechanism distinct from that of serotonin-reuptake inhibitors.

It will be of great interest to identify the molecular target of gliotoxin. Our genetic analysis suggests that this target will function in serotonin signaling downstream of serotonin release. Our data further indicate that it is unlikely that gliotoxin acts as a modulator or activator of serotonin receptors themselves because it affects serotonin signaling pathways that use different serotonin receptors. Gliotoxin activates egg laying in a manner that requires SER-1/SER-7 GPCRs, and it causes paralysis in a manner that strongly requires the SER-4 GPCR and partly requires the MOD-1 ligand-gated ion channel. Genetic studies of egg laying and other serotonin-dependent behaviors have identified many molecular components of serotonin signaling pathways that function in the generation or modulation of behavior (13, 19, 59, 60). An exciting possibility is that the molecular target of gliotoxin is a novel molecular constituent of neuromodulator and neurotransmitter signaling pathways.

**The Nervous System as a Focus of Host–Microbe Interactions.** Two compounds recovered by our screen—gliotoxin and lysergol—are microbial metabolites with strong and specific effects on the nematode nervous system. *C. elegans* lives in microbe-rich environments where it feeds on nutritive microbes and can be infected by pathogens. The nematode nervous system generates a number of critical microbe-response behaviors, including positive chemotaxis toward microbial prey and innate and learned avoidance of pathogens (61–64). In addition to behavior, the nematode nervous system is an essential regulator of stress responses, including host responses to physiological stress caused by infection (65–67). It is tempting to speculate that, within the contexts of predator–prey and host–pathogen interactions, microbes have evolved the ability to influence the nematode nervous system via the production of neuroactive metabolites. Previous studies have shown that metabolites produced by gut bacteria can have neuroprotective effects or modulate sensory abilities in *C. elegans* (68, 69). The neuromuscular system that generates egg-laying behavior might be a particularly attractive target for microbial small molecules. Prey microbes could slow the growth of populations of nematode predators by inhibiting the neurochemical signals required for egg laying. Pathogenic microbes, on the other hand, could elicit egg laying by nematode hosts by activating the same neurochemical signaling pathways, thereby ensuring access to hosts when the eggs hatch. The observation in our study of widespread resistance to gliotoxin in wild populations of *C. elegans* lends credence to this idea.

A

| *C. elegans* protein | mammalian homolog |
|---|---|
| TPH-1 | tryptophan hydroxylase |
| EGL-3 | prohormone convertase |
| UNC-31 | calcium-dependent activator protein for secretion (CAPS) |
| MOD-5 | 5-HT reuptake transporter |
| EGL-30 | $G_q\alpha$ |
| EGL-8 | phospholipase C β |

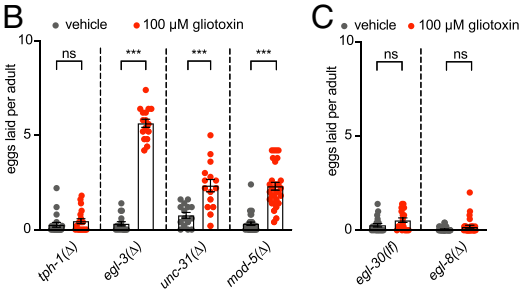


**Fig. 5.** Endogenous serotonin and $G_q$ signaling are required for sensitivity to gliotoxin. (*A*) *C. elegans* genes that were tested for roles in gliotoxin sensitivity and their mammalian homologs. (*B* and *C*) Egg-laying responses of *tph-1*, *egl-3*, *unc-31*, *mod-5*, *egl-30*, or *egl-8* mutants to 100 μM gliotoxin. Data are presented as mean ± SEM. Each point represents the mean of 20 to 30 trials of cohorts of five animals. Data were analyzed by two-way ANOVA. ***$P \leq 0.0001$. ns, not significant.

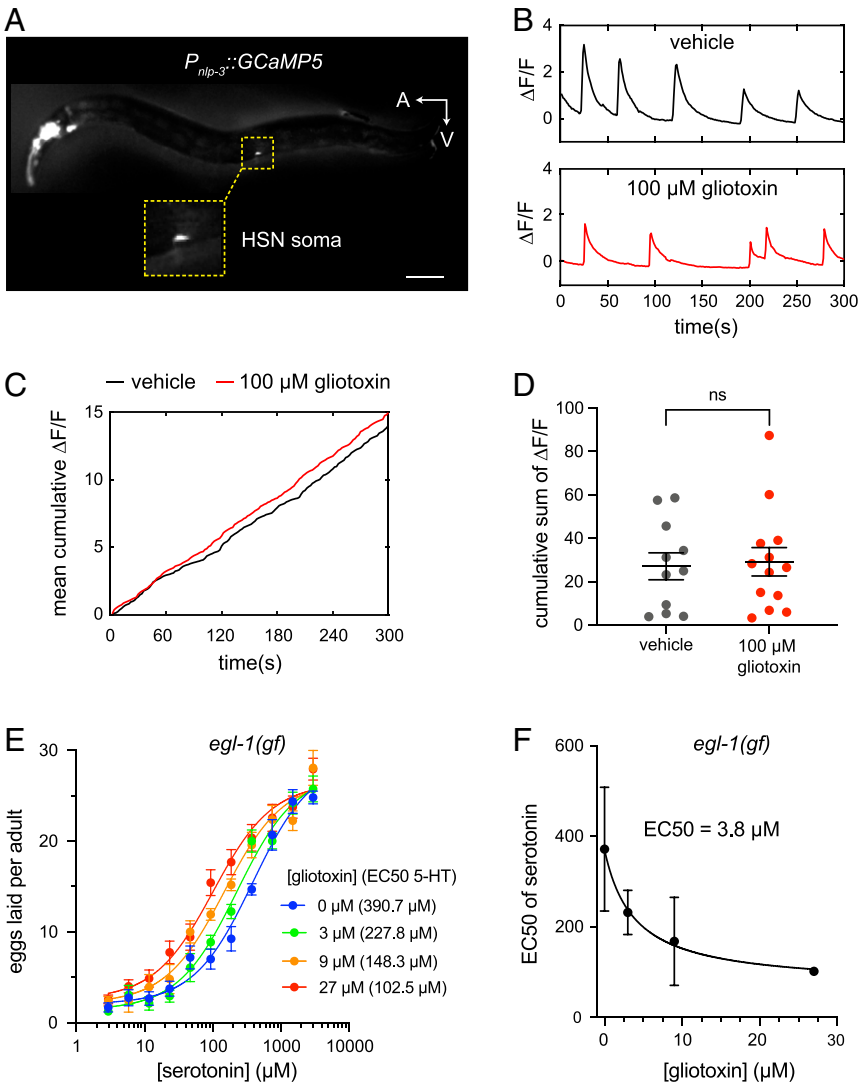


**Fig. 6.** Exogenous serotonin restores gliotoxin-sensitivity to animals that lack HSNs. (*A*) Expression of a $P_{nlp-3}$::*GCaMP5* transgene used to measure HSN activity. Dashed lines indicate a region of interest containing the HSN cell body, shown at higher magnification in the *Inset*. A, anterior. V, ventral. Scale bar, 100 μm. (*B*) Representative traces of GCaMP5 signals in HSN treated with vehicle or 100 μM gliotoxin. (*C*) Mean cumulative GCaMP5 signals of HSNs treated with vehicle or 100 μM gliotoxin. $n = 11$–13 trials. (*D*) Scatter plot of cumulative GCaMP5 signals from individual recordings. Data are presented as mean ± SEM. Data were analyzed by unpaired *t* test. $n = 11$–13 trials. ns, not significant. (*E*) Dose–response curves of serotonin-evoked egg laying by *egl-1(gf)* mutants dosed with different concentrations of gliotoxin. Data are presented as mean ± SEM. Numbers in parentheses are computed $EC_{50}$s of serotonin at indicated gliotoxin concentration. $n = 16$ trials of cohorts of five animals. (*F*) The effect of gliotoxin on serotonin-evoked egg laying by *egl-1(gf)* mutants plotted showing the $EC_{50}$ for gliotoxin as a modifier of serotonin sensitivity. Data are presented as mean ± SEM, $n = 3$.

The interactions between environmental microbes and the nematode nervous system that we hypothesize are likely recapitulated in other animals in the context of host–microbe interactions. A host of neuropsychiatric disorders are accompanied by changes in the host microbiome, and manipulation of the microbiome impacts nervous system development and function in many ways (70). Recently, progress has been made toward identifying specific chemical mediators of the effects of the microbiome on the host nervous system. Bacteria resident to the insect gut, for example, control locomotion and foraging by influencing the production of nutrient signals that are, in turn, potent modulators of aminergic signaling (71). The vertebrate microbiome can produce the amino acid metabolites taurine and 5-aminovaleric acid, which interact with γ-aminobutyric acid (GABA) and glycine receptors to influence social behaviors (72) and can produce the neurotransmitter histamine and the psychoactive compound phenethylamine, each of which activates GPCRs expressed in the host nervous system (73). The microbiome has also been shown to produce novel compounds, including cannabinoid-like compounds, that interact specifically with host GPCRs (74, 75). The microbes found within animal hosts (commensals) radically differ from the microbes found in soil ecosystems inhabited by *C. elegans* and other free-living nematodes. It is likely that environmental microbes and commensals have evolved different molecular mechanisms to influence the animal nervous system. We suggest that environmental microbes that have coevolved with nematodes will be a rich source of novel psychoactive compounds. Because the nematode nervous system uses mechanisms of neurochemical signaling that are conserved between nematodes and vertebrates, some of these compounds might also affect the vertebrate brain and be the basis for new psychopharmacology.

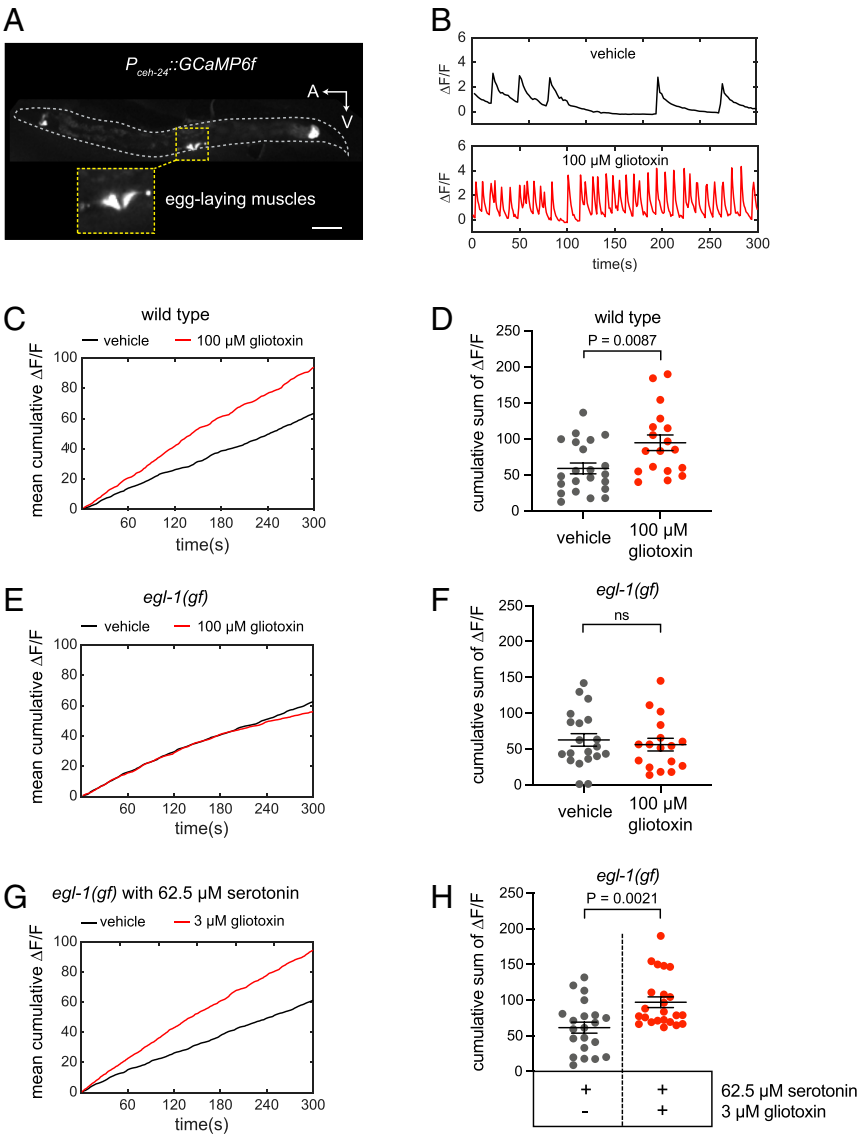


**Fig. 7.** Gliotoxin activates the synaptic targets of HSNs in a serotonin-dependent manner. (*A*) Expression of a $P_{ceh-24}$::*GCaMP6f* transgene used to measure the activity of ELMs that receive synaptic input from HSNs. Dashed white lines outline the animal. Dashed yellow lines indicate a region of interest containing ELMs, shown at 2.5-fold magnification in the *Inset*. A, anterior. V, ventral. Scale bar, 100 μm. (*B*) Representative GCaMP6f signals in ELMs treated with vehicle or 100 μM gliotoxin. (*C*) Mean cumulative GCaMP6f signals of ELMs of wild-type animals treated with vehicle or 100 μM gliotoxin. $n = 18–22$ trials. (*D*) Scatter plot of cumulative GCaMP6f signals in ELMs of the wild type. Data are presented as mean ± SEM. Data were analyzed by unpaired *t* test. $n = 18–22$ trials. (*E*) Mean cumulative GCaMP6f signals of ELMs of *egl-1(gf)* mutants treated with vehicle or 100 μM gliotoxin. $n = 17–20$ trials. (*F*) Scatter plot of cumulative GCaMP6f signals in ELMs of *egl-1(gf)* mutants. Data are presented as mean ± SEM. Data were analyzed by unpaired *t* test. $n = 17–20$ trials. ns, not significant. (*G*) Cumulative GCaMP6f signals of ELMs treated with 62.5 μM serotonin or 62.5 μM serotonin plus 3 μM gliotoxin in *egl-1(gf)* animals. $n = 21–23$ trials. (*H*) Scatter plot of cumulative GCaMP6f signals in *egl-1(gf)* ELMs. Data are presented as mean ± SEM. Data were analyzed by unpaired *t* test. $n = 21–23$ trials. ns, not significant.

## Materials and Methods

Dataset S1 contains source data for all scatterplots in main and supplementary figures. The following compounds were purchased from commercial sources: gliotoxin (Cayman Chemical), bis(methylthio)gliotoxin (Cayman Chemical), lysergol (AK Scientific), methylergometrine maleate (ApexBio), dPPA (MilliporeSigma), PMA (MilliporeSigma), IDB (Santa Cruz Biotechnology), and serotonin hydrochloride (MilliporeSigma).

***C. elegans* Genetics.** Strains were maintained at 20 °C as described by Brenner (76). The following strains were used in this study: N2 (wild-type strain), DA2109 *ser-7(tm1325) ser-1(ok345)*, MT1082 *egl-1(n487)*, IK130 *pkc-1(nj3)*, VC127 *pkc-2(ok328)*, MJ563 *tpa-1(k530)*, RB745 *ser-4(ok512)*, MT9668 *mod-1(ok103)*, LX1834 *ser-4(ok512); mod-1(ok103)*, MT14984 *tph-1(n4622)*, VC671 *egl-3(ok979)*, CB928 *unc-31(e928)*, MT8944 *mod-5(n822)*, MT1434 *egl-30(n686)*, MT1083 *egl-8(n488)*, MT7929 *unc-13(e51)*, CB113 *unc-17(e113)*, LX2004 *lin-15AB(n765) lite-1(ce314) vsIs183[$P_{nlp-3}$::GCaMP5::nlp-3 3′UTR; $P_{nlp-3}$::mCherry::nlp-3 3′UTR; lin-15(+)]*, FQ1194 *lin-15AB(n765); wzEx335 [$P_{ceh-24}$::GCaMP6f; lin-15(+)]*, FQ1924 *egl-1(n487); wzEx335[$P_{ceh-24}$::GCaMP6f; lin-15(+)]*. One hundred fifty-four wild *C. elegans* strains from the CeNDR (30) are listed in *SI Appendix*, Table S1.

**Screening Chemical Libraries for Activators of Egg Laying.** To identify chemicals that can stimulate egg-laying behavior, 5-d-old animals were placed in each well of a 96-well microtiter plate with 100 μL of S basal buffer (77) and exposed to chemicals of interest at a final concentration of 100 μM. The library consisted of structurally and functionally diverse commercial natural products and recently isolated ones; these were of approximately equal proportion of plant, bacterial, and fungal origin. After dosing the animals for 2 h, released eggs were counted using a standard dissection stereomicroscope. Chemicals that elicited release of two or more eggs per animal

were retested at least twice. For these compounds, a behavioral response index was calculated by subtracting the SEM number of eggs laid from the mean to identify compounds that reproducibly stimulate egg laying. The $EC_{50}$ of compounds was computed using nonlinear regression of dose–response data as described (78).

**Analysis of Drug-Induced Egg Laying.** To test the effect of select chemicals on egg-laying behavior, 5-d-old animals were placed in each well of a 96-well microtiter plate with 100 μL of S basal buffer (77) and exposed to vehicle (1% DMSO) plus the indicated concentration of compound. After 2 h, released eggs were counted using a standard dissection stereomicroscope. Each data point represents the mean of a trial of five animals. For time-course experiments, released eggs were counted every 20 min. To test the requirement of disulfide bonds in gliotoxin, the indicated concentration of gliotoxin was treated with 1 mM DTT for 1 h prior to the behavioral assay.

**Analysis of *C. elegans* Pharyngeal Pumping.** To test the effect of chemical of interest on pharyngeal pumping, day-old animals were placed in a drop of polystyrene beads (2.5% by volume, 0.1-μm diameter; Polyscience) on a 10% agarose pad in nematode growth medium (NGM) buffer with the indicated concentration of chemicals, as previously described (79). Animals were dosed for 10 min prior to imaging, and videos were acquired using a 40× oil objective (numerical aperture [N.A.] 1.6) on a Zeiss Axio Imager M2. Pumps were counted manually. Data were analyzed by one-way ANOVA.

**Analysis of Serotonin-Induced Immobilization.** Five-day-old animals were placed in 100 μL of S basal buffer (77) with the indicated concentrations of chemicals of interest. Animals were scored as immobile if they did not show smooth swimming motion; small head and tail movements were not considered. Animals were scored every 10 min over a 1-h period. Data were analyzed by two-way ANOVA.

**Calcium Imaging of Serotonin Neurons and ELMs.** Day-old transgenics were dosed for 2 h in S basal buffer (77) containing either vehicle (1% DMSO) or vehicle plus drug. After dosing, animals were recovered, mounted on 5% agarose pads made in M9 buffer, immobilized using focal application of VetBond surgical glue to the dorsal surface, and placed under a coverslip. Videos were taken using a 10× objective on a Zeiss Axio Imager M2. GCaMP5 and GCaMP6f were excited by 473 nm light using a metal halide epifluorescence light source (EXFO), and images were acquired with a cooled charge-coupled device (CCD) camera (Andor Clara). Images were acquired at 0.5 or 1 Hz for 15 min. The microscope, camera, and excitation light source were controlled by Micromanager (80). GCaMP fluorescence was analyzed as previously reported (50). Briefly, for each frame, the difference between mean fluorescence in a region of interest (ROI), including cells and a comparable ROI containing only background fluorescence, was computed. The baseline fluorescence F used to compute ΔF/F was determined using a linear regression of the entire time series of background-corrected fluorescence measurements.

Statistical analyses of all data were performed using GraphPad Prism 8 for macOS, version 8.4.3 (471).

**Data Availability.** All study data are included in the article, *SI Appendix*, and Dataset S1.

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