A Genetic Survey of Fluoxetine Action on Synaptic Transmission in Caenorhabditis elegans

Andrey Kullyev,*,1 Catherine M. Dempsey,*,†,1 Sarah Miller,1 Chih-Jen Kuan,*, Vera M. Hapiak,† Richard W. Komuniecki,† Christine T. Griffin† and Ji Ying Sze*,2

*Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461 1Department of Biology, National University of Ireland, Maynooth, County Kildare, Ireland 2Department of Biological Sciences, University of Toledo, Toledo, Ohio 43606

Manuscript received May 13, 2010 Accepted for publication August 13, 2010

ABSTRACT

Fluoxetine is one of the most commonly prescribed medications for many behavioral and neurological disorders. Fluoxetine acts primarily as an inhibitor of the serotonin reuptake transporter (SERT) to block the removal of serotonin from the synaptic cleft, thereby enhancing serotonin signals. While the effects of fluoxetine on behavior are firmly established, debate is ongoing whether inhibition of serotonin reuptake is a sufficient explanation for its therapeutic action. Here, we provide evidence of two additional aspects of fluoxetine action through genetic analyses in Caenorhabditis elegans. We show that fluoxetine treatment and null mutation in the sole SERT gene mod-5 eliminate serotonin in specific neurons. These neurons do not synthesize serotonin but import extracellular serotonin via MOD-5/SERT. Furthermore, we show that fluoxetine acts independently of MOD-5/SERT to regulate discrete properties of acetylcholine (Ach), gamma-aminobutyric acid (GABA), and glutamate neurotransmission in the locomotory circuit. We identified that two G-protein–coupled 5-HT receptors, SER-7 and SER-5, antagonistically regulate the effects of fluoxetine and that fluoxetine binds to SER-7. Epistatic analyses suggest that SER-7 and SER-5 act upstream of AMPA receptor GLR-1 signaling. Our work provides genetic evidence that fluoxetine may influence neuronal functions and behavior by directing targeting serotonin receptors.

FLUOXETINE is a selective serotonin reuptake inhibitor (SSRI) and has made a major impact on the treatment of many behavioral disorders. The empirical action of SSRIs is blocking the serotonin reuptake transporter (SERT). SERT is localized in the plasma membrane and transports extracellular serotonin (5-HT) into the cytoplasm (Blakey et al. 1991; Hoffman et al. 1991), this being the major mechanism of terminating 5-HT signaling. Consequently, SSRIs are thought to exert therapeutic effects by blocking SERT from removal of 5-HT in the synaptic cleft, thereby increasing the level of 5-HT signals (Schatzberg and Nemeroff 2004). However, several observations point to additional actions of SSRIs on the 5-HT system and neuronal functions. First, knockout of SERT in mouse caused a marked reduction of 5-HT in the brain (Bengel et al. 1998). Second, a variety of studies with cultured mammalian cells and mouse brain slices showed that SSRIs and tricyclic antidepressant agents (TCAs) have high affinities to many 5-HT receptor subtypes and act as agonists or antagonists depending on particular receptors being tested (Ni and Miledi 1997; Kroese and Roth 1998; Eisensamer et al. 2003). Third, genetic analyses of the nematode Caenorhabditis elegans in our laboratory and others showed that fluoxetine and the TCA imipramine (Tofrani) could influence behavior independent of SERT function (Weinshenker et al. 1995; Ranganathan et al. 2001; Dempsey et al. 2005). In this study, we carried out a systematic survey of SSRIs treatment in C. elegans to gain new insights into actions of SSRIs on the 5-HT system and other neurotransmitter systems.

In both vertebrates and invertebrates, 5-HT functions as a neuromodulator to either facilitate or inhibit synaptic transmission of other neurotransmitters (Fink and Gothert 2007). Modulation of synaptic activity by 5-HT signaling underscores the synaptic plasticity involved in stress responses, learning, adaptation, and memory (Kandel 2001; Zhang et al. 2005). The role of 5-HT in C. elegans was initially identified through pharmacological experiments showing that exogenous 5-HT can promptly induce changes in a variety of behaviors, including feeding, egg laying, and locomotion (Avery and Horvitz 1990; Weinshenker et al. 1995; Nurrish et al. 1999). The relevance of these behaviors to endogenous 5-HT has since been validated.

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.110.118877/DC1.

*These authors contributed equally to this work.
†Corresponding author: 202 Golding Bldg., 1300 Morris Park Ave., Bronx, NY 10461. E-mail: jiying.sze@einstein.yu.edu

Copyright © 2010 by the Genetics Society of America
DOI: 10.1534/genetics.110.118877

Genetics 186: 929–941 (November 2010)
through studies of mutants of 5-HT signaling. Importantly, multiple 5-HT receptors may function in distinct cell types synergistically or antagonistically to regulate a specific behavior (Carnell et al. 2005; Dernovici et al. 2007; Murakami and Murakami 2007; Hapiak et al. 2009). In nearly all tested paradigms, fluoxetine and imipramine induce behavioral changes similarly to exogenous 5-HT (Weinshenker et al. 1995; Nurrish et al. 1999), implying that fluoxetine regulates 5-HT inputs to these neural circuits. However, the tryptophan hydroxylase gene tph-1 is required for 5-HT biosynthesis in C. elegans (Sze et al. 2000), mod-5 encodes its sole SERT (Ranganathan et al. 2001), and yet fluoxetine could stimulate egg laying and inhibit locomotion in mod-5 and tph-1 mutants (Weinshenker et al. 1995; Choy and Thomas 1999; Ranganathan et al. 2001; Dempsey et al. 2005). These findings provided a basis for further investigation into genes and synaptic functions regulated by 5-HT and the impact of fluoxetine on 5-HT signaling.

Here we present genetic evidence of multifaceted effects of fluoxetine on the 5-HT system and its downstream targets in C. elegans. We show that fluoxetine treatment and loss of MOD-5/SERT function do not simply increase presynaptic 5-HT signals. Rather, they may eliminate 5-HT in specific neurons. Furthermore, fluoxetine acts independently of SERT to regulate 5-HT serotonin receptors and their downstream targets involved in acetylcholine (ACh), gamma-aminobutyric acid (GABA), and glutamate neurotransmission.

MATERIALS AND METHODS

Strains: The maintenance of C. elegans strains, nematode growth media (NGM), and standard buffers (M9, S-basal, and S-medium) used to handle worms have been described (Brenner 1974). Wild type (WT) was the Bristol strain N2. Mutant strains used were as follows: dop-6(ok2090), cha-1(p1152), pha-1(e2123);mdf-18;Punc-17::GFP;pha-1(+); (ACh::gfp) (from J. Rand, University of Oklahoma Health Sciences Center, Oklahoma City, OK), eat-4(ad572), eat-4(e2744), glr-1(t2461), KP987 lin-15B(n765) wax1 X[lin-15(+); PV6 glr-1::GFP]; mod-5(n3314), mod-5(n822) (from R. Horvitz, Massachusetts Institute of Technology, Cambridge, MA), mod-1(ok103), myo-3(s386);exE30[Pmyo-3::GFP;rol-6(su1006)]; (MYO-3::GFP), mrf-6 (sa525), ser-1(ok345), ser-3 (ok955), ser-3(ad1774), ser-3(k0027), ser-1(ok512), ser-3(tm2654), ser-3(tm2647), ser-7(tm1325), ser-7(tm1548), ser-7(tm1728), slo-1(s379), slo-1(by2999), tag-24 (sk71), tan-1(k825), tph-1(mg280), unc-25(e156), unc-29(e193), unc-43(e408), unc-43(h498), unc-49(e07), and EG1653 (lin-15(n765)); ods-22[pEk3(lin-15 +); UNC-49B::GFP] (from E. Jorgensen, University of Utah, Salt Lake City, UT).

Constructions of ser-5(+/-) and ser-7(+/-) transgenes: The constructs were generated by PCR, and purified PCR products were used to generate transgenic worms. eb-2::gfp, which is expressed in the gut (Fukunaga et al. 1999), was used as a transgenic marker. ser-5 was a genomic fragment amplified from the WT genome encompassing a 6305-bp 5’-upstream promoter sequence, exons/introns, and a 1118 bp 3’-UTR of the ser-5 gene.

Indirect immunofluorescence histochemistry and microscopy: Whole-mount staining of C. elegans with anti-5-HT antibodies was performed as described previously (Sze et al. 2002). To analyze the effect of fluoxetine and imipramine on 5-HT immunoreactivity, the drugs were dissolved in water and the solution was poured onto NGM plates to give the final concentration of 0.4 mg/ml imipramine or 0.5 mg/ml fluoxetine. The plates were dried under a hood for 2–3 hr and used immediately. Well-fed mixed-staged worms were incubated on the drug plates overnight and then fixed for staining. The control animals were raised and stained in parallel but without drug treatment. Stained animals were viewed using an AxioImager Z1 microscope equipped with proper filters and an Axiocam MR digital camera (Zeiss, Northwood, NY).

Drug and behavioral assays: Sensitivity to fluoxetine and other SSRIs was scored by calculating the percentage of animals paralyzed after being incubated in a liquid medium containing a drug. In all experiments except that described in Figure 2, a and c, the assays were carried out in culture media containing food. Food used in this assay was Escherichia coli OP50. A single colony of the bacteria was inoculated in 250 ml of 2xYT medium with shaking at 37° overnight. The resulting bacteria were concentrated by centrifugation, and the pellet was resuspended in 3 ml of M9 buffer. Worms were washed off NGM plates with M9 and the number of worms in the suspension was adjusted to ~1000 worms/ml. Drug assays were carried out in 15 ml polypropylene conical tubes. Each assay contained 50 μl of the bacteria concentrate and 50 μl of the 1000/μl worm suspension in 2 ml of S-medium. Unless specified, the final concentrations of drugs in the media were 5 mg/ml 5-HT and 0.5 mg/ml fluoxetine. The number of immobile animals was scored following incubation on a rocking platform at 20°. The incubation time for fluoxetine assays was 24 hr unless indicated otherwise. Animals remaining completely immobile for 10 sec were recorded as paralyzed. WT and mutant strains were always assayed in parallel and the difference in the percentage of paralyzed WT and mutant animals was determined. Fluoxetine sensitivities of strains grown and assayed in parallel were compared.

In experiments described in Figure 2, a and c, worms were incubated in 5 mg/ml 5-HT or 0.5 mg/ml fluoxetine in M9 without food for 30–60 min and were then either transferred to NGM plates to recover or examined immediately.

Resistance to aldicarb-induced paralysis was assayed as described (Nurrish et al. 1999). Briefly, 20 1-day-old adults per strain were transferred onto a NGM plate containing 1 mm aldicarb, and the number of animals paralyzed on the plate was scored at various time points. An animal was scored as being paralyzed if no movement was detected after prodding with a platinum wire. To assay the inhibition of aldicarb effects by 5-HT and fluoxetine, animals were preincubated for 2 hr on plates containing 5-HT (5 mg/ml), fluoxetine (0.5 mg/ml), or no drug control, before being exposed to aldicarb.

Imaging analysis and quantification of GFP reporters: One-day-old young adult hermaphrodites were examined, unless specified otherwise. For evaluation of ACh::gfp expression level, images of the 9th and 10th ACH neurons along the ventral nerve cord were captured at a fixed exposure time of 150 ms to 100% UV level, and the fluorescence intensity over a 25 × 25-pixel area within a neuron was quantified, using Adobe Photoshop 7.0 software. For evaluation of the abundance of UNC-49::GFP, images along the ventral nerve cord were captured at a fixed exposure time of 300 ms and the...
fluorescence intensity over a 50 × 5-µm area as illustrated in Figure 3e was quantified using Image J software.

GLR-1::GFP was evaluated by counting the number of GFP puncta along the ventral cord in live, genotype-blinded animals under the fluorescence scope.

[^3]Lysergic acid diethylamide (LSD) binding assays: The SER-7b expression vector and the binding assays with the membrane fraction of COS-7 cells expressing SER-7b have been published (Honson et al. 2003, 2006). Briefly, COS-7 cells were transiently transfected with the SER-7b expression construct for 48 hr. The transfected cells were harvested and lysed, and the membrane fraction of the lysate was collected after centrifugation. [H]LSD saturation binding and inhibition of [H]LSD binding by 5-HT and fluoxetine were assayed in a 100-µl reaction volume in a well of 96-well microtiter plates at room temperature. For saturation binding, the membrane extract containing 15 µg proteins was incubated for 1 hr in the dark with various concentrations of [H]LSD (1–50 nm) to obtain B_max and K_d values. To test the affinity of 5-HT and fluoxetine to SER-7b, the membrane extract was incubated with 10 nm [H]LSD and various concentrations of 5-HT and fluoxetine, determining the ability to replace [H]LSD binding. For both saturation and inhibition binding assays, nonspecific bindings were evaluated by incubation of the reaction mixture with a 1000-fold excess of unlabeled LSD. The binding assays were terminated by filtration with GF/B filters (Perkin-Elmer, Wellesley, MA) previously soaked with 0.3% polyethylenimine. The filters were washed three times with ice-cold TEM buffer and dried overnight, and the radioactivity was quantified by liquid scintillation counting. The binding data were analyzed by nonlinear regression analysis using DeltaGraph (DeltaGraph Version 4.0; DeltaPoint, Chicago).

Statistics: Statistical analyses were performed using Minitab 12.1 (Minitab Inc., 1998). For comparisons between two test groups, Student’s t-tests were carried out. Comparisons between more than two groups used ANOVA (one-way) followed by a Tukey’s pairwise multicomplication procedure. Results that could not be normalized were tested with nonparametric Mann–Whitney U-tests or Kruskal–Wallis tests.

RESULTS

Fluoxetine and mutations in mod-5/SERT eliminate 5-HT in specific neurons: In C. elegans 5-HT in specific neurons can be precisely discerned by whole-mount staining of the entire animal with antibodies raised against 5-HT. In WT larvae 5-HT immunoreactivity can be detected in four classes, a total of seven neurons in two-test regions: a pair of the ADF chemosensory neurons, and a pair of the AIM and the single RIH interneurons. In addition, 5-HT immunoreactivity can be detected in a pair of the HSN motorneurons in adults (Figure 1b).

One explanation for these results could be less efficient expression of the transgenes in the AIM and RIH neurons. Alternatively, AIMS and RII do not synthesize 5-HT. To distinguish between these possibilities, we analyzed mutants of the sole SERT gene mod-5. In both mod-5(n3314) deletion and mod-5(n822) opal mutants, we observed 5-HT immunoreactivity in NSMs, ADFs, and adult HSNs but not in AIMS and RIH (Figure 1c). We concluded that AIMS and RIH use MOD-5/SERT to absorb extracellular 5-HT, but do not synthesize it.

We therefore used 5-HT immunoreactivity in the AIM and RIH neurons as a measurement to assess the efficacy of MOD-5/SERT inhibitors in C. elegans. In WT animals incubated for 4 hr on culture plates containing 0.5 mg/ml fluoxetine or 0.4 mg/ml imipramine, 5-HT immunoreactivity in AINS and RIH was substantially reduced (data not shown). 5-HT immunoreactivity in AIMS and RIH became undetectable in most animals following 24-hr drug treatments (Figure 1d). Relatively high drug concentrations are commonly used in experiments with C. elegans because drugs are supplied in growth media and only small amounts of a drug enter animals (Lewis et al. 1980). It has been reported that in the CNS of rodents and humans SERT is expressed in many neurons that do not express the enzymes essential for 5-HT biosynthesis (D’Amato et al. 1987; Hoffman et al. 1998; Lebrand et al. 1998; Verney et al. 2002). Thus, in mammals as well as in C. elegans there are two distinctive populations of serotonergic neurons: neurons synthesizing...
5-HT and neurons absorbing 5-HT from extracellular space. Our results indicate that for those 5-HT–absorbing neurons fluoxetine treatment may result in a reduction, rather than an increase, in 5-HT signals. This intriguing finding prompted us to further investigate the effects of SSRIs on neuronal functions regulated by 5-HT. The conditions for fluoxetine to inhibit 5-HT immunoreactivity in the AIM and RIH neurons were used as a guideline in studies described below.

A behavioral assay for genetic survey of fluoxetine action in living animals: The locomotory control circuit of C. elegans affords a simple paradigm for delineation of the genetic basis and molecular mechanisms of SSRIs on 5-HT downstream targets in a defined neural circuit. C. elegans locomotion is a reproducible behavior reflecting integrated signaling of multiple neurotransmitters and neuromodulators, including ACh, GABA, glutamate, neuropeptides, dopamine, and 5-HT. In either solid or liquid medium, C. elegans moves continuously in a sinusuous fashion. It has been well established that 5-HT and fluoxetine inhibit locomotion (Choy and Thomas 1999; Nurrish et al. 1999). We reasoned, if we could identify the genes and locomotory properties regulated by 5-HT and fluoxetine, that may give us some clues to the actions of fluoxetine on 5-HT downstream targets.

In this study, we monitored C. elegans locomotion in liquid media because of the ease to recognize paralyzed worms in liquid. When bathed in liquid media containing 5-HT or fluoxetine, C. elegans stopped moving within 1 hr and the paralyzed animals could resume locomotion when they were immediately transferred to drug-free media (Figure 2a). Fluoxetine-induced paralysis was dose dependent (Figure 2b).

We next sought cellular markers correlating with paralysis induced by 5-HT and fluoxetine. C. elegans may become paralyzed either by hypercontraction of the body-wall muscles due to excessive stimulatory signaling or by hyperrelaxation due to a paucity of stimulatory signaling (Reiner et al. 1995). To distinguish between these possibilities and to compare the effect of 5-HT and fluoxetine, we examined the body-wall muscle sarcomeres in living animals using GFP-tagged myosin heavy chain protein MYO-3 (MYO-3::GFP). In untreated animals the muscle fibers were interdigitated, reflecting a normal muscle contractile tone; however, in animals exposed to either 5-HT or fluoxetine for <1 hr the sarcomeres were elongated and muscle fibers were stretched apart, remaining parallel to each other and giving a relaxed appearance (Figure 2c). By contrast, animals treated with the cholinesterase inhibitor aldicarb, which causes hypercontraction of the body-wall muscles, showed densely packed muscle fibers (Figure 2c). Interestingly, animals incubated in media containing 5-HT for 4 hr became adapted to the drug and resumed muscle contractile tone, whereas the muscle fibers in animals incubated with fluoxetine remained relaxed (Figure 2c). Incidentally, animals exposed to 5-HT resumed locomotion, but animals exposed to fluoxetine remained paralyzed even after 24 hr and died. These results suggest that both 5-HT and fluoxetine caused profound body-wall muscle relaxation; however, their actions are not identical.

To test whether paralysis induced by fluoxetine is a result of inactivation of MOD-5/SERT, we analyzed mod-5 mutants. The mod-5(n3314) and mod-5(n822) mutants grown on NGM plates exhibited superficially normal locomotion, as previously reported (Ranganathan et al. 2001). Both mutants remained sensitive to fluoxetine in the paralysis assay, although their sensitivities were slightly reduced compared to WT animals (Figure 2d). Furthermore, the tph-1(mg280) mutant was fully sensitive to fluoxetine-induced paralysis (Figure 2d). These results are in agreement with a published study (Ranganathan et al. 2001). MYO-3::GFP in untreated mod-5 mutants was similar to that in WT animals, although the muscle fibers in the untreated tph-1 mutant were often thicker and more branching (supporting information, Figure S1). Fluoxetine caused muscle relaxation in both mod-5 and tph-1 mutants (Figure S1).

To further characterize this fluoxetine assay, we tested mutants of type II calcium- and calmodulin-dependent protein kinase (CaMKII) unc-43. It has been reported that unc-43 loss- and gain-of-function mutations cause, respectively, hypo- and hypercontraction of the body-wall muscles (Reiner et al. 1995, 1999). Indeed, the unc-43(n498)gf mutant displayed densely packed MYO-3::GFP muscle fibers (Figure 2c). Fluoxetine did not cause muscle relaxation in the unc-43gf mutant, although the muscle fibers were packed less densely compared to untreated unc-43gf animals (Figure 2c). The unc-43gf mutant was more resistant, whereas the unc-43(n409)lf mutant was hypersensitive to fluoxetine-induced paralysis (Figure 2c). These results showed that the sensitivity to fluoxetine-induced paralysis could be influenced by a particular component in the locomotory system, suggesting that this assay may allow us to identify additional genes and functional pathways regulated by fluoxetine.

5-HT and fluoxetine regulate ACh, GABA, and glutamate neurotransmission: To characterize the impact of 5-HT and fluoxetine on synaptic transmission of the locomotory circuit, we examined ACh, GABA, and glutamate signaling in living C. elegans. We assayed ACh release at the body-wall neural muscular junctions (NMJs) by measuring the sensitivity to the acetylcholinesterase inhibitor aldicarb. Using GFP reporters, we analyzed the expression of genes involved in ACh, GABA, and glutamate neurotransmission and examined the morphology of the lomomotory neurons. We also tested fluoxetine sensitivity in mutants of ACh, GABA, and glutamate neurotransmission, using the paralysis assay.

Aldicarb causes paralysis in C. elegans due to the accumulation of ACh at the body-wall NMJs and is
therefore frequently used to measure steady-state ACh release in living animals (Mahoney et al. 2006). 5-HT and fluoxetine reduced the paralytic effect of aldicarb (Figure 3a), as previously reported (Nurrish et al. 1999). By contrast, 5-HT and fluoxetine did not inhibit sensitivity to levamisole, a specific agonist of the nicotinic ACh receptor UNC-29 in the body-wall muscles (data not shown) (Nurrish et al. 1999), suggesting that 5-HT regulates presynaptic ACh neurotransmission. We next examined a GFP reporter under the control of a common promoter element shared by the vesicular ACh transporter gene unc-17 and the choline acetyltransferase gene cha-1 (ACh::gfp) (Alfonso et al. 1994). 5-HT and fluoxetine did not cause any discernible changes in the morphology of cholinergic neurons, but they both reduced ACh::gfp expression. Following 2 hr exposure to either 5-HT or fluoxetine, there was a modest 10% reduction in GFP fluorescence in the ventral cord motorneurons, compared to age-matched, untreated controls (Student’s t-test, \( P < 0.05 \) for 5-HT and \( P < 0.06 \) for fluoxetine, \( N = 30 \) per treatment). Twenty-four hours of exposure to fluoxetine resulted in a \(~30\%\) reduction in ACh::gfp intensity (Figure 3b). Fluoxetine also reduced aldicarb sensitivity and downregulated ACh::gfp expression in mod-5 mutants (Figure 3, a and b). These results
indicate that the drug treatments did not damage the ACh neurons. Rather, both 5-HT and fluoxetine may reduce ACh signaling at the body-wall NMJs and the downregulation of cha-1 and unc-17 could account for part of the fluoxetine effect; however, this action of fluoxetine is independent of MOD-5/SERT.

An unc-29 loss-of-function mutant was hypersensitive to fluoxetine-induced paralysis, although a cha-1 reduction-
of-function allele did not significantly alter fluoxetine sensitivity (Figure 3c). The *slo-1* BK potassium channel gain-of-function allele *ky399*, which reduced ACh release (Davies et al. 2003), was hypersensitive to fluoxetine in the paralysis assay (Figure 3d). Conversely, a *slo-1* loss-of-function allele and a mutant of the negative ACh neurotransmission regulator *tom-1* both exhibited an increase in ACh neurotransmission (Wang et al. 2001; Gracheva et al. 2006) and were both more resistant to fluoxetine as compared to WT (Figure 3d). These data suggest a model in which 5-HT signaling inhibits ACh neurotransmission and a deficit in ACh signaling may facilitate muscle relaxation following 5-HT and fluoxetine treatments.

We next examined GABAergic neurons. Since GABA is the major inhibitory input to the body-wall muscles, 5-HT and fluoxetine could cause muscle relaxation by increasing GABA signaling. 5-HT and fluoxetine did not produce an appreciable change in the expression of a GFP reporter for the GABA biosynthesis enzyme glutamic acid decarboxylase *unc-29* (data not shown). However, the GFP-tagged GABA receptor protein *unc-49* (UNC-49::GFP) was significantly increased at the body-wall NMJs (Figure 3e) and in the head muscles in animals treated with 5-HT or fluoxetine, with the fluorescence level higher in animals treated with fluoxetine (Figure 3f). If increased *unc-49* activity were responsible for the paralytic effect of fluoxetine, we would expect *unc-49* mutants to be more resistant to fluoxetine. However, both *unc-25* and *unc-49* mutants were hypersensitive to fluoxetine in the paralysis assay (Figure 3e). Thus, the increase in *unc-49* expression cannot account for paralysis induced by fluoxetine.

We also analyzed the relation of glutamate neurotransmission to fluoxetine sensitivity. Glutamate neurotransmission produces fast inhibitory inputs to the locomotory circuit (Jørgensen 2005). Glutamate receptors have been detected in the locomotory command interneurons and the motor neurons (Hart et al. 1995; Dent et al. 1997, 2000). Glutamate neurotransmission is thought to function as a pattern generator by regulating reciprocal inhibition between the forward and the backward command neurons (Chalfie et al. 1985; Zheng et al. 1999; Brodie et al. 2001), and excessive glutamate signaling may disrupt the neuronal circuitry, resulting in a cessation of movement (Yates et al. 2003). The *eat-4* glutamate transporter is implicated in glutamate neurotransmission (Lee et al. 1999; Rand et al. 2000). The AMPA type glutamate receptor *glr-1* is the best-studied *C. elegans* GLR receptor: it is expressed in the locomotory command interneurons (Hart et al. 1995; Maricq et al. 1995) and controls duration and direction of the movement (Zheng et al. 1999). We did not detect a significant change in the expression and localization of the GFP-tagged EAT-4 protein (EAT-4::GFP) and GLR-1 protein (GLR-1::GFP) in animals treated with 5-HT or fluoxetine (data not shown, see below). But, mutants of *eat-4* and *glr-1* were more resistant to fluoxetine-induced paralysis compared to WT animals (Figure 3c).

Collectively, these experiments demonstrated a complex of interactions between fluoxetine treatment and synaptic functions of ACh, GABA, and glutamate. The downregulation of ACh receptors and the upregulation of 5-HT receptors SER-7 and SER-5 antagonistically regulate fluoxetine sensitivity: While the common effects of 5-HT and fluoxetine on MYO-3::GFP and the neuronal markers were suggestive of fluoxetine actions on 5-HT targets in the locomotory system, our analyses of the *mod-5* mutants showed that the fluoxetine effects were largely independent of MOD-5/SERT function (Figures 2d and 3, a and b). To gain more insights into the molecular mechanisms by which fluoxetine regulates synaptic functions and the relation to 5-HT, we took advantage of available deletion mutants of predicted 5-HT receptors and tested their ability to block fluoxetine-induced paralysis. None of tested 5-HT receptor mutants showed obvious defects in locomotion per se. Sensitivity to fluoxetine-induced paralysis was not significantly changed in mutants of *mod-1* (two alleles), *ser-3* (three alleles), *ser-4*, T02E9 and C24A8.1 (data not shown). The *ser-4 (ok512)* allele modestly slowed down paralysis (Figure S2). By contrast, three alleles of *ser-7*, which is most related to the mammalian 5-HT7 receptor (Hobson et al. 2006), were all significantly resistant to fluoxetine-induced paralysis compared to WT animals (Figure 4a, Figure S2). The *ser-1;ser-7* double mutant did not show stronger resistance than the *ser-7* single mutant, as measured after 40 min (Figure S2) and 24 hr (Student’s t-test, *P* = 0.13, *n* = 3 replicates) of exposure to fluoxetine. Transgenic expression of the WT *ser-7* gene restored fluoxetine sensitivity of the *ser-7* mutants (Figure 4a), indicating that SER-7 function mediates this fluoxetine action. However, the *ser-7* mutants were not completely resistant to fluoxetine-induced paralysis, although all three *ser-7* alleles are deletion mutations predicted to be functional null (Hobson et al. 2006). It is possible that fluoxetine could influence other, SER-7-independent functions in the locomotory circuit.

To determine whether SER-7 specifically mediates fluoxetine action or is a target shared by SSRIs, we analyzed two other SSRIs, sertraline (Zoloft) and escitalopram (Lexapro, Cipralex). Both sertraline and escitalopram induced paralysis in *C. elegans*, and the *ser-7* mutants were more resistant than WT (Figure 4b).

In contrast to the *ser-7* mutants, two *ser-5* deletion alleles both predicted to be functional null (Harris et al. 2009) were both hypersensitive to fluoxetine-induced paralysis compared to WT (Figure 4c). The *ser-5* mutants were also hypersensitive to inhibition of locomotion by 5-HT (Figure S3). Interestingly, two trans-
genic arrays of the WT ser-5 gene, Ex[ser-5(+)], not only reversed the hypersensitivity of the ser-5 mutant, but also conferred resistance to fluoxetine in both ser-5 and WT backgrounds (Figure 4, c and d). These results suggest that SER-5 activity antagonizes the inhibitory effects of fluoxetine.

We further tested genetic interaction between ser-7 and ser-5. The ser-5;ser-7 double mutant was more resistant to fluoxetine compared to WT, although the resistance was slightly weaker than that of the ser-5 single mutant (Figure 4d). Overexpression of Ser-5 further enhanced resistance to fluoxetine in ser-7 mutants (Figure 4d). Taken together, these results suggest that SER-5 signaling and SER-7 signaling antagonistically regulate fluoxetine action on the locomotory circuit.

**Mutations in ser-7 do not disrupt fluoxetine actions on the ACh and GABA systems:** We asked whether SER-7 is required for fluoxetine to regulate synaptic functions in the locomotory system. Two experimental results indicate that the action of fluoxetine on ACh neurotransmission is unaffected in the absence of the ser-7 single mutant (Figure 5). First, fluoxetine inhibited aldicarb sensitivity in the ser-7 mutants as in WT animals (Figure 5a). Second, fluoxetine reduced ACh::gfp expression in the ser-7 mutants (Figure 5b).

SER-7 is also not required for fluoxetine action on the GABA system (Figure 6). Fluoxetine increased UNC-49::GFP abundance in ser-7 mutants as in WT animals (Figure 6a). Furthermore, if SER-7 acts in the GABA signaling pathway, a double mutant of ser-7 and GABA synthesis mutant unc-25 would be either hypersensitive to fluoxetine as is the unc-25 mutant or resistant to fluoxetine as is the ser-7 mutant. In contrast, we found that the fluoxetine sensitivity of the ser-7; unc-25 double mutant was in between that of the two single mutants (Figure 6b). Collectively, the data suggest that SER-7 regulates the activity of the locomotory system via a mechanism other than ACh and GABA.

**SER-5 and SER-7 act in the same pathway as GLR-1 to regulate fluoxetine sensitivity:** We also explored the relation of SER-5 and SER-7 to glutamate neurotransmission. GLR-1::GFP is expressed in puncta along the axons of the locomotory command neurons extending the full length of the ventral nerve cord, each punctum corresponding to a single synapse, and the density of GLR-1 synapses along the ventral cord is almost invariant between animal and animal (Rongo et al. 1998). Although GFP intensity of individual puncta was not significantly changed (data not shown), the number of GLR-1 synapses was reduced in ser-7 mutants, compared to age-matched WT animals (Figure 7a).

To explore the functional relationship between glutamate neurotransmission and ser-7, we generated double mutants. Single mutants of ser-7, glr-1, and eat-4, as well as double mutants of ser-7;glr-1 and ser-7;eat-4, all exhibited similar resistance to fluoxetine (Figure 7b). To test if glutamate signaling is a downstream target of SER-7, we attempted to overexpress SER-7. Expressing the ser-7(+) transgene in a WT background caused hypersensitivity to fluoxetine-induced paralysis (Figure 7b). However, the glr-1 mutant expressing the same ser-7(+) transgene was as resistant to fluoxetine as the glr-1

![Figure 4.—SSRI-induced paralysis in ser-5 and ser-7 mutants. Larval stage four (L4) worms were incubated for 24 hr in liquid media supplemented with a drug: 0.5 mg/ml fluoxetine (a and d); 0.5 mg/ml fluoxetine, 0.25 mg/ml sertraline, and 2.25 mg/ml escitalopram (b); and 0.25 mg/ml fluoxetine (c). Drug sensitivities were calculated as described in Figure 2d. The values of the mutants are normalized to that of WT assayed in parallel. Two transgenic lines each for Ex[ser-7(+)] and Ex[ser-5(+)] were assayed. Each bar represents at least three independent experiments ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test.](image-url)
mutant (Figure 7b), suggesting that GLR-1 activity is required for SER-7 to promote paralysis by fluoxetine. Furthermore, the fluoxetine sensitivity of the ser-5;glr-1 double mutant was similar to that of the glr-1 single mutant (Figure 7b), suggesting that the fluoxetine hypersensitivity of the ser-5 mutants also depends on GLR-1 function.

Fluoxetine binds to SER-7: We tested whether fluoxetine directly binds SER-7 expressed heterologously in mammalian COS-7 cells, using a standard approach (PERT et al. 1973). One group of us previously established that the membrane of COS-7 cells transiently expressing the SER-7b isoform exhibits saturable specific binding to the 5-HT-like ligand [3H]LSD (HOBSON et al. 2003, 2006). 5-HT could efficiently displace [3H]LSD binding, while dopamine, histamine, tyramine, and octopamine did not show a significant affinity to SER-7b (HOBSON et al. 2003). Like 5-HT, fluoxetine effectively competed [3H]LSD for binding SER-7b expressed in the COS-7 cells with IC50 of 0.42 ± 0.03 μM (Figure 8), suggesting that fluoxetine can directly bind to SER-7.

**DISCUSSION**

SSRIs are useful in the treatment of a wide spectrum of behavioral and psychiatric disorders. Increasingly it has become clear that many of these disorders are inheritable, with each involving multiple genetic loci contributing small and additive effects to a complex of symptomatic traits. While the action of SSRIs as SERT inhibitors is well established, the cellular and molecular mechanisms by which SSRIs alleviate specific symptoms are poorly understood. In this study, we identified two additional aspects of fluoxetine action on the serotonegic system through genetic analyses in *C. elegans*. We showed that fluoxetine eliminated 5-HT in specific neurons. These neurons absorb extracellular 5-HT via MOD-5/SERT but do not synthesize it. Furthermore, we demonstrated that fluoxetine directly targets G-protein-coupled 5-HT receptors to regulate a behavioral circuit. These findings could shed some light on therapeutic effects of fluoxetine and our understanding of 5-HT neurotransmission.

**The effects of fluoxetine are multifacrotal:** There is an ongoing debate on the effects of SSRIs on 5-HT signaling. In rodents, administration of fluoxetine immediately induces a significant increase in the extracellular levels of 5-HT in the brain (SCHATZBERG and NEMEROFF 2004). Intriguingly, a SERT knockout mouse showed reduced brain 5-HT (BENGEL et al. 1998). Furthermore, an allele of reduced SERT expression is found in patients suffering bipolar disorder and autism (BARTLETT et al. 2005; BRUNE et al. 2006; BARNETT and SMOLLER 2009). In this study, we identified two distinctive populations of serotonergic neurons in *C. elegans*: the NSM, ADF, and HSN neurons producing 5-HT and the RIH and AIM neurons absorbing 5-HT from extracellular space but unable to synthesize it. We showed that fluoxetine and imipramine, like the mod-5/SERT mutations, eliminated 5-HT in the AIM and RIH neurons.

5-HT–absorbing neurons are likely an evolutionary conserved feature of the serotonergic system. In rodents SERT is expressed in a range of CNS neurons that do not express 5-HT biosynthesis enzymes (GASPARI et al. 2003). In particular, SERT is expressed transiently in the thalamus and in all primary sensory areas including visual, auditory, and somatosensory areas in early postnatal rodents (D’AMATO et al. 1987; BENGEL et al. 1997; HANSSON et al. 1999) and in nonmonoaminergic neurons in developing human cerebral cortex (VERNEY et al. 2002). How does blocking SERT function in 5-HT–absorbing neurons possibly affect 5-HT signaling? One possible
role of 5-HT–absorbing neurons could be to scavenge 5-HT spillover, thereby enhancing the specificity of the signaling of 5-HT transmission. Indeed, the axons of the AIM and RIH neurons are close to the axons of ADF and NSM (White et al. 1986). This model is consistent with published studies showing that mod-5/SERT-null mutants exhibit certain behavioral phenotypes of increased 5-HT signaling (Ranganathan et al. 2001). However, it is possible that 5-HT–absorbing neurons use 5-HT as borrowed transmitter, serving as "relay stations" to pass 5-HT from the original neuronal sources to distant targets. The support for this idea is the presence of the vesicular monoamine transporter (VMAT) CAT-1 in the AIM and RIH neurons (Duerr et al. 1999; Sze et al. 2002), indicating the capability to store imported 5-HT in the synaptic vesicles. If this model is correct, that would imply that fluoxetine reduces 5-HT signaling mediated by these neurons.

Our genetic and biochemical analyses indicate that fluoxetine acts in part by targeting 5-HT receptors. This result is consistent with the biochemical studies showing that fluoxetine and other SSRIs bind to many subtypes of mammalian 5-HT receptors (Kroeze and Roth 1998). Interestingly, our data suggest that the actions of fluoxetine and 5-HT are not identical. Fluoxetine appeared to produce greater effects than exogenous fluoxetine. *P < 0.05, Student’s t-test. (b) Fluoxetine-induced paralysis. Fluoxetine sensitivity was calculated as described in Figure 2d. Each bar represents three replicate experiments ± SEM. **P < 0.01, ***P < 0.001. There is no significant difference between WT and ser-7; unc-25 (P > 0.9, Student’s t-tests).

Figure 6.—Action of fluoxetine on GABA in ser-7 mutants. (a) UNC-49::GFP expression in ser-7 mutants. Intensity of the fluorescence along the locomotory NMJs was quantified as described in Figure 3e. The values of WT treated with fluoxetine (0.5 mg/ml, 2 hr) and ser-7(tm1325) animals untreated and treated with fluoxetine are normalized to that of untreated WT. The experiment was performed six times. These data represent one set of experiments with genotype and treatment blinded. The number of animals examined: WT, 9 untreated and 10 treated with fluoxetine; ser-7, 12 untreated and 6 treated with fluoxetine. *P < 0.05, Student’s t-test.
5-HT on examined neuronal markers. Furthermore, animals treated with serotonin but not with fluoxetine could recover and resume locomotion. The recovery is unlikely due to 5-HT being unstable (Figure S4), suggesting animals can adapt to 5-HT but not to fluoxetine. It will be interesting to define the binding sites of 5-HT and SSRIs on SER-7 in the future.

It has been established that fluoxetine, but not 5-HT, can induce C. elegans nose contraction, demonstrating that fluoxetine can target genes outside of 5-HT signaling pathways to regulate behavior (Choy and Thomas 1999; Choy et al. 2006). However, we found that the nrf-6 mutant, which is strongly resistant to fluoxetine-induced nose contraction (Choy and Thomas 1999), did not affect fluoxetine-induced paralysis (Figure 3c). Thus, systematic characterization of fluoxetine effects on individual behaviors in C. elegans may identify its gene targets in 5-HT signaling pathways, as well as the genes mediating other aspects of SSR1 actions.

Interaction between 5-HT and other neurotransmitters: Our analysis of GFP reporters revealed that fluoxetine influences discrete steps of synaptic transmission of ACh, GABA, and glutamate. Regulation of synaptic function by 5-HT signaling is thought to play myriad roles in the modulation of emotion, cognition, and motor behavior in mammals (Millan 2003). Increased central cholinergic tone induces depression in humans (Janowsky and Overstreet 1990). Fluoxetine is useful in the treatment of congenital myasthenic syndromes, which is characterized by increased ACh neurotransmission at NMJs (Harper et al. 2003; Colomer et al. 2006).

Our genetic analyses suggest that SER-5 and SER-7 act antagonistically to regulate GLR-1 signaling in the locomotory circuit. Opposing 5-HT receptors have been found to modulate other behaviors in C. elegans. For example, ser-7 and ser-1 stimulate egg laying in opposition to ser-1 (Hapiak et al. 2009), and ser-1 and ser-7 antagonistically regulate aging (Murakami and Murakami 2007). However, SER-5 and SER-7 have not been detected in neurons directly involved in locomotion (Tsalk et al. 2003; Carre-Piererrat et al. 2006; Harris et al. 2009) and ser-7 mutants do not exhibit defects in GLR-1-mediated nose-touch response (data not shown). It is likely that SER-7 and SER-5 are not required for glutamate neurotransmission but act indirectly to influence its activity in the locomotory circuit. Interestingly, fluoxetine, imipramine, and several other antidepressants can increase AMPA receptor phosphorylation in the mouse brain (Svenningsson et al. 2002, 2007; Du et al. 2007).

We showed that loss of ser-7 function did not block fluoxetine action on ACh and GABA neurotransmission in the locomotory circuit. It has been reported that the G-protein–coupled 5-HT receptors SER-1 (Dernovici et al. 2007) and SER-4 (Govorunova et al. 2010) are also involved in the control of locomotion. ser-1 is predicted to regulate the locomotory circuit indirectly through interneurons (Dernovici et al. 2007). Therefore, in C. elegans, as in mammals (Lucki 1992), 5-HT is probably not required for behavior to occur. Rather it may act via distinct 5-HT receptors in multiple cellular sites to coordinate synaptic functions of behavioral circuits.

We thank R. Horvitz, E. Jorgensen, J. McGhree, J. Rand, and the Caenorhabditis Genetics Center for worm strains and the C. elegans Knockout Consortium for deletion mutants. We specially thank Shohei Mitani for 5-HT receptor deletion alleles. We are grateful to R. Patel for assistance in some behavioral assays. This work was supported by a Marie Curie Outgoing International Fellowship (to C.M.D.), an award from the Department of Defense Autism Spectrum Disorder Research Program, and grants from the National Institute of Mental Health (to J.Y.S.).

LITERATURE CITED


Figure 8.—Fluoxetine binds to SER-7. The membrane fraction of COS-7 cells transiently expressing the SER-7b isoform was incubated with 10 nM [3H]LSD alone or in the presence of 5-HT or fluoxetine at the indicated concentrations. The data are representative of at least three experiments, each performed in triplicate. Each point is the mean ± SEM.
Asparagine synthetase with fluoxetine. Neuromuscul. Disord. 25:
1999 Direct visualization of the elt-2 gut-specific GATA factor
Action of SSRIs on Synaptic Transmission 941


Communicating editor: P. G. Phillips
A Genetic Survey of Fluoxetine Action on Synaptic Transmission in Caenorhabditis elegans

Andrey Kullyev, Catherine M. Dempsey, Sarah Miller, Chih-Jen Kuan, Vera M. Hapiak, Richard W. Komuniecki, Christine T. Griffin and Ji Ying Sze

Copyright © 2010 by the Genetics Society of America
DOI: 10.1534/genetics.110.118877
FIGURE S1.—Photomicrographs of MYO-3::GFP in the body-wall muscle sarcomeres in mod-5 and tph-1 mutants. Animals were incubated for 60 min in S-medium containing 0.5 mg/ml fluoxetine and the drug-free controls. The inset in lower left panel shows branches crossing the muscle fibers, which were frequently observed in untreated tph-1 mutants, but not in WT and mod-5 mutant animals. One-day old adults were examined.
FIGURE S2.— The time course of fluoxetine-induced paralysis. The assay has been performed multiple times, and the data from one experiment are presented.
FIGURE S3.—Effects of 5-HT treatment on locomotion in WT, ser-5 and ser-7 mutant animals. One-day old animals were transferred onto NGM plates supplemented with 5 mg/ml 5HT (hatched bars) or without 5-HT (black bars) for 15 min. The number of body bends of individual animals was scored by continuously monitoring individual animals for 20 seconds. In the presence of 5-HT, locomotion of ser-5 mutants was slower than that of WT (p < 0.05). However, the difference between WT and the ser-7 mutant animals is not significant (p > 0.05). Student’s t-test.
FIGURE S4.—Analysis of 5-HT stability in solution. The stability of 5-HT in solution was evaluated by testing its ability to induce egg laying in WT animals. 5-HT was dissolved in M9 buffer to give the final concentration of 5 mg/ml 5HT, and the solution was stored in dark at 20°C. To test the response to 5-HT, animals were placed individually into wells of a 96-well plate, with each containing 100 ml of M9 buffer or M9 buffer containing 5-HT. The number of eggs laid was scored after 60 min. Animals did not laid egg in M9 buffer (data not shown) (Trent et al. 1983). There is no significant difference in the number of eggs laid between animals incubated in the solution at these four time points (p > 0.4 Student’s t-test).

Reference