Behavioral/Cognitive

Cannabinoids Stimulate the TRP Channel-Dependent Release of Both Serotonin and Dopamine to Modulate Behavior in *C. elegans*

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Cannabis sativa alters sensory perception and exhibits potential medicinal benefits. In mammals, cannabinoids activate two canonical receptors, CB_1/CB_2, as well additional receptors/ion channels whose overall contributions to cannabinoid signaling have yet to be fully assessed. In *Caenorhabditis elegans*, the endogenous cannabinoid receptor agonist, 2-arachidonoylglycerol (2-AG) activates a CB_1 ortholog, NPR-19, to modulate behavior (Oakes et al., 2017). In addition, 2-AG stimulates the NPR-19 independent release of both serotonin (5-HT) and dopamine (DA) from subsets of monoaminergic neurons to modulate locomotory behaviors through a complex monoaminergic signaling pathway involving multiple serotonin and dopamine receptors. 2-AG also inhibits locomotion in remodeled monoamine receptor mutant animals designed to measure the acute release of either 5-HT or DA, confirming the direct effects of 2-AG on monoamine release. 2-AG-dependent locomotory inhibition requires the expression of transient receptor potential vanilloid 1 (TRPV1) and TRPN-like channels in the serotonergic or dopaminergic neurons, respectively, and the acute pharmacological inhibition of the TRPV1-like channel abolishes both 2-AG-dependent 5-HT release and locomotory inhibition, suggesting the 2-AG may activate the channel directly. This study highlights the advantages of identifying and assessing both CB_1/CB_2-dependent and independent cannabinoid signaling pathways in a genetically tractable, mammalian predictive model, where cannabinoid signaling at the molecular/neuronal levels can be correlated directly with changes in behavior.

Key words: cannabinoids; dopamine; serotonin; TRP channels

Significance Statement

This study is focused on assessing CB_1/CB_2-independent cannabinoid signaling in a genetically tractable, whole-animal model where cannabinoid signaling at the molecular/neuronal levels can be correlated with behavioral change. *Caenorhabditis elegans* contains a cannabinoid signaling system mediated by a canonical cannabinoid receptor, NPR-19, with orthology to human CB_1/CB_2 (Oakes et al., 2017). The present study has characterized an NPR-19-independent signaling pathway that involves the cannabinoid-dependent release of both serotonin and dopamine and the expression of distinct TRP-like channels on the monoaminergic neurons. Our work should be of interest to those studying the complexities of CB_1/CB_2-independent cannabinoid signaling, the role of TRP channels in the modulation of monoaminergic signaling, and the cannabinoid-dependent modulation of behavior.

Introduction

Cannabis or marijuana alters sensory perception and has been purported to exert a wide range of recreational and medicinal effects (Pacher et al., 2006; Grotenhermen and Müller-Vahl, 2012). *Cannabis sativa* contains >60 bioactive compounds, or phytocannabinoids (pCBs), including cannabidiol (CBD) and the hallucinogen Δ^9-tetrahydrocannabinol (THC). These pCBs and the endogenous cannabinoids (eCBs), 2-arachidonoylglycerol (2-AG), and *N*-arachidonoylethanolamine (AEA) differentially activate a canonical cannabinoid (CB) signaling pathway initiated by two receptors, CB_1 and CB_2. CB_1 and CB_2 are differentially expressed and, in part, mediate a retrograde signal from postsyn-

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aptic neurons to inhibit neurotransmitter release. CB_1 is localized primarily to presynaptic nerve terminals in the brain and CNS. CB_2 is also found in the CNS, but is most robustly expressed in the immune system (Herkenham et al., 1990; Glass et al., 1997; Tsou et al., 1998; Ohno-Shosaku and Kano, 2014). CBs also modulate the release of dopamine (DA) and serotonin (5-HT), and some of the behavioral effects of CBs are mediated, either directly or indirectly, through changes in serotonergic, adrenergic, and dopaminergic signaling (Kurihara et al., 2001; Cheer et al., 2004, 2005; Sagredo et al., 2006; McLaughlin et al., 2009; Fitzgerald et al., 2012; Winters et al., 2012; Romero et al., 2013; Gantz and Bean, 2017). However, in many cases, it is unclear whether the CB-dependent modulation of monoaminergic signaling involves CB_1/CB_2 directly or CB_1/CB_2-independent signaling pathways. For example, CBs also differentially activate the recently deorphanized $G\alpha_{i/o}$-coupled receptors GPR18 and GPR55, and a range of transient receptor potential receptors, including the transient receptor potential vanilloid 1 (TRPV1) receptor (Di Marzo et al., 1998; De Petrocellis et al., 2001; Starowicz et al., 2007; Di Marzo and Maccarrone, 2008; Maccarrone et al., 2008). However, less is known about how these noncanonical CB receptors/ion channels interact with CB_1/CB_2 signaling in the modulation of CB-dependent behaviors.

Given the complexity of the mammalian nervous system, it has been difficult to correlate studies of CB signaling at the molecular/neuronal levels with changes in individual behaviors. In contrast, we have focused on the genetically tractable, nematode model *Caenorhabditis elegans*, with the goal of examining the effects of CBs on the modulation of sensory integration and decision-making. Our recent work has demonstrated that CB signaling likely has ancient evolutionary origin and has identified a canonical CB signaling system in *C. elegans* (Oakes et al., 2017). For example, the CB-dependent modulation of nociception and feeding requires the human CB receptor ortholog NPR-19 (Oakes et al., 2017). CBs activate NPR-19 directly with affinities similar to human CB_1, and CB-dependent phenotypes can be rescued in *npr-19*-null animals by the expression of the human CB_1, confirming the proposed orthology of the two receptors (Oakes et al., 2017). CBs also extensively modulate monoaminergic signaling through NPR-19 independent pathways (Oakes et al., 2017).

Therefore, the present study is focused on characterizing the effects of CBs on monoaminergic signaling and has used the 2-AG-dependent modulation of locomotory behavior to probe NPR-19-independent CB signaling. Specifically, we have demonstrated that 2-AG inhibits forward locomotion and increases turning through NPR-19-independent pathways that involve the activation of multiple TRP channels and the endogenous release of both 5-HT and DA from subsets of monoaminergic neurons. For example, 2-AG-dependent locomotory inhibition is dramatically reduced or absent in *tph-1*- and *cat-2*-null animals that lack key enzymes required for 5-HT and DA biosynthesis, respectively and in *ser-4*- and *dop-4*-null animals that encode 5-HT and DA receptors, respectively. In contrast, 2-AG still inhibits locomotion in *mod-5*- or *dat-1*-null animals that lack reuptake transporters for either 5-HT or DA, respectively, suggesting that the primary role of 2-AG in locomotory inhibition involves the stimulation of monoamine release, not the inhibition of reuptake. Indeed, 2-AG also inhibits locomotion in remodeled monoamine receptor mutant animals designed to measure the acute release of either 5-HT or DA. This 2-AG-dependent monoamine release and locomotory inhibition requires the expression of TRPV1 and TRPN-like channels in the serotonergic or dopaminergic neurons, respectively, and the acute pharmacological inhibition of the $TRPV_1$-like channel abolishes both 2-AG-dependent 5-HT release and locomotory inhibition, suggesting that the 2-AG may activate the channel directly. Together, these studies highlight the potential utility of *C. elegans* as a model to study CB_1/CB_2-independent CB signaling.

Materials and Methods

Strains and transgene construction. Strains were grown and maintained at 16°C and room temperature (22°C) on nematode growth media (NGM) agar plates with OP50 *E. coli* as a food source (Brenner, 1974). All strains were purchased from the Caenorhabditis Genetics Center at the University of Minnesota (St. Paul, MN) and the National Bioresource Project at Tokyo's Women's Medical University (Tokyo, Japan). The following strains were used: *N2* (*Bristol*), *cat-1(ok411)*, catalog #RB681; *cat-2(n4547)*, catalog #MT15620; *cat-4(ok342)*, catalog #LC35; *dat-1(ok157)*, catalog #RM2702; *dop-3(ok295)*, catalog #BZ873; *dop-4(tm1392)*, catalog #FG58; *DA receptor quadruple (DA quad)*-null: *(dop-2(vs105) V*, *dop-4(ok1321)*, *dop-1(vs100)*, *dop-3(vs106) X)*; *mod-1(ok103)*, catalog #MT9668; *mod-5(n3314)*, catalog #MT9772; *npr-19(ok2068)*, catalog #RB1668; *octr-1(ok371)*, catalog #CX13079; *osm-9(ky10)*, catalog #CX10; *ser-1(ok345)*, catalog #DA1814; *ser-4(ok512)*, catalog #AQ866; *tph-1(mg280)*, catalog #MT15434; *trp-4(sy695)*, catalog #TQ296; and *5-HT receptor quintuple (5-HT quint)*-null [*ser-5(tm2654);ser-4(ok512)*: *mod-1(ok103); ser-7(tm1325)*, *ser-1(ok345)*]. The *ADF::tph-1* rescue strain used was CX13571 [*tph-1(mg280); kySi56; kyEx4077=srh-142::nCre* (95 ng/μl); *myo-3::mCherry*; 5 ng/μl)] and was a gift from Dr. Cornelia Bargmann, The Rockefeller University, New York, NY. RNAi transgenes were generated by PCR fusion, as described in the study by Esposito et al. (2007), and coinjected with *f25b3.3::gfp* (to 100 ng) as a selection marker. PCR fusions were performed as described by Hobert (2002) (Esposito et al., 2007).The *unc-17β::ser-4* and *unc-17β::dop-3* transgenes were generated by three-piece PCR fusion of the 562 bp *unc-17* promoter *ser-4* and *dop-3* cDNA, respectively, and *gfp::unc-54* 3′UTR and were injected into 5-HT quintuple- and DA quadruple-null animals, respectively.

Octanol avoidance assays. Octanol avoidance assays were performed as described in the study by and as modified by Harris et al. (2011). For all assays, L4 stage animals were picked 24 h before assay. To measure aversive responses to 1-octanol, the blunt end of a hair is briefly dipped in 1-octanol and placed in front of a forward-moving worm, and the time taken to initiate backward locomotion is recorded. 2-Aminoethoxydiphenyl borate (2-APB) plates were prepared 10 min before assay by spreading 60 μl of 10 μM 2-APB (in H_2O) on fresh NGM plates. During the assay, animals were first transferred to intermediate (nonseeded) plates and left for 30 s to remove residual bacteria. The animals were then transferred to assay plates and tested after a 10 min of incubation. In all assays, 20–25 worms were examined for each strain and condition, and each assay was performed at least three times. Statistical analysis was performed using mean ± SE and Student's *t* test.

Locomotory assay. Locomotion was assayed as described by Sawin et al. (2000). Well fed, young adult hermaphrodite animals are picked before assay and maintained on NGM plates with *E. coli* OP50. During the assay, seven animals were transferred to the assay plate. Motility was assessed as the number of body bends/20 s at 5 min intervals for either 15 or 20 min, starting as soon as animals are transferred. Time 0 was recorded ~20–30 s after transfer to 2-AG plates. Reversal assays were performed as described in WormBook, and were assessed as the number of spontaneous reversals per 3 min following 10 min incubation in control (vehicle), 2-AG (0.4, 8, or 320 μM), 2-APB (10 μM), 5-HT (2 mM), arachidonic acid (320 μM), DA (5 mM), and JZL184 (320 μM; Hart, 2006). For all assays, each strain was assayed at least three times with seven animals/assay and statistical analysis was performed using mean ± SE and Student's *t* test.

Confocal imaging and transcriptional fluorescence. To assess potential 2-AG-dependent changes in *cat-2p::mCherry* fluorescence, a *cat-2p::mCherry* transcriptional transgene was generated by overlap PCR fusion of 1.5 kb of the *cat-2* promoter to *mCherry* and was coinjected at 50 ng with *rol-6* (*su1106*; 50 ng) into wild-type (N2) animals (Hobert, 2002). Well fed L4 animals were picked 24 h before the assay. For assay, wild-

type animals expressing *cat-2p::mCherry* were picked from plates containing OP50 and placed onto a fresh NGM plate without food to allow any residual bacteria to be removed. Animals were then picked from the transfer plates onto fresh NGM plates (10 animals/plate) containing either vehicle (water) as a control or 300 μM 2-AG for 1 h. Following treatment, animals were immobilized on 3% agarose pads with 20 mM sodium azide and imaged using an Olympus IX81 inverted confocal microscope. The resulting images were analyzed using ImageJ, in which each ADE and CEP neuron was isolated and *mCherry* fluorescence within each neuron was quantified independently using ImageJ and Microsoft Excel.

Results

Both serotonergic and dopaminergic signaling are required for 2-AG-dependent locomotory inhibition

Previously, we demonstrated that 2-AG inhibited forward locomotion in *C. elegans* through a pathway that did not involve the human CB_1 receptor ortholog NPR-19, but instead required serotonergic signaling (Oakes et al., 2017). For example, 2-AG-dependent inhibition was reduced in *tph-1*-null animals that lack a key enzyme required for 5-HT biosynthesis and *ser-4*-null animals that lack a 5-HT receptor involved in 5-HT-dependent locomotory inhibition (Law et al., 2015; Oakes et al., 2017). In contrast, inhibition was enhanced in *mod-5*-null animals that lack a key 5-HT reuptake transporter, suggesting that 2-AG increased endogenous 5-HT levels by stimulating 5-HT release, not inhibiting reuptake (Oakes et al., 2017).

As noted in Figure 1, the 2-AG inhibition of forward locomotion was dose dependent with an IC_{50} of ~3 μM (Fig. 1*a*,*b*). However, 2-AG did not cause complete paralysis even at high 2-AG concentrations, instead initiating a "locomotory confusion" phenotype, as described recently for the 5-HT inhibition of locomotion (Law et al., 2015). These relatively high exogenous 2-AG levels were required to overcome the relative impermeability of the nematode cuticle and do not reflect the sensitivity of the endogenous *C. elegans* CB signaling system. For example, the two nematode G-protein-coupled receptor (GPCRs) identified previously in the CB-dependent modulation of aversive behavior, NPR-19 and the α_2-adrenergic-like OA (octopamine) receptor OCTR-1, both exhibited affinities for 2-AG in the nM range that were similar to those reported for human CB_1 and significantly below those required for the 2-AG inhibition of aversive behaviors (Oakes et al., 2017).

Since 2-AG is degraded to arachidonic acid in *C. elegans* by monoacylglycerol lipase (MAGL), it was also important to confirm that the effects of 2-AG did not result from its breakdown to arachidonic acid. As predicted, (1) arachidonic acid alone had no effect on the rate of forward locomotion in this assay system, in contrast to 2-AG (Fig. 1*c*); (2) JZL184, a MAGL inhibitor demonstrated previously to stimulate 2-AG-dependent behaviors in *C. elegans*, also inhibited locomotion, presumably by increasing endogenous 2-AG levels (Fig. 1*c*; Oakes et al., 2017); and (3) the effects of exogenous 2-AG were enhanced in the presence of JZL184 (Fig. 1*c*). Importantly, this JZL184-dependent inhibition was also NPR-19 independent and SER-4 dependent, supporting a role for endogenous 2-AG signaling in locomotory modulation (Fig. 1*c*). To confirm this *npr-19* result, we also examined locomotion in wild-type animals expressing an *npr-19* RNAi driven by an *npr-19* promoter. The effectiveness of this *npr-19* RNAi construct has been validated previously and abolishes the 2-AG-dependent inhibition of aversive responses, mimicking the *npr-19*-null animals (Oakes et al., 2017). As predicted, the expression of the *npr-19* RNAi had no effect on either the 2-AG- or JZL184-dependent inhibition of locomotion (Fig. 1*c*).

2-AG-dependent locomotory inhibition also required the release of DA, in addition to 5-HT (Fig. 1*d*,*e*; Oakes et al., 2017). For example, 2-AG-dependent inhibition was absent in *cat-2*-null animals that lack tyrosine hydroxylase, a key enzyme involved in DA biosynthesis (Fig. 1*d*). DA levels are dramatically reduced in *cat-2*-null animals (Sanyal et al., 2004). Importantly, 2-AG-dependent inhibition was still present in *dat-1*-null animals that a lack key DA reuptake transporter, suggesting that the primary role of 2-AG in locomotory inhibition involves the stimulation DA release, not the inhibition of reuptake, as also reported for 5-HT (Fig. 1*d*,*e*; Oakes et al., 2017). Similarly, 2-AG-dependent inhibition was absent in *dop-2; dop-4 dop-1 dop-3* DA receptor quadruple-null animals (DA quads) that lack key DA receptors and in *dop-4*-null animals that lack a $G\alpha_s$-coupled DA receptor (Fig. 1*e*). Additionally, RNAi knockdown of DOP-4 (a α_s-coupled DA receptor) in wild-type animals mimicked the dop-4-null phenotype (Fig. 1*e*). In contrast, *dop-3*-null animals lacking a $G\alpha_o$-coupled DA receptor were hypersensitive to 2-AG inhibition (Fig. 1*e*), highlighting the antagonistic interaction between DOP-3 and DOP-4 signaling described previously for the DA modulation of aversive behaviors (Ezak and Ferkey, 2010; Ezcurra et al., 2011).

Interestingly, the temporal pattern of 2-AG modulation differed significantly in the 5-HT- and DA-deficient signaling mutants. For example, in mutants with disrupted serotonergic signaling, *tph-1*, *ser-4*, and 5-HT receptor quintuple-null animals (5-HT quints), 2-AG-dependent inhibition was initially delayed, but eventually these mutant animals began to slow (Fig. 1*d*). Importantly, RNAi knockdown of SER-4 in the AIB interneurons of wild-type animals mimicked *ser-4*-null animals, and 2-AG sensitivity was restored to 5-HT quints following neuron-specific rescue of SER-4 in the AIB interneurons (Fig. 1*d*). In contrast, in mutants with disrupted DA signaling, 2-AG never inhibited locomotion and, in fact, 2-AG rapidly (<30 s) and significantly (>25%) stimulated locomotion in these mutants, compared with the same mutants in the absence of 2-AG (Fig. 1*e*).

As expected, 2-AG-dependent inhibition also was markedly reduced in *cat-1*- and *cat-4*-null animals with compromised DA and 5-HT signaling (Fig. 1*f*). *cat-1* encodes a synaptic vesicular monoamine transporter and *cat-4* an ortholog of human GTP cyclohydrolase 1 that contains reduced levels of both 5-HT and DA (Sulston et al., 1975; Desai et al., 1988; Nurrish et al., 1999). Interestingly, 2-AG did not initially stimulate locomotion in the *cat-4*-null animals, as observed for *cat-2* or DA quad mutants with disrupted DA signaling, suggesting that serotonergic signaling might be involved in this CB-dependent stimulation. Interestingly, 2-AG inhibition could be rescued in the *cat-4*-null animals by preincubation in either 5-HT or DA (Fig. 1*f*), suggesting that the overstimulation of either 5-HT or DA signaling compensated for reduced levels of the other. Similar compensatory responses in both *C. elegans* monoaminergic and peptidergic signaling have been reported previously, where the overexpression of one gene compensates for the absence of another.

2-AG also stimulated reversals, in addition to inhibiting forward locomotion (Fig. 2*a*). This 2-AG-dependent stimulation did not require *npr-19* or *cat-2*, but was absent in *tph-1*-, *mod-1*-, and *ser-1*-null animals (Fig. 2*a*). *mod-1* and *ser-1* encode a 5-HT-gated Cl^- channel and a $G\alpha_q$-coupled 5-HT receptor, respectively, that have previously been implicated in modulation of reversal behavior associated with nociception (Harris et al., 2009, 2011). Together, these data suggest that 2-AG stimulates the release of both 5-HT and DA to activate a complex monoaminergic signaling network to inhibit locomotion and increase turning.

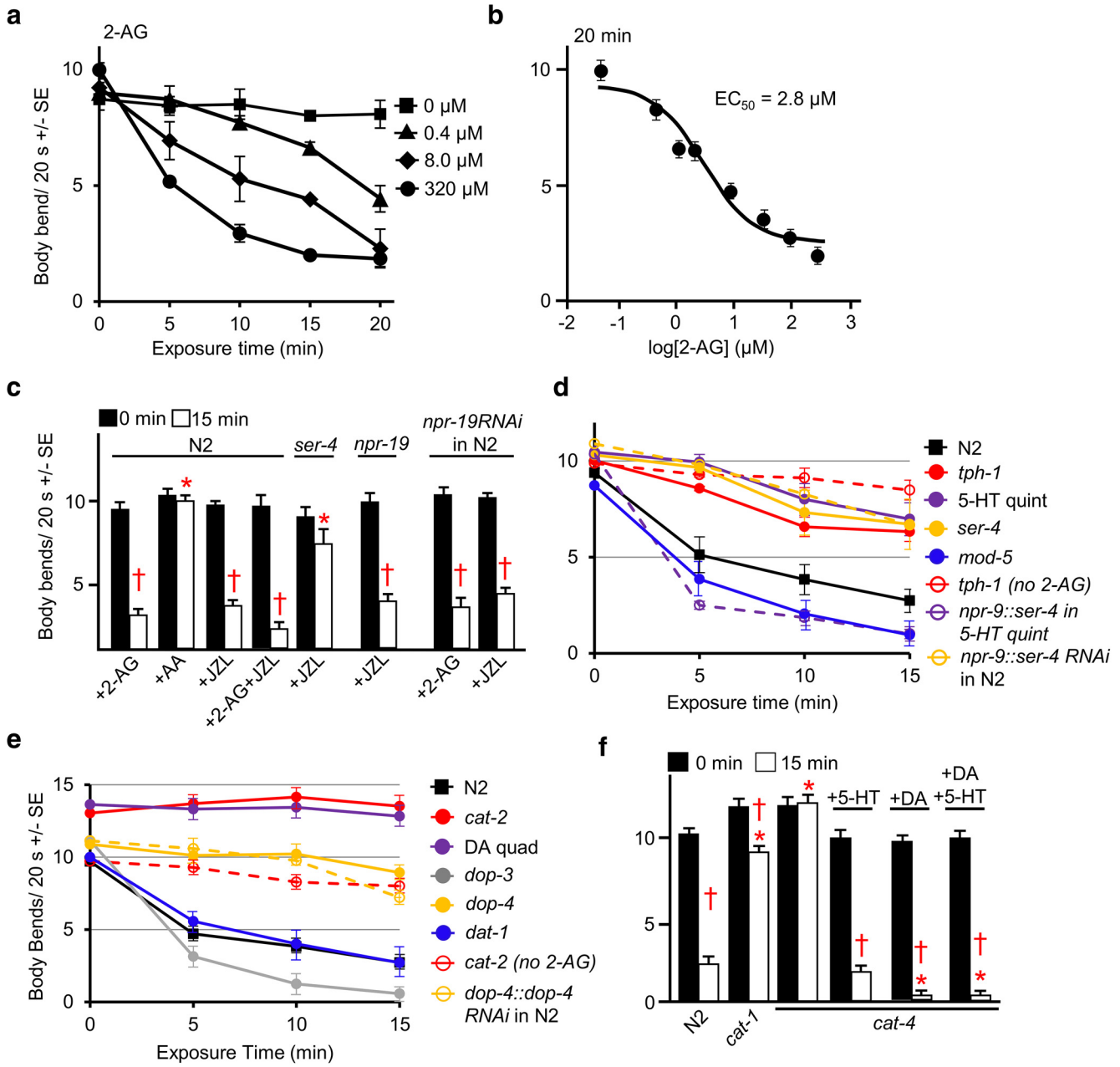

Figure 1. 2-AG-dependent locomotory inhibition requires both serotonergic and dopaminergic signaling. Wild-type and mutant animals were incubated in 2-AG, and locomotion was assayed as body bends/20 s, as described in Materials and Methods. ***a***, 2-AG-dependent locomotory inhibition in wild-type (N2) animals. ***b***, 2-AG dose–response curve for wild-type animals. ***c***, JZL184 (JZL) inhibits locomotion in wild-type animals. ***d***, ***e***, 2-AG-dependent locomotory inhibition requires both serotonergic and dopaminergic signaling. ***f***, Exogenous 5-HT or DA restores 2-AG-dependent locomotory inhibition in 5-HT- and DA-deficient *cat-4*-null animals. The mutant animals used were as follows: *cat-1(ok411)*, *cat-2(n4547)*, *cat-4(ok342)*, *dat-1(ok157)*, *dop-3(ok295)*, *dop-4(tm1392)*, *DA receptor quadruple (DA quad)*-null [*dop-2(vs105) V*, *dop-4(ok1321) dop-1(vs100)*, *dop-3(vs106) X*], *mod-5(n3314)*, *npr-19(ok2068)*, *ser-4(ok512)*, *tph-1(mg280)*, and *5-HT quint*-null [*ser-5 (tm2654);ser-4(ok512)*, *mod-1(ok103);ser-7(tm1325)*, and *ser-1(ok345)*]. The *npr-19* RNAi strain are wild-type animals expressing an *npr-19::npr-19* RNAi transgene driven by the native *npr-19* promoter. * Indicates significantly different from wild-type animals at 15 min; † indicates significantly different from the same animals at time 0 ($p \leq 0.05$). Data are presented as the mean ± SE (*n*) and were analyzed by two-tailed Student's *t* test using GraphPad software.

2-AG stimulates the release of DA from subsets of DA neurons

To measure the acute, 2-AG-dependent release of DA directly, we created mutant, transgenic animals expressing the $G\alpha_o$-coupled DA receptor DOP-3 directly in the cholinergic motor neurons of DA quad mutants, on the assumption that any 2-AG-dependent DA release would activate DOP-3 on the motor neurons, inhibit acetylcholine release onto the muscle, and inhibit locomotion (Fig. 3*a*). We used a similar approach previously to measure acute 5-HT release (Oakes et al., 2017). DA or 2-AG had no effect on forward locomotion in the DA quad mutants, but both rapidly inhibited locomotion after the expression of the inhibitory DOP-3 in the motor neurons of the remodeled quad mutants (Fig. 3*b*). Together, these data demonstrate that the 2-AG-dependent inhibition of forward locomotion requires the release of DA.

Identification of the neurons involved in 2-AG-dependent 5-HT and DA release

To identify the monoaminergic neurons involved in 2-AG-dependent locomotory inhibition, we used a combination of se-

lective RNAi knockdown/rescue in the serotonergic neurons, where selective promoters were available. Indeed, the selective expression of *tph-1* in the ADF neurons rescued 2-AG-dependent locomotory inhibition in *tph-1*-null animals (Fig. 4*a*). In contrast, but in agreement with the ADF-selective rescue, ADF-selective RNAi knockdown of *tph-1* in wild-type animals abolished 2-AG-dependent inhibition (Fig. 4*a*). Together, these data suggest that 2-AG stimulates the release of 5-HT from serotonergic ADF neurons to mediate CB-dependent locomotory inhibition.

To identify the dopaminergic neurons involved in the 2-AG-dependent locomotory inhibition, we measured CB-dependent increases in *cat-2::gfp* fluorescence, on the assumption that, following 2-AG-dependent DA release, DA biosynthesis would be upregulated to replenish DA stores. As predicted, *cat-2* expression was observed in the two ADE and four CEP neurons in the anterior of the transgenic animals (Fig. 4*b*), confirming the predicted *cat-2* expression pattern (Suo et al., 2003). 2-AG robustly increased *cat-2::mCherry* fluorescence in the ADEs, but not the CEPs, suggesting that 2-AG may stimulate DA release selectively from the ADEs at a minimum (Fig. 4*c*).

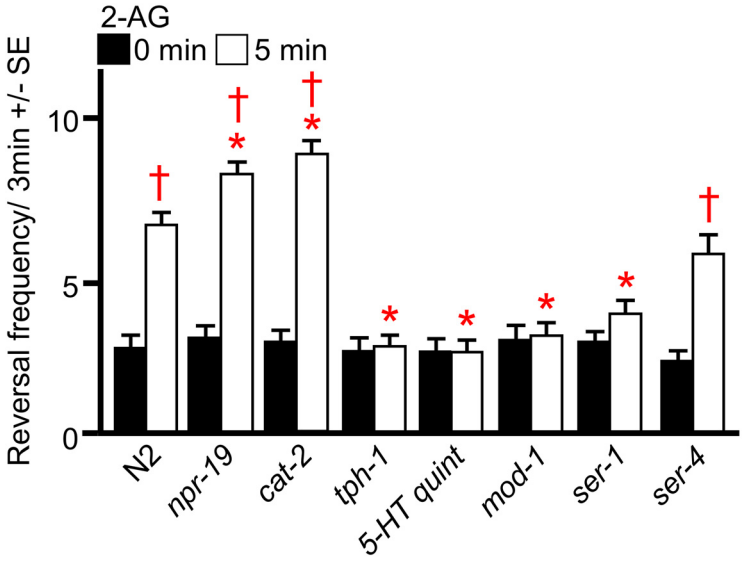

Figure 2. 2-AG increases the frequency of spontaneous reversals. Wild-type and mutant animals were incubated in 2-AG, and spontaneous reversals were assayed after 5 min on NGM plates containing 32 μM 2-AG, as described in Materials and Methods. The mutant animals used were as follows: *cat-2(n4547)*, *npr-19(ok2068)*, *tph-1(mg280)*, *5-HT quint*-null [*ser-5(tm2654); ser-4(ok512); mod-1(ok103); ser-7(tm1325)*, *ser-1(ok345)*], *mod-1(ok103)*, *ser-1(ok345*), and *ser-4(ok512)*. * Indicates significantly different from wild-type animals at 5 min; † indicates significantly different from the same animals at time 0 ($p \leq 0.05$). Data are presented as the mean ± SE (*n*) and were analyzed by two-tailed Student's *t* test using GraphPad software.

TRP channels are required for the 2-AG-dependent inhibition of locomotion

To identify the pathways involved in 2-AG-dependent locomotory inhibition and monoamine release, we screened for reduced 2-AG-dependent inhibition in a range of signaling mutants. Using this approach, we identified a number of TRP channel mutants that were resistant to 2-AG-dependent inhibition, including *trp-4* that encodes the pore-forming subunit of a mechanosensitive TRPN [NOMPC (mechanoreceptor potential channel)] channel that localizes to the cilia of the dopaminergic neurons and *osm-9* that encodes a $TRPV_1$-like subunit expressed in many sensory neurons (Fig. 5*a*). Interestingly, the temporal pattern of slowing in the *trp-4* and *osm-9* mutants mimicked those of dopamine- and serotonin-deficient animals, respectively (Fig. 5*a*). Many CBs activate mammalian TRP channels with affinities in the low-nanomolar range, although little is known about the physiological role of these channels in CB-dependent behavioral modulation (Di Marzo et al., 1998; De Petrocellis et al., 2001; Di Marzo and Maccarrone, 2008; Maccarrone et al., 2008; De Petrocellis and Di Marzo, 2010).

2-AG stimulated locomotion in the *trp-4*-null animals, a phenotype also observed in animals deficient in dopaminergic signaling, and *trp-4* RNAi knockdown in the dopaminergic neurons mimicked the *trp-4*-null phenotype, suggesting that TRP-4 functioned in the dopaminergic neurons to stimulate DA release (Fig. 5*b*,*c*). In contrast, as mentioned previously, the temporal pattern of slowing in the *osm-9* mutants mimicked that observed in animals with compromised serotonergic signaling, as outlined above, and *osm-9* RNAi knockdown in the serotonergic neurons, using the *tph-1* promoter mimicked the *osm-9*-null phenotype, suggesting that *osm-9* functioned directly in the serotonergic neurons (Fig. 5*c*). OSM-9 and OCR-2 form a heteromeric channel in many sensory neurons, and *osm-9*- or *ocr-2*-null animals do not accumulate significant 5-HT in the two serotonergic ADF sensory neurons, identified above in the 2-AG-dependent locomotory inhibition (Zhang et al., 2004). The activation of an OSM-9/OCR-2 $TRPV_1$-like channel appears to be essential to activate ADF *tph-1* transcription (Zhang et al., 2004; Sokolchik et al., 2005; Sze, 2007). This observation suggested that the lack of 2-AG inhibition in *osm-9* mutant animals could result from the absence of 5-HT in the ADFs and not a failure of 2-AG to activate an ADF $TRPV_1$-like channel to stimulate 5-HT release. Unfortunately, *ocr-2*-null animals could not be assessed in this assay, as *ocr-2*-null animals slowed significantly in the absence of 2-AG (Colbert et al., 1997).

Therefore, to confirm a role for the acute activation of an OSM-9/$TRPV_1$-like channel in 2-AG-dependent inhibition, we incubated animals in 2-APB, which at low concentrations inhibits TRPV channels in other systems (Xu et al., 2005; Togashi et al., 2008). As anticipated, acute exposure to 2-APB had no effect on locomotion in wild-type animals but, as predicted, dramatically decreased aversive responses to dilute 1-octanol to levels observed in *osm-9*-null animals (Fig. 6*a*,*b*). Aversive responses to dilute 1-octanol require the activation of a heteromeric OSM-9/OCR-2 $TRPV_1$-like channel in the two ASH sensory neurons, validating the use of 2-APB as a potential $TRPV_1$-like channel inhibitor in these studies (Colbert et al., 1997; Tobin et al., 2002). 2-ABP inhibited 2-AG-dependent locomotory inhibition in wild-type animals and in the remodeled 5-HT quints designed to measure acute 5-HT release, but, as predicted, had no effect on 5-HT-dependent locomotory inhibition in these same remodeled mutant animals, supporting our hypothesis that the activation of a $TRPV_1$-like channel in the serotonergic neurons was required for 5-HT release (Fig. 6*b*). In contrast, 2-APB had no effect on either DA- or 2-AG-dependent locomotory inhibition in remodeled DA quad mutants designed to measure acute DA release (Fig. 6*c*). Whether the TRP-4 channel is insensitive to

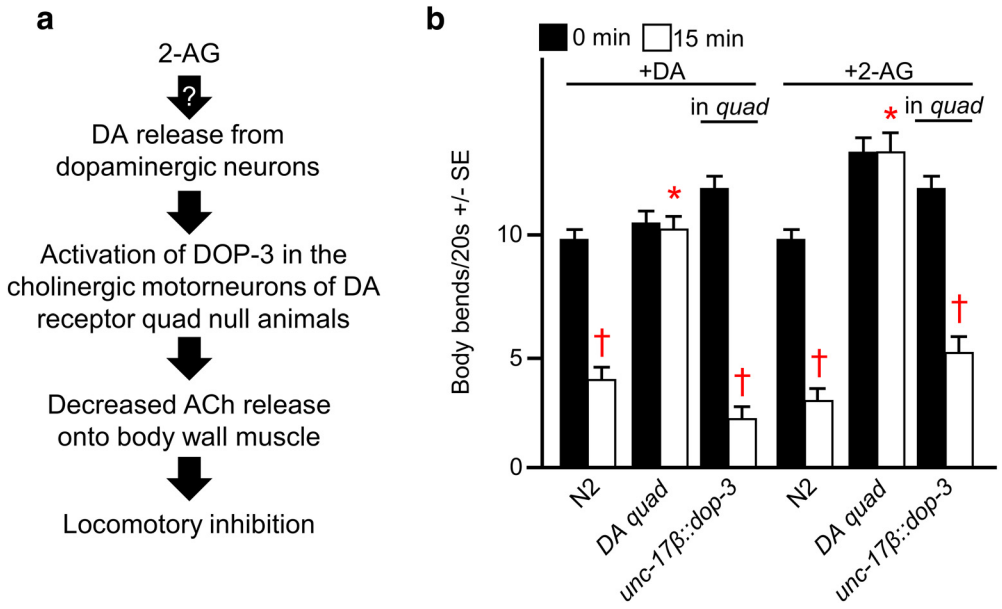

Figure 3. 2-AG inhibits locomotion in *C. elegans* quadruple dopamine receptor mutants remodeled to detect acute dopamine release. Wild-type, mutant, and remodeled animals were incubated in 2-AG, and locomotion was assayed as body bends/20 s, as described in Materials and Methods. ***a***, To detect acute 2-AG-stimulated DA release, the $G\alpha_o$-coupled D_2-like receptor DOP-3 was expressed in the cholinergic motor neurons of DA receptor quadruple-null (DA quad) animals under the assumption that any 2-AG-dependent DA release would activate DOP-3 on the motor neurons, inhibit acetylcholine release, and inhibit locomotion. ***b***, DA or 2-AG-dependent locomotory inhibition was restored in DA quad animals designed to measure acute DA release. The *unc-17β* promoter drives expression in the cholinergic motor neurons. * Indicates significantly different from wild-type animals at 15 min; † indicates significantly different from the same animals at time 0 ($p \leq 0.05$). Data are presented as the mean ± SE (*n*) and were analyzed by two-tailed Student's *t* test using GraphPad software.

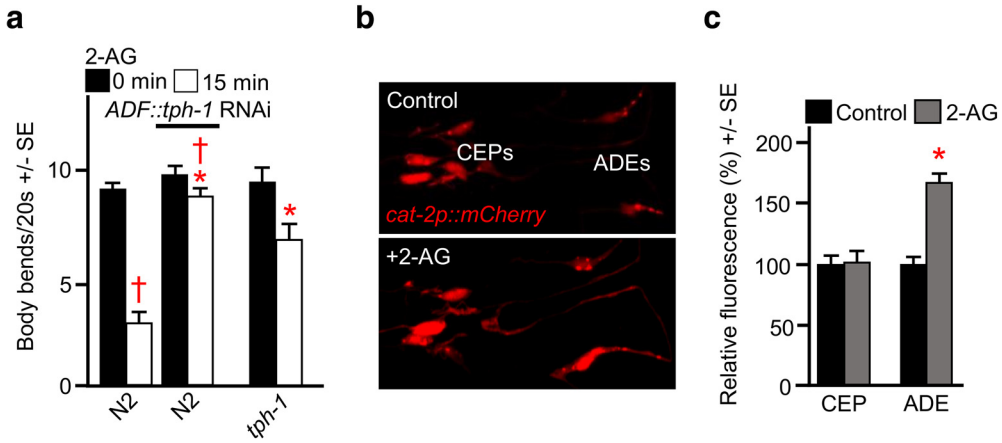

Figure 4. Identification of the monoaminergic neurons involved in 2-AG-dependent locomotory inhibition. ***a***, ADF-selective RNAi knockdown of *tph-1* in wild-type (N2) animals abolished 2-AG-dependent locomotory inhibition and ADF-selective expression of *tph-1* restored 2-AG sensitivity of *tph-1* (*mg280*)-null animals. ***b***, Fluorescence from a *cat-2p::mCherry* transgene confirms mCherry expression in the dopaminergic CEPs and ADEs. ***c***, *cat-2p::mCherry* fluorescence in the dopaminergic CEP and ADE neurons was analyzed after 2-AG incubation, as described in Materials and Methods. Images were captured using an Olympus IX81 confocal microscope and analyzed using ImageJ. * Indicates significantly different from wild-type animals at 15 min; † indicates significantly different from the same animals at time 0 ($p \leq 0.05$). Data are presented as the mean ± SE (*n*) and analyzed by two-tailed Student's *t* test using GraphPad software.

2-APB or requires higher 2-APB concentrations for inhibition is unclear. Cilia of the dopaminergic neurons, where TRP-4 is expressed, are embedded directly in the cuticle, potentially making them less responsive to perturbations in the external environment, in contrast to dendrites from sensory neurons found in the amphids that are directly exposed to the external environment (Inglis et al., 2007). Unfortunately, higher 2-APB concentrations (10-fold) inhibited locomotion in wild-type animals. Together, observations support the hypothesis that 2-AG-dependent inhibition requires the activation of TRP channels on the monoaminergic neurons and monoamine release to inhibit forward locomotion and increase turning.

Discussion

Cannabis alters sensory perception and has been purported to be useful for the treatment of a range of disorders, including anxiety, epilepsy, nausea, loss of appetite, and pain management (Gross et al., 2004; Svendsen et al., 2004; Wissel et al., 2006; Haney et al., 2007; Bedse et al., 2017). Although initial reports suggested that nematodes lacked canonical CB receptors, our recent work demonstrates that *C. elegans* contains an endogenous CB signaling system mediated by a CB receptor, NPR-19 (Oakes et al., 2017). NPR-19 responds directly to the endogenous CBs 2-AG and AEA with affinities similar to human CB_1 (Lehtonen et al., 2011; Oakes

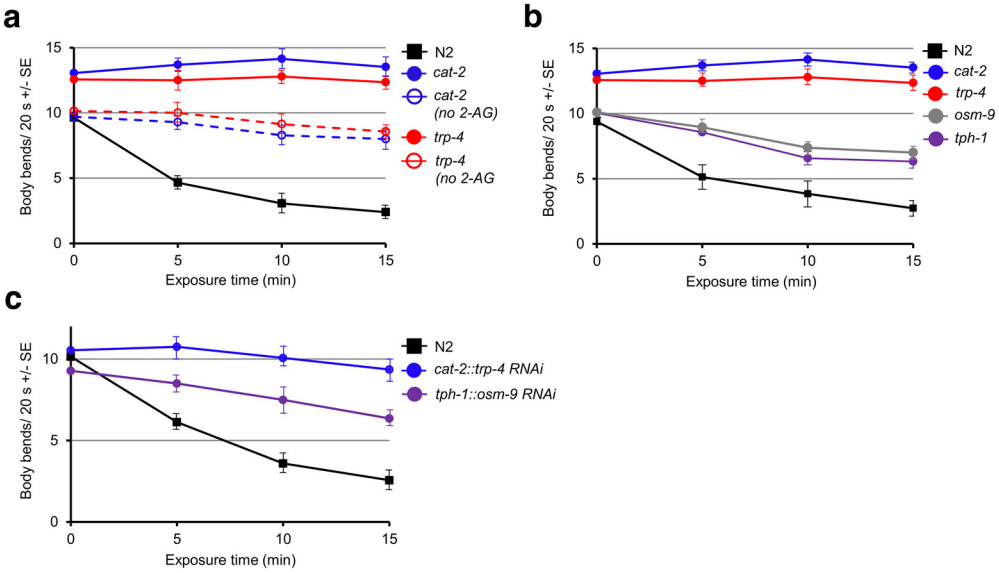

Figure 5. 2-AG-dependent locomotory inhibition requires the TRP-like channels OSM-9 and TRP-4 in the serotonergic and dopaminergic neurons, respectively. Animals were incubated in 2-AG and locomotion was assayed as body bends/20 s as described in Materials and Methods. ***a***, *osm-9* (*ky10*)- and *trp-4* (*sy695*)-null animals are resistant to 2-AG-dependent locomotory inhibition and the temporal patterns of resistance to 2-AG-dependent locomotory inhibition in *osm-9*- and *trp-4*-null animals mimics similar patterns in *tph-1* (*mg280*)- and *cat-2* (*n4547*)-null animals, respectively. ***b***, Locomotion in *trp-4*- and *cat-2*-null animals is stimulated in the presence of 2-AG. ***c***, Selective *osm-9* and *trp-4* RNAi knockdown in the serotonergic and dopaminergic neurons of wild-type animals, driven by the *tph-1* and *cat-2* promoters, respectively, abolishes 2-AG-dependent locomotory inhibition. Data are presented as the mean ± SE (*n*) and analyzed by two-tailed Student's *t* test using GraphPad software.

et al., 2017). As demonstrated in this and our previous study, CBs cause a "dazed and confused" phenotype, inhibiting aversive behavior, feeding, and locomotion, and increasing turning. CBs also modulate additional processes in *C. elegans*, including neuronal migration, dauer formation, and cholesterol metabolism (Lucanic et al., 2011; Reis Rodrigues et al., 2016; Galles et al., 2018). The effects of CBs on aversive behavior, feeding, and axon regeneration require NPR-19 (Pastuhov et al., 2016; Oakes et al., 2017). In contrast, 2-AG signals through NPR-19-independent pathways to inhibit forward locomotion and increase turning. This NPR-19-independent signaling requires multiple TRP channels and the release of both 5-HT and DA from subsets of monoaminergic neurons.

TRP channels are essential for 2-AG-dependent 5-HT and DA release

2-AG-dependent locomotory inhibition and monoamine release are also absent in *osm-9*- and *trp-4*-null animals that lack a $TRPV_1$-like channel subunit expressed in the ADFs and the pore-forming subunit of a mechanosensitive TRPN (NOMPC) channel expressed in the dopaminergic neurons, respectively. 2-AG actually stimulates locomotion in the *trp-4*-null animals, a phenotype also observed in animals deficient in dopaminergic signaling and *trp-4* knockdown in the dopaminergic neurons mimicked the *trp-4*-null phenotype, suggesting that TRP-4 functions directly in the dopaminergic neurons to stimulate DA release. Functional correlations between TRP-4 signaling in the dopaminergic neurons and DA-dependent locomotory behaviors have been demonstrated previously. For example, *trp-4*-null animals are deficient in the basal slowing behavior that is dependent on DA release (Kindt et al., 2007). In contrast, the temporal pattern of slowing in the TRPV1-like mutants mimics that observed in animals with compromised serotonergic signaling, and osm-9 knockdown in the serotonergic neurons mimics the *osm-9*-null phenotype, suggesting that *osm-9* functions in the serotonergic neurons. Importantly, 2-APB that at low concentrations inhibits TRPV channels in both nematodes and mammals also acutely inhibits 2-AG-dependent locomotory inhibition in both wild-type animals and remodeled 5-HT receptor mutant animals designed to measure 5-HT release. Together, these data suggest that 2-AG may activate multiple TRP channels in *C. elegans* to modulate the release of monoamines from distinct subsets of serotonergic and dopaminergic neurons, as also observed in mammals (Fig. 7; Sagredo et al., 2006: De Luca et al., 2014).

Similarities between CB signaling in nematodes and mammals

Our recent work has identified many similarities between CB signaling in mammals and nematodes. Indeed, the utility of *C. elegans* as a translational tool for understanding basic processes and drug action in the mammalian nervous system is well documented (Engleman et al., 2016). Although *C. elegans* contains a compact nervous system (only 302 neurons and ~7000 synapses), it still exhibits complex behaviors modulated by serotonergic, dopaminergic, adrenergic (octopaminergic), and opioid signaling that are mediated by receptors with clear orthology to their mammalian counterparts (Allen et al., 2011; Law et al., 2015; Mills et al., 2016; Oakes et al., 2017). Similarly, the noradrenergic/ octopaminergic inhibition of pain/aversive responses is also similar in mammals and nematodes, with α_2-adrenergic receptors inhibiting primary nociceptors and α_1-like adrenergic receptors stimulating the release of an array of inhibitory neuropeptides (Pertovaara, 2006; Komuniecki et al., 2012; Mills et al., 2012).

Aspects of CB signaling are conserved between mammals and nematodes, and both systems contain an eCB signaling system mediated by canonical CB receptors. For example, mammalian and nematode systems do the following: (1) synthesize identical CBs, 2-AG, and AEA, and express orthologous receptors CB_1/CB_2 in mammals and NPR-19 in nematodes (Lehtonen et al.,

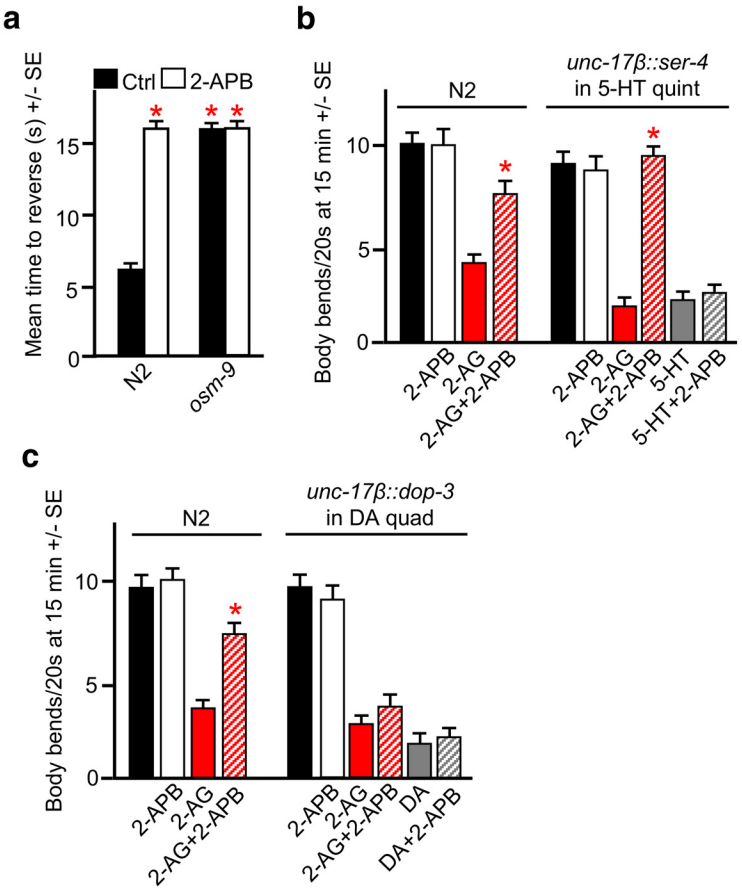

Figure 6. The TRPV channel blocker 2-APB abolishes 2-AG-dependent locomotory inhibition in wild-type and remodeled *C. elegans* designed to detect acute 5-HT release. Wild-type and mutant animals were incubated in 2-AG and/or 2-APB, and locomotory and 1-octanol avoidance assays were performed as described in Materials and Methods. ***a***, 2-APB inhibits basal aversive responses to 100% 1-octanol and mimics the octanol-resistant phenotype of *osm-9*-null animals. ***b***, 2-APB has no effect on locomotion in wild-type animals, but abolishes 2-AG-dependent locomotory inhibition in remodeled *C. elegans* designed to detect acute 5-HT release. ***c***, 2-APB has no effect on 2-AG-dependent locomotory inhibition in remodeled *C. elegans* designed to detect acute DA release. The *unc-17β* promoter drives expression in the cholinergic motor neurons. * Indicates significantly different from wild-type animals at 15 min; † indicates statistically significant from the same animals at time 0 ($p \leq 0.05$). Data are presented as the mean ± SE (*n*) and analyzed by two-tailed Student's *t* test using GraphPad software.

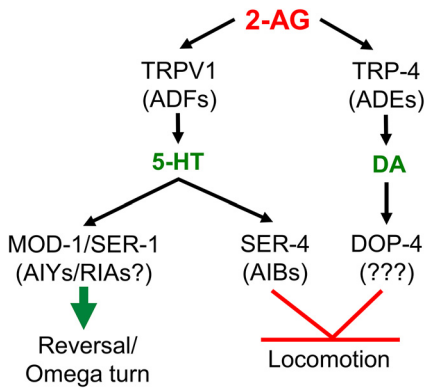

Figure 7. Model of 2-AG-dependent modulation of locomotory behavior. 2-AG stimulates the release of 5-HT from the serotonergic ADF neurons and DA from the dopaminergic ADE neurons to stimulate spontaneous reversal and inhibit forward locomotion through a pathway requiring the TRPV-like channel subunit OSM-9 and the TRPN-like channel subunit TRP-4, respectively. SER-1, $G\alpha_q$ coupled 5-HT receptor; SER-4, $G\alpha_o$-coupled 5-HT receptor; MOD-1, 5-HT-gated chloride channel.

2011; Oakes et al., 2017); (2) degrade CBs through similar pathways mediated by MAGL for 2-AG and fatty acid amide hydroxylase (FAAH) for AEA hydrolysis, and both nematode-degradative enzymes Y97E10AL and FAAH-1 appear to be inhibited by antagonists specific for their mammalian counterparts (Oakes et al., 2017); and (3) exhibit significant CB_1/CB_2 or NPR-19-independent CB signaling, mediated by additional GPCRs and TRP channels. For example, in mammals CBs also differentially activate the mammalian GPCRs, GPR18, and GPR55 (Begg et al., 2005; Kohno et al., 2006; Overton et al., 2006; Ryberg et al., 2007; Lauckner et al., 2008; McHugh et al., 2012; Martínez-Pinilla et al., 2014). However, CB/GPCR interactions are complex and often antagonistic. For example, 2-AG, AEA, and THC function as agonists for GPR55, while CBD functions as an antagonist (Ryberg et al., 2007). CBs also activate at least five different TRP channels with affinities in the nanomolar range, including TRPV1 and a range on TRP channels in *C. elegans* (De Petrocellis and Di Marzo, 2010; Zygmunt et al., 2013). CB/TRP channel interactions are also complex. For example, AEA inhibits nociception by activating CB_1 and at higher concentrations TRPV1 (Morisset et al., 2001; Iskedjian et al., 2007; Horvath et al., 2008; Clapper et al., 2010; Zygmunt et al., 2013). In contrast, *N*-arachidonoyl-5-HT, an eCB that functions as a dual blocker of FAAH and the TRPV1 channel, induces anxiolytic effects by stimulating CB_1 and inhibiting TRPV1, highlighting the potentially complex interactions between these different CB-dependent signaling pathways (Gobira et al., 2017).

CBs also modulate the release of both 5-HT and DA in both nematodes and mammals, and some of the behavioral effects of CBs are mediated, either directly or indirectly, through the complex modulation of serotonergic and dopaminergic signaling. For example, CBs extensively modulate serotonergic signaling in mammals. However, the behavioral consequences of CB-dependent modulation of serotonergic signaling have not been fully characterized. In general, CBs modulate the synthesis, release, and turnover of 5-HT, inhibit 5-HT reuptake and modulate the efficacy of 5-HT receptors, although many of these observations are dependent on the neurons and CBs involved. For example, CBs inhibit 5-HT reuptake in rats and THC increases 5-HT levels in the rat prefrontal cortex (Steffens and Feuerstein, 2004; Sagredo et al., 2006). In addition, the pCB Δ^9-tetrahydrocannabivarin appears to act through 5-HT_{1A} receptors to increase the efficacy, but not the EC_{50} of the 5-HT_{1A} agonist 8-OH-DPAT (Cascio et al., 2015). In contrast, the inhibition or genetic knockdown of CB_1 increases endogenous 5-HT levels, suggesting that eCBs inhibit 5-HT release and CB_1 knockdown in mice increases 5-HT_{1A} and 5-HT_{2A} receptor efficacy, but decreases serotonergic neuronal activation (Darmani et al., 2003; Mato et al., 2007; Aso

et al., 2009). In *C. elegans*, CBs do not appear to activate 5-HT receptors directly, increase their efficacy for 5-HT, or block 5-HT reuptake, but instead stimulate the release of endogenous 5-HT via a mechanism requiring $TRPV_1$-like channel activation (Oakes et al., 2017).

Similarly, 2-AG and AEA increase the firing rate of dopaminergic neurons in mammals and stimulate DA release directly by activating TRP channels expressed on dopaminergic neurons and indirectly by inhibiting GABA release upstream of dopaminergic neurons via CB_1-dependent activation (Cheer et al., 2005; Starowicz et al., 2007; Zygmunt et al., 2013; De Luca et al., 2014; Wang et al., 2015). In *C. elegans*, CB-dependent DA release is NPR-19 independent and requires a TRPN-like channel expressed on the dopaminergic neurons, although a role for CBs in modulating inputs into the *C. elegans* dopaminergic neurons remains to be examined. Given the complexity of the mammalian nervous system and the range of potential CB receptor ligands, generalizations about specific signaling pathways are complicated and the relative significance of CB_1/CB_2-dependent and CB_1/CB_2-independent signaling in behavioral modulation in mammals is not fully understood. The present study highlights similarities between CB signaling in mammals and nematodes and the potential benefit for the additional study in *C. elegans*, especially the examination of pCBs. For example, CBD appears to be involved in many of the proposed medicinal benefits of CB signaling, suggesting that the study of CBD signaling in *C. elegans* is warranted (Pertwee, 2005). In addition, the effects of CBs are rapid in *C. elegans*, and TRP channel activation is essential for the release of both 5-HT and DA, suggesting that CBs might begin to alter sensory processing almost immediately upon exposure. Indeed, CBs gate at least five distinct TRP channels in mammals, suggesting that the relationship between CB-dependent TRP channel activation and monoaminergic signaling in mammals might benefit from additional examination (De Petrocellis et al., 2001; Bandell et al., 2004; McIntosh et al., 2007). Finally, much remains to be learned about how eCB and pCB signaling interact and how ligands with similar affinity for canonical CB receptors initiate such dramatic differences in downstream signaling and ultimately behavior. *C. elegans* may prove to be a useful model to decipher these subtle interactions operating in more complex nervous systems.

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