Behavioral/Cognitive

Cannabinoids Activate Monoaminergic Signaling to Modulate Key *C. elegans* Behaviors

Mitchell D. Oakes,[1] Wen Jing Law,[1] Tobias Clark,[1] Bruce A. Bamber,[1] and Richard Komuniecki[1]
Department of Biological Sciences, University of Toledo, Toledo, Ohio 43606

Cannabis sativa, or marijuana, a popular recreational drug, alters sensory perception and exerts a range of potential medicinal benefits. The present study demonstrates that the endogenous cannabinoid receptor agonists 2-arachidonoylglycerol (2-AG) and anandamide (AEA) activate a canonical cannabinoid receptor in *Caenorhabditis elegans* and also modulate monoaminergic signaling at multiple levels. 2-AG or AEA inhibit nociception and feeding through a pathway requiring the cannabinoid-like receptor NPR-19. 2-AG or AEA activate NPR-19 directly and cannabinoid-dependent inhibition can be rescued in *npr-19*-null animals by the expression of a human cannabinoid receptor, CB_1, highlighting the orthology of the receptors. Cannabinoids also modulate nociception and locomotion through an NPR-19-independent pathway requiring an α_{2A}-adrenergic-like octopamine (OA) receptor, OCTR-1, and a 5-HT_{1A}-like serotonin (5-HT) receptor, SER-4, that involves a complex interaction among cannabinoid, octopaminergic, and serotonergic signaling. 2-AG activates OCTR-1 directly. In contrast, 2-AG does not activate SER-4 directly, but appears to enhance SER-4-dependent serotonergic signaling by increasing endogenous 5-HT. This study defines a conserved cannabinoid signaling system in *C. elegans*, demonstrates the cannabinoid-dependent activation of monoaminergic signaling, and highlights the advantages of studying cannabinoid signaling in a genetically tractable whole-animal model.

Key words: cannabinoid; *C. elegans*; monoamine; neuromodulation; nociception

Significance Statement

Cannabis sativa, or marijuana, causes euphoria and exerts a wide range of medicinal benefits. For years, cannabinoids have been studied at the cellular level using tissue explants with conflicting results. To better understand cannabinoid signaling, we have used the *Caenorhabditis elegans* model to examine the effects of cannabinoids on behavior. The present study demonstrates that mammalian cannabinoid receptor ligands activate a conserved cannabinoid signaling system in *C. elegans* and also modulate monoaminergic signaling, potentially affecting an array of disorders, including anxiety and depression. This study highlights the potential role of cannabinoids in modulating monoaminergic signaling and the advantages of studying cannabinoid signaling in a genetically tractable, whole-animal model.

Introduction

Cannabis sativa, or marijuana, has long been a popular recreational drug because of its unique ability to alter sensory perception and cause euphoria. More importantly, marijuana also has been reported to exert a wide range of medicinal effects (Pacher et al., 2006). *Cannabis* contains >60 bioactive compounds, or phytocannabinoids, the two most common being Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). In addition, the endogenous cannabinoids 2-arachidonoylglycerol (2-AG) and *N*-arachidonoylethanolamine (anandamide or AEA) are synthesized within the brain and CNS. Cannabinoids primarily activate $G\alpha_o$-coupled cannabinoid receptors 1 and 2 (CB_1 and CB_2). CB_1 is localized primarily to the brain and CNS (Herkenham et al., 1990; Glass et al., 1997; Martin et al., 1998), whereas CB_2 is restricted to the periphery and certain leukocytes (Munro et al., 1993). Endocannabinoids and phytocannabinoids activate the same receptors and elicit similar cellular responses despite their structural differences. Both 2-AG and AEA mediate retrograde inhibition of synaptic neurotransmission via activation of CB_1 on presynaptic membranes (Ohno-Shosaku and Kano, 2014). 2-AG or AEA inhibition is terminated by monoacylglycerol lipase (MAGL) or fatty acid amide hydroxylase (FAAH), respectively, with inhibition of either, eliciting analgesic and antinociceptive behavior (Piomelli et al., 2006; Long et al., 2009a; Long et al., 2009b).

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Correspondence should be addressed to Richard Komuniecki, Department of Biological Sciences, University of Toledo, 2801 W. Bancroft Street, Toledo, OH 43606. E-mail: rkomuni@uoft02.utoledo.edu.

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Thus far, the majority of studies on cannabinoids have been conducted at the cellular level, sometimes using mammalian tissue explants to observe receptor activation. In contrast, our goal was to examine the role of cannabinoids on whole-animal behavior and to dissect the role of cannabinoid signaling in the modulation of sensory integration and downstream decision making. Therefore, the present study was designed to examine the effects of cannabinoid receptor agonists on nociceptive behaviors in the nematode (*C. elegans*) model system because CB_1 appears to suppress pain in mammals (Sofia et al., 1973; Yaksh and Reddy, 1981; Tsou et al., 1995; Walker and Huang, 2002).

Our results demonstrate that mammalian cannabinoid receptor ligands activate a conserved cannabinoid signaling system in *C. elegans* and also modulate monoaminergic signaling, potentially affecting an array of disorders, including anxiety and depression. In contrast to published reports, *C. elegans* contains an endogenous canonical cannabinoid signaling system (McPartland and Glass, 2001; Pastuhov et al., 2016). Inhibiting the breakdown of endogenous 2-AG or AEA mimics 2-AG or AEA addition and inhibits nociception and feeding through a pathway that requires the cannabinoid-like receptor NPR-19. Cannabinoids activate NPR-19 directly and *npr-19*-null animals can be rescued by the expression of human CB_1, confirming the orthology of the two receptors. In addition, higher exogenous cannabinoid levels also activate an α_{2A}-adrenergic-like receptor (OCTR-1) and a 5-HT_{1A}-like receptor (SER-4) to modulate both nociception and locomotion through NPR-19-independent pathways. Cannabinoids activate OCTR-1 directly when expressed heterologously in *Xenopus laevis* oocytes. In contrast, 2-AG does not activate SER-4 directly and cannabinoids appear to enhance SER-4-dependent serotonergic signaling by increasing endogenous serotonin (5-HT). This study highlights the potential role of cannabinoids in modulating monoaminergic signaling and the advantages of studying cannabinoid signaling in a genetically tractable, whole-animal model.

Materials and Methods

Nematode strains and construction of C. elegans *transgenes*. Strains were maintained as described in Brenner (1974). The following strains were used: *N2* (*Bristol*), *ckr-2* (*tm3082*), *dop-1* (*ok298*), *mod-5* (*n3314*), *npr-3* (*tm1583*), *npr-5* (*ok1583*), *npr-19* (*ok2068*), *npr-24* (*ok3192*), *octr-1* (*ok371*), *ser-2* (*pk1357*), *ser-4* (*ok512*), and *tph-1* (*n4622*). RNAi transgenes were generated by PCR fusion as described in Esposito et al. (2007) and coinjected with *f25b3.3::gfp* (to 100 ng). The *octr-1* (+), *npr-19* (+) full-length genomic and *npr-19::npr-19::gfp* transcriptional transgenes were generated by PCR fusion and coinjected with *f25b3.3::gfp* (to 50 ng). The *npr-19::gfp* transcriptional transgene was constructed by PCR fusion of 1.5 kb *npr-19* promoter including the first intron fused to *gfp::unc-54* 3′′-UTR and coinjected with *unc-122::rfp* (to 50 ng). The *npr-19::CNR1::gfp* transgene was generated by 3-piece PCR fusion of the *npr-19* promoter including the first intron, full-length human *CNR1* cDNA, and *gfp::unc-54* 3′′-UTR and were coinjected with *unc-122::rfp* (to 50 ng). *unc-17β*-driven transgenes were generated by PCR fusion of the *unc-17β* promoter (562 bp) to GPCR cDNA and *gfp::unc-54* 3′′-UTR and coinjected with *unc-122::rfp* (to 50 ng). *npr-9::ser-4::gfp* transgene was generated by PCR fusion using native *npr-9* promoter and coinjected with *unc-122::rfp* (to 50 ng). PCR fusions were performed as described in Hobert (2002).

Octanol avoidance assays. Octanol avoidance assays were performed as described in Chao et al. (2004) and as modified by Harris et al. (2011). For all behavioral assays, L4 stage animals were picked 24 h before assaying. 2-AG and AEA plates were prepared 10 min before assay by spreading 60 μl of 2-AG or AEA (in H_2O) on fresh NGM plates. To measure aversive responses to 1-octanol, the blunt end of a hair was briefly dipped in 1-octanol and placed in front of a forward-moving worm and the time taken to initiate backward locomotion was recorded. Animals were first transferred to intermediate (nonseeded) plates, left for 30 s, transferred to assay plates, and tested after 10 min. 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.

Heterologous expression and electrophysiology in X. laevis *oocytes*. The human CB_1 (CNR1), α_{2A}-adrenergic (ADRA2A), 5-HT_{1A} (HTR1A), GIRK1, GIRK2, and *C. elegans npr-19*, *octr-1*, and *ser-4* cDNAs were cloned between Not1 and Age1 restriction enzyme sites into a *Xenopus* expression vector containing a T7 promoter and the *Xenopus* 5′ and 3′ β-globin UTRs to generate *pxGIRK1*, *pxGIRK2*, *pxCNR1*, *pxADRA2A*, *pxHTR1A*, *pxser-4*, *pxoctr-1*, and *pxnpr-19*, respectively. Linearized plasmids were transcribed using an Ambion mMessage mMachine T7 kit (Applied Biosystems). ADRA2A, CNR1, GIRK1, and GIRK2 cDNAs were from Addgene and HTR1A cDNA was from GE Healthcare. *X. laevis* oocytes were from Xenopus One and Nasco. Oocytes were separated mechanically before incubation in ND-96 (Ca^{2+} free) medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6) containing 1 mg/ml collagenase type 1A (Sigma Aldrich) for 30 min. Defolliculated oocytes were separated and incubated in modified Barth's medium with 1 mM Na pyruvate, 0.01 mg/ml gentamicin, and 1× antibiotic–antimycotic (Invitrogen) at 16°C overnight. Receptor cRNAs were injected at 50 ng/50 nl and GIRK1 and GIRK2 channel cRNAs were injected at 0.5 ng/50 nl. Oocytes were incubated at 16°C for 48–72 h after injection and then transferred to 4°C. Two-electrode voltage-clamp (TEVC) recordings were performed 72 h after injection using an Axon Gene Clamp 500 Amplifier (Molecular Devices) as described previously (Stühmer, 1998; Bamber et al., 2003). For TEVC recordings, standard low K^+ Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2) and a high K^+ Ringer's solution (96 mM KCl, 2 mM NaCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2) were applied by gravity perfusion. Ligands were applied by gravity perfusion initially at 1 μM. Oocytes coexpressing GIRK1/2 and GPCRs were perfused with intervals of increasing concentrations of 2-AG and AEA to determine ligand specificity and EC_{50}. 2-AG and AEA dose–response curves were fitted with the equation: $I - I_{max}/(1 + 10^{(\log EC50 - [agonist]) \times n})$, where I is the current at a given 2-AG or AEA concentration, I_{max} is current at saturation, EC_{50} is the 2-AG and AEA concentration required to elicit half-maximal current, and n is the slope coefficient. Curve fitting was performed using GraphPad Prism software.

Confocal imaging. To localize NPR-19, a transcriptional *npr-19::gfp* transgene was generated using 1.5 kb upstream of the predicted *npr-19* start site, including the first intron. To identify a subset of amphid sensory neurons, animals were incubated in 5 μM 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD; Invitrogen) for 1 h and then transferred to a standard NGM plate seeded with OP50 for 1 h to destain. For neuronal identification, *npr-19::gfp* was coinjected with *tph-1::rfp*, *tdc-1::rfp*, *flp-8::rfp*, *flp-18::rfp*, or *ceh-36::rfp*. All imaging was performed on an Olympus IX81 inverted confocal microscope. Animals expressing the *npr-19::gfp* transgene were immobilized on agarose pads with 20 mM sodium azide and imaged for GFP/RFP/DiD fluorescence.

Pharyngeal pumping assay. Pharyngeal pumping was assayed on NGM plates. 2-AG plates were prepared 10 min before assay by spreading 60 μl of 320 μM 2-AG (in H_2O) on fresh, predried NGM plates. For all pumping assays, L4 animals were picked 24 h before assay. Animals were moved from food plates to either a nonseeded NGM plate for control or 2-AG plates and incubated for 10 min. During assay, locomotion was recorded using a Sony Exwave HAD color-video digital camera for 2 min. Videos were played back in slow motion and the number of pharyngeal pumps per minutes was counted. Statistical analysis was performed using mean ± SE and Student's *t* test.

Feeding assay. Uptake of fluorescently labeled latex beads was performed as described in Kiyama et al. (2012). Fluoresbrite YG Microspheres were from Polysciences (1.00 μm; catalog #17154-10), diluted in ethanol, and stored at 4°C. Feeding plates were made by spreading 150 μl of M9 bead solution (1×10^8 microspheres/plate) and drying for 30 min. Wild-type and *npr-19*-null animals were incubated for 10 min on plates containing 2-AG, AEA, or no drug. Animals were transferred to bead plates ± 2-AG or AEA, allowed to feed for 30 min at room temperature, and then removed, washed

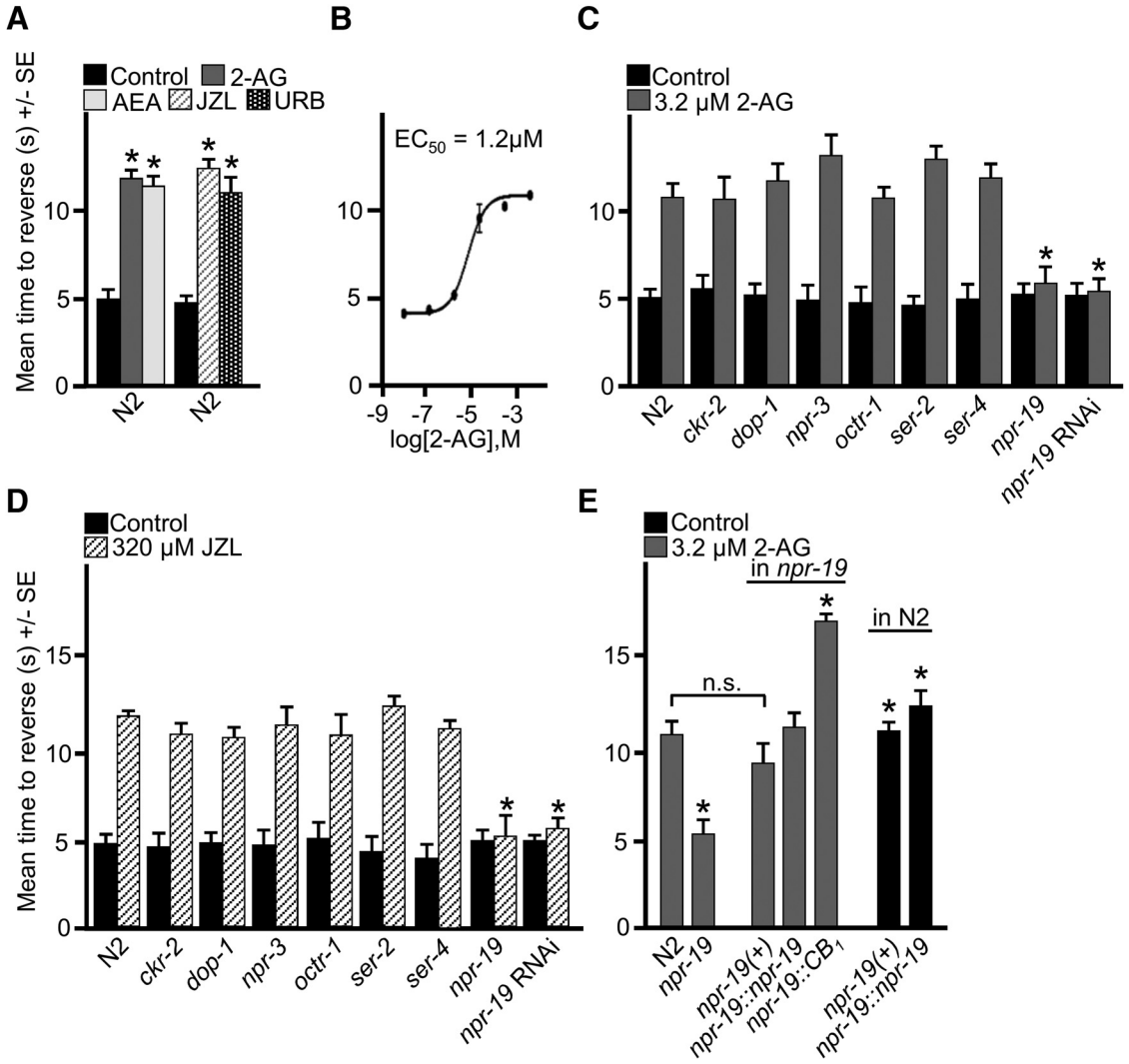

Figure 1. *C. elegans* contains an endogenous cannabinoid signaling system requiring the cannabinoid receptor NPR-19. The initiation of aversive responses to 1-octanol was examined as described by Harris et al. (2009). ***A***, 2-Arachidonoylglycerol (2-AG), anandamide (AEA), JZL184 (JZL), or URB697 (URB) inhibition of aversive responses to 1-octanol. ***B***, 2-AG dose–response curve for wild-type animals. ***C***, Screen for receptor-null animals resistant to 2-AG-dependent inhibition of aversive responses. ***D***, Screen for receptor-null animals resistant to JZL184-dependent inhibition of aversive responses. ***E***, *npr-19* or CB_1 expression driven by a minimal *npr-19* promoter in *npr-19*-null animals. *Significantly different from wild-type animals in the absence of effector ($p \leq 0.05$). Data are presented as a mean ± SE (*n*) and were analyzed by two-tailed Student's *t* test.

with M9 to remove excess beads, and immobilized on agarose pads with 20 mM Na azide for imaging using an Olympus IX81 inverted confocal microscope. Images were analyzed using ImageJ. Statistical analysis was performed using mean ± SE and Student's *t* test.

Locomotory (*body bend*) *assay*. Freshly poured agar plates (non-NGM) containing either 320 μM 2AG/AEA were used for assay. Well-fed, young adult hermaphrodite animals are picked before assay and maintained on NGM plates with *E. coli* OP50. During assay, seven animals were transferred to the assay plate. Motility was assessed as number of body bend/20 s at 5 min intervals for 30 min starting as soon as animals were transferred. Each strain was assayed at least three times with seven animals per assay. Statistical analysis was performed using mean ± SE and Student's *t* test.

Endocannabinoid compounds. 2-AG, AEA, JZL184, and URB597 were all from Tocris Bioscience and stock solutions are in DMSO or ethanol at 100 mM and are stored at −80°C.

Results

Endocannabinoids 2-AG and AEA inhibit aversive behavior

2-AG and AEA have been identified recently in *C. elegans* extracts by mass spectrometry (Lehtonen et al., 2011), but a simple BLAST search using the human cannabinoid receptor CB_1 failed to identify any *C. elegans* receptors with significant identity to CB_1, consistent with previous reports that *C. elegans* lacks clear mammalian cannabinoid receptor orthologs (McPartland and Glass, 2001; Pastuhov et al., 2016). In mammals, 2-AG and AEA exert antinociceptive action in models of acute inflammatory and neuropathic pain; therefore, we examined their effects on aversive responses to 1-octanol in *C. elegans* (Iskedjian et al., 2007; Clapper et al., 2010). This aversive decision-making circuit is mediated primarily by the two ASH sensory neurons and has been characterized extensively (Wragg et al., 2007; Harris et al., 2011; Mills et al., 2012). 2-AG and AEA inhibited the more rapid initiation of aversive responses to 100% 1-octanol in *C. elegans* (2-AG: $t = 17.5$, df = 16, $p < 0.0001$; AEA: $t = 7.8$, df = 8, $p < 0.0001$), with 2-AG exhibiting an EC_{50} of ~1 μM (Fig. 1*A*,*B*). These relatively high concentrations of ligands were probably necessary to overcome the relative impermeability of the nematode cuticle.

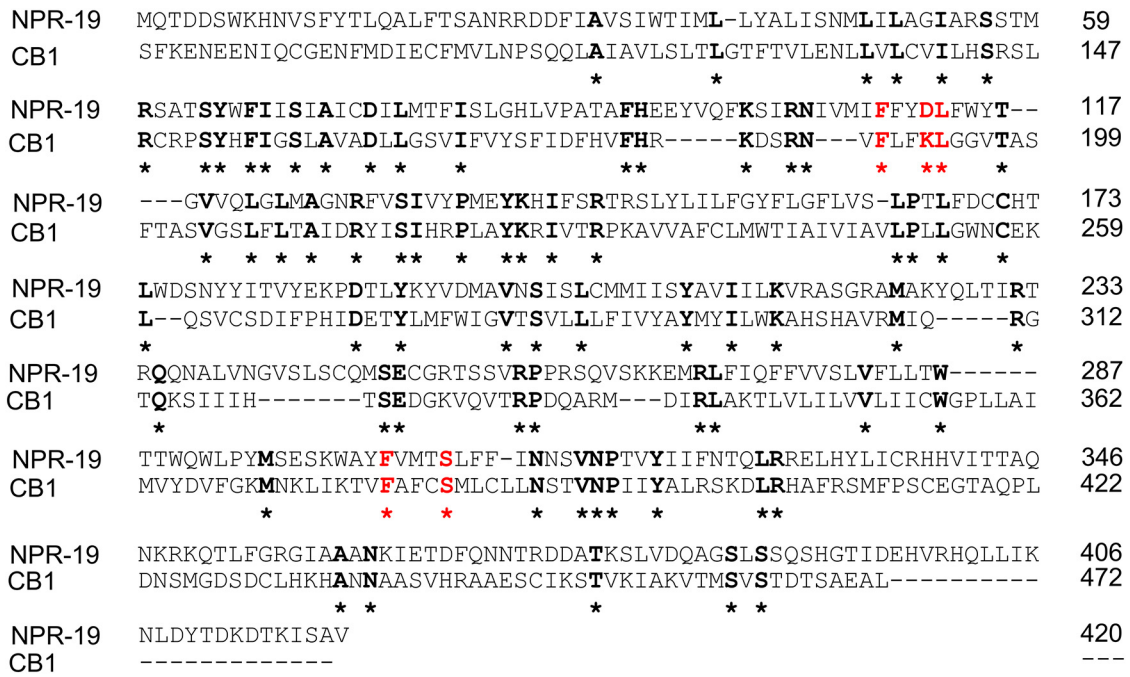

Figure 2. Comparison of CB1 and NPR-19 aa sequences. CB1/NPR-19 protein alignment. Conserved key amino acid residues involved in AEA binding (F_{189}, L_{193}, L_{192}, F_{379}, and S_{383}) are highlighted in red; identical residues are bolded and indicated with an asterisk.

In mammals, the degradation of 2-AG and AEA and termination of signaling are initiated by a membrane-bound MAGL and FAAH, respectively (Long et al., 2009b). The predicted *C. elegans* proteins, Y97E10AL.2 and FAAH-1, exhibit significant sequence identity to human MAGL (39%) and FAAH (38%), respectively, and selective inhibitors are available for both predicted mammalian orthologs (Piomelli et al., 2006; Long et al., 2009a). As anticipated, the inhibition of either MAGL with JZL184 or FAAH with URB597, predicted to inhibit the degradation of 2-AG or AEA, respectively, mimicked 2-AG or AEA addition and inhibited aversive responses to 1-octanol (JZL184: $t = 10.1$, df $= 11$, $p < 0.0001$; URB597: $t = 20.9$, df $= 7$, $p < 0.0001$; Fig. 1*A*). Together, these results suggest that *C. elegans* contains an endogenous cannabinoid signaling system.

2-AG/JZL184 inhibition of aversive responses is absent in *npr-19*-null animals

To identify potential *C. elegans* cannabinoid receptors, we reexamined protein BLAST data using human CB_1 and identified a number of previously characterized *C. elegans* monoamine receptors and predicted neuropeptide receptors, including NPR-19, with limited identity to CB_1 (McPartland and Glass, 2001). To determine whether any of these receptors were required for the cannabinoid-mediated inhibition of aversive responses, we screened the appropriate null animals for loss of JZL184 or 2-AG-dependent inhibition of aversive responses (Fig. 1).

JZL184 or 2-AG still inhibited aversive responses in *ckr-2*-, *dop-1*-, *npr-3*-, *octr-1*-, *ser-2*-, and *ser-4*-null animals. In contrast, JZL184 or 2-AG inhibition was dramatically reduced in *npr-19*-null animals (2-AG: $t = 10.8$, df $= 17$, $p < 0.0001$; JZL184: $t = 10.3$, df $= 12$, $p < 0.0001$; Fig. 1*C*,*D*). Similarly, JZL184 or 2-AG inhibition was absent after *npr-19* RNAi knockdown (2-AG: $t = 10.2$, df $= 13$, $p < 0.0001$; JZL184: $t = 5.1$, df $= 7$, $p < 0.0001$) using a predicted 1.5 kb *npr-19* promoter (Fig. 1*C*,*D*). 2-AG inhibition could be rescued in *npr-19*-null animals by expression of a full-length *npr-19* transgene driven by the predicted 1.5 kb promoter, including 1 kb of the *npr-19* 3′-UTR (Fig. 1*E*). In addition, wild-type animals overexpressing this *npr-19* transgene mimicked the addition of 2-AG and initiated aversive responses more slowly than wild-type animals in the absence of 2-AG ($t = 2.8$, df $= 12$, $p < 0.001$; Fig. 1*E*). Importantly, 2-AG sensitivity in *npr-19*-null animals could also be rescued by the expression of CNR1 cDNA, the human CB_1-encoding gene, driven by the *npr-19* promoter described above, confirming the orthology of the two receptors ($t = 8.8$, df $= 12$, $p < 0.0001$; Fig. 1*E*).

As predicted, although NPR-19 and human CB_1 exhibited only 23% sequence identity, many key amino acids involved in AEA binding appear to be conserved (Fig. 2). The residues delimiting the AEA-binding pocket are largely hydrophobic, based on both modeling and site-directed mutagenesis (Reggio, 2010), and include F_{189}, L_{193}, F_{379}, and S_{383}. All four residues were conserved in NPR-19 (Fig. 2). F_{189} interacts with the AEA amide oxygen and an F_{189A} mutation in CB_1 decreases AEA binding sixfold (McAllister et al., 2004). The AEA amide oxygen also interacts with a charged residue at position 192 (K in CB_1, D in NPR-19) and the AEA hydroxyl forms a hydrogen bond with S_{383} (McAllister et al., 2003).

These data highlight the effective coupling of a human G-protein-coupled receptor to endogenous *C. elegans* G-proteins and strongly support the hypothesis that NPR-19 is a mammalian cannabinoid receptor ortholog.

2-AG and AEA activate NPR-19 heterologously expressed in *Xenopus* oocytes directly

To demonstrate that 2-AG/AEA activate NPR-19 directly, *Xenopus* oocytes were coinjected with *npr-19* and *GIRK1/2* cRNAs. GIRK1/2 encode inwardly rectify potassium channel subunits activated by G-protein $\beta\gamma$ subunits and were coexpressed on the assumption that NPR-19 would be $G\alpha_o$-coupled, based on the observation above that the $G\alpha_o$-coupled human CB_1 rescued aversive phenotypes in *npr-19*-null animals. As expected, 2-AG

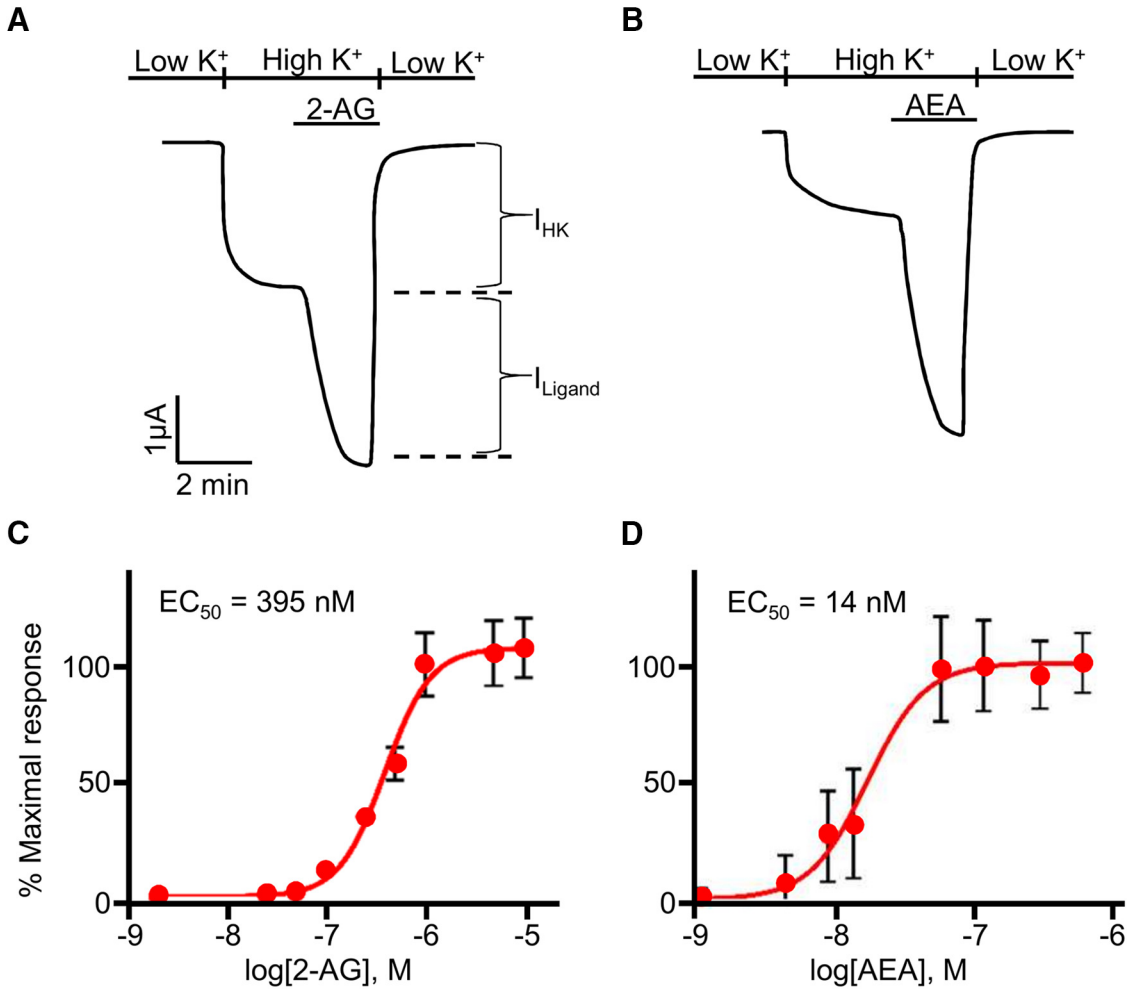

Figure 3. 2-Arachidonoylglycerol (2-AG) and anandamide (AEA) activate NPR-19 expressed in *Xenopus laevis* oocytes. Two-electrode voltage-clamp (TEVC) recordings were performed on oocytes expressing NPR-19 and GIRK1/2 subunits 72 h after injection, as described previously (Stühmer, 1998; Bamber et al., 2003). Representative traces are shown for NPR-19 activation by 2-AG (***A***) and AEA (***B***). I_{HK} represents the current induced upon a switch from low-K^+ to high-K^+ Ringer's solution. I_{Ligand} represents the current induced after ligand application. NPR-19 dose–response curve is shown for 2-AG (***C***) and AEA (***D***). Data are represented as a mean ± SE (*n*).

and AEA had no effect on oocytes expressing GIRK1/2 alone, but initiated robust inwardly rectifying currents in oocytes expressing NPR-19 (Fig. 3*A*,*B*), with EC_{50}s of 395 ± 5.1 nM (Fig. 3*C*) and 14 ± 2.4 nM (Fig. 3*D*), respectively. The EC_{50}s for 2-AG and AEA are in the range of EC_{50}s reported for human CB_1, 125 nM (Luk et al., 2004) and 89 nM (McAllister et al., 1999), respectively. Together, these data demonstrate that NPR-19 is a cannabinoid receptor.

NPR-19 is expressed in a limited number of neurons and inhibits pharyngeal pumping and feeding

Based on fluorescence from an *npr-19::gfp* transgene, NPR-19 is only expressed in a limited number of neurons, including the two inhibitory, glutamatergic M3 pharyngeal motorneurons (Fig. 4*A*,*B*) and the two URX sensory neurons (Fig. 4*A*,*C*) that play key modulatory roles in regulating pharyngeal pumping and avoidance behavior, respectively (Raizen and Avery, 1994; McGrath et al., 2009). As predicted, *npr-19* RNAi knockdown in the URXs, using either the URX-selective *gpa-8* or *flp-8* promoters, mimicked the *npr-19*-null phenotype and significantly decreased 2-AG-dependent inhibition of aversive responses to 100% 1-octanol (*gpa-8*: $t = 13.6$, df = 10, $p < 0.0001$; *flp-8*: $t = 16.8$, df = 11, $p < 0.0001$; Fig. 4*D*). The inhibitory M3s repolarize pharyngeal muscle after contraction and ablation of the M3s decreases the rate of pharyngeal pumping and feeding (Raizen and Avery, 1994). Indeed, 2-AG or AEA also inhibited pharyngeal pumping of food (2-AG: $t = 5.2$, df = 5, $p < 0.001$; AEA: $t = 5.7$, df = 6, $p < 0.001$; Fig. 4*E*), although at higher concentrations than those required for the inhibition of nociception (320 vs 3.2 µM). In contrast to nociception, JZL184 or URB597 had no effect on pumping (Fig. 4*E*), presumably because of the higher cannabinoid levels required for inhibition.

These higher cannabinoid levels also inhibited feeding, as assessed by the uptake of fluorescently labeled latex beads (2-AG: $t = 6.8$, df = 4, $p < 0.001$; AEA: $t = 16.6$, df = 4, $p < 0.0001$; Fig. 4*G*) in wild-type animals. The cannabinoid-dependent inhibition of both pumping and feeding were *npr-19* dependent and, as predicted, could be rescued by the expression of a full-length *npr-19* transgene driven by the predicted 1.5 kb promoter, including 1 kb of the *npr-19* 3′-UTR [pumping (2-AG): $t = 9.5$, df = 11, $p < 0.001$; pumping (AEA): $t = 10.3$, df = 10, $p < 0.0001$; feeding (2-AG): $t = 8.2$, df = 10, $p < 0.001$; feeding (AEA): $t = 9.0$, df = 13, $p < 0.0001$; Figure 4*F*,*G*]. More specifically, *npr-19* RNAi knockdown in the M3s using either the M3-selective *glt-1* or *egl-36* promoters, mimicked the *npr-19*-null

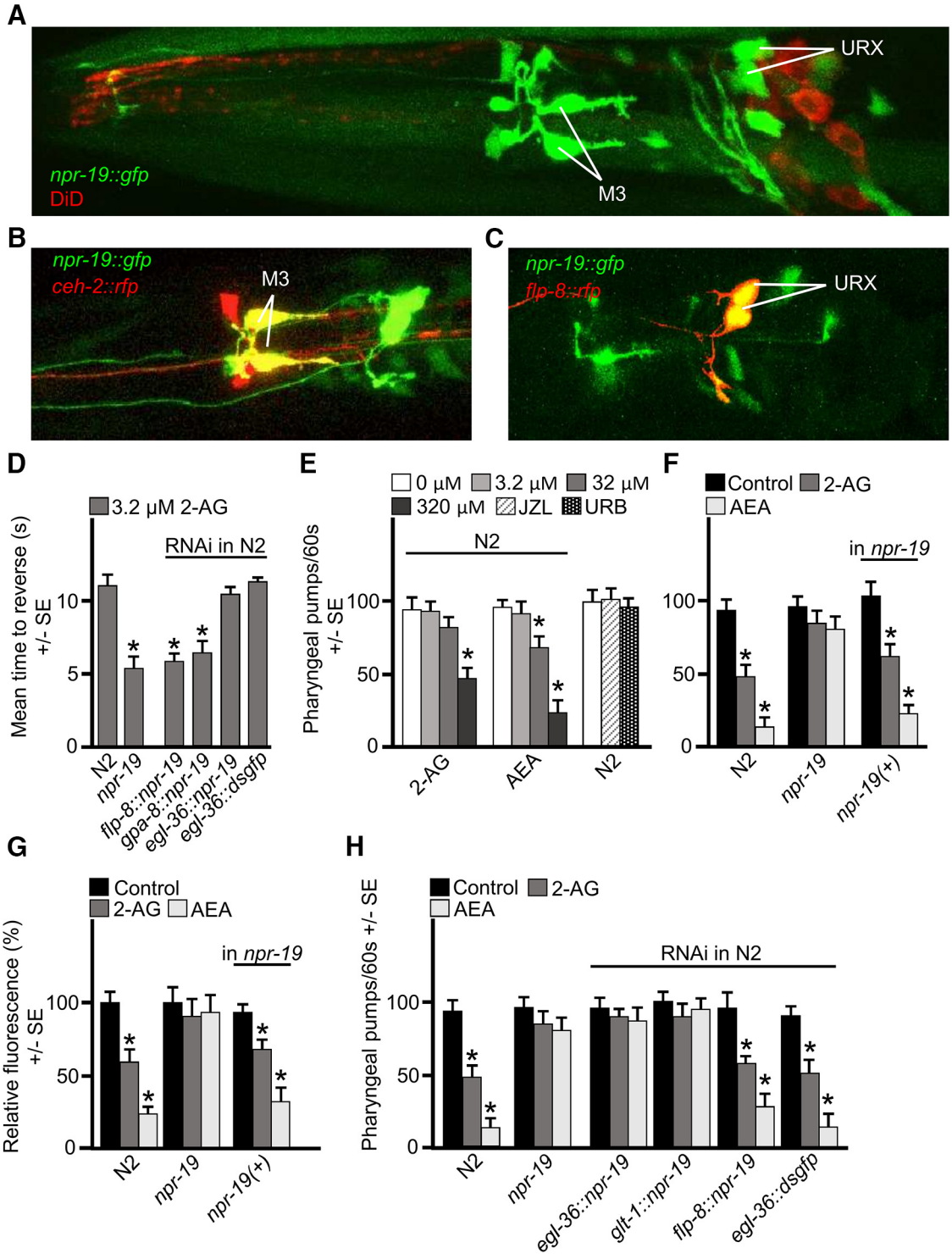

Figure 4. NPR-19 is expressed in a limited number of neurons, including URX and M3. ***A–C***, A transcriptional *npr-19::gfp* transgene was generated using 1.5 kb upstream of the predicted *npr-19* start site, including the first intron. ***A***, DiD-stained wild-type animal expressing *npr-19::gfp*. ***B***, ***C***, For neuronal identification, *npr-19::gfp* was coinjected with either *flp-8::rfp* or *ceh-36::rfp*. Wild-type animals coexpressing *npr-19::gfp* and either *ceh-2::rfp* (***B***) or *flp-8::rfp* (***C***) were used to identify M3s and URXs, respectively. ***D***, Aversive responses to 1-octanol after selective *npr-19* RNAi knockdown in the URXs via *flp-8* or *gpa-8* promoters. ***E***, Concentration-dependent 2-arachidonoylglycerol (2-AG) and anandamide (AEA) inhibition of pharyngeal pumping. ***F***, Pharyngeal pumping of *npr-19*-null and rescue animals. ***G***, Effects of 2-AG/AEA on feeding as measured by the uptake of fluorescent beads, as described in Kiyama et al. (2012). ***H***, Pharyngeal pumping after selective *npr-19* RNAi knockdown in the M3s via *egl-36* or *glt-1* promoters. *flp-8* and *gpa-8* promoters drive expression in the two URXs and a limited number of other neurons; *egl-36* and *glt-1* promoters drive expression in the two M3s and a limited number of other neurons (Wormbase). *Significantly different from wild-type animals in the absence of effector ($p \leq 0.05$). Data are presented as a mean ± SE (*n*) and were analyzed by two-tailed Student's *t* test.

phenotype and significantly decreased the 2-AG-dependent inhibition of pharyngeal pumping (Fig. 4*H*). To ensure that the neuron-specific RNAi phenotypes did not result from transgene overexpression, we expressed a *dsgfp* RNAi using the same promoters. As predicted, these RNAi transgenes had no effect on nociception or feeding (Fig. 4*D*,*H*). These data demonstrate key neuron-specific roles for cannabinoids and NPR-19 in the modulation of aversive behavior, pharyngeal pumping, and feeding.

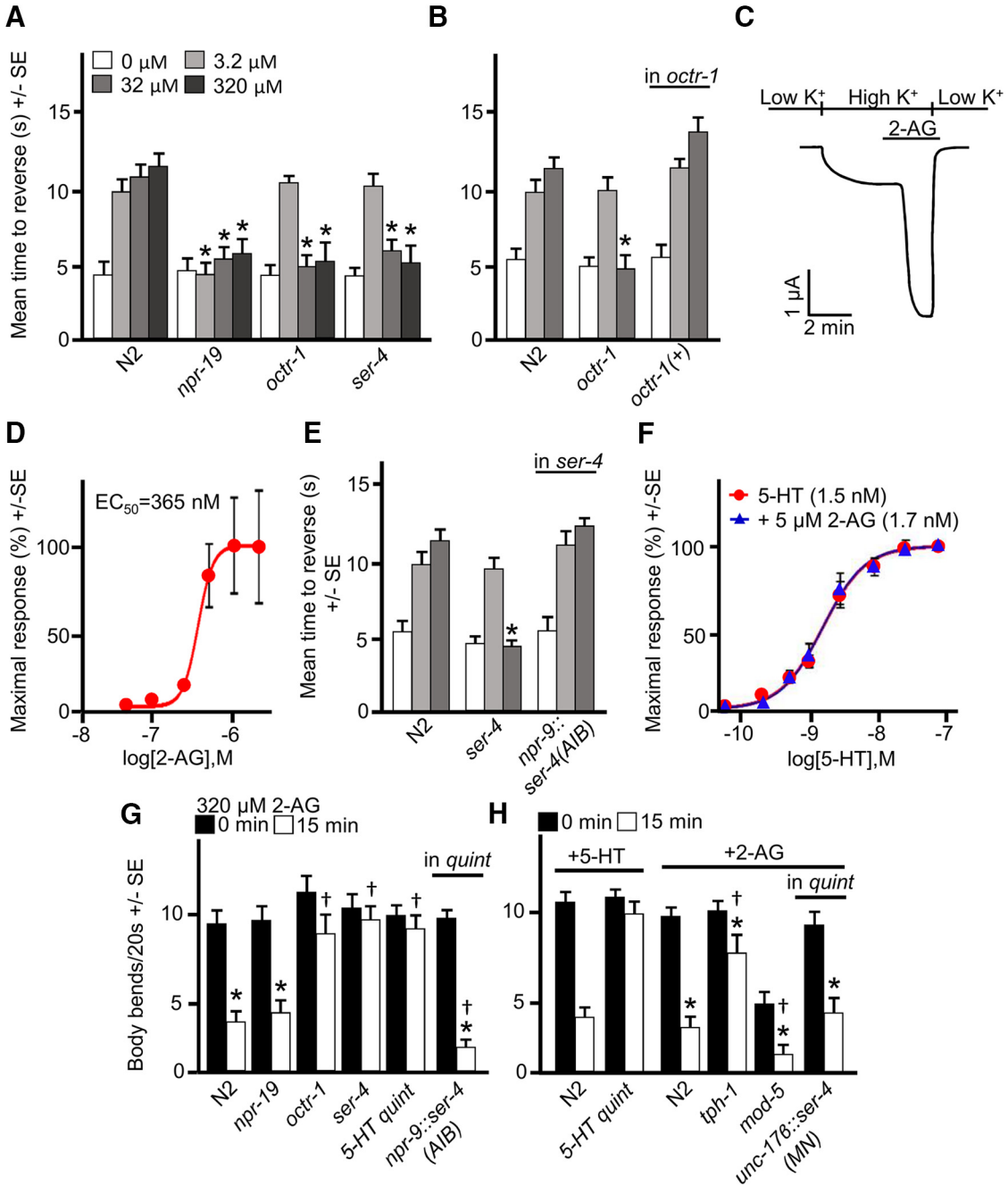

Figure 5. SER-4 and OCTR-1 are required for cannabinoid-dependent inhibition of nociception and locomotion at higher exogenous cannabinoid concentrations. Aversive responses to 1-octanol were examined as described by Harris et al. (2009). ***A***, Concentration-dependent 2-arachidonoylglycerol (2-AG) inhibition of aversive responses. ***B***, *octr-1* rescue of 2-AG inhibition of aversive responses in *octr-1*-null animals. ***C***, ***D***, ***F***, Two-electrode voltage-clamp (TEVC) recordings performed on oocytes coexpressing OCTR-1 or SER-4 and GIRK1/2 subunits 72 h after injection, as described previously (Stühmer, 1998; Bamber et al., 2003). ***C***, Representative trace of direct OCTR-1 activation by 2-AG. ***D***, OCTR-1 dose–response curve for 2-AG. ***E***, 2-AG inhibition of aversive responses to 1-octanol in *ser-4*-null animals. ***F***, SER-4 dose–response curves for serotonin (5-HT) and 5-HT + 5 μM 2-AG after SER-4 expression in *Xenopus* oocytes. ***G–I***, 2-AG-dependent locomotory inhibition. ***H***, 5-HT- and 2-AG-dependent inhibition of locomotion and 2-AG-dependent locomotory inhibition in 5-HT receptor quintuple-null animals expressing *ser-4* off-target in the cholinergic motorneurons (MNs). Data are represented as mean ± SE (*n*) and were analyzed by two-tailed Student's *t* test. *Significantly different from 0 min; †significantly different from N2 at 15 min ($p \leq 0.05$).

At higher exogenous cannabinoid concentrations, both serotonin and octopamine (OA) receptors are required for the cannabinoid-dependent inhibition of nociception and locomotion

Because the recreational use of cannabinoids might elevate total cannabinoid levels beyond what were normally observed endogenously, we examined the effects of elevated 2-AG and AEA levels on worm behavior. Surprisingly, at higher exogenous cannabinoid concentrations (32 vs 3.2 μM) the α_{2A}-adrenergic-like receptor OCTR-1 and the 5-HT$_1$-like receptor SER-4 are both required for the 2-AG inhibition of nociception in addition to NPR-19 because *octr-1* and *ser-4*-null animals are also resistant to 2-AG inhibition (*octr-1*: $t = 7.4$, df = 49, $p < 0.0001$; *ser-4*: $t = 5.8$, df = 48, $p < 0.0001$; Fig. 5*A*). The monoaminergic modulation of aversive responses is complex and involves the synergistic and antagonistic interactions of multiple monoamine receptors interacting at multiple levels in the locomotory decision-making circuit modulating nociception (Wragg et al., 2007; Harris et al.,

2011; Mills et al., 2012). An *octr-1::gfp* transgene is broadly expressed, including both the ASHs and the ventral nerve cord (Wragg et al., 2007), and 2-AG sensitivity could be restored in *octr-1* animals by *octr-1* expression driven by the predicted 5 kb *octr-1* promoter (Fig. 5*B*). 2-AG activated OCTR-1 directly after heterologous expression, with an EC_{50} of 365 ± 24 nM (Fig. 5*C*,*D*). Interestingly, NPR-19 and OCTR-1 exhibited similar EC_{50}s for 2-AG, although OCTR-1-dependent phenotypes were only observed at higher exogenous 2-AG concentrations. These differences could be explained in a number of ways, including differential modulation of the receptors *in vivo*, differential localization of the receptors relative to ligand entry, or the degree of receptor activation required for the phenotype. In contrast, *ser-4* is only expressed in a limited number of neurons and 2-AG sensitivity could be restored in *ser-4*-null animals by *ser-4* expression in the two AIB interneurons (Fig. 5*E*). In contrast to OCTR-1, 2-AG (5 μM) did not activate SER-4 directly and had no effect on SER-4 affinity for 5-HT (Fig. 5*F*).

Although MAGL or FAAH inhibition with JZL184 or URB597, respectively, had no effect on locomotion, increasing exogenous cannabinoid levels even further (to 320 μM) also caused animals to become sluggish and stop moving for brief periods ($t = 6.7$, df = 36, $p < 0.0001$; Fig. 5*G*). Both SER-4 and OCTR-1 were involved in this cannabinoid-dependent locomotory inhibition, but, in contrast to aversive responses, NPR-19 was not involved because *npr-19*-null animals behaved as wild-type animals and were similarly inhibited by 2-AG ($t = 3.9$, df = 40, $p < 0.0001$; Fig. 5*G*). In fact, this cannabinoid-dependent locomotory phenotype mimicked the 5-HT-dependent "locomotory confusion" phenotype mediated by the 5-HT activation of the $G\alpha_o$-coupled 5-HT_1-like receptor SER-4 in the two AIB interneurons (Law et al., 2015). Indeed, *ser-4* and 5-HT receptor quintuple-null animals were both resistant to cannabinoid-dependent locomotory inhibition and, as predicted, could be rescued by *ser-4* expression in the AIBs of *ser-4*-null animals ($t = 6.2$, df = 12, $p < 0.0001$; Fig. 5*G*). Interestingly, *octr-1*-null animals were also resistant, potentially involving the direct 2-AG activation of the inhibitory OCTR-1 in the motorneurons (Fig. 5*G*). Indeed, *octr-1* overexpression inhibited locomotion in the absence of 2-AG compared with wild-type animals (6.5 vs 9.6 body bends/20 s; data not shown). These results demonstrate that elevated cannabinoid levels have the potential to stimulate both octopaminergic and serotonergic signaling to modulate an array of key behaviors.

Cannabinoids increase endogenous 5-HT levels

As noted above, SER-4 was only required at higher exogenous cannabinoid concentrations and 2-AG did not activate SER-4 directly. Therefore, we hypothesized that cannabinoids might increase endogenous 5-HT levels, leading to the locomotory inhibition, either by increasing 5-HT release or inhibiting reuptake. To examine this hypothesis directly, we examined *tph-1*-null animals that lack tryptophan hydroxylase, the rate-limiting enzyme in 5-HT biosynthesis. As anticipated, *tph-1*-null animals that lack endogenous 5-HT were resistant to 2-AG-dependent locomotory inhibition ($t = 5.1$, df = 12, $p < 0.001$), suggesting that 5-HT is required for 2-AG inhibition and that 2-AG may increase endogenous 5-HT (Fig. 5*H*). Indeed, the 2-AG and 5-HT-dependent locomotory confusion phenotypes are similar and 2-AG-dependent locomotory inhibition mimics that observed in *mod-5*-null animals that lack a key 5-HT reuptake transporter and also display elevated 5-HT levels and inhibited locomotion (Fig. 5*H*). However, 2-AG still inhibits locomotion in these already slowed *mod-5*-null animals ($t = 8.2$, df = 26, $p < 0.0001$; Fig. 5*H*), suggesting additional 2-AG targets, including direct effects on 5-HT release. In addition, 5-HT or 2-AG also inhibit locomotion in transgenic quintuple 5-HT receptor-null animals expressing SER-4 off-target in the cholinergic motorneurons ($t = 3.8$, df = 40, $p < 0.001$; Fig. 5*I*), supporting the observation that 2-AG stimulates global increases in 5-HT. These mutant transgenic animals have been used previously to identify SER-4 agonists for use as potential anthelmintics because the SER-4/$G\alpha_o$-mediated inhibition of the cholinergic motorneurons leads to a rapid flaccid paralysis (Law et al., 2015).

The increased 5-HT levels might also explain the requirement for OCTR-1 in the inhibition of nociception at higher cannabinoid levels because 5-HT stimulates aversive responses in part by activating serotonergic signaling in an array of additional neurons that is antagonized by ASH OCTR-1. Indeed, this complex serotonergic/octopaminergic antagonism in the modulation of ASH-dependent aversive responses has been characterized previously, with at least three different 5-HT receptors, SER-1, SER-5, and MOD-1, involved in stimulating the initiation of aversive responses (Wragg et al., 2007; Harris et al., 2009; Mills et al., 2012). Together, these data highlight the complex interaction among cannabinoid, serotonergic, and octopaminergic signaling and suggest that they may also be relevant to understanding the role of exogenous cannabinoids in the modulation of human behavior because *C. elegans* has proven previously to be a useful model for understanding monoaminergic modulation in mammals (Komuniecki et al., 2012; Mills et al., 2012) (Fig. 6).

Discussion

The present study demonstrates that *C. elegans* contains an endogenous cannabinoid signaling system that modulates an array of key behaviors (Fig. 6). For example, the endocannabinoids 2-AG and AEA inhibit both aversive behavior and feeding. 2-AG and AEA have been identified previously in *C. elegans* extracts by GC/MS (Lehtonen et al., 2011). In contrast to previous reports suggesting that *C. elegans* does not contain a canonical cannabinoid receptor (McPartland et al., 2001), although a mutant screen did identify that the predicted neuropeptide receptor, NPR-19 was involved in the effects of cannabinoids on axon regeneration *in C. elegans* (Pastuhov et al., 2016). In the present study, we have demonstrated that NPR-19 is essential for many cannabinoid-dependent behaviors and responds directly to cannabinoid ligands with high affinity. Indeed, although $G\alpha_o$-coupled NPR-19 exhibits only 23% identity to the human $G\alpha_o$-coupled cannabinoid receptor CB_1, many of the key amino acids involved in ligand binding are conserved in the two receptors and phenotypes in *npr-19*-null animals could be rescued by the expression of human CB1, confirming the orthology of the two receptors (McPartland et al., 2001).

These cannabinoid ligands also activate octopaminergic and serotonergic signaling by functioning as agonists for the α_{2A}-adrenergic-like OA receptor OCTR-1 and increasing endogenous 5-HT. This is based on the observations that 5-HT mimics the inhibitory effects of 2-AG on locomotion and that the 2-AG inhibition of both nociception and locomotion is significantly reduced in *tph-1*-null animals that lack a key 5-HT biosynthetic enzyme and have dramatically reduced 5-HT levels. The monoaminergic modulation of aversive responses to 1-octanol is complex and involves multiple sensory neurons and an array of monoamine receptors. For example, 5-HT stimulates the initiation of an ASH-dependent aversive

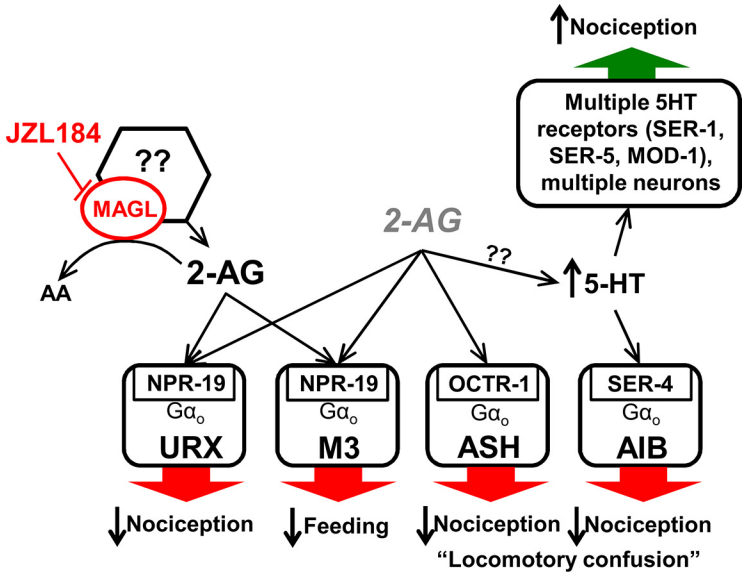

Figure 6. Model of 2-arachidonoylglycerol (2-AG)-dependent modulation of behavior. Endogenous 2-AG activates NPR-19 in the URXs to inhibit nociception. In addition, at higher 2-AG levels via exogenous application, 2-AG (italic gray) also activated NPR-19 to inhibit pharyngeal and feeding and also activated directly and indirectly a number of monoamine receptors to inhibit locomotion through an NPR-19-independent mechanism. These elevated 2-AG levels appear to increase endogenous serotonin (5-HT) and activate SER-4 in the two AIB interneurons to initiate locomotory confusion and paralysis that has been characterized previously (Law et al., 2015). Interestingly, 5-HT stimulates the initiation of aversive responses by activation of at least three additional 5-HT receptors, each operating at different levels within the ASH-dependent aversive circuit (Harris et al., 2011). However, this potential 5-HT stimulation appears to be overcome by the direct 2-AG activation of the α_{2A}-adrenergic-like OA receptor OCTR-1 in the ASHs to inhibit 5-HT-stimulated aversive responses, as demonstrated previously (Wragg et al., 2007). MAGL degrades 2-AG and terminates signaling and JZL184, an MAGL inhibitor, increases endogenous 2-AG levels and mimics the application of low levels of 2-AG.

response and requires three distinct 5-HT receptors operating at different levels within the locomotory circuit (Harris et al., 2009), with 5-HT decreasing ASH calcium but increasing ASH depolarization and activity (Zahratka et al., 2015). In contrast, OA antagonizes this 5-HT-dependent stimulation via the $G\alpha_o$-coupled α_2-adrenergic-like OA receptor OCTR-1, inhibiting the ASHs directly and the $G\alpha_q$-coupled OA receptor SER-6, which stimulates the release of an additional layer of inhibitory monoamines and neuropeptides (Mills et al., 2012). Indeed, this octopaminergic modulation of nociception, with a $G\alpha_o$-coupled receptor inhibiting the primary nociceptor and a $G\alpha_q$-coupled receptor stimulating the release of multiple inhibitory neuropeptides, mimics the noradrenergic modulation of chronic pain in humans (Komuniecki et al., 2012). The levels of OA appear to be critical for the inhibitory response because OA inhibition is masked at higher OA concentrations by activation of a second antagonistic $G\alpha_q$-coupled OA receptor, SER-3, in the ASHs, highlighting the delicate and dynamic balance of this modulatory system (Wragg et al., 2007). At low levels of cannabinoid receptor ligands, achieved by either inhibition of their endogenous breakdown or exogenous application, behaviors appear to be modulated exclusively by NPR-19. However, at higher levels of exogenous addition, both the octopaminergic and serotonergic signaling systems are activated. Indeed, it was puzzling at first why SER-4 and OCTR-1 were only required for the inhibition of nociception at higher cannabinoid concentrations. However, we propose that these higher cannabinoid concentrations increase endogenous 5-HT and its potential stimulation of aversive responses must be antagonized by ASH OCTR-1 for the cannabinoid-dependent inhibition of nociception to be realized. *C. elegans* contains multiple serotonergic neurons that function independently in the modulation of key behaviors. For example, global 5-HT released by the two neurosecretory NSMs stimulates the initiation of aversive responses to 1-octanol, whereas more local 5-HT from the ADFs appears to inhibit this response (Song et al., 2013). In contrast, ADF 5-HT is responsible for the increased pharyngeal pumping associated with the presence of food, whereas the NSMs located directly above the pharynx apparently are not involved. Interestingly, 2-AG dramatically inhibited the 5-HT quintuple-null animals expressing the inhibitory 5-HT_{1A}-like receptor SER-4 off target in the cholinergic motor neurons, suggesting that cannabinoids initiate a more global increase in 5-HT. Whether this results from the direct and/or indirect stimulation of secretion by the NSMs or the inhibition of 5-HT reuptake is unclear, but the locomotory confusion phenotype initiated by either 5-HT or 2-AG is mimicked by knockdown of the key 5-HT reuptake transporter MOD-5. Cannabinoid receptor agonists also modulate α-adrenergic and serotonergic signaling in mammals and function as agonists for the human α_{2A}-adrenergic receptor. For example, cannabinoid receptor stimulation activates the hypothalamic–pituitary–adrenal axis because central administration of THC in rats leads to an increase in plasma adrenocorticotrophin hormone levels (Corchero et al., 1999) and in the expression of corticotrophin releasing hormone mRNA in the anterior pituitary (Corchero et al., 2001). 2-AG increases norepinephrine (NE) release (Kurihara et al., 2001) and AEA or HU-210, a synthetic CB_1 agonist, significantly increases the level of circulating corticosterone (McLaughlin et al., 2009). In fact, interactions between endogenous cannabinoid and noradrenergic signaling have been observed in a number of organ systems. For example, cannabinoid receptor signaling plays a role in noradrenergic splenic contraction and interacts with adrenergic systems in the prefrontal cortex (Simkins et al., 2016). In addition, cannabinoids can block neuronal NE uptake and the phytocannabinoid cannabigerol functions as a α_2-adrenoceptor agonist in isolated mouse vas deferens (Cascio et al., 2010). Cannabinoids also modulate the synthesis, release, and turnover of 5-HT and appear to inhibit 5-HT reuptake and enhance 5-HT_{1A} signaling (Egashira et al., 2002; Sagredo et al., 2006). For example, both endogenous and synthetic cannabinoids inhibit 5-HT reuptake in rats and chronic THC administration increases endogenous 5-HT levels in the prefrontal cortex of rats. In addition, the phytocannabinoid Δ^9-tetrahydrocannabivarin appears to act through 5-HT_{1A} receptors to produce antipsychotic effects by functioning as an allosteric modulator, increasing the efficacy but not the EC_{50} of the potent 5-HT_{1A} agonist 8-OH-DPAT (Cascio et al., 2015). Together, these observations highlight the similarities between the nematode and mammalian cannabinoid signaling system and the potential of the *C. elegans* whole-animal model for the study of cannabinoid/monoamine interactions.

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