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Pharmacological identification of cholinergic receptor subtypes: modulation of locomotion and neural circuit excitability in *Drosophila* larvae

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Abstract—Acetylcholine (ACh) is an abundant neurotransmitter and neuromodulator in many species. In *Drosophila melanogaster* ACh is the neurotransmitter used in peripheral sensory neurons and is a primary excitatory neurotransmitter and neuromodulator within the central nervous system (CNS). The receptors that facilitate cholinergic transmission are divided into two broad subtypes: the ionotropic nicotinic acetylcholine receptors (nAChRs) and the metabotropic muscarinic acetylcholine receptors (mAChRs). This receptor classification is shared in both mammals and insects; however, both the pharmacological and functional characterization of these receptors within the *Drosophila* nervous system has lagged behind its mammalian model counterparts. In order to identify the impact of ACh receptor subtypes in regulating the performance of neural circuits within the larval CNS, we used a behavioral and electrophysiological approach to assess cholinergic modulation of locomotion and sensory-CNS-motor circuit excitability. We exposed intact and semi-intact 3rd instar larvae to ACh receptor agonists and antagonists to observe their roles in behavior and regulation of neural circuit excitability and to investigate AChR pharmacological properties *in vivo*. We combined this with targeted AChR RNAi-mediated knockdown to identify specific receptor subtypes facilitating ACh modulation of circuit efficacy. We identify a contribution by both mAChRs and nAChRs in regulation of locomotor behavior and reveal they play a role in modulation of the excitability of a sensory-CNS-motor circuit. We further reveal a conspicuous role for mAChR-A and mAChR-C in motor neurons in modulation of their input-output efficacy.

Key words: acetylcholine, Drosophila melanogaster, pharmacology, electrophysiology, behavior.

INTRODUCTION

Acetylcholine (ACh) has long been recognized as a primary neurotransmitter and neuromodulator in the nervous system of *Drosophila melanogaster*. It is the principal neurotransmitter used in sensory neurons and is a prominent excitatory neurotransmitter and neuromodulator within the CNS (Lee and O'Dowd, 1999; Yasuyama and Salvaterra, 1999; Su and O'Dowd, 2003). The primary enzymes involved in ACh metabolism, choline acetyltransferase and the degradative enzyme acetylcholinesterase (AChE), are highly expressed in sensory neurons and within the CNS (Buchner, 1991). Null mutations in these genes impart embryonic lethality, signaling the importance of ACh in *Drosophila* development and nervous system function (Buchner, 1991). Despite this documented significance, little is known regarding the modulatory role of ACh in modifying neural circuit and network activity in this species. While elegant work has enhanced our understanding of ACh signaling and the specific receptor subtypes that regulate a number of behaviors in adult flies, including in olfactory information processing (Gu and O'Dowd, 2006; Silva et al., 2015), motion detection (Takemura et al., 2011) in mediating giant fiber escape response (Fayyazuddin et al., 2006)

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Abbreviations: ACh, Acetylcholine; AChE, Acetylcholinesterase; AChRs, Acetylcholine receptors; m6, Abdominal muscle 6; BD, Benzoquinonium dibromide; BDSC, Bloomington Drosophila Stock Center; CS, Canton S; CNS, Central nervous system; CPG, Central pattern generator; EJP, Excitatory junction potential; GluR, Glutamate receptor; HL-3, Hemolymph-like 3 (Saline); MLA, Methyllycaconitine citrate salt; mEJPs, Mini Excitatory junction potentials; mAChRs, Muscarinic acetylcholine receptors; nAChRs, Nicotinic acetylcholine receptors; Pestanal, Piperonyl butoxide; SEM, Standard error of the mean; curare, Tubocurarine.

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and in stimulating grooming, jumping, and hyperactive geotaxis ability (Bainton et al., 2000; Hou et al., 2003), much less is known about its respective function in larvae. Recent analysis has highlighted cholinergic modulation of nociception (Hwang et al., 2007; Titlow et al., 2014) feeding (Gorczyca et al., 1991; Hückesfeld et al., 2016; Schlegel et al., 2016) and locomotion (Song et al., 2007; Hasegawa et al., 2016) in larval *Drosophila*; however, the receptor subtype contribution to these behaviors is not fully known. Moreover, while the pharmacological properties of acetylcholine receptors (AChRs) have been investigated widely *in vitro*, their functional properties *in vivo* remain largely indeterminate. Here, we present a behavioral and electrophysiological approach aimed at advancing knowledge of larval *Drosophila* AChR regulation of central circuits underlying larval locomotor behavior and sensory-CNS-motor neural circuit excitability.

The *Drosophila* cholinergic system is remarkably complex and this complexity has contributed to the difficulty in classifying the function of cholinergic receptor subtypes within the *Drosophila* CNS. As in mammals, *Drosophila* AChRs are categorized into two major subtypes: the metabotropic muscarinic acetylcholine receptors (mAChRs), and the ionotropic nicotinic acetylcholine receptors (nAChRs), both of which are activated by ACh and the agonists, muscarine and nicotine, respectively. The nicotinic receptor is part of the cys-loop family of ligand-gated ion channels that facilitates fast synaptic transmission. Muscarinic receptors are metabotropic and act indirectly with ion channels through second messenger G proteins to modulate cell physiology, including changes in intrinsic excitability (Collin et al., 2013). The *Drosophila* genome contains ten nAChR (Dα1-Dα7 and Dβ1-Dβ3) subunits and three mAChRs: A-type (encoded by gene CG4356), B-type (encoded by gene CG7918), and C-type (CG12796) have been cloned in this organism (Collin et al., 2013; Xia et al., 2016). In heterologous expression systems, mAChR-A and C types, like mammalian M1/M3/M5 receptors, signal via Gq/11 and are generally excitatory, while the B-type receptor, like M2/M4 receptors, signals via Gi/o and is generally inhibitory (Caulfield and Birdsall, 1998; Collin et al., 2013; Xia et al., 2016). While recent studies have shed light on the pharmacological properties of mAChRs that are useful in aiding insight into their impact in neurons and circuits, the characterization of nAChRs has not been as fruitful, as reconstitution of functional receptors has proven difficult in heterologous expression systems. Additionally, although recent findings have illuminated the pharmacological properties and intracellular transduction pathways activated by mAChRs *in vitro*, their *in vivo* properties and impact on behavior, neuronal and circuit excitability is poorly understood (Collin et al., 2013; Ren et al., 2015).

In addition to the general complexity of the system, another factor contributing to the lack of insight into cholinergic transmission in *Drosophila* larvae is the difficulty in performing electrophysiological recordings from neurons within the CNS. The relative inaccessibility for individual cell recordings makes it challenging to assess regulation of synaptic transmission at inter-neuronal synapses in an intact nervous system. Much of the current understanding of the receptor subtype contribution to central synaptic transmission stems from *in vitro* work from dissociated neurons in culture (Lee and O'Dowd, 1999). While substantial insights emerged utilizing these techniques, relying on *in vitro* analysis in the absence of *in vivo* studies prohibits a full understanding of the contribution of AChRs to neurotransmission and circuit function in this species. Therefore, we have utilized an *in situ* approach that allows for the observation of activity changes in a sensory-CNS-motor circuit in the presence of applied AChR agonists and antagonists to assess cholinergic modulation of motor output associated with alterations in sensory-CNS input. Additionally, we have utilized a compound delivery paradigm that may prove efficacious in probing the impact of pharmacological agents on larval behavior and circuit physiology in a model that provides unique challenges. While injection procedures have been employed extensively, the stress of injections at the larval stage may confound assessment of the rapid effects of the injected compound. For intact analysis, we have used a feeding paradigm that provides information regarding the time course of compound action with influence over short-term (20- minute) and longer exposure (24-hour) periods through consistent food consumption. We couple this with an electrophysiological approach in application of cholinergic agonists and antagonists, which provides a powerful combination enabling insight into the pharmacological properties of AChRs in an intact nervous system.

Larval locomotion represents a common behavior that is widely studied as a model Central Pattern Generator (CPG). Forward locomotion is guided by rhythmic motor output, regulated by synchronized signaling of a host of interneurons that control activity within individual segments and permit intrasegmental coordination (see review Kohsaka et al., 2017). Both sensory and central neuronal activity are crucial in regulating rhythmic motor neuron firing that underlies synchronized muscle contraction during crawling (Hughes and Thomas, 2007; Song et al., 2007; Cheng et al., 2010; Titlow et al., 2014). The importance of sensory feedback and interneuronal activity controlling the behavior highlights the likelihood of a significant role for ACh in modulating larval crawling speed. While the cellular components that make up the neural networks regulating locomotor behavior are being unraveled (Clark et al., 2018), the influence of neuromodulators, including ACh, in altering the efficacy of these circuits warrants investigation. In the present study we utilize a pharmacological approach to enhance understanding of the receptor subtypes that are important in facilitating ACh regulation of larval locomotion and add insight into their pharmacological properties in an intact nervous system. Based on the identification of behavioral alterations in response to select pharmacological agents, we performed analysis with RNAi-mediated knockdown of specific receptor subtypes at various levels of the network known to contribute to regulation of the behavior. To reconcile behavioral observations with alterations in the excitability of central circuits driving output to a muscle critical in locomotor behavior, we recorded synaptic potentials at larval abdominal muscle 6 (m6), a longitudinal, ventral

abdominal muscle involved in propelling larvae during forward and backward locomotion. Our electrophysiological recordings measuring glutamatergic induced excitatory junction potential (EJP) frequency in response to contralateral sensory stimulation shed light on acute AChR modulation of a functional circuit recruiting motor neuron firing during larval crawling. We show here a significant contribution by both mAChRs and nAChRs in regulating larval locomotor behavior. We reveal that pharmacological manipulation of these receptor subtypes alters sensory-CNS-motor circuit excitability suggesting the intrinsic properties of neurons that make up the network recruiting motor neurons targeting m6 are likely modulated by muscarinic and nicotinic ACh signaling. Furthermore, we identify the most robust influence in muscarinic modulation of motor output in response to sensory-CNS input is by mAChR-A and mAChR-C directly in motor neurons.

EXPERIMENTAL PROCEDURES

Fly maintenance and stocks

Canton S (CS) flies were used in all behavioral assays using pharmacological agents and in select electrophysiological experiments. This strain has been isogenic in the lab for several years and was originally obtained from Bloomington Drosophila Stock Center (BDSC). In order to drive mAChR RNAi knockdown in select neurons, the Gal4/UAS system was used. The following Gal4 driver lines were utilized: D42-Gal4 (w[*]; P{w[+mW.hs]=GawB}D42 BDSC stock number: 8816), ChaT-Gal4 (w[*]; P{w[+mC]=ChAT-GAL4.7.4}19B P{w[+mC]=UAS-GFP.S65T}Myo31DF[T2]; BDSC stock number: 6793 (henceforth Cha-Gal4), and Elav-Gal4 (P{w[+mW.hs]=GawB}elav[C155], P{w[+mC]=UAS-mCD8::GFP.L}Ptp4E[LL4], P{ry[+t7.2]=hsFLP}1, w[*]; BDSC stock number: 5146). F1 progeny produced from a cross with males from each Gal4 driver line with virgin females collected from the following UAS effector lines were used for analysis: UAS-mAChR-A RNAi (y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02725}attP2; BDSC stock number: 27571) and UAS-mAChR-C RNAi (y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03291}attP2; BDSC stock number: 29612). F1 progeny from a cross between w1118 flies and the appropriate UAS effector lines were used for genetic controls. All flies were raised on standard cornmeal-agar-dextrose-yeast medium in vials kept at room temperature (22-23°C) under a 12 hour light/dark cycle

Pharmacology

Acetylcholine (CAS # : 60-31-1), nicotine (CAS #: 65-31-6), clothianidin (CAS#:210880-92-5) muscarine (CAS #: 2936-25-6), atropine (CAS #: 51-55-8),scopolamine (CAS #: 6533-68-2), piperonyl butoxide (Pestanal) (CAS#:51-03-6) and methyllycaconitine citrate salt (MLA)(CAS#: 112825-05-5) were purchased from Sigma-Aldrich (St. Louis MO, USA) (Milwaukee WI, USA). Tubocurarine (curare) (Cat #:2820) and benzoquinonium dibromide (BD) (Cat #:0424), were purchased from Tocris Bioscience (Minneapolis, MN, USA). Fly saline, modified Hemolymph-like 3 (HL3) (Stewart et al., 1994; de Castro et al., 2014) containing: (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25 N,N-Bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) was used. Saline was maintained at pH 7.1.

Larval development and maintenance

To control for variation in age of flies tested, 6-hour egg collections were employed and embryos were selected and moved to vials housed at room temperature (22-23° C). Larvae were raised until early 3rd instar stage on standard cornmeal-agar-dextrose-yeast medium. They were then selected and moved to food containing various concentrations of food mixed with the compound being assayed at early 3rd instar (pre-wandering larval) stage.

Compound delivery and behavioral analysis in 3rd instar larvae

Ensuring larvae are exposed to a desired concentration of compound during intact feeding is difficult. However, a study by van Swinderen and Brembs (2010) in which flies were fed 0.5mg/mL methylphenidate showed that this concentration was effective in initiating physiological responses similar in time and efficacy to human administration. Thus, controlled concentrations of each compound were added to a food mixture and larvae were placed in this mixture for two time periods to assess the time effect of exposure. Specifically, the compounds were dissolved in one milliliter (mL) of distilled water and mixed with 2 grams of standard fly food. Multiple concentrations were used to generate a dose-response effect and are indicated in molar (M) in the Figure legends. The concentrations used for each compound were kept consistent unless noted. A control (water only), 0.001M, 0.01M, and 0.1M concentration of each compound were utilized in order to maintain a relatively high concentration under the assumption that a diluted concentration would be exposed to the nervous system. Additionally, separate populations of larvae were subjected to two different feeding durations: an acute 20-minute duration and a 24-hour duration, which has been shown to induce molecular alterations that may manifest in alterations in circuit performance (Ping and Tsunoda, 2012), in order to assess time-course of action. In each behavioral test, for each time period, the populations of 3rd instar larvae were collected and fed each concentration plus a control to account for intra-population variability.

For behavioral tests, AChR agonists, nicotine, clothianidin, muscarine, and acetylcholine were assayed. AChR antagonists curare, benzoquinonium dibromide (BD), scopolamine, and atropine were tested. For electrophysiological recordings of the larval sensory-CNS-motor circuit, additional compounds were screened, including acetylcholinesterase inhibitor piperonyl butoxide (Pestanal) and nAChR antagonist, methyllycaconitine (MLA) in addition to the aforementioned compounds.

Locomotor behavioral analysis (body wall contraction rate)

Early 3rd instar locomotor behavior was evaluated as described in Neckameyer (1996) and Li et al. (2001). In brief, single animals were moved to an apple-juice agar (1% agar) surface following exposure to a controlled concentration of ACh agonist, or antagonist in a food vial. The number of body wall contractions, quantified by recording posterior to anterior peristaltic contractions, was counted for 1 minute under dim lighting in room temperature (22°C-23°C). All behavioral analyses took place between 2-5 pm. Larvae were age-matched as previously described.

Electrophysiology in 3rd instar larvae

The technique utilized is described in Dasari and Cooper (2004). In short, a longitudinal dorsal midline cut was made in 3rd instar larvae to expose the CNS. Two of the last segmental nerves were cut and sucked into a suction electrode filled with HL-3 saline and connected to an AxoClamp 2B amplifier. Sharp microelectrodes (~40 MΩ resistance) filled with 3M KCl were used for monitoring larval muscle fiber 6. Proximal stumps of the severed segmental nerves were stimulated to drive sensory neurons that activate central neural circuits, ultimately generating action potentials in motor neurons and EJPs in muscle. The segmental nerves were stimulated with trains of pulses, with the paradigm maintained at 10 pulses per train at 40-60Hz and a train rate of 0.1Hz (S88 Stimulator, Astro-Med, Inc., GRASS Co., USA) (See Fig. 1 A and B for illustration). The stimulus voltage was dependent on the initial observation of evoked responses in muscle, and generally varied between 0.4-1.0 volts based on slight variations in seal resistance in the stimulating electrodes. Segmental nerves were stimulated with a controlled frequency and voltage until a response was observed from an intracellular microelectrode in muscle fiber 6 (m6) contralateral (across the midline) to the stimulus. This allows for the examination of activity within the CNS associated with a controlled afferent nerve stimulus and the associated motor output. The intracellular recording from a defined muscle provides a clear signal in the frequency of evoked responses from CNS input to the motor neurons innervating the muscle. Excitatory junction potentials (EJPs) were observed and analyzed with LabChart 7.0 (ADInstruments, USA). The traces were

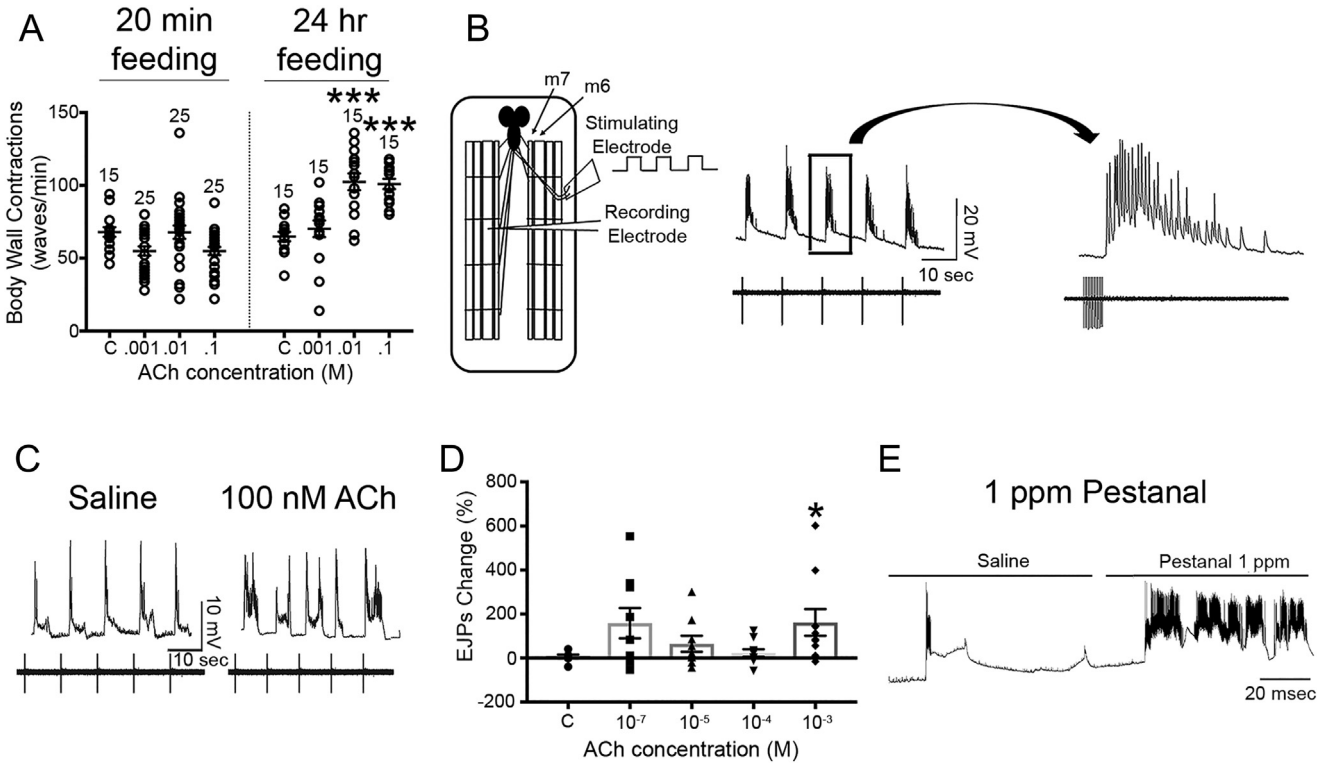

Fig. 1. ACh modulation of locomotion and sensory-CNS-motor circuit excitability A) Average number of body wall contractions for different concentrations of ACh observed over 20-minute and 24-hour feedings. Aligned dot plot is representative of responses among the larval population. Feeding ACh over a 24-hour period generated a significant increase in locomotion, Kruskal-Wallis Test H = 32.49. n = number of larvae and is indicated above each respective dot plot. Ordinary One-Way ANOVA used for 20-minute analysis and Kruskal-Wallis analysis used for 24-hour analysis. Significance was probed relative to control. $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). B) Diagram of sensory-CNS-motor *in situ* larval preparation. EJPs were evoked with 5 stimulus trains to sensory afferents which were recorded in larval muscle 6. C) Sample trace of EJPs in response to 100nM application of ACh. D) Average percent change in EJP frequency in response to application of ACh. n = 7 (control),9,9,10,10 increasing in concentration respectively. Mann-Whitney Rank Sum used for analysis and significance was probed relative to control. $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). E) Sample trace of spontaneous EJPs in response to application of 1ppM (0.00296mM) Pestanal. Pestanal induced an increase in spontaneous EJPs and muscle depolarization in the absence of sensory stimulation.

measured by averaging the EJP frequency in 5 stimulus trains made with normal saline and 5 stimulus trains after exchanging saline with various compounds after 10 minutes of exposure. Individual trains of pulses elicit bursts of EJPs that were quantified through manual counting (see Fig. 1). Once the saline was exchanged, the solution was left on the preparation for 2 minutes before analyzing EJPs, unless responses were observed more rapidly. In some cases, solution was left on the preparation to observe potential changes over a longer time course and is noted in the Results. To ensure preparation viability following the application of each compound, the compounds were washed out and replaced with normal saline. The average frequency of EJPs from each animal and the means from each treatment group were compared. In recordings monitoring EJP changes in the mAChR RNAi knockdown lines (UAS-mAChR-A-RNAi and UAS-mAChR-C-RNAi), an exposure to muscarine (1mM) was used. This concentration represented the most concentrated dosage used for larval recordings. Data were recorded as percent change from a saline solution to a saline solution containing the compound of varying concentration in order to generate a dose-response relationship.

Statistical Analysis

The data presented is expressed as mean +/- standard error of the mean (SEM). The program, SigmaPlot (version 13.0) was for statistical analysis and GraphPad Prism 7 was used for graphing. For locomotor behavioral analyses a One-way ANOVA (ordinary), or One-Way ANOVA on Ranks (Kruskal-Wallis) was used to compare mean body wall contractions in response to each dosage of the compounds of interest and were corrected for multiple comparisons with Dunnett's test or Dunn's test, respectively. The use of parametric (ordinary) or non-parametric (Kruskal-Wallis) analysis was determined after testing for normal distribution in the data using the D'Agostino & Pearson normality test (alpha level = 0.05). Non-parametric statistics were used if the data failed normality testing. Means and significance for the groups at each dosage were compared to controls. Groups fed compounds for different time periods (ie 20-minute feeding groups and 24-hour feeding groups) were analyzed separately and the respective statistical test used to compare means among these groups is indicated in the associated figure legends.

The electrophysiological analysis is presented as percent change from control (saline only), as there is considerable variation among baseline EJP frequencies from preparation to preparation. The average percent changes for the given samples were calculated and compared via Mann-Whitney U Rank Sum analysis for comparison of each percentage change at each concentration relative to a control (saline to saline exchange). The significance indicator above each concentration represents significant difference relative to control. P of ≤ 0.05 is considered as statistically significant. The number of asterisks indicate significance levels: $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***) for all analyses.

RESULTS

Impact of acetylcholine on larval locomotion and sensory-CNS-motor circuit excitability

As stated, a number of techniques to increase circulating concentrations of endogenous modulators and/or exogenous compounds that may mimic or block modulator action have been attempted. We utilized a feeding technique that enables larvae to be consistently exposed to the added compound via normal feeding by mixing 1ml. of compound containing solution with two grams of food. Given that ACh is a primary excitatory transmitter within the *Drosophila* CNS (Lee and O'Dowd, 1999), we predicted to see a significant influence on locomotion using this technique. We found that acute feeding of ACh (20 minutes) did not impact larval locomotor behavior at the doses tested (Fig. 1A; ordinary One-Way ANOVA; $F(3,86)=4.166$, $p \geq 0.05$). Conversely, after a 24-hour feeding, high dose (.01M and .1M) ACh significantly increased locomotion in a dose-dependent manner (Fig. 1A; Kruskal-Wallis test; $H(15,15)=32.49$, $p \leq 0.001$) relative to control.

The longer exposure period likely represents sufficient time for the ingested compound to permeate the gastrointestinal tract (GI) and access neural tissue. However, using the feeding paradigms, while effective in analyzing compound influence over extended periods, it is difficult to decipher the concentration of the compounds gaining access to the CNS. Moreover, is the alteration in locomotor behavior indicative of changes in intrinsic excitability of neurons making up the network driving the behavior? To address this question and to circumvent uncertainties regarding the concentration of compounds that are ultimately exposed to the CNS, we utilized an *in situ* electrophysiological approach in which 3rd instar larvae were dissected and the CNS exposed directly to an added saline containing a known concentration of the compound of interest. We found that low dose ACh (100nM) did not affect EJP frequency (Fig. 1D; Mann-Whitney U test; $U(7,9)=22$, $p \geq 0.05$). As we increased the concentration of ACh to 10μM and 100μM, a reduction in the percent change was observed relative to the lowest dose. An average percent change of 72.4 ± 36.5% and 22.79 ± 16.8% for 10μM and 100μM concentrations, respectively, occurred, which did not represent statistically significant differences relative to control (Fig. 1D). However, at the highest dosage tested, 1mM, 9 out of 10 preparations displayed a positive percent change, averaging 161.89 ± 60.63%, a statistically significant increase relative to control. (Fig. 1D; Mann-Whitney U test; $U(7,10)=10$, $p \leq 0.05$). The overall average percent change exchanging saline to saline was 6.6 ± 9.1% ($n=7$). Therefore, each concentration of applied ACh induced an overall positive percent change in EJP frequency with the 1mM dosage significantly increasing EJP frequency relative to control.

As a means of comparison with responses observed by augmenting ACh concentration through exogenous application, we tested the ability of a specific acetylcholinesterase (AChE) inhibitor to alter the activity within the larval CNS. We tested a specific organophosphate compound, Pestanal, which serves as a prominent commercial insecticide.

Because previous work investigating AChE inhibitor influence on nervous system development in *Drosophila* larvae suggests the use of lower concentrations, we used concentrations ranging from 1ppm to 1000ppm (0.00296mM-2.96mM) (Kim et al., 2011). As 1ppm Pestanal was bath applied, a burst of spontaneous EJPs was observed (see sample Fig. 1E). Heightened activity persisted throughout the experimental time-course, and was present in the absence of sensory stimulation. This was consistent in 5 preparations tested. Depolarization followed by repolarization to membrane potentials close to resting potential were interspersed during Pestanal application. Because of this, we could not assess EJP frequency changes associated with sensory stimulation due to the persistent spontaneous activity and depolarization (Fig. 4E). Higher doses of Pestanal also enhanced activity in a similar manner (data not shown). Thus, the exposure to Pestanal stimulated a substantial increase in spontaneous activity, producing a

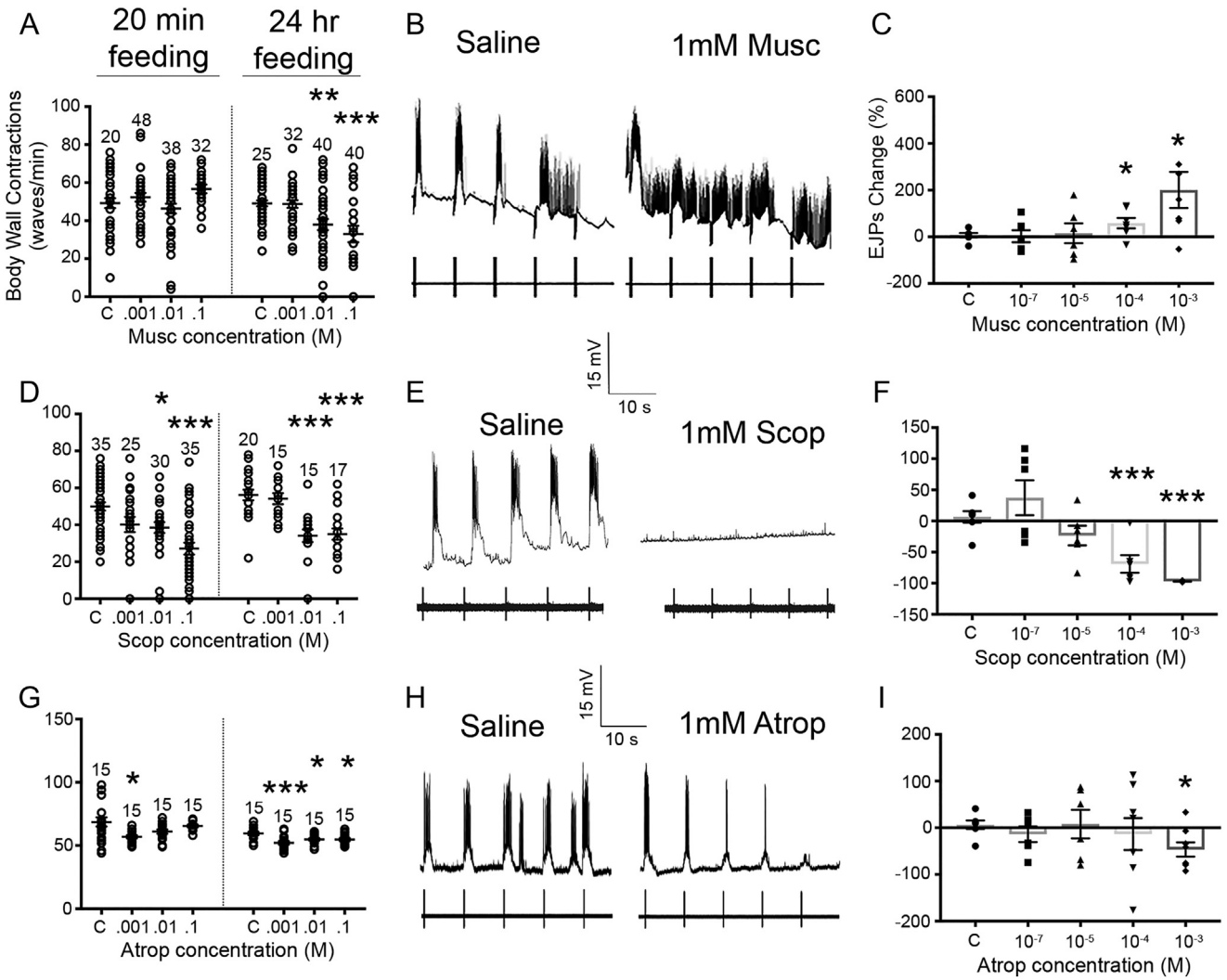

Fig. 2. Muscarinic modulation of locomotion and sensory-CNS-motor circuit excitability A) Average number of body wall contractions for different concentrations of muscarine observed over 20-minute and 24-hour feedings. Aligned dot plot is representative of responses among the larval population. Feeding muscarine over a 24-hour period generated a significant decrease in locomotion One Way ANOVA $F=8.32$. n= number of larvae and is indicated above each respective aligned dot plot. Ordinary One-Way ANOVA used for locomotor analysis and significance was probed relative to control. $p< 0.05$ (*), $p< 0.01$ (**), and $p< 0.001$ (***). B) Sample *in situ* sensory-CNS-motor circuit trace. Application of 1mM muscarine induces a dose-dependent significant increase in EJP frequency. C) Average percent change in EJP frequency in response to application of muscarine n=7 (control),6,6,7,7 increasing in concentration respectively. Mann-Whitney Rank Sum used for *in situ* analysis and significance was probed relative to control. $p< 0.05$ (*), $p< 0.01$ (**), and $p< 0.001$ (***). D) Average number of body wall contractions for different concentrations of scopolamine. Feeding scopolamine generated a significant decrease in locomotion after 20-minute and 24-hour feeding, Kruskal-Wallis Test $H=25.19$, $H=30.0$ respectively. E) Sample trace of EJPs in response to 1mM scopolamine. F) Average percent change in EJP frequency in response to application of scopolamine. Scopolamine reduced EJP frequency. n=7 (control),6,6,6,6 increasing in concentration respectively. G) Average number of body wall contractions for different concentrations of atropine. Feeding atropine generated a significant decrease in locomotion after 20-minute and 24-hour feeding ordinary One-Way ANOVA, $F=3.83$, $F=6.064$, respectively. H) Sample trace of EJPs in response to 1mM atropine. I) Average percent change in EJP frequency in response to application of atropine. Atropine reduced EJP frequency at 1mM. n=7 (control),6,6,8,8 increasing in concentration respectively.

relatively more robust increase in CNS activity compared with acute exposure of exogenous ACh application. Taken together, enhancement of ACh tone through application of an inhibitor of synaptic degradation or by exogenous application produced an enhancement in motor output to m6, indicative of increased excitation of CNS circuits, consistent with previous analysis as measured in motor neurons (Rohrbough and Broadie, 2002). This increase in circuit excitability correlated with increased locomotor speed following chronic ACh exposure.

Muscarinic cholinergic modulation of locomotion and sensory-CNS-motor circuit excitability

Similar to acute ACh treatment, muscarine did not significantly alter locomotor behavior after 20-minute feeding (Fig. 2A; ordinary One-Way ANOVA; $F(3,126)=2.472$ $p \geq 0.05$). However, after a 24-hour feeding, muscarine reduced larval locomotor speed in a dose-dependent manner, with the 0.01M solution significantly decreasing locomotion relative to control (Fig. 2A; ordinary One-Way ANOVA; $F(3,137)=6.729$, $p \leq 0.01$). The most concentrated, 0.1M solution, exhibited a more efficacious influence in significantly decreasing locomotion after chronic exposure (Fig. 2A; ordinary One-Way ANOVA; $F(3,137)=6.729$, $p \leq 0.001$). Therefore, compared to ACh treatment, augmenting muscarinic cholinergic signaling with the mAChR agonist, muscarine, induced the reverse effect, opposing the increase in locomotion observed in response to chronic ACh treatment.

Additionally, we tested the ability of non-selective mAChR antagonists in the intact animal to alter circuit function to further shed light on the pharmacological properties of AChR receptors that influence locomotion. We assayed the competitive mAChR antagonists scopolamine and atropine in our analysis, both of which have been shown to block ACh and muscarine action on *Drosophila* mAChRs in heterologous expression systems (Collin et al., 2013; Xia et al., 2016), and *in vivo* in analysis of olfactory associative learning (Silva et al., 2015). While we predicted to see responses that opposed our agonist-induced behavioral outcomes, we instead observed a number of interesting results. Both acute and 24-hour feeding of scopolamine produced a dose-dependent significant reduction in locomotion (Fig. 2D; Kruskal-Wallis test $H=25.19$, $p \leq 0.001$; Kruskal-Wallis test $H=30$; $p \leq 0.001$). At 0.01M, scopolamine significantly diminishing locomotor behavior after just 20 minutes of feeding (Fig. 2D; Kruskal-Wallis Test $H(37,30)=25.19$, $p \leq 0.05$). Likewise, atropine reduced larval locomotion after both acute and long-term exposure, mimicking scopolamine (Fig. 2G; ordinary One-Way ANOVA; $F(3,64)=3.83$, $p \leq 0.01$; ordinary One-Way ANOVA; $F(3,56)=6.064$, $p \leq 0.01$). Thus, both scopolamine and atropine treatment surprisingly mirrored muscarinic impact on locomotion, decreasing locomotor speed significantly relative to control (Fig. 2A,G).

The suppressive effects on locomotion observed in response to both mAChR antagonists as well as muscarine was surprising and may highlight the potential that persistent exposure may desensitize receptors. Additionally, the particular potency of scopolamine and atropine may also bring to light the potential for off-target effects, as no significant alteration in behavior was observed in response to acute feedings of ACh or muscarine. To address some of these uncertainties and to assess whether these compounds altered the excitability a sensory-CNS-motor circuit upon application directly to the CNS, we recorded their impact *in situ*. We identified a dose-dependent increase in sensory-CNS-motor excitability in response to bath application of muscarine. Both the 100μM and 1mM doses induced a significant increase in EJP frequency relative to control (Fig. 2C; Mann-Whitney U test $U(7,7)=7$, $p \leq 0.05$; Mann-Whitney U test $U(7,7)=7$, $p \leq 0.05$). While 100nM and 10μM concentrations produced variable responses, increasing the concentration to 100μM and 1mM enhanced circuit activity, with the highest dosage producing a robust positive percent change of 200.6 ±77.4%, representing a significant increase relative to control (Fig. 2C; Mann-Whitney U test $U(7,7)=7$, $p \leq 0.05$). Thus, in response to acute, *in situ* exposure, in a manner similar to ACh, muscarine increased the excitability of the evoked sensory-CNS-motor circuit. Moreover, unlike in the intact animal, both mAChR antagonists opposed the excitatory influence induced by muscarine. Upon exposure to high dose (1mM) scopolamine, sensory-CNS-motor circuit activity rapidly shut down, reducing EJP frequency to 0 within 20 seconds (Fig. 2E). Moreover, the presence of 100μM and 1mM scopolamine produced an average percent change of -69.0 ±14.1% and -97.1 ±0.4% respectively, representing statistically significant reductions relative to control (Fig. 2F; Mann-Whitney U test $U(7,6)=1$, $p \leq 0.001$; Mann-Whitney U test $U(7,6)=0$, $p \leq 0.001$). Importantly, we noted that spontaneous quantal events (miniature EJPs; mEJPs) were present throughout the recording, suggesting the reduction in activity observed at m6 was not a result of post-synaptic GluR inhibition (Fig. 2E). While high dose scopolamine reduced activity reliably, atropine exposure did not elicit consistent effects. Exposing the nervous system to 100nM-100μM atropine resulted in increased EJP frequency in half the preparations tested and decreased in half, displaying substantial variability (Fig. 2I). However, 1mM atropine did reduce activity in 7 out of 8 preparations, inducing a significant percent change of -46.7 ±15.2% (Fig. 2I; Mann-Whitney U test $U(7,8)=8$, $p \leq 0.05$). Therefore, at 1mM, both mAChR antagonists reduced CNS activity, with scopolamine inducing a more consistent reduction at lower concentrations, opposing the excitatory influence of acute muscarine and ACh treatment. Taken together, activation of mAChRs by muscarine application enhanced sensory-CNS-motor excitability while both non-selective mAChR competitive antagonists opposed this effect. This influence is at odds with the action of muscarine in the intact animal, which induced a general inhibition of locomotor behavior.

Muscarinic receptor RNAi-mediated knockdown impact on larval locomotion

As noted, we identified some interesting discrepancies in muscarinic cholinergic influence of CNS activity in the intact

and semi-intact analysis. Most notably, augmenting muscarinic cholinergic signaling with muscarine and blocking mAChRs induced a similar trend in reducing locomotor speed. Additionally, while muscarine treatment enhanced circuit excitability *in situ*, it reduced locomotor behavior after chronic exposure in the intact animal. To address these questions and to identify specific mAChRs that may be prominent in altering the intrinsic properties of neurons within the locomotor network, we utilized a RNAi-mediated approach. The *Drosophila* genome contains three separate mAChR genes: mAChR-A, B, and C (Collin et al., 2013; Xia et al., 2016). The A and C-type receptors are closely homologous with mammalian mAChRs and display similar pharmacological properties, including activation by muscarine and blockade by scopolamine and atropine (Collin et al., 2013; Xia et al., 2016), while the B-type receptors display little affinity for these compounds. Given the significant influence on network activity observed upon exposure to the assayed compounds, we focused our attention on the mAChR-A and C-type receptors. These receptors have both been shown to be expressed in the 3rd instar larval brain (Silva et al., 2015; Xia et al., 2016) and display similar pharmacological properties in heterologous expression systems (Ren et al., 2015; Xia et al., 2016). Moreover, since the impact on locomotor behavior can be modulated at multiple levels of the network, we used the Gal4-UAS system to drive mAChR-A and C knockdown pan-neuronally with the Elav-Gal4 driver line, in sensory and CNS cholinergic neurons using the ChaT-Gal4 driver, and in motor neurons, directly, using the D42-Gal4 driver line. A previous study utilizing the UAS-mAChR-A-RNAi line used in the present study shows a ~65% reduction in expression in neural tissue upon knockdown using the pan-neuronal, Elav-Gal4, driver (Bielopolski et al., 2018). Likewise, driving knockdown via the UAS-mAChR-C-RNAi line has been shown to be efficacious in reducing mAChR-C activity in response

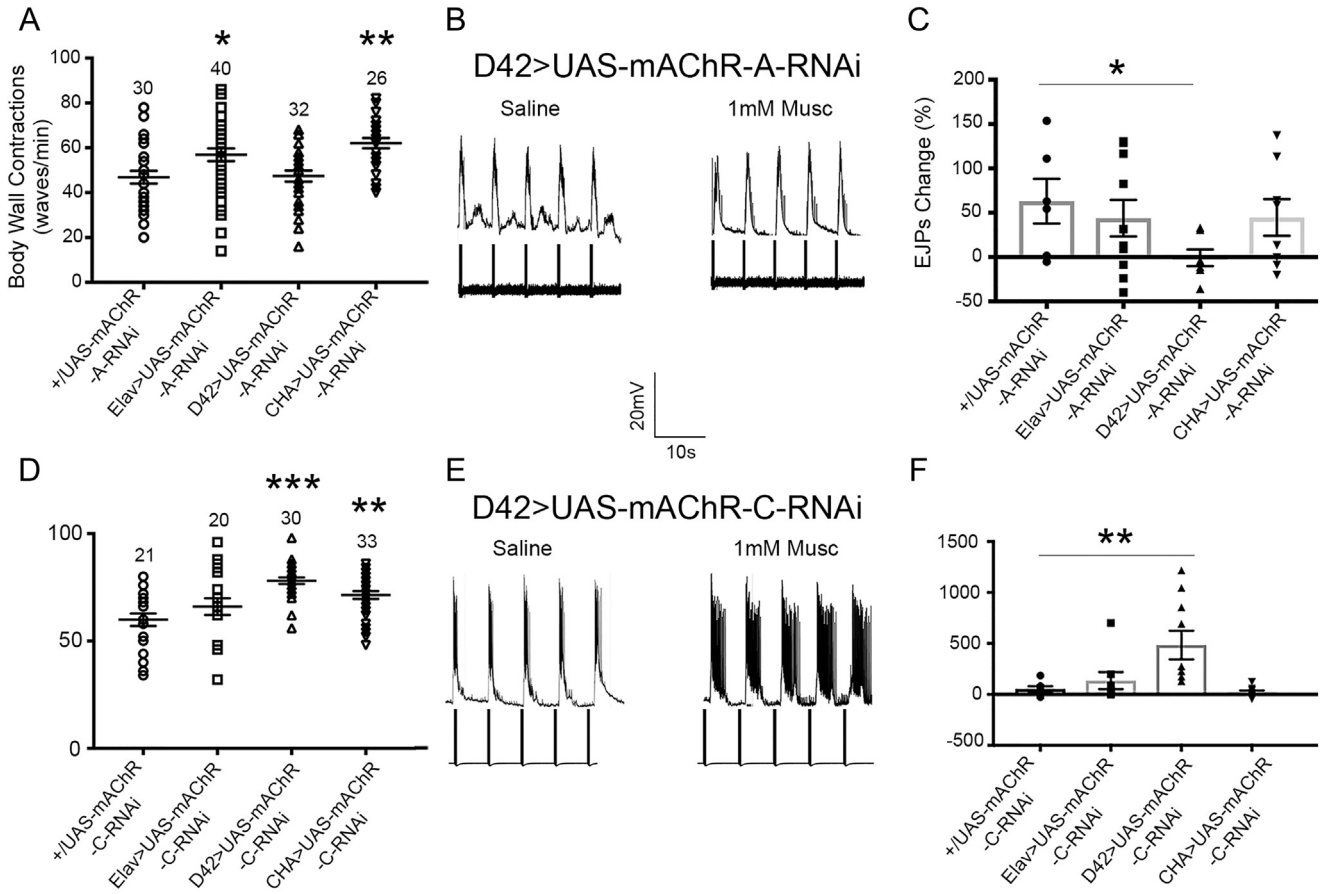

Fig. 3. Muscarinic receptor knockdown impact on locomotion and sensory-CNS-motor circuit excitability. A) Average number of body wall contractions in RNAi-mediated knockdown of mAChR-A in select neurons. Aligned dot plot is representative of responses among the larval population. Reduced expression of mAChR-A pan-neuronally and in cholinergic neurons resulted in increased locomotive speed. n= number of larvae and is indicated above each respective dot plot. Ordinary One-Way ANOVA used for locomotor analysis and significance was probed relative to control. $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). B) Sample *in situ* sensory-CNS-motor circuit trace in D42-Gal>UAS-mAChR-A-RNAi larvae. C) Average percent change in EJP frequency in response to application of muscarine. Reduced expression of mAChR-A in motor neurons attenuates the increase in EJP frequency in response to 1mM muscarine. n=6 (control),9,7,8 from left to right. D) Average number of body wall contractions in RNAi-mediated knockdown of mAChR-C in select neurons. Reduced expression of mAChR-C in motor neurons and cholinergic neurons resulted in increased locomotive speed. E) Sample *in situ* sensory-CNS-motor circuit trace in D42-Gal>UAS-mAChR-C-RNAi larvae. F) Average percent change in EJP frequency in response to application of muscarine. Reduced expression of mAChR-C in motor neurons increases EJP frequency in response to 1mM muscarine. n=6 (control),8,7,8 from left to right. Mann-Whitney Rank Sum used for *in situ* analysis and significance was probed relative to control. $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

to endogenous ACh activation in behavioral assays (Xia et al., 2016).

We first assessed the impact of receptor knockdown on locomotor behavior. Driving mAChR-A knockdown pan-neuronally, a technique we predicted to mirror network inhibition by pharmacological blockade, produced a significant increase in locomotor speed relative to control (56.9 ±2.85 vs 46.9 ±2.82 mAChR-A-RNAi vs control; Fig. 3A; ordinary One-Way ANOVA; $F(3,124)=6.92$, $p \leq 0.05$). Similarly, pan-neuronal knockdown of mAChR-C produced an increase in locomotion, although this difference relative to control was not statistically significant (66.0 ±3.89 vs 59.9 ±2.9; Fig. 3D; ordinary One-Way ANOVA, $F(3,100)=10.24$ $p \geq 0.05$).

While pan-neuronal knockdown most closely mimics broad pharmacological network manipulation, the complexity of the circuits driving locomotion make interpretation of the impact of single receptors on the behavior quite challenging. To pare down the influence on different levels of the network, we targeted mAChR knockdown in cholinergic neurons providing motor input (sensory-CNS) and also on motor output by knocking the receptors down in motor neurons, directly. The rationale for driving knockdown in cholinergic neurons, aside from their known prominence in the CNS relaying sensory input to motor output relevant to our electrophysiological analysis (Hasegawa et al., 2016), is that mAChRs are often expressed pre-synaptically, acting as autoreceptors to modulate synaptic release probability (Nordstrom and Bartfai, 1980). As observed in pan-neuronal knockdown, reduction of mAChR-A receptor expression in cholinergic neurons induced a significant increase in larval locomotion relative to control (62.1 ±2.28 vs 46.9 ±2.82; Fig. 3A; ordinary One-Way ANOVA; $F(3,124)=6.92$, $p \leq 0.01$). Likewise, knockdown of mAChR-C in these neurons also significantly increased locomotion relative to matched control (71.4 ±1.79 vs 59.9 ±2.91; Fig. 3D; ordinary One-Way ANOVA, $F(3,100)=10.24$ $p \leq 0.01$). Thus, knocking down both mAChRs in neurons providing sensory and central input shifted the intact network toward a more excitable state, enhancing the behavior. Additionally, locomotion may be impaired if the intrinsic properties of motor neurons are altered, as their input-output efficacy is crucial in guiding rhythmic muscle contraction. Knockdown of mAChR-A in motor neurons did not significantly alter locomotor behavior (Fig. 3A; ordinary One-Way ANOVA; $F(3,124)=6.92$, $p \geq 0.05$). However, reducing expression of mAChR-C receptors in motor neurons drastically increased locomotor speed relative to control, inducing a robust increase to an average of 78.1 ±1.45 waves/min (Fig. 3D; ordinary One-Way ANOVA, $F(3,100)=10.24$ $p \leq 0.001$). Therefore, taken together, knockdown of mAChRs at multiple levels of the network driving locomotion revealed a crucial role for both mAChRs, and their influence appears to be primarily inhibitory under physiological conditions. The general inhibitory influence exhibited by mAChR-C and mAChR-A in the intact animal supports the suppressive effects identified after pharmacological augmentation of network activity by muscarinic signaling through persistent muscarine exposure.

Muscarinic receptor RNAi-mediated knockdown impact on sensory-CNS-motor circuit excitability

In order to address how mAChR knockdown may impact the excitability of an evoked circuit driving muscle contraction, we again turned to an *in situ* electrophysiological approach using the same RNAi knockdown lines. We exposed each line to 1mM muscarine, a concentration that induced a significant increase in EJP frequency in CS preparations (see Fig. 2), to identify how reduced mAChR expression may alter sensitivity to the compound when exposed to the nervous system. We anticipated a general attenuation of the enhancement EJP frequency in response to 1mM muscarine treatment in the RNAi lines; however, behavioral analysis utilizing the RNAi lines suggests the potential for a more inhibitory influence of these receptors on network activity. We first noted that both effector control lines (UAS-mAChR-A-RNAi/ +, UAS-mAChR-C-RNAi/+) and a control driver line (Elav-Gal4/+, data not shown) exhibited a positive percent change in EJP frequency when exposed to 1mM muscarine, as was observed in CS preparations (Fig. 3C, F). Pan-neuronal knockdown of mAChR-A induced a slight, but non-statistically significant reduction in positive percent change in EJP frequency upon muscarine application in relation to matched control (43.91 ±20.6% vs 63.0 ± 25.1%; Fig. 3C; Mann-Whitney U test $U(6,10)=25$, $p \geq 0.05$). Likewise, a similar reduction in EJP frequency was observed when mAChR-A was knocked down in cholinergic neurons, although this, again, did not reach a level of statistical significance (44.58 ±20.7% vs 63.0 ±25.1%; Fig. 3C; Mann-Whitney U test $U(6,8)=20$, $p \geq 0.05$). However, when mAChR-A expression was reduced in motor neurons, a significant attenuation of the positive percent change in response to muscarine treatment was observed, as bath application of muscarine did not increase EJP frequency in the D42-Gal4 > UAS-mAChR-A-RNAi larvae (Fig. 3C; Mann-Whitney U test $U(6,7)=6$, $p \leq 0.05$). Specifically, an average negative percent change of 0.91 ±9.37% was observed in these preparations (Fig. 3C). Therefore, mAChR-A knockdown did not significantly alter muscarine sensitivity when targeted pan-neuronally or in sensory-CNS cholinergic neurons, but did induce a significant reduction in motor neuron release probability in response to evoked input when altered in motor neurons.

Furthermore, while there was a similarity in the effect of reducing expression of both receptor subtypes in the intact animal, we identified a distinct difference *in situ.* Pan-neuronal knockdown of mAChR-C induced an enhancement in sensitivity to muscarine application relative to matched control, although this increase did not reach a statistically significant level (134.52 ±84.1% vs 52.83 ±27.4%; Mann-Whitney U test $U(7,8)=24$, $p \geq 0.05$). While mAChR-C knockdown in cholinergic neurons did not significantly alter EJP frequency change in response to muscarine treatment (Fig. 3F; Mann-Whitney U test $U(7,8)=19$, $p \geq 0.05$) reducing expression in motor neurons significantly increased muscarine sensitivity, inducing an average positive percent change in EJP frequency of 484.62 ±140.4, with all 7 preparations increasing in frequency (Fig. 3F; Mann-Whitney

U test U(7,7)=2, p≤0.001). Therefore, just as we observed in the analysis of mAChR-A, our electrophysiological analysis revealed a conspicuous role for the C-type receptor in regulating the input-output efficacy of motor neurons in response to synaptic input. The reverse in direction of EJP frequency change in response to muscarine exposure implies an opposing influence of the A and C-type receptors in motor neurons, with reduction of expression of one subtype shifting the relative weight of influence toward the other. Taken together, we reveal that both A-type C-type receptors contribute to the modulation of excitability of a functional sensory-CNS-motor circuit recruited during larval crawling, with particularly intriguing opposing roles in regulation of the excitability of motor neurons innervating m6.

Nicotinic cholinergic modulation of locomotion

While the neuromodulatory influence of muscarinic cholinergic signaling on neural circuit efficacy is poorly understood, the vast majority of knowledge regarding cholinergic transmission in *Drosophila* larvae centers on nicotinic acetylcholine transmission (Lee and O'Dowd, 1999; Rohrbough and Broadie, 2002). It is established that the bulk of excitatory transmission within the larval CNS is mediated by ACh through nAChRs (Lee and O'Dowd, 1999); however, a comprehensive pharmacological screen and detection of how they may alter intrinsic properties of the neural network driving locomotion in the intact or semi-intact animal warrants investigation. Due to the complexity of the nicotinic cholinergic system, we chose to utilize a strictly pharmacological approach to investigate the impact of nicotinic cholinergic signaling on behavior and sensory-CNS-motor circuit excitability. Unlike ACh and muscarine treatment, we found nicotine to be potent in altering locomotor behavior after an acute, 20-minute feeding, reducing larval locomotion after both acute (at 0.1M) and 24-hour feeding (at 0.01M) (Fig. 4A; Kruskal-Wallis test; H(23,27)=25.61, p≤0.001; Kruskal-Wallis test; H(31,25)=88.9, p≤0.001) suggesting a conspicuous role for nAChRs in regulating locomotion. It is noted that the 24-hour exposure to 0.1M nicotine represents a lethal dosage, with 53 out of a total 55 (96%) larvae tested in each behavioral paradigm dying after 24-hour exposure (Fig. 4A); however, data were included in order to depict the continued reduction/abolishment in behaviors as concentration was increased. We considered the possibility that a high dose of nicotine was rapidly desensitizing nAChRs within the CNS, given the potency with which we observed inhibition of the behaviors of interest. Additionally, it is known to be highly lipophilic and may act to alter cell physiology by means other than via activation of membrane nAChRs, including through the stimulation of Ca^{2+} release from intracellular stores (Hukkanen et al., 2005). Thus, to observe if our rapid shutdown of activity was unique to nicotine, we tested an additional non-selective nAChR agonist, clothianidin in the intact animal. Clothianidin, like other neonicotinoids, acts with high specificity to at least three different subtypes of insect nAChRs (Simon-Delso et al., 2015) and does not readily cross the blood-brain barrier in vertebrates due to its highly charged nature (Sheets et al.,

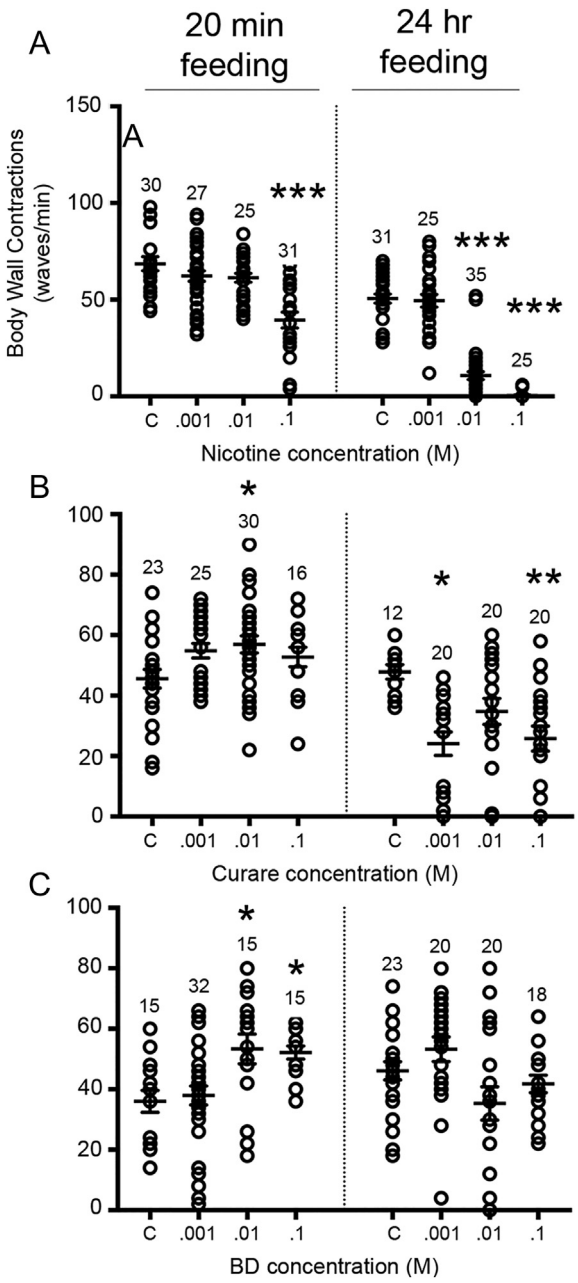

Fig. 4. Nicotinic cholinergic modulation of locomotion. A) Average number of body wall contractions for different concentrations of nicotine observed over 20-minute and 24-hour feedings. Aligned dot plot is representative of responses among the larval population. Feeding nicotine over a 20-minute and 24-hour period generated a significant decrease in locomotion, Kruskal-Wallis Test H=25.61, H=88.9, respectively. n= number of larvae and is indicated above each respective dot plot. Significance was probed relative to control. p< 0.05 (*), p< 0.01 (**), and p< 0.001 (***). B) Average number of body wall contractions for different concentrations of curare observed over 20-minute and 24-hour feedings. Acute feeding of curare increased locomotion while feeding over 24-hour period generated a significant decrease in locomotion, Kruskal-Wallis Test H=7.087, H=16.07, respectively. C) Average number of body wall contractions for different concentrations of BD observed over 20-minute and 24-hour feedings. Acute feeding of BD increased locomotion while feeding over 24-hour period did not alter locomotive speed, ordinary One-Way ANOVA F=5.765, F=3.485, respectively.

2016). Surprisingly, we identified 100% lethality at each concentration tested after 24-hour exposure, showing clothianidin displays relatively higher toxicity than nicotine. Moreover, a significant reduction in locomotion was observed within 20 minutes after exposure, as locomotion was abolished at each concentration tested (data not depicted as all individuals exhibited zero body wall contractions). Thus, clothianidin proved more potent in inhibiting locomotor behavior.

The inhibitory influence of nAChR agonists at each dosage tested was a bit surprising given the predominate excitatory influence within central neurons in larvae (Lee and O'Dowd, 1999). To address how blocking nAChRs *in vivo* altered locomotor behavior, we tested the role of two non-selective, competitive nAChR antagonists, tubocurarine (curare) and benzoquinonium dibromide (BD), which have previously been tested on the larval heart (Malloy et al., 2016) and in additional experimentation in cultured embryonic neurons in *Drosophila* in order to block synaptic transmission (Lee and O'Dowd, 1999; Ping and Tsunoda, 2012). While we predicted to see responses that opposed our agonist-induced behavioral outcomes, we instead observed a number of interesting results. Acute feeding (20 minutes) of both curare and BD produced an increase in locomotion, opposing the inhibitory influence induced by acute nicotine feeding, with significant increases in response to 0.01M curare treatment and 0.01M and 0.1M BD (Fig. 4B,C; Kruskal-Wallis Test $H(23,30)=7.087$, $p \leq 0.05$; ordinary One-Way ANOVA $F(3,73)=5.765$, $p \leq 0.05$, $p \leq 0.05$ respectively). However, after 24-hour exposure to high doses of both compounds, body wall contractions were reduced, with significant reductions induced by curare at 0.001M and 0.1M and a trend suggesting reduced locomotion induced by BD that was not statistically significant (Fig. 4B,C; Kruskal-Wallis Test H $(12,20)=16.07$, $p \leq 0.05$; Kruskal-Wallis Test $(12,20)=16.07$, $p \leq 0.01$; ordinary One-Way ANOVA $F=3.85$, $p \geq 0.05$ respectively). Therefore, the responses to the two nAChR antagonists tested were similar, but the likeness in regard to nicotine action in regulating the circuit performance was surprising and points to the potential for nicotine-induced nAChR desensitization at high doses, particularly after chronic exposure.

Nicotinic cholinergic modulation of sensory-CNS-motor circuit excitability

We detected a reduction in locomotion that was enhanced as the concentration was increased when larvae were exposed to nicotine; however, it is noted that the concentration utilized was high in order to ensure the compound permeated the GI tract and accessed neural tissue. To probe the impact of a larger range of concentrations in a more controlled condition, we utilized the *in situ* electrophysiological approach. We considered the potential for a reverse in direction of influence on circuit excitability as the concentration of nicotine was increased so we extended our analysis, adding a 10nM nicotine treatment. Upon application we noted that low doses of nicotine (10nM-10μM) induced an enhancement in EJP frequency, causing a high frequency burst of activity upon sensory stimulation. The percent change of 36.1 ± 13.9% induced by 10μM nicotine application represented a significant increase relative to control (Fig. 5B; Mann-Whitney U test $U(7,5)=4$, $p \leq 0.05$). However, we noticed a drastic change as we increased the concentration 10-fold from 10μM to 100μM. Upon application of 100μM nicotine, we observed an initial high frequency burst of EJPs and then a rapid shutdown, which lasted throughout the experimental period (Fig. 5CB,C). Subsequent stimulations did not elicit EJPs in m6 and we observed a significant average percent change of -98.1 ±0.7% (Fig. 5B; Mann-Whitney U test $U(7,5)=0$, $p \leq 0.01$). Likewise, we noticed a similar response at 1mM, where a significant average percent change of -97.52 ±0.7% was observed (Fig. 5B; Mann-Whitney U test $U(7,5)=0$, $p \leq 0.01$). In each case, the reduction of activity was detected within 20 seconds following application. Again, to observe if this rapid shutdown of EJP activity was unique to nicotine, we tested the non-selective nAChR agonist, clothianidin, on the exposed CNS. As observed in the behavioral assessment, we found clothianidin to more potent in impacting circuit activity, abolishing activity in response to sensory stimulation at lower concentration than nicotine. Low dose clothianidin (100nM) induced an average percent change of 11.0 ± 28.5% (Fig. 5D; Mann-Whitney U test $U(7,6)=19$, $p \geq 0.05$) which did not represent a statistically significant change relative to control; however, as the concentration was increased 10-fold, a vigorous shutdown of activity was observed within 30 seconds of compound application. Specifically, 10μM application induced an average percent change of -92.1 ±2.1% and 1mM application induced an average percent change of -94.5 ±.8%, each of which represented significant reductions relative to control (Fig. 5D; Mann-Whitney U test $U(7,5)=0$, $p \leq 0.001$; Mann-Whitney U test $U(7,5)=0$, $p \leq 0.001$). Since we observed an abolishment of activity as low as 10μM that persisted in the presence of 1mM, we omitted the 100μM concentration. Thus, consistent with intact behavioral analysis, we found that clothianidin was more potent in reducing circuit excitability relative to nicotine, abolishing activity at a concentration as low as 10μM. Taken together, experiments with semi-intact preparations show low-dose nicotine significantly enhances sensory-CNS-motor circuit excitability while high dose nicotine reduced activity in the CNS, inhibiting circuit activity with clothianidin mirroring the effect of nicotine on the exposed CNS.

In light of these data, we predicted that the two assayed non-selective nAChR antagonists would reduce activity driving input to m6 with high potency through inhibition of excitatory nAChRs in the CNS. However, again, we observed responses inconsistent with predicted outcomes. Both curare and BD were not potent in reducing circuit activity. Only 1mM application of BD induced a reduction of EJP frequency at m6, representing an average percent change of -97.6 ±9.7%, a significant reduction relative to control (Fig. 5H; Mann-Whitney U test $U(7,6)=20$, $p \geq 0.05$; Mann-Whitney U test $U(7,7)=2$, $p \leq 0.001$). Curare application induced positive average percent changes at each

concentration tested; however, in each case, these did not represent significant differences relative to control (Fig. 5F; Mann-Whitney U test, U = 20,16,10.5,7), p ≥ 0.05). Since we observed similar enhancement of activity in our behavioral analysis following acute exposure, which ultimately reduced after 24 hours, we tested the efficacy of both antagonists after a 15-minute bathing period. After this time, we noticed a slight reduction in excitability relative to the previous time points, but activity only returned to baseline with little attenuation in the frequency of evoked EJPs (data not shown). Therefore, although longer exposure may reduce activity as indicated in our behavioral assessment, curare was not potent in blocking nAChR-mediated modulation of circuit activity, and in the time course of experimentation, only 1mM BD significantly reduced EJP frequency relative to control (Fig. 5H; Mann-Whitney U test U(7,7)=2, p ≤ 0.001).

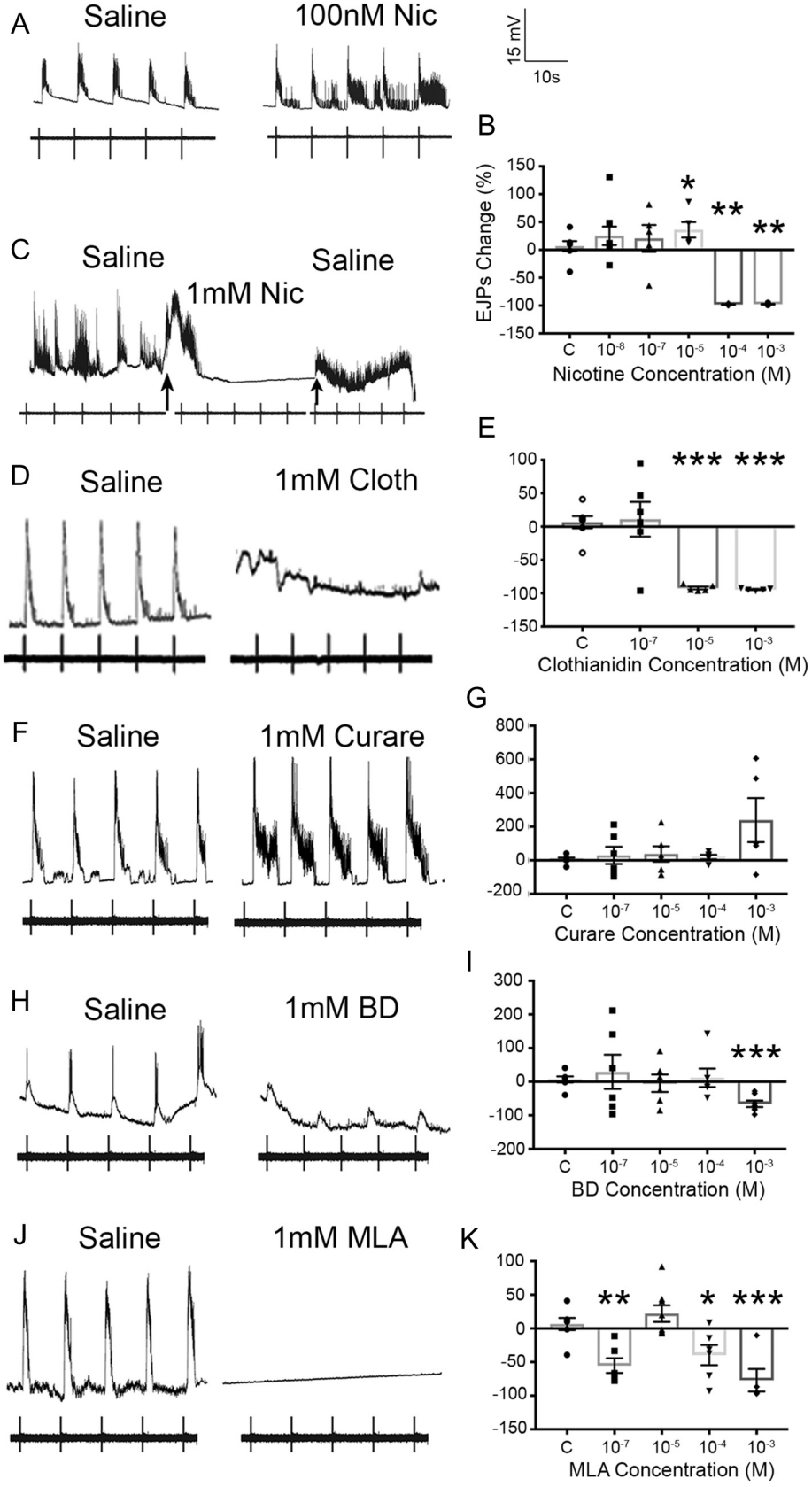

Fig. 5. Nicotinic cholinergic modulation of sensory-CNS-motor circuit excitability. A) Sample trace of 100 nM nicotine and saline. At 100 nM nicotine increased EJP frequency. B) Average percent change EJPs in response to nicotine application. Low dose nicotine increased EJP frequency while high dose nicotine decreased frequency. n = 7(control),8,5,5,5 increasing in concentration, respectively Mann-Whitney Rank Sum used for *in situ* analysis and significance was probed relative to control. p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***). C) Sample trace displaying response to 1mM nicotine exposure. 1 mM nicotine abolishes CNS activity after brief excitation (note ~10sec post application). The first arrow indicates when nicotine was added. The second arrow shows when saline was used to wash out the nicotine. Upon the saline wash, activity returned. D) Sample trace displaying response to 1mM clothianidin. E) Average percent change in EJPs in response to clothianidin application. High dose of clothianidin also shut down activity similar to nicotine (trace not shown). n = 7 (control), 6, 5, 5 increasing in concentration respectively. F) Sample trace displaying response to 1mM curare. G) Average percent change in EJPs in response to curare application. Curare does not alter EJP frequency. n = 7(control), 6,6,5,5 increasing in concentration respectively H) Sample trace displaying response to 1mM BD. I) Average percent change in EPJP in response to BD application. High does BD reduces EJP frequency. n = 7(control), 6,6,6,7 increasing in concentration respectively. J) Sample trace displaying response to 1mM MLA. K) Average percent change in EPJP in response to MLA application. MLA application reduces EJP frequency. n = 7(control), 6,8,6,5 increasing in concentration respectively.

Additionally, we tested the nAChR antagonist, MLA, which has been shown in vertebrates to be more subtype specific, with relatively higher affinity for the α7 and α6 subunits in the rodent CNS (Albuquerque et al., 2009; Halff et al., 2014). The Dα7 receptor in *Drosophila* is highly expressed in the CNS (Chintapalli et al., 2007; Celniker et al., 2009; Gramates et al., 2017) and displays high sequence similarity (~42-43%) with its mammalian counterpart (Grauso et al., 2002). Thus, the impact of MLA exposure on sensory-CNS-motor circuit activity may highlight the importance of the Dα7 in regulating this circuit; however, the selectivity of this compound and receptor-specific affinity is poorly defined in this model. Upon bathing the preparation in high dose (1mM) MLA we noticed rapid and robust shutdown of activity, similar to what was observed with high concentration nicotine (Fig. 5C,I). Evoked activity pertaining to the sensory stimulation and spontaneous EJPs were completely abolished within 10 seconds post-application, suggesting rapid reduction of endogenous, tonic activity in addition to evoked sensory-CNS-motor activity. We also noted mEJPs were still present during the recording suggesting that this compound was not blocking GluRs at the NMJ. This was observed in 7 out of 7 preparations tested and lasted the entirety of the experimental time-course (continued abolishment of activity after 2-minutes post-application). The overall average percent change of -77.0 ± 16.8% represented a significant reduction in EJP frequency relative to control (Fig. 5J; Mann-Whitney U test $U(7,5)=1$, $p \leq 0.001$). As the concentration was reduced, EJPs were still present but a significant reduction in EJP frequency was observed in response to 100nM and 100μM treatments (Fig. 5J; Mann-Whitney U test $U(7,6)=2$, $p \leq 0.01$;Mann-Whitney U test $U(7,6)=5$, $p \leq 0.05$). Therefore, of the nAChR antagonists tested, MLA was the most potent in reducing circuit excitability. Taken together, we identify a crucial role for nAChRs in altering the excitability of a sensory-CNS-motor circuit driving abdominal muscle 6 contraction and reveal their significant influence in regulating locomotor behavior.

DISCUSSION

While strides have been made in identifying the pharmacological properties and the contribution of cholinergic receptor subtypes to neural circuit activity in the *Drosophila* model, considerable work remains. Insights into acetylcholine receptor (AChR) properties have expanded through the use heterologous expression systems; however, how these properties are translated to the level of neural circuits and networks have not yet been fully addressed. In this study, we utilized a primarily pharmacological approach to investigate the role of nicotinic cholinergic and muscarinic cholinergic signaling in larval *Drosophila*. We have provided a comprehensive pharmacological assessment of the role of both ACh receptor subtypes in larval locomotion and in modulation of the excitability of an evoked sensory-CNS-motor circuit recruiting abdominal motor neuron firing. The role of important neuromodulators in altering neural circuit properties in the *Drosophila* CNS warrants further investigation, as much of our current knowledge stems from *in vitro* analysis. This work provides enhanced resolution into ACh influence on circuit efficacy regulating larval locomotor behavior. Combined with recent endeavors mapping circuit connections underlying this behavior, determining how neuromodulators modify these neural connections is essential in gaining a full understanding of network function.

Acetylcholine treatment enhances sensory-CNS-motor circuit excitability and locomotor speed

It is apparent that both peripheral and central neuronal activity are integral in contributing to the locomotor circuit during larval crawling (Hughes and Thomas, 2007; Song et al., 2007; Cheng et al., 2010). This signifies the likelihood of a prominent influence of ACh in modulating the efficacy of sensory to motor information transfer. We have identified that, while acute feeding did not induce a significant change relative to control, chronically enhancing ACh tone through feeding significantly increased crawling speed. This suggests ACh is integral in regulating excitatory input onto motor neurons and/or within central circuits mediating coordinated motor neuron firing, which influence body wall wave propagation. Indeed, Hasegawa et al. (2016) identified premotor cholinergic neurons that synapse directly with motor neurons and regulate intersegmental bursting activity during fictive locomotion. These neurons cause local, intersegmental muscle contraction when activated, suggesting they excite motor neurons intersegmentally and contribute to the regulation of timing of muscle contraction during locomotion (Hasegawa et al., 2016). We have shown here that prolonged exposure to ACh maintains this excitatory influence on larval speed. Moreover, we identified a similar response of increased the excitation of evoked sensory-CNS input onto m6. The general excitatory responses we uncovered in response to acute ACh application supports previous electrophysiological analysis activating this circuit in a similar manner (Baines et al., 1999; Rohrbough and Broadie, 2002; Hasegawa et al., 2016). A surprising outcome, however, was the identification of variability in responses as the dosage was increased. For instance, at 100μM, in 10 preparations tested, 3 exhibited a reduction in EJP frequency. It is possible that nAChRs may be blocked or desensitized as previously suggested as ACh dosage is increased, as channel block may occur at high doses (Barik and Wonnacott, 2009). The increase in activity in response to exogenous ACh application was recapitulated following AChE inhibitor (Pestanal) exposure; however, a drastic difference in activity was observed. While exposing the semi-intact preparation to exogenous ACh did not induce a substantial increase in spontaneous activity, Pestanal application significantly enhanced EJP frequency even in the absence of sensory stimulation. This illuminates the potential that the endogenous concentration at synapses within the CNS may be higher than the applied doses when CNS circuits are spontaneously active, and the 1mM dosage may represent, more closely, the concentration at active synapses.

Muscarinic receptors modulate locomotor behavior and sensory-CNS-motor circuit excitability

While it has become apparent that ACh plays an important role in regulating network activity driving larval locomotion, deciphering how the components underlying ACh signaling (ie specific receptor subtypes) differentially contribute to circuit activity remains largely indeterminate. Thus, our primary goal was to parse the components of cholinergic signaling apart and to illuminate the receptor subtypes that facilitate ACh regulation of circuit excitability while exposing important pharmacological properties in the larval brain. Our pharmacological analysis revealed a critical role for muscarinic cholinergic signaling in the locomotor network. We have shown here that mAChR-A and C-type receptors both contribute to modulation of locomotion and likely induce opposing effects on membrane excitability at multiple levels of the network. While we observed consistency in the impact of ACh in the intact and semi-intact animal, some variation in responses were observed with muscarinic pharmacological treatment, prompting further investigation using receptor RNAi knockdown.

We first noted that some interesting discrepancies arose in the pharmacological and RNAi knockdown results. The overall impact on locomotor behavior through mAChR signaling appears to be inhibitory in the intact animal. Network activation of mAChRs through pharmacological exposure to muscarine reduced locomotor speed, while reduction of expression of both A and C-type receptors generally increased locomotion. A notable and curious exception was the similar response observed with scopolamine and atropine treatment in the intact animal. Scopolamine, particularly in the *in-situ* preparation, displayed a higher potency and efficacy in reducing circuit activity, when considering both time course of action and concentration. Could this represent off-target effects? We considered that the overall more robust influence induced by scopolamine may signal that mAChRs are activated strongly in the network under normal physiological conditions, and thus, reduction in receptor activity would induce a more robust change relative to gain of function. Moreover, the pharmacological manipulation may induce a more robust blockade of mAChRs, particularly at high doses. The slight alteration in degree of modification in receptor activity may tip the scales on network function enough to educe obvious differences. We do, however, note that similar confounding outcomes have been identified in previous *in situ* experiments utilizing these pharmacological agents (Malloy et al., 2016). Thus, the potential for scopolamine, particularly, to alter the conductance of other ion channels or central neuromodulatory systems is possible. Voltage-clamp recordings in central neurons measuring scopolamine impact on specific ionic currents may help to address these uncertainties.

Secondly, while the excitatory effect on circuit and network activity in response to ACh was consistent in the behavioral and *in situ* electrophysiological analysis, muscarine displayed inhibitory effects *in vivo* and excitatory effects *in situ*. Moreover, RNAi knockdown of mAChRs revealed some discrepancy in their impact. A remarkable similarity in the behavioral and electrophysiological analysis was revealed upon manipulation of expression of mAChR-C, with this receptor subtype exhibiting a particularly robust influence in inhibition of motor neurons. Conversely, manipulating mAChR-A expression revealed ambiguity, as this receptor seemingly inhibited network activity in the intact animal, but provided excitatory influence *in situ.* We suspect this difference to lie in the manner with which mAChRs regulate intersegmental signaling vs intrasegmental signaling. In measuring motor output in response to afferent sensory stimulation, it can be difficult to directly correlate our electrophysiological results with modulation of intrasegmental coordinated signaling, which aides in timing of muscle contraction during crawling. The neural circuits within each segment are repeated, and activation of these neurons in sequence are guided by specific interneurons and sensory feedback (Fushiki et al., 2016). It is probable that muscarinic modulation of these select neurons may underlie what was observed in the intact animal. The sensory-motor stimulation used here may not recruit these neurons in a manner mimicking their influence when the circuits are constitutively active (Titlow et al., 2014). We do note, however, that for ACh and nicotinic treatments, the impact on excitability of the evoked sensory-CNS-motor circuit reliably predicted the change in direction in larval crawling speed. To further evaluate the potential for muscarinic modulation of segment-to-segment propagation requires additional experimentation, perhaps utilizing a technique described by Pulver et al. (2015).

Muscarinic receptors are prominent in modulation of motor output in a functional sensory-CNS-motor neural circuit

Recording EJPs in muscle in response to contralateral sensory nerve stimulation involves the recruitment and activity of a host of interneurons. Therefore, making definitive inferences regarding individual receptor contribution to cellular mechanisms underlying complex network activity is quite difficult. However, by limiting our recordings to abdominal m6, we isolated motor output from two identifiable neurons that innervate the muscle from the same nerve branch: type Ib and type Is (Atwood et al., 1993; Choi et al., 2004; Schaefer et al., 2010). Our identification of a significant contribution of mAChRs in modulating release probability of these motor neurons in response to sensory-CNS stimulation of a controlled magnitude signifies their prominent role in shaping excitatory post-synaptic potential to action potential (spike) coupling (E-S). As stated, it has been shown in heterologous expression systems that both the A-type and C-type signal via a Gq/11-PIC$_\beta$ mechanism to augment intracellular Ca^{2+}, a pathway expected to enhance neuronal excitability (Ren et al., 2015; Xia et al., 2016). Thus, we anticipated a reduction in sensitivity to muscarine in the RNAi knockdown lines relative to control. While this is indeed what we observed upon A-type knockdown, knockdown of mAChR-C elicited the opposite effect. Motor neurons became more responsive to synaptic input in the

presence of muscarine with reduced mAChR-C expression, suggesting an inhibitory influence on excitability and a shift in the relative contribution of the excitatory A-type receptor. Although signaling through 'M1'-like mAChRs in mammals typically leads to excitation of neurons through suppression of M-current generated by Kv7/KCNQ containing channels (Jentsch, 2000; Delmas and Brown, 2005) and reduction in conductance of subthreshold Kv4-containing A-type K+ channels (Akins et al., 1990), there is abundant evidence of inhibitory Gq signaling. For instance, Gq signaling through mammalian mAChRs can reduce voltage-gated Na^+ currents (Cantrell et al., 1996) inhibit voltage-dependent Ca^{2+} channels (Kammermeier et al., 2000; Gamper et al., 2004; Suh et al., 2010; Keum et al., 2014), or activate SK-type calcium-activated potassium conductance (Gulledge and Stuart, 2005; Gulledge and Kawaguchi, 2007) through inositol trisphosphate (IP_3)-mediated calcium release. mAChR-C may very well act via a mechanism described here in larval motor neurons, which could influence propagation of synaptic inputs and synaptic release probability. We have shown here mAChR-A and mAChR-C may induce opposing effects in motor neurons, and may act in concert to regulate activation of m6. To better understand how these receptors impact synaptic release probability in type Ib and Is neurons, whole-cell current and voltage clamp recordings, as described by Rohrbough and Broadie (2002) can be performed in the presence of mAChR agonists and antagonists to further probe regulation of motor neuron electrical properties. To our knowledge, this is the first study suggesting an inhibitory influence of mAChR-C in *Drosophila* larvae and, along with a recent study in the adult fly, adds to emerging evidence of mAChR inhibition in select neurons (Bielopolski et al. 2018).

Oral supplementation of nicotinic receptor agonists and antagonists reduce larval locomotor speed

We identified an overall excitatory effect in both the intact and semi-intact animal on the locomotor network when exposed to ACh with the muscarinic contribution displaying varying effects. Based on previous analysis *in vitro* and *in situ*, we suspected a primarily excitatory influence of network activity by nAChRs (Lee and O'Dowd, 1999; Rohrbough and Broadie, 2002; Su and O'Dowd, 2003). While the excitatory effect produced by ACh exposure would imply that ACh acts through either nAChRs or mAChRs to excite components of the circuit, we found that both nicotine and muscarine exposure significantly reduced larval crawling speed. Interestingly, we identified both nicotine and clothianidin to be significantly more potent than either ACh or muscarine in altering locomotion in the intact animal and in altering the efficacy of the evoked sensory-CNS-motor circuit. This suggests a particularly prominent role for nAChRs in modulation of the locomotor circuit. Indeed, a recent RNAi screen uncovered multiple nAChR subunits involved in regulation of larval locomotion (Aleman-Meza et al., 2017). Knockdown of a few nAChR subunits, including α1 and α3 induced severe locomotor defects (Aleman-Meza et al., 2017). Moreover, as stated, nicotine is highly lipophilic and the rapid permeation of the GI tract after consumption is likely. This may underlie the relatively faster time-course of action; however, exposure to a less membrane permeable agonist in clothianidin induced similar effects, suggesting the impact of circuit activity may be through alteration in fast synaptic transmission. A surprising outcome in our analysis was the identification of a significant decrease in locomotor speed in response to nicotine treatment, which was enhanced in a dose-dependent manner. Although this inhibition was opposed by nAChR antagonists after acute exposure, chronic exposure to curare also inhibited locomotor behavior. It is possible that some inhibitory signaling may be potentiated in response to nicotine treatment, which may explain the impact of both competitive antagonists. Indeed, motor neurons receive considerable inhibitory input that may be recruited by cholinergic transmission (Rohrbough and Broadie, 2002; Itakura et al., 2015; Kohsaka et al., 2014). However, as we have shown in the *in situ* analysis, it is likely the assayed antagonists require significant exposure time to impart a substantial influence on circuit/network activity. Thus, it is more likely that the results reveal nicotine-induced desensitization following chronic exposure as persistent nicotine intake coupled with increased concentration may enhance the probability of receptor inactivation through desensitization (Picciotto et al., 2008).

High dose nicotine and clothianidin application rapidly abolishes activity of an evoked sensory-CNS-motor circuit

We have shown here that sensory-CNS-motor circuit excitability is enhanced by low-dose nicotine, ACh, and augmenting endogenous ACh concentration. The current work shows that two competitive nAChR antagonists elicit effects that are opposite to those of ACh and low-dose nicotine, albeit with varying potencies. We have shown that curare is not potent in reducing sensory-CNS-motor circuit excitability, and, as previously shown, requires significant exposure time to block nAChR activity *in vivo* (Gorczyca et al., 1991; Rohrbough and Broadie, 2002). Of the nAChR antagonists tested, BD and MLA significantly reduced circuit activity, with MLA exhibiting greater potency. While we noted that MLA typically acts as a subtype-specific antagonist in the mammalian nervous system, with preferential affinity for the α7 and α6 subunits in the rodent CNS (Albuquerque et al., 2009; Halff et al., 2014), we temper the suggestion that this indicates, definitely, the presence of receptors containing these subunits within the circuit given the relative lack of pharmacological characterization in heterologous systems. However, previous studies have illuminated an abundance of the Dα7 subunit in the *Drosophila* CNS (Chintapalli et al., 2007; Celniker et al., 2009; Gramates et al., 2017) and analysis of cultured embryonic motor neurons show expression of this subunit (Ping and Tsunoda, 2012). Whether this expression pattern is maintained in 3rd instar larvae is uncertain, but we show that an antagonist specific to this subunit in mammals significantly reduces excitability of a sensory-CNS-motor circuit.

Therefore, consistent with previous analyses, pharmacological blockade of nAChRs within the larval CNS reduces the efficacy of sensory-CNS-motor circuit function. An obstinate ambiguity, however, in both semi-intact and intact analysis was the likeness with which high-dose nicotine and clothianidin mimicked this action. We noted that exposure to both low-dose nicotine (10nM-10μM) and clothianidin (100nM) enhanced sensory-CNS-motor circuit excitability relative to control, with the former representing a significant increase. Moreover, we noticed a burst in EJPs at m6 prior to shut-down when high dose nicotine was bath applied, suggesting the circuit was initially excited. nAChRs are known to desensitize following channel opening and display enhanced affinity for agonist binding in this state (Picciotto et al., 2008). The reverse in direction of excitation (from enhanced excitability to reduced excitability as the dosage increased) coupled with the initial increase in circuit activity upon bath application strengthens the hypothesis that reduced circuit efficacy can be traced to nAChR desensitization. Furthermore, in previous *in vitro* analysis in dissociated CNS neurons from adult flies, desensitization of nAChRs was more pronounced in response to nicotine treatment than to ACh, a result consistent with our analysis (Wegener et al., 2004). Although we acknowledge we cannot definitively confirm this supposition, these lines of evidence allude to it. Therefore, we suggest, in concert with previous analyses, that ACh likely signals through nAChRs to provide excitatory input to motor neurons, which is necessary for normal locomotion. The reduction in circuit efficacy in response to high doses of nicotine and clothianidin and the similarity in impact of multiple competitive antagonists is likely due to rapid desensitization of nAChRs. Altogether, we show here that low concentrations of nicotine enhance activity driving input to m6, which is reversed as the dosage is increased. The receptor subtypes mediating this response are acutely sensitive to MLA and BD at high concentrations.

In summary, we have shown that both nAChRs and mAChRs are integral in regulating ACh modulation of larval *Drosophila* locomotion and sensory-CNS-motor circuit excitability. We show both muscarinic and nicotinic cholinergic signaling plays a prominent role in regulating the efficacy of synaptic input to type Is and Ib motor neurons innervating larval m6, a muscle critical in propelling larvae during crawling. While we have shown that ACh exposure, both *in vivo* and *in situ*, enhanced the excitability of the locomotor network suggesting that activation of both nicotinic and muscarinic signaling in concert increases network excitability, we reveal variability in the relative contribution of these components. Muscarinic cholinergic signaling appears primarily inhibitory in the intact animal, but does enhance excitability of the circuit driving input to m6. Identifying how mAChRs may impact intrasegmental signaling during crawling, more specifically, is a topic of future interest. Moreover, we have identified that A-type and C-type receptors act at multiple levels of the neural network to regulate the efficacy of motor output guiding larval crawling. Perhaps most intriguingly, we have shown that both of these receptors play a particularly prominent role in motor neuron input-output efficacy. Furthermore, while identifying the mechanisms through which nicotinic cholinergic signaling imparts its influence on larval locomotion requires additional experimentation, we have provided evidence, particularly through semi-intact electrophysiological analysis, that nAChRs appear to be required for normal locomotion. We suggest signaling through nAChRs provides necessary excitatory input to drive the behavior. Moreover, nicotine-induced potent inhibition of circuit excitability may be through receptor desensitization at high doses. Thus, endogenous ACh signaling likely acts primarily through MLA, BD, and curare-sensitive nAChRs with additional influence from mAChR-A and C-type receptors in regulating locomotor behavior.

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