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SHORT COMMUNICATION

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Synaptic vesicle fusion is modulated through feedback inhibition by dopamine auto-receptors

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Abstract

Mechanisms of synaptic vesicular fusion and neurotransmitter clearance are highly controlled processes whose finely-tuned regulation is critical for neural function. This modulation has been suggested to involve pre-synaptic auto-receptors; however, their underlying mechanisms of action remain unclear. Previous studies with the well-defined *C. elegans* nervous system have used functional imaging to implicate acid sensing ion channels (ASIC-1) to describe synaptic vesicle fusion dynamics within its eight dopaminergic neurons. Implementing a similar imaging approach with a pH-sensitive fluorescent reporter and fluorescence resonance after photobleaching (FRAP), we analyzed dynamic imaging data collected from individual synaptic termini in live animals. We present evidence that constitutive fusion of neurotransmitter vesicles on dopaminergic synaptic termini is modulated through DOP-2 auto-receptors via a negative feedback loop. Integrating our previous results showing the role of ASIC-1 in a positive feedback loop, we also put forth an updated model for synaptic vesicle fusion in which, along with DAT-1 and ASIC-1, the dopamine auto-receptor DOP-2 lies at a modulatory hub at dopaminergic synapses. Our findings are of potential broader significance as similar mechanisms are likely to be used by auto-receptors for other small molecule neurotransmitters across species.

KEYWORDS

auto-receptor, *C. elegans*, dopamine, FRAP, neurotransmitter, synaptic modulation

1 | INTRODUCTION

Precise modulation of synaptic neurotransmitter levels is essential for neural function. Mechanisms of synaptic vesicular fusion and neurotransmitter clearance are highly controlled processes whose finely-tuned regulation is critical for functioning of the nervous system, and appear to be regulated, in part, by pre-synaptic auto-receptors. While auto-receptors for various neurotransmitters have been known for decades (Göthert, 1985), their *in vivo* mechanisms of action remain to be fully elaborated. The complexity of mammalian CNS circuits makes it particularly challenging to disentangle inter-digitated mechanisms of synaptic homeostasis. The invertebrate nematode *Caenorhabditis elegans* model with a well-defined 302-neuron *C. elegans* nervous system provides with a simple-yet-powerful model to dissect synaptic homeostasis. We have previously used (NT) the nematode model and its powerful genetics and ability to image functional synaptic termini in live animals in order to describe synaptic vesicle fusion dynamics within its eight dopaminergic neurons (Voglis & Tavernarakis, 2008). Implementing a similar imaging approach with a pH-sensitive fluorescent marker, we present evidence that constitutive fusion of neurotransmitter vesicles in the *C. elegans* dopaminergic synaptic termini is modulated through negative feedback via DOP-2 auto-receptors. These findings are of

potential broader significance as similar modulatory mechanisms are likely to be used by auto-receptors for other small molecule neurotransmitters across species including humans.

Dopamine is an amine neurotransmitter that influences motor control, cognition, motivation and reward. This neurotransmitter is synthesized by dopaminergic neurons whose cell bodies are located in the midbrain with cortical projections (reviewed in Liss & Roeper, 2008; Schultz, 2007). There are eight dopaminergic neurons in the *C. elegans* hermaphrodite: two anterior deirid neurons (ADE) and four cephalic neurons (CEP) located toward the anterior, and two posterior deirid neurons (PDEs), plus an additional six neurons that are present only in the male tail (Chase & Koelle, 2007; White, Southgate, Thomson, & Brenner, 1986). Tyrosine hydroxylase (encoded by *cat-2*) catalyzes tyrosine to levodopa, which is then converted to dopamine by an aromatic amino acid decarboxylase (encoded by *bas-1*). Dopamine is packaged for release into acidified vesicles by a vesicular monoamine transporter (encoded by *cat-1)* (Duerr et al., 1999; Loer & Kenyon, 1993). Dopamine-laden vesicles fuse with the plasma membrane constitutively at a low basal rate to release dopamine, with increased rates evoked by depolarization (Chase & Koelle, 2007; Ramirez & Kavalali, 2011).

Dopamine receptors are 7-transmembrane proteins that are classified into D1-like and D2-like, based on their mode of action. D1-type transduce signals by coupling to stimulatory G-proteins ($G\alpha_s$) while D2-type signaling is transduced through inhibitory $G\alpha_{i/o}$ class of G-proteins (Missale, Nash, Robinson, Jaber, & Caron, 1998). Although the majority of DA receptors (both D1- and D2-types) are expressed in non-dopaminergic neurons and function post-synaptically, dopamine auto-receptors are expressed on dopaminergic neurons, and form a subgroup within the D2-type of receptors (Beaulieu & Gainetdinov, 2011). There are four confirmed *C. elegans* dopamine receptors (DOP-1 to 4); *dop-1*, *dop-3* and *dop-4* are expressed in non-dopaminergic neurons (Chase & Koelle, 2007). *dop-2* codes for a D2-type receptor whose expression is limited exclusively to dopaminergic neurons (Suo, Sasagawa, & Ishiura, 2003), representing a delimited auto-receptor, thereby providing a clear model to study the general role of pre-synaptic auto-receptors in regulating neurotransmitter release.

Our earlier studies established that DOP-2 physically interacts with GPA-14, a $G_{\alpha i}$ subunit (Pandey & Harbinder, 2012) and its deletion affects associative as well as non-associative learning (Mersha et al., 2013; Voglis & Tavernarakis, 2008). Previous work from one of our labs (NT) has shown that an acid-sensing ion channel (ASIC-1) provides a feed-forward loop for synaptic vesicle fusion in dopaminergic neurons (Voglis & Tavernarakis, 2008). Considering potential cross talk between ASIC-1 and D2 auto-receptors, we set out to uncover the influence of the *C. elegans* DOP-2 auto-receptor on synaptic vesicle fusion at the level of individual synapses in live animals. An analogous fluorescence resonance after photobleaching (FRAP)-based approach was used, exploiting a pH-sensitive fluorescent reporter to quantitate synaptic vesicle fusion as a surrogate for synaptic dopamine levels. Analyses of our FRAP imaging data from individual synaptic termini in live animals provide evidence that the DOP-2 auto-receptor provides a feedback loop to dopaminergic neurons. These findings are of potential broader significance as similar mechanisms are likely to be used by auto-receptors for other small molecule neurotransmitters.

2 | METHODS

SEpHluorin is a pH-sensitive green fluorescent optical indicator protein that displays very limited fluorescence inside the acidic environment of synaptic vesicles; however, upon vesicle fusion with the synaptic membrane, the fluorescent domain of SEpHluorin is exposed to the near neutral pH of the extracellular environment (of the synaptic cleft), and its bright fluorescence is switched on (Miesenbock, De Angelis, & Rothman, 1998); A *dop-2(vs105)* deletion mutant strain (obtained from CGC, University of Minnesota) was crossed into a transgenic strain expressing SNB-1::SEpHluorin (described in Voglis & Tavernarakis, 2008). Fusion rates of SNB-1::SEpHluorin sensor protein at individual synapses originating from worm dopaminergic neurons were examined in wild-type, and *asic-1(ok415)* and *dop-2(vs105)* deletion mutant backgrounds, and assessed using FRAP. FRAP experiments were carried out essentially as previously described in the worm system (Samuel, Silva, & Murthy, 2003; Voglis & Tavernarakis, 2008). Briefly, well-fed young adults from the SNB-1::SEpHluorin carrying transgenic strains, grown at 20 °C, were mounted onto a 5% agarose pad and anesthetized with 2 microL of 20 mM Levamisol. Individual dopaminergic synapses were imaged in real time and baseline synaptic fluorescence was determined using a Zeiss LM 710 confocal microscope. Distinct synapses of axons originating from worm CEP, ADE or PDE dopaminergic neurons were located with the 10X objective and then switched to 40X (C-Apochromat) to capture images. After taking three control images to measure initial intensity, the synapse under observation was bleached to about 50% of initial intensity using 15mW argon laser power (488nm wavelength), and emission was filtered with a 500–550nm BP filter. Sixty images were captured every 2 s starting immediately after photobleaching until 2 min to monitor fluorescence recovery. Qualitative representations of raw images are shown in supplementary Figure S1. A pixel depth of 16-bit was used to obtain emission intensity data from the Zeiss microscope associated *Zen* digital imaging software (Carl Zeiss AG, Oberkochen, Germany). The fluorescence of the selected synapses was quantified by measuring the fluorescence intensity in three regions: at the synapse of interest *F*i; at an unbleached region *F*c (another fluorescent synapse from the dopaminergic cell body region) and at a background region *F*b (a non-fluorescent region). The data were transferred to the open-source *Fiji* software for image alignment so as to correct for any XY movement (Schindelin, Arganda-Carreras, & Frise,). Images corrected for XY displacement, if any, with *Fiji* software were converted into numerical data using ZEN and fluorescence intensity values for each synapse were normalized and then quantitated with consideration to unbleached and background non-fluorescent regions. Data from each region of

interest were averaged and used to normalize intensity points such that: Normalized Value = (*F*i − *F*b Ave)/(*F*i * Bleach Correction). Fluorescence intensity was normalized for background and photobleaching and then to a zero to 1 scale in which the bleached intensity is equal to zero and the maximum fluorescence intensity (the pre-bleach intensity) is equal to 1. Data sets were fitted to a two-phase exponential function that captures the sum of a fast and slow recovery $M\left(1-fe^{-\frac{t}{\tau_1}}+(1-f)\,e^{-\frac{t}{\tau_2}}\right)$, where *M* denotes the maximum recovery, τ_1, τ_2 are the two distinct time constants observed during recovery, and *f* quantifies their relative contributions. The slope of the slow recovery phase (stating around 10 s to the end of the experiment) was calculated by performing linear regression with respect to time, and 95% confidence intervals for slopes was calculated through bootstrapping.

3 | RESULTS AND DISCUSSION

FRAP measurements on constitutive vesicle fusion rates in live, anesthetized animals are presented and discussed here. A qualitative representation of FRAP performed on a single synapse of an anterior CEP in wild-type worms, showing images before photobleaching and at different times of fluorescence recovery is provided (in supplementary information, Figure S1). Comparison of our results obtained with the *dop-2* (vs105) deletion mutant, the *asic-1* (ok415) deletion mutant and wild-type animals show distinct recovery dynamics (Figure 1). To begin with, we replicated previously reported FRAP experiments confirming that removal of ASIC-1 compromises neurotransmitter vesicle fusion at dopaminergic synapses. Specifically, the FRAP recovery at dopaminergic synapses labeled with SNB-1::SEpHluorin in *asic-1* (ok415) deletion mutants is clearly reduced, as compared to wild type animals supporting the previously suggested role for ASIC-1 as part of a feed-forward modulator in synaptic vesicle fusion (Voglis & Tavernarakis, 2008). Furthermore, FRAP recovery at dopaminergic synapses in *dop-2* (vs105) deletion mutants shows a reverse trend, indicating that DOP-2 provides an inhibitory component in synaptic vesicle fusion (Figure 1). Examination of the wild type as well as the mutant curves shown in Figure 1 shows biphasic nature of the FRAP data: a rapid initial increase in fluorescence recovery (τ_1), followed by a slower recovery (τ_2). The FRAP data were fitted to a two-phase exponential function as described in Methods section above to capture the sum of a fast and slow recovery, and the maximum recovery rate (*M*) for wild type was set to 100%. The

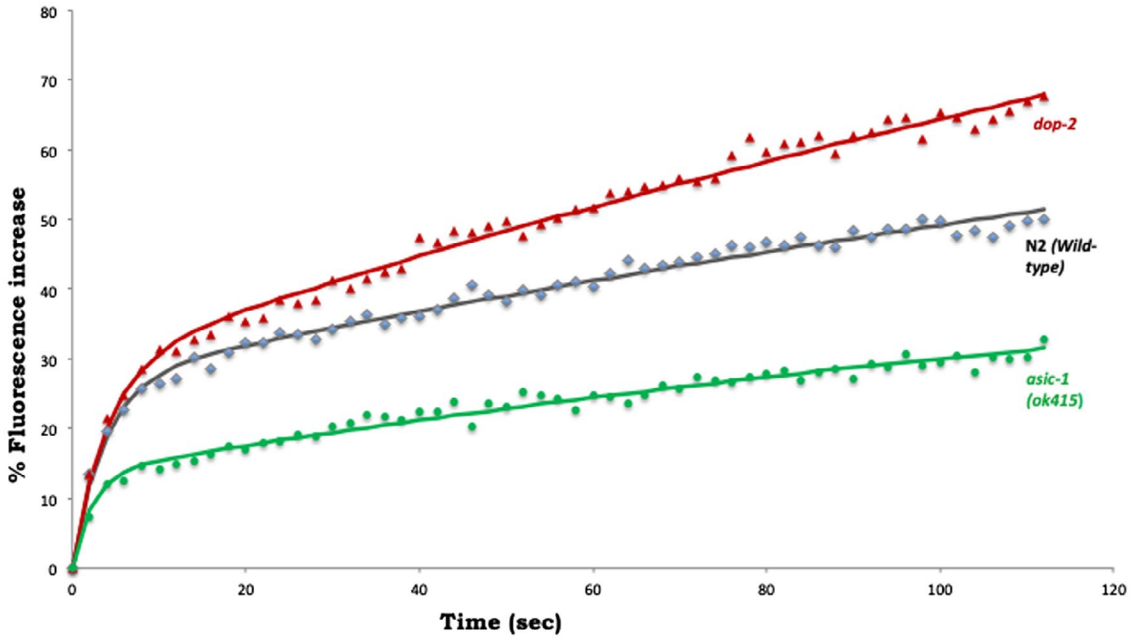

FIGURE 1 Fluorescence recovery after photobleaching (FRAP) at dopaminergic synapses labeled with SNB-1::SEpHluorin is significantly increased in animals carrying a lesion in the *dop-2* gene (red), compared with wild-type N2 animals (gray). FRAP recovery rate in animals carrying a lesion in the *asic-1* gene (encodes an acid sensing ion channel) is markedly reduced (green) as also reported previously (Voglis & Tavernarakis, 2008). While ASIC-1 has been proposed to facilitate a positive feedback loop that reinforces constitutive dopamine release results presented in the current study indicate the existence of a distinct negative feedback mediated through DOP-2 auto-receptors. Given the biphasic nature of recovery, the data were fitted to an exponential recovery with two time constants (solid lines; see Methods). Setting complete FRAP recovery for wild-type N2 animals (*M* = 100%), we calculated the two time constants to be τ_1 = 3.8 s, τ_2 = 274 s and *f* = 0.27. The *dop-2* deletion animals show a higher recovery, *M* = 150%, while their time scale of recovery is very similar to WT (τ_1 = 3.8 s, τ_2 = 274 s, *f* = 0.2). The *asic-1* deletion mutants display both lesser recover and slower recovery dynamics (*M* = 45%, τ_1 = 2.3 s, τ_2 = 192 s, *f* = 0.25). Sample size of *n* = 30 synapses for wild-type N2 animals, *n* = 28 synapses for *dop-2* deletion mutant, and *n* = 28 synapses for *asic-1* deletion mutant

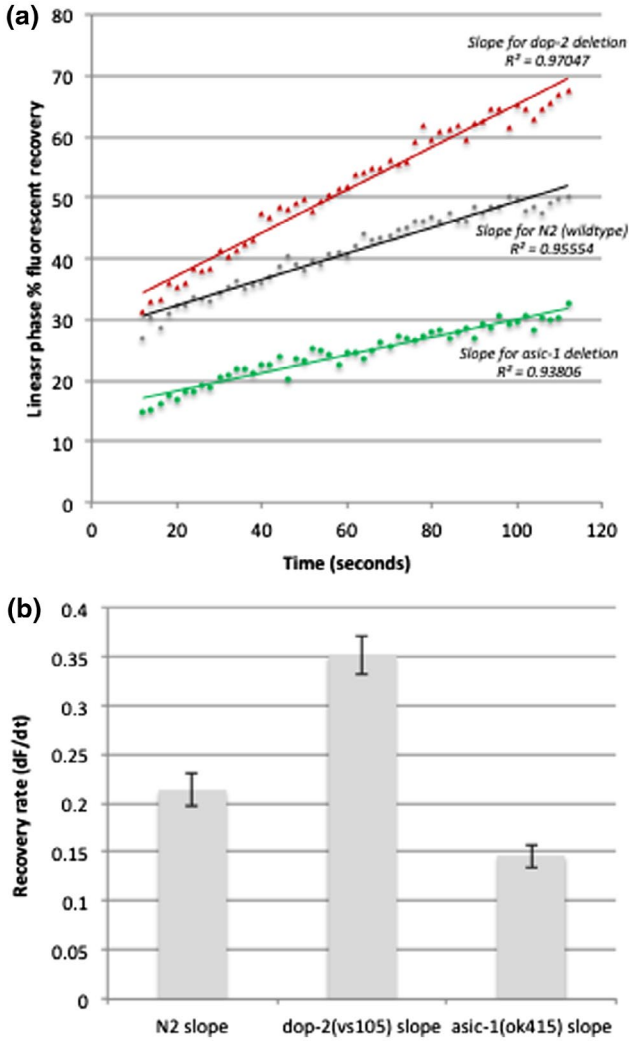

FIGURE 2 Slow recovery phase of wild-type animals and both *dop-2* and *asic-1* deletion mutants display distinct slopes as determined by linear regression (a). As compared to wild type, a significantly higher recovery for *dop-2* deletion mutants and lower recovery for *asic-1* deletions are observed (b). Error bars shown in the bar chart denote 95% confidence intervals for the slopes estimated using bootstrapping ($p < 10^{-4}$)

fitting shows similar recovery dynamics between wild-type N2 and *dop-2* deletion animals, but slower dynamics for *asic-1* deletion mutants for the initial fast recovery phase (Figure 1). The dominant slow recovery phase shows a significantly higher slope with respect to time for *dop-2* deletions, indicating a faster rate of vesicle fusion compared to WT, while the lower slope of *asic-1* deletion mutants indicates a lower rate of vesicle fusion (Figures 1 and 2). The faster rate of vesicle fusion in *dop-2* deletion mutants suggests that *dop-2* deletion mutants lack a mechanism that down-regulates the release of dopamine at synapses. In other words, DOP-2 provides an inhibitory component in synaptic vesicle fusion which is in line with previously reported observations from our lab that DOP-2 physically associates with an inhibitory $G_{\alpha i}$ subunit (Pandey & Harbinder, 2012) as well as the previously described D2-like pharmacological properties reported for DOP-2 (Suo, Ishiura, & Van Tol, 2004). Analyses of results from our FRAP data indicate that there may be accumulation of extracellular synaptic dopamine in *dop-2* mutants, a high release of dopamine and/or due to a decreased activity of the dopamine transporter DAT-1.

In summary, our results from quantitative visualization of neurotransmitter vesicle fusion rates in individual synapses in a living, intact organism, provide *in vivo* evidence for the participation of D2 auto-receptors in modulation of synaptic dopamine levels. Behavioral and genetic evidence suggests that DOP-2 influences synaptic vesicular fusion through functional interactions with dopamine modulators including the membrane dopamine transporter (DAT; Bermingham et al., 2016). DAT works as a symporter by coupling dopamine uptake along with intracellular translocation of one Cl^- and two Na^+ ions which tends to depolarize the neuronal membrane. Studies on DAT in striatal synaptosomes have revealed a voltage-dependent regulation mechanism for DAT activity; membrane depolarization has been shown to decrease DAT activity while hyperpolarization causes reduction in DAT activity (Giompres & Delis, 2005). This, in turn, is likely to cause increased activation of presynaptic D2 auto-receptors coupled to a $G_{\alpha i}$ subunit of an inhibitory G-protein, which would reduce vesicle fusion and DA release. Testing the functional interaction of DOP-2 auto-receptors with the membrane transporter, DAT, would provide additional insight into synaptic dopamine

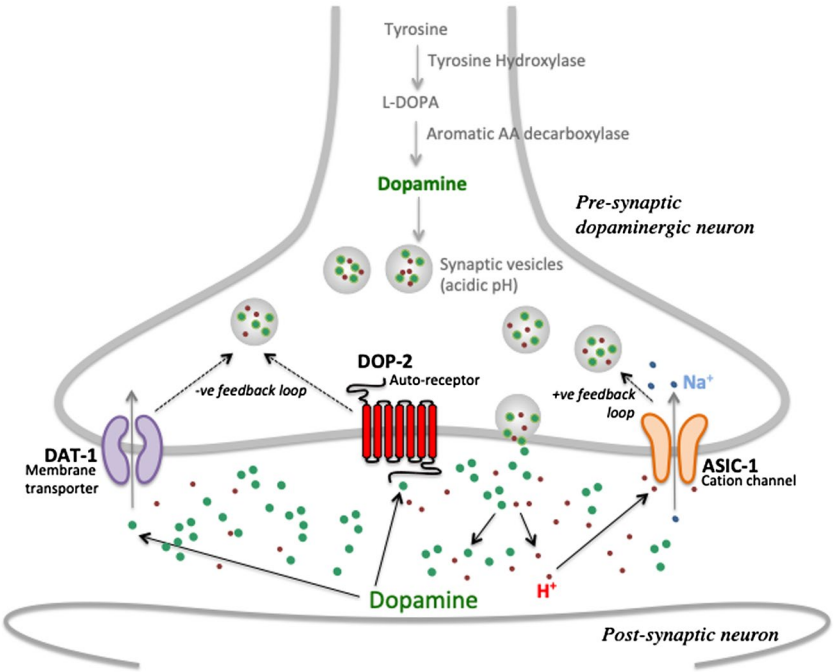

FIGURE 3 *An updated model for modulation of synaptic vesicle release at dopaminergic neuron synapses*. Fusion of acidified neurotransmitter vesicles with the synaptic membrane causes release of dopamine and H^+ ions. Dopamine activates a feedback loop through auto-receptors (DOP-2), and possibly through the membrane transporter (DAT-1), while ASIC-1 functions to facilitate a positive feedback loop that reinforces dopamine release in response to a local pH drop in the synaptic cleft. Thus, DOP-2, DAT-1 and ASIC-1 form a modulatory hub responsible for fine-tuning synaptic dopamine levels

modulation. Release of dopamine and H^+ ions upon fusion of acidified neurotransmitter vesicles with the synaptic membrane activates dopamine auto-receptors, DAT transporters and ASIC channels. The *C. elegans* auto-receptors (DOP-2) and the membrane transporter (DAT-1) likely act as feedback components, while ASIC-1 functions to facilitate a positive feedback loop that reinforces dopamine release in response to a local pH drop in the synaptic cleft. Thus, DOP-2, DAT-1 and ASIC-1 may form a modulatory hub responsible for fine-tuning synaptic dopamine levels. We put forth an updated model for modulation of synaptic vesicle fusion at dopaminergic neuron synapses (Figure 3).

Given that D2 receptors are highly conserved across phyla, our results with the worm model provide a foundational understanding on similar mechanisms that may modulate synaptic vesicle fusion in vertebrates (Ford, 2014). It will be interesting to dissect out the role of other potential modulators such as DAT on synaptic vesicle fusion and its cross talk with D2 auto-receptors and ASIC channels. We remain mindful that we have measured synaptic vesicle fusion as an indirect surrogate for dopamine levels. As of currently available technology, it is not feasible to directly measure synaptic dopamine levels. Recent advances in false fluorescent neurotransmitters (FFNs) have shown promise; however, they are non-specific for monoamines (Sames, Dunn, Karpowicz, & Sulzer, 2013). Newer FFNs which are dopamine specific have the potential to open up an avenue for direct measurement of vesicular contents.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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