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Drosophila Dopamine2-like Receptors Function as Autoreceptors

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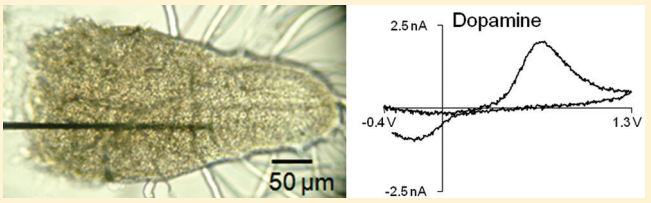
ABSTRACT: Dopaminergic signaling pathways are conserved between mammals and *Drosophila*, and D2 receptors have been identified in *Drosophila*. However, it has not been demonstrated whether *Drosophila* D2 receptors function as autoreceptors and regulate the release of dopamine. The goal of this study was to determine if *Drosophila* D2 receptors act as autoreceptors by probing the extent to which D2 agonists and antagonists affect evoked dopamine release. Fast-scan cyclic voltammetry was used to measure stimulated dopamine release at a carbon-fiber microelectrode implanted in an intact, larval *Drosophila* nervous system. Dopamine release was evoked using 5 s blue-light stimulations that open Channelrhodopsin-2, a blue-light-activated cation channel that was specifically expressed in dopaminergic neurons. In mammals, administration of a D2 agonist decreases evoked dopamine release by increasing autoreceptor feedback. Similarly, we found that the D2 agonists bromocriptine and quinpirole decreased stimulated dopamine release in *Drosophila*. D2 antagonists were expected to increase dopamine release, and the D2 antagonists flupenthixol, butaclamol, and haloperidol did increase stimulated release. Agonists did not significantly modulate dopamine uptake, although the modulatory effects of D2 drugs on release were affected by prior administration of the uptake inhibitor nisoxetine. These results demonstrate that the D2 receptor functions as an autoreceptor in *Drosophila*. The similarities in dopamine regulation validate *Drosophila* as a model system for studying the basic neurobiology of dopaminergic signaling.



KEYWORDS: *Dopamine, autoreceptor, Drosophila, fast-scan cyclic voltammetry*

The monoamine neurotransmitter dopamine plays a major role in many human behaviors such as movement, cognition, reward, addiction, and motivation. Abnormalities in dopaminergic signaling are implicated in diseases such as schizophrenia, Parkinson's disease, and drug addiction. Dopaminergic signaling is mediated by receptors that are located either postsynaptically, where they regulate downstream signaling, or presynaptically, where they act as autoreceptors regulating release.[1] D2 receptors (D2Rs) are the predominant dopamine autoreceptor, and dysfunction of D2 autoreceptors is involved in disease etiology.[2] Therefore, D2 receptors are important drug target sites.[2] For example, patients with schizophrenia have a higher level of expression of D2 receptors and higher basal levels of dopamine; thus, many antipsychotics target the D2 receptor.[3] Other studies have shown that mice without the D2R gene have significant neurological impairments and Parkinson-like symptoms.[4] Consequently, D2Rs are targets for Parkinson treatment.[5] In addition to their implication in specific diseases, D2Rs have also been shown to modulate locomotion.[6,7] Thus, autoreceptors are critical for regulating dopamine release and maintaining dopaminergic function.

Drosophila is a popular biological model system because of its short life span, high fecundity, and facile genetics. Genetic mutants mimicking human diseases can be constructed and studied more rapidly in *Drosophila* than in mammals. Cellular machinery that controls dopamine regulation, such as transporter proteins, synthesis enzymes, and vesicles, is conserved between *Drosophila* and mammals.[8,9] Our lab has recently developed a method for directly measuring dopamine release in *Drosophila* and has verified that release and reuptake rates are similar to those of mammals.[10,11] However, the extent to which dopamine receptors in *Drosophila* act as autoreceptors has not been tested.

Three mammalian isoforms of D2R, differing by up to 29 amino acids, have been isolated: D2 short ($D2_S$), D2 long ($D2_L$), and D2 extra long.[12,13] The $D2_S$ receptor subtype is located presynaptically and functions as an autoreceptor, while the $D2_L$ receptor subtype is located postsynaptically.[14] Both isoforms are found in many species: human, rat, mouse, bovine, *Caenorhabditis elegans*, and *Xenopus*.[15−19] Eight isoforms of a *Drosophila* D2-like receptor (DD2R) have been identified. These DD2Rs are G-protein-coupled receptors with a high affinity for dopamine that have amino acid sequences homologous to those of mammalian D2-like receptors.[20] It is unclear whether these receptors are $D2_L$ or $D2_S$, and identifying the cellular locations and function of these DD2R receptors is difficult. Immunohistochemistry studies have identified DDR2 localization in larva, and DD2R staining is colocalized with both dopaminergic cell bodies and projections, although the expression presynaptically or postsynaptictically has not been determined.[21] DD2Rs were expressed in HEK293 cells, and pharmacological evaluation with mammalian D2R agonists and antagonists showed that the agonist bromocriptine and the antagonists flupenthixol and butaclamol exhibited high-affinity binding. In contrast, the agonist quinpirole and the antagonist haloperidol had little to

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no affinity for the DD2Rs.[20] However, some drugs with poor affinity cause behavioral effects in *Drosophila*. For example, the agonist quinpirole increases locomotor activity in adults.[22,23] Molecular biology and behavioral results suggest that D2 autoreceptor functionality may be conserved in *Drosophila*. Chemical measurements of dopamine release would provide direct evidence and establish the relative effectiveness of DD2R drugs in an intact *Drosophila* central nervous system (CNS).

In this study, we used fast-scan cyclic voltammetry at carbon-fiber microelectrodes to characterize changes in evoked dopamine release in *Drosophila* larvae following pharmacological manipulation with different D2 agonists and antagonists. We show D2 agonists decreased stimulated dopamine release and D2 antagonists increased dopamine release. These studies demonstrate that the *Drosophila* D2 receptor functions as an autoreceptor and regulates dopamine release, thus validating *Drosophila* as a model system for studying dopaminergic diseases.

RESULTS AND DISCUSSION

Fast-scan cyclic voltammetry (FSCV) at carbon-fiber microelectrodes has been used extensively to measure electrically stimulated dopamine release in animal models in vivo, in brain slices, and at single cells.[24−26] In mammals, FSCV has been used to measure the effects of D2 receptor agonists and antagonists on evoked dopamine release.[27,28] For example, in rat brain slices, the dopamine agonist quinpirole decreases stimulated dopamine release,[28] and in anesthetized rats, the dopamine antagonist flupenthixol increases stimulated dopamine release.[29] The combination of specific stimulation of the dopaminergic terminals and rapid measuring techniques allow presynaptic effects of the drugs to be probed, so these studies verify that D2 receptors act as autoreceptors, regulating a feedback loop that controls release.[30] While electrical stimulation works well in mammalian experiments, the *Drosophila* ventral nerve cord is smaller than a typical stimulating electrode. Therefore, optical stimulations are used instead of electrical stimulations. Channelrhodopsin-2, a blue-light sensitive ion channel, is specifically expressed in dopaminergic neurons. Dopamine release is measured with FSCV at a carbon-fiber microelectrode implanted in an isolated *Drosophila* larva nerve cord after blue-light stimulation.[10,11] Presynaptic effects of the drugs are investigated as this experimental protocol is analogous to the mammalian protocols because dopaminergic terminals are specifically activated and release is measured on a rapid time scale.[30] The effects of D2R agonists and antagonists were tested on ChR2-mediated dopamine release to determine autoreceptor function.

Dopamine Agonists Decrease Evoked Dopamine Release. In humans, bromocriptine is a potent D2R agonist used in the treatment of Parkinson's disease. Administering bromocriptine to mammals decreases the extent of striatal dopamine synthesis through activation of the D2R.[31] In *Drosophila*, bromocriptine restores locomotion to mutants exhibiting Parkinsonian-like behavior.[21] Bromocriptine had a similar affinity for the *Drosophila* D2R (DD2R) and for the human D2R and has the highest-affinity binding of several common dopamine receptor agonists for DDR2 receptors transfected into HEK293 cells.[32]

To test the effects of drugs in *Drosophila*, an electrode was implanted into a ventral nerve cord dissected from a 5-day-old larva and an initial, 5 s blue-light stimulation performed to assess dopamine release before drug administration. Two, subsequent 5 s blue-light stimulations were performed 15 and 30 min after administration of the drug. Figure 1A shows that in a control experiment where buffer is added instead of drugs, the stimulations after 15 and 30 min produced the same dopamine signal as the initial stimulation. While there was a large variation in the extent of release between samples because of variance in ChR2 expression [average evoked dopamine release was 490 ± 60 nM for all flies (n = 42)], stimulated release at 15 min intervals within each sample was stable.

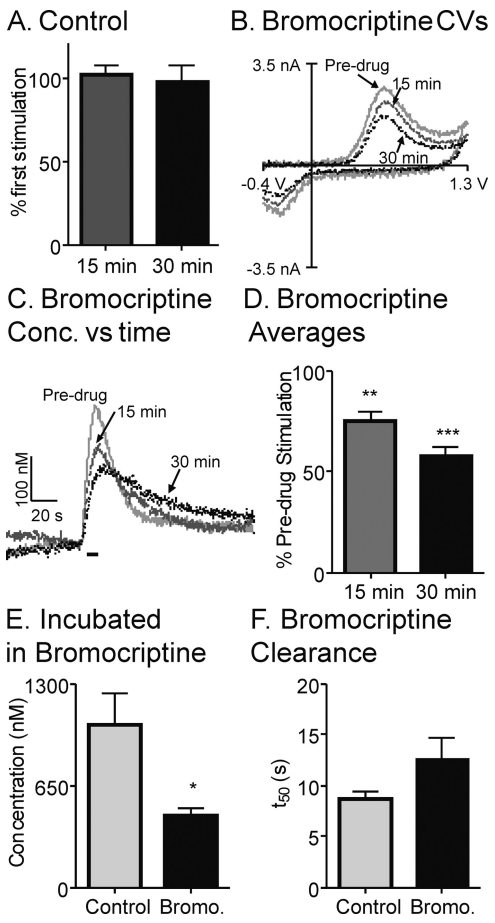

Figure 1. Effect of D2 agonist bromocriptine on evoked dopamine. (A) Control data show that when buffer is added instead of drug, stimulations evoked 15 and 30 min after the initial stimulation are stable (n = 5). (B) Background-subtracted cyclic voltammogram from a single nerve cord comparing evoked dopamine release before the addition of drug and 15 and 30 min after application of 50 μM bromocriptine. (C) Concentration vs time profile showing the effect of bromocriptine on stimulated dopamine release in a single nerve cord. The bar underneath marks the duration of the blue-light stimulation. (D) Pooled data (n = 7) show 50 μM bromocriptine decreased evoked dopamine release. Data are normalized to the initial stimulation in each animal. Statistics were determined via comparison of evoked release before and after addition of drug using paired t tests. $^{**}p < 0.01$; $^{***}p < 0.001$. (E) Preincubation with 50 μM bromocriptine also decreased evoked dopamine release (n = 6). Significance is determined using an unpaired t test. $^{*}p < 0.05$. (F) Dopamine clearance (t_{50}) is not significantly different in the presence of bromocriptine (paired t test; n = 6).

Panels B and C of Figure 1 show example cyclic voltammograms and concentration−time profiles for evoked release in the fly before and after bromocriptine was administered to bring the concentration in the bath around the nerve cord to 50 μM. The peak concentration decreased, and there was a larger effect 30 min (47% decrease) after addition of bromocriptine than after 15 min (24% decrease). Pooled data are plotted as a percentage of the initial stimulation (Figure 1D), and bromocriptine

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decreased evoked dopamine release. To test significance, levels of evoked release before and after addition of drug were compared with a paired *t* test, and release was significantly decreased both 15 and 30 min after addition of drug.

To ensure that prestimulation did not deplete the population of dopamine-loaded vesicles or change synaptic physiology, nerve cords were also incubated with bromocriptine 15−20 min before any stimulations were performed. Figure 1E shows evoked dopamine release is significantly lower after bromocriptine incubation than in control samples. The effect of bromocriptine is the same regardless of prior stimulation, although the decrease in release (57%) is slightly larger. The larger decrease after incubation could be due to the experimental protocol facilitating more bromocriptine diffusion, as the optic lobes were removed in bromocriptine and more drug could have entered during the cutting when no glial barrier would have formed. However, the concentration of stimulated release after addition of bromocriptine was about the same for both the prestimulated and unstimulated groups. The control data for this experiment had larger than average releases, so these data show why it is useful to conduct prestimulation to be able to directly compare drug to control data in the same sample.

In addition to evoked concentration, dopamine clearance kinetics can be determined from current−time plots. The time from the end of the stimulus until the signal decays to the half-maximal concentration is reached, t_{50}, can be used as an estimate of uptake by the dopamine transporter. The t_{50} increased slightly for nerve cords after addition of bromocriptine (Figure 4F), although the effect was not significant.

The D2R agonist quinpirole was also tested. In mammalian brain slice experiments, quinpirole significantly decreases stimulated dopamine release, while in behaving rats, quinpirole modulates locomotion in a concentration-dependent manner.[21,33] In *Drosophila*, administration of quinpirole to the nerve cord of decapitated adult flies stimulates locomotion and grooming responses.[22] However, quinpirole had no significant affinity for DD2R in cells transfected with *Drosophila* D2R isoforms.[20] Quinpirole (50 μM) was administered, the same dose for bromocriptine and similar to that used in behavioral experiments.[22,28,34] Figure 2 shows that stimulated dopamine release decreased after quinpirole administration. On average, release was 35 and 69% less 15 and 30 min after addition of quinpirole, respectively, which is consistent with mammalian results.[35,36] We observed a significant effect on evoked release with quinpirole, whereas significant binding was not seen in transfected cells.[20] It is common for drug binding in vivo to be different than in transfected cells; thus, an in vivo method of testing drugs is valuable.[37,38] For quinpirole, the t_{50} did not significantly change (Figure 2C), and the trend toward a higher t_{50} was similar to that observed with bromocriptine.

In summary, both D2R agonists bromocriptine and quinpirole significantly decreased dopamine release. This decrease in release suggests that the *Drosophila* D2R functions as an autoreceptor, regulating dopamine release.

Dopamine Antagonists Increase Evoked Dopamine Release. If the DD2R is an autoreceptor, then D2 antagonists should increase stimulated dopamine release. Flupenthixol is a D2 antagonist used to treat schizophrenia.[39] Acute flupenthixol upregulates dopamine synthesis in mammals[40] and increases stimulated dopamine release in rats.[29] In HEK293 cells transfected with DD2R isoforms, flupenthixol showed the highest affinity among the antagonists tested.[20] Figure 3A shows an example concentration−time profile for evoked dopamine after addition of 5 μM flupenthixol. The highest release was observed 15 min after antagonist administration, although release was still elevated after 30 min. Figure 3B demonstrates that on average endogenous dopamine release doubled 15 min after flupenthixol administration, a significant increase. The increase in dopamine release after addition of flupenthixol is similar to that found mammalian studies and is consistent with the DD2R acting as an autoreceptor. The t_{50} increased significantly for nerve cords after addition of flupenthixol (Figure 3B). Flupenthixol is a weak human dopamine transporter inhibitor; therefore, it is not surprising that clearance was significantly decreased.[41]

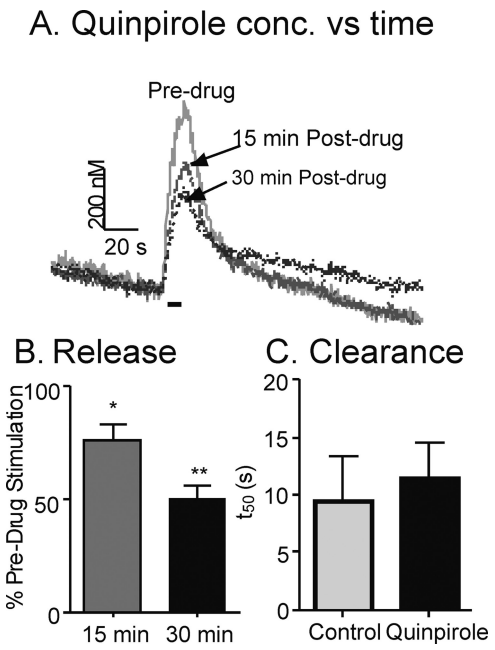

Figure 2. Effect of D2 agonist quinpirole. (A) Concentration−time traces show 50 μM quinpriole decreased evoked dopamine release. (B) Averaged data demonstrate (n = 7) evoked release is significantly decreased 15 and 30 min after addition of quinpirole (paired t test comparing evoked release before and after addition of drug). (C) Dopamine clearance (t_{50}) is not significantly reduced in the presence of quinpirole (paired t test; n = 6). *p < 0.05. **p < 0.01.

Butaclamol is another D2R antagonist that was developed for the treatment of schizophrenia but showed a high incidence of extrapyramidal side effects and was never marketed.[42] In mammalian models, butaclamol has a high affinity for D2 receptors, and in DDR2-transfected cells, butaclamol has a significant affinity for DD2R.[20,43] Butaclamol significantly increased stimulated dopamine release 15 min after its administration at a concentration of 5 μM but not after 30 min (Figure 3C). The t_{50} was not significantly different after addition of butaclamol (Figure 3C).

Haloperidol is a common antipsychotic used in humans, with significant affinity for D2R.[44] Haloperidol has been used extensively in mammalian autoreceptor studies.[20] For example, haloperidol increases electrically evoked dopamine release in freely moving animals.[45,46] In cells transfected with DD2R, haloperidol had a lower affinity than flupenthixol and butaclamol.[20] Evoked dopamine release was significantly increased 15 min after addition of 5 μM haloperidol but not at 30 min (Figure 3D). Figure 3D shows that t_{50} did not significantly change after addition of haloperidol.

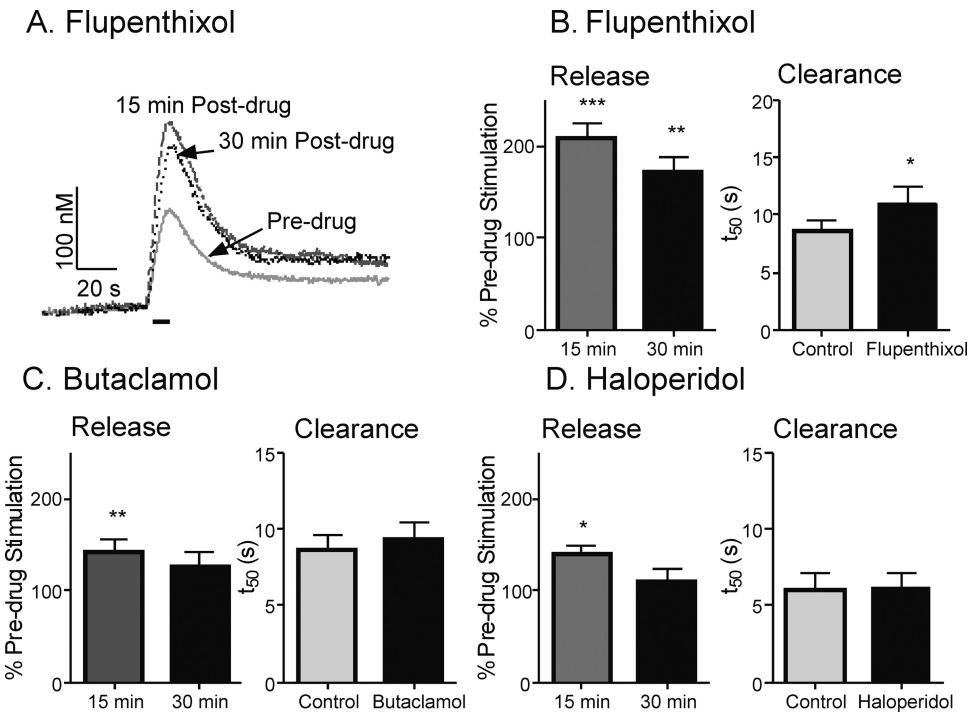

Figure 3. Effect of dopamine D2 antagonists. (A) An example concentration−time profile shows 5 μM flupenthixol increased dopamine release. Release is higher 15 min than 30 min after addition of drug. (B) Averaged data for 5 μM flupenthixol show that release was significantly increased 15 and 30 min after addition of drug and that the time for dopamine clearance significantly increases after addition of 5 μM flupenthixol (n = 6). (C) Averaged data for 5 μM butaclamol show that evoked release is significantly increased 15 min after addition of drug but not 30 min, and t_{50} is not significantly different (n = 5). (D) Averaged data for 5 μM haloperidol show a significant increase in release after 15 min and no change in t_{50} (n = 6). All statistics are from paired t tests of release or clearance before and after addition of drug. *p < 0.05. **p < 0.01. ***p < 0.001.

The increase in stimulated dopamine release after addition of flupenthixol, butaclamol, or haloperidol provides further evidence that the DD2Rs act as autoreceptors and that *Drosophila* is a model system homologous to mammals. The similarity of the response in *Drosophila* and mammals suggests that while the D2 receptor isoforms may be different, basic biology is conserved. Interestingly, the effect of the D2 antagonist was greatest after 15 min. This could be caused by D2-mediated increases in basal dopamine levels, which could deplete the pool for stimulated release. Another cause for the greater effect at 15 min could be that receptor density is increased after prolonged exposure to the antagonist, which is observed in mammals on a longer time scale.[47,48] Flupenthixol had the greatest effect of all antagonists tested, consistent with flupenthixol having the highest affinity for DD2R in transfected cells.[20] Butaclamol and haloperidol produced very similar results, even though haloperidol had a lower affinity for DDR2 in transfected cells. Thus, electrochemical detection of dopamine release in *Drosophila* provides an easy test for pharmacological efficacy of D2 agonists and antagonists.

Interactions of D2 Receptors and DAT. In mammals, an interaction between D2 receptors and the dopamine transporter (DAT) has been postulated because D2 agonists have been found to increase the kinetics of DAT, accelerating clearance.[49] The extent of this effect is debated and varies by brain region and electrochemical detection method. Meiergerd et al.[50] found a large effect of agonists on clearance in the striatum using rotating disk voltammetry, while Joseph et al.[51] and Mathews et al.[33] found no effect using FSCV in the striatum and nucleus accumbens, respectively. Our study provides no evidence that D2 agonists increase DAT kinetics in *Drosophila*. For the agonists, bromocriptine and quinpirole nonsignificantly increase t_{50}, the opposite of the expected effect. For the antagonists, only flupenthixol slowed clearance while butaclamol and haloperidol did not, suggesting this is not a widespread effect of antagonists. An interaction between the D2 receptor and DAT may not occur, or the effect may not be able to be measured via FSCV.

To examine this possible interaction further, nisoxetine, an inhibitor of the *Drosophila* dopamine transporter (dDAT), was administered before a D2 agonist or antagonist (Figure 4). The t_{50} increases significantly after addition of nisoxetine and bromocriptine, similar to data for bromocriptine alone, and this is the opposite of the effect expected if the D2 agonist had a facilitatory effect on DAT. Therefore, these data do not reveal any cooperative effect of an agonist on DAT activity. After addition of nisoxetine and bromocriptine, there is a nonsignificant (p = 0.0523) trend toward increased release compared to that with nisoxetine alone (Figure 4A,B). Uptake inhibition causes the D2 agonist to have the opposite effect it had when administered alone. Increased basal dopamine levels after addition of nisoxetine may saturate D2 receptors, and thus, there is no decrease in release after administration of the D2 agonist.

When the same experiments were repeated with a D2 antagonist, there was no difference in either release or clearance after the addition of flupenthixol compared to nisoxetine alone (Figure 4C,D). Thus, there does not appear to be any facilitatory interaction of D2 receptors for uptake that is blocked by the antagonist. However, administration of an uptake inhibitor does inhibit the effect of a D2 antagonist on release. In mammals, D2R administration after DAT inhibition

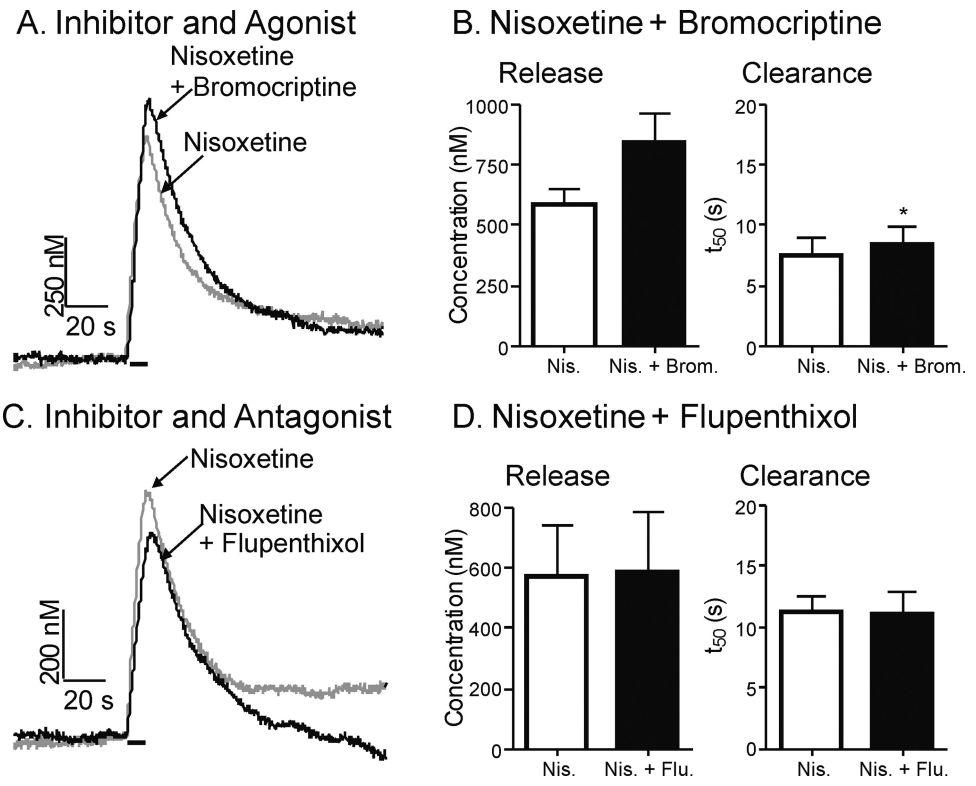

Figure 4. Effects of the DAT inhibitor with D2R agonists and antagonists. (A) Concentration−time profile for a VNC incubated in 50 μM nisoxetine followed by addition of 50 μM bromocriptine. (B) Clearance of dopamine is significantly increased in the presence of both nisoxetine and bromocriptine (n = 6). The effect of 50 μM bromocriptine is suppressed in the presence of 50 μM nisoxetine (n = 6). (C) Concentration−time profile for a VNC incubated in 5 μM nisoxetine followed by addition of 5 μM flupenthixol. (D) Clearance is not significantly changed in VNCs incubated in nisoxetine followed by flupenthixol. The effect of flupenthixol is suppressed in the presence of nisoxetine. All statistics are from paired t tests of release or clearance before and after addition of drug. *p < 0.05. **p < 0.01. ***p < 0.001.

did not increase high-frequency stimulated release in rats,[52] and DAT inhibitors and D2 antagonists increase basal levels of dopamine, which may lead to less DA being available for stimulated release.[53] Similarly, our results show that nisoxetine blocked the ability of the antagonist to increase dopamine release in *Drosophila*. While DAT inhibition affected the regulation of stimulated release by autoreceptors, a direct interaction of D2 receptors and DAT for clearance was not identified. Future experiments could further probe the complex regulation of release governed by uptake and autoreceptors in *Drosophila*.

CONCLUSIONS

We have chemically investigated the effect of the *Drosophila* dopamine-2 receptor on regulating dopamine release. The decrease in stimulated dopamine release in the presence of dopamine agonists and the increase in release in the presence of antagonists are consistent with DDR2 acting as an autoreceptor. These studies were modeled after mammalian studies, which probed autoreceptor functionality by electrical stimulation of dopaminergic fibers and detection of dopamine with FSCV.[27,33,52,54] This specific stimulation protocol and the fast nature of the detection led to a probing of presynaptic effects.[30] Similarly, in our study, optical stimulation of ChR2 located specifically in dopaminergic terminals would also allow investigation of primarily presynaptic regulation. Thus, the pharmacological effects are unlikely to be due to downstream effects caused by activation of postsynaptic dopamine receptors. The effects of D2 agonists and antagonists on stimulated dopamine release in *Drosophila* are analogous to results in mammals; this supports the conclusion that the DD2R is functioning as an autoreceptor, regulating the release of dopamine. While no interaction facilitating uptake was observed for D2 receptors and DAT, disruption of dopamine signaling with an uptake inhibitor did alter the effects of D2 drugs on dopamine release. Because autoreceptors play such an important role in human disease etiology, the conservation of autoreceptors between species makes *Drosophila* a useful model for studying dopaminergic diseases.

METHODS

Chemicals. Unless specified, all chemicals were from Sigma-Aldrich (St. Loius, MO). All solutions were made using Milli-Q water (Millipore, Billerica, MA). All calibrations and dissections were conducted in a modified Schneider's buffer [15.2 mM $MgSO_4$, 21 mM KCl, 3.3 KH_2PO_4, 36 mM NaCl, 5.8 mM NaH_2PO_4, 5.4 mM $CaCl_2$, 11.1 mM glucose, and 5.3 mM trehalose (pH 6.2)]. Stock solutions (10 mM) of all agonists and antagonists were made in DMSO and were diluted with modified Schneider's buffer. The final concentration in the bath around the *Drosophila* CNS was 50 μM bromocriptine, 50 μM quinpirole, 5 μM flupenthixol, 5 μM butaclamol, 5 μM haloperidol, or 5 μM raclopride. For nisoxetine experiments, the final concentration in the bath was 50 μM nisoxetine for agonist and 5 μM nisoxetine for antagonist experiments. A 10 mM stock solution of dopamine for electrode calibration was made in 0.1 M perchloric acid and diluted to 1 μM with modified Schneider's buffer. Larvae were fed 10 mM all-*trans*-retinal mixed with Red Star yeast (Red Star, Milwaukee, WI) and water.

Electrochemical Measurements. Carbon-fiber microelectrodes were made by aspirating single T-650 carbon fibers (Cytec Engineering Materials, West Patterson, NJ) into 1.2 mm × 0.68 mm glass capillaries (A-M Systems, Carlsburg, WA). After pulling in a

vertical pipet puller (Narishige PE-21, East Meadow, NY), electrodes were trimmed to 40−60 μm. Electrodes were then dipped for 30 s into heated (85 °C) Epon Resin 828 (Miller-Stephenson, Danbury, CT) mixed with 14% (w/w) *m*-phenylenediamine hardener (Fluka, Milwaukee, WI). After curing in an oven at 100 °C for 2 h followed by 150 °C overnight, the electrodes were soaked in 2-propanol and backfilled with 1 M KCl before being used.

Tar Heel CV software (gift of M. Wightman, University of North Carolina, Chapel Hill, NC) was used to collect and analyze data from a Dagan Chem-Clamp potentiostat (Dagan, Minneapolis, MN). A triangular waveform was generated, and data were digitized with a homemade breakout box with PCI 6052 and 6711 boards (National Instruments, Austin, TX). Every 100 ms, the electrode was scanned from −0.4 to 1.3 V and back at a scan rate of 400 V/s. A Ag/AgCl reference electrode was placed in the Petri dish near the ventral nerve cord.

Electrodes were allowed to cycle for 15 min prior to being implanted. The peak oxidation current from the collected cyclic voltammograms was converted into concentration using a postelectrode calibration with 1 μM dopamine. For all drug experiments, a second calibration was performed in the presence of drug to account for possible effects of the drug on the electrode sensitivity.

Preparation of Ventral Nerve Cords. Flies containing UAS-ChR2 were crossed to flies expressing *th-GAL4* (a gift from J.Hirsh, University of Virginia) to generate homozygous lines with a *th-GAL4*;UAS-ChR2 genotype. Three-day-old, wandering third instar *th-GAL4*;UAS-ChR2 larvae were selected on the basis of size and activity level, fed *trans*-retinal for 2 days, and kept in the dark. Five-day-old larvae were selected on the basis of size; the central nervous systems were dissected in modified Schneider's buffer and the optic lobes removed as previously described.[10,11] Once isolated, the ventral nerve cord (VNC) was adhered to the bottom of a Petri dish (Becton Dickinson, Franklin Lakes, NJ) with 3 mL of buffer. Using a 40× water immersion lens, an electrode was inserted into the neuropil, a region dense with dopamine cell bodies and terminals. For control experiments, an initial 5 s blue-light stimulation was used to evoke dopamine release followed by the addition of 1 mL of buffer and 5 s stimulations were repeated at 15 min intervals. For drug experiments, an initial 5 s blue-light stimulation was performed, then 1 mL of a solution containing the drug was added to the buffer around the nerve cord, and 5 s stimulations were repeated at 15 min intervals.[10] For preincubation experiments, dissected nerve cords were incubated with drug in the dark for 15−20 min prior to blue-light stimulation.

Statistical Analysis. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Paired *t* tests were used to compare release before and after the addition of drugs with the exception of preincubation with drug, where unpaired *t* tests were used. Data were considered different at a 95% confidence level. Error bars are standard error of the mean.

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