Mechanisms of amphetamine action illuminated through optical monitoring of dopamine synaptic vesicles in Drosophila brain

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Amphetamines elevate extracellular dopamine, but the underlying mechanisms remain uncertain. Here we show in rodents that acute pharmacological inhibition of the vesicular monoamine transporter (VMAT) blocks amphetamine-induced locomotion and self-administration without impacting cocaine-induced behaviours. To study VMAT’s role in mediating amphetamine action in dopamine neurons, we have used novel genetic, pharmacological and optical approaches in Drosophila melanogaster. In an ex vivo whole-brain preparation, fluorescent reporters of vesicular cargo and of vesicular pH reveal that amphetamine redistributes vesicle contents and diminishes the vesicle pH-gradient responsible for dopamine uptake and retention. This amphetamine-induced deacidification requires VMAT function and results from net H+ antiport by VMAT out of the vesicle lumen coupled to inward amphetamine transport. Amphetamine-induced vesicle deacidification also requires functional dopamine transporter (DAT) at the plasma membrane. Thus, we find that at pharmacologically relevant concentrations, amphetamines must be actively transported by DAT and VMAT in tandem to produce psychostimulant effects.

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Prescribed and illicit amphetamines, including amphetamine and methamphetamine derivatives, are some of the most widely used and abused drugs: Total prescriptions number over 15 million yearly in the US, with ~56 million users globally. Amphetamines' psychostimulant effects are generally thought to result from increased extracellular dopamine mediated by efflux of cytoplasmic dopamine through the dopamine transporter (DAT). How amphetamines mobilize dopamine from vesicles to the cytoplasm for subsequent efflux is less clear. Dopamine is synthesized in the cytosol and concentrated into synaptic vesicles ~105-fold (~0.1 M intraluminal dopamine) relative to cytoplasmic dopamine (~1 μM) by the vesicular monoamine transporter (VMAT)1–3. Whether amphetamines also act directly on VMAT to redistribute dopamine from vesicles into the cytoplasm has been debated, and numerous mechanisms have been proposed.

Amphetamines interact with VMAT in vitro, leading some investigators to conclude that they act as non-substrate inhibitors that elevate cytoplasmic dopamine by simply blocking its accumulation in vesicles and thus making most available for efflux. Others have inferred that amphetamines are substrates of VMAT that drive carrier-mediated exchange of vesicular monoamines into the cytoplasm5,6. A third proposed mechanism is that amphetamines deplete synaptic vesicle dopamine stores through mechanisms akin to lipophilic weak bases and protonophores, which degrade the vesicular pH gradient (ApH). This ApH, generated by the vacuolar H+-ATPase (V-ATPase), not only drives loading of monoamines into the vesicle lumen by VMAT, but also promotes intravesicular retention of monoamines through their protonation11. Amphetamines are weak bases (pKₐ 8.8–9.9) and have been proposed to decrease the vesicular ΔpH by buffering luminal free protons, thereby depleting vesicles of stored dopamine and indirectly blocking substrate import. This is the mechanism of action for weak bases such as chloroquine, which is lipophilic enough to diffuse across lipid bilayers (LogP (4.72)12–14. Although amphetamines are less lipophilic (for example, LogP of amphetamine = 1.41)15, they have been hypothesized both to enter vesicles by diffusion as well as by VMAT-mediated transport2. Evidence for these diverse mechanisms has come in large part from in vitro studies of isolated vesicles, cells and brain slices, but the actual relevance of these proposed mechanisms to amphetamines’ in vivo actions has not been ascertained.

Earlier work by Dwoskin and colleagues showed that tetrabenazine and lobeline analogues, which are inhibitors of the neuronal VMAT isoform, VMAT2, blocked methamphetamine’s behavioural action in rodents16,17. While these results suggested that VMAT is instrumental in mediating methamphetamine’s effects, several of these compounds have modest selectivity for VMAT2 and also inhibit DAT. To address this, we developed a new VMAT blocker, (+)-CY477, with improved selectivity and show that acute VMAT inhibition in rodents blocks locomotor and self-administration behaviours stimulated by amphetamines without affecting those induced by cocaine. This selective antagonism indicates that VMAT function is required for the acute actions of amphetamines to release dopamine from intraluminal stores.

To elucidate how amphetamines act on synaptic vesicles to release dopamine into the cytoplasm, we developed an experimental system in Drosophila melanogaster using a functionally viable ex vivo brain preparation16. Actions of amphetamines were studied in this whole-brain preparation using small synthetic and genetically-encoded fluorescent reporters visualized by multiphoton microscopy. We used the second-generation fluorescent false neurotransmitter FFN206 (ref. 17) in combination with genetic manipulations to monitor in real time the dynamics of dopaminergic vesicle cargo in whole fly brains. To examine the effects of amphetamines on vesicle pH, we expressed the synaptic vesicle pH biosensor, dVMAT-pHluorin18, in dopaminergic neurons, which allowed us to monitor real-time changes in synaptic vesicle pH in whole living brain.

Our findings in flies demonstrate that at pharmacologically relevant concentrations, amphetamines, as well as other VMAT substrates, must be transported by VMAT to diminish vesicular ΔpH and redistribute vesicular contents. Our data suggest that VMAT-mediated substrate-coupled H⁺ antiport provides the critical mechanism for amphetamine-induced vesicular decacidification.

**Results**

**VMAT mediates acute amphetamine-induced rodent behaviours.** To validate VMAT2’s role in acute behavioural actions of amphetamines in rodents, we developed (+)-CY477, a novel derivative of dihydrotetrabenazine that retains high VMAT2 affinity but has better selectivity (Supplementary Note 1). (+)-CY477 potently inhibited dopamine uptake by rat VMAT2 expressed in cultured cells (half-maximal inhibitory concentration (IC₅₀) = 26 nM; Supplementary Fig. 1a). (+)-CY477 also exhibited good selectivity for vesicular over plasma membrane monoamine transporters. It inhibited binding to mammalian VMAT2 (Kᵢ = 7.18 ± 1.14 nM), but was inactive at DAT, the serotonin transporter and the norepinephrine transporter (see Supplementary Methods, Supplementary Table 1). Thus, given (+)-CY477’s potency and selectivity for VMAT2, we used it to examine effects of acute pharmacological inhibition of VMAT2 on psychostimulant actions in both mice and rats (see Supplementary Methods, Fig. 1, Supplementary Fig. 2). In these protocols, (+)-CY477 was administered ≤5 min preceding psychostimulant treatments, allowing us to avoid potential confounds associated with gene knockdown or chronic pharmacological depletion of monoamine stores. Acute (+)-CY477 pretreatment (0.3–10 mg kg⁻¹) significantly diminished methamphetamine (3 mg kg⁻¹)-induced hyperlocomotion in mice (Fig. 1a). In contrast, (+)-CY477 failed to attenuate cocaine (20 mg kg⁻¹)-induced hyperlocomotion (Fig. 1b). Preservation of the locomotor response to cocaine indicates that vesicular dopamine stores were functionally intact using these (+)-CY477 doses. Notably, the acute (+)-CY477 doses used in this mouse hyperlocomotion assay were substantially lower than those that produced catalepsy (Supplementary Fig. 1b, Supplementary Discussion). Furthermore, in psychostimulant self-administration assays performed in rats, acute (+)-CY477 pretreatment inhibited self-administration of both methamphetamine and amphetamine in a dose-dependent manner (Fig. 1c,d), whereas cocaine self-administration was again unaffected (Fig. 1e; Supplementary Discussion). Both cocaine and amphetamines are expected to produce locomotor and self-administration behaviours through the common step of elevating extracellular dopamine levels. However, that (+)-CY477 selectively antagonized amphetamine and methamphetamine-induced behaviours but not those of cocaine, clearly illustrates that VMAT2 plays a critical role in the acute actions of amphetamines but not those of cocaine.

**dVMAT is essential for amphetamine behaviour in Drosophila.** Building on these findings in rodents, we established a novel experimental system in D. melanogaster to dissect mechanisms of monoaminergic neurotransmission in vivo. The genetic tractability of Drosophila permits targeted manipulation of gene expression to determine contributions of individual genes to amphetamine’s actions and the neuronal pathways in which they function. Using a behavioural assay of amphetamine-induced hyperlocomotion20,21, we first examined whether...
the sole Drosophila VMAT isoform, dVMAT, is required for amphetamine to produce its behavioural effects in larvae. Vehicle-fed dVMAT null mutants had significantly diminished baseline crawling velocity relative to wildtype (WT) controls (approximately sixfold decrease, $P<0.001$; Fig. 2a). The amphetamine-induced increase in crawling velocity was approximately fivefold greater in WT than in dVMAT null mutants, demonstrating that VMAT is essential for amphetamine-induced hyperlocomotion.

To identify the presynaptic neuronal populations that mediate dVMAT’s contribution to amphetamine-induced locomotion, we restricted dVMAT expression to different monoaminergic neurons using the tyrosine hydroxylase (TH)-GAL4 expression driver23 in the null background. We used the tyrosine hydroxylase (TH)-GAL4 expression driver23 in the null background to restore dVMAT expression to different monoaminergic neuron populations. We restricted dVMAT expression to different monoaminergic neuron populations using the tyrosine hydroxylase (TH)-GAL4 expression driver23 (Tdc Rescue, Fig. 2b) in the dVMAT null background. In contrast to TH Rescue animals, Tdc Rescue larvae exhibited no significant amphetamine-stimulated hyperlocomotion ($\Delta = 0.01 \text{ mm s}^{-1}$, $P>0.05$) even though their rates of basal locomotion (0.73 mm s$^{-1}$) were comparable to WT (0.83 mm s$^{-1}$, $P>0.05$). Thus, dVMAT expression specifically in dopamine neurons plays a critical role in mediating amphetamine-stimulated behaviour in flies, consistent with voluminous data that dopamine is the primary mediator of amphetamine’s psychomotor stimulatory action in mammals2,25.

FFN206 labels monoaminergic nerve terminals in whole brain. To analyse the mechanisms of monoamine loading and release from synaptic vesicles, we used an ex vivo whole fly brain preparation to optically monitor monoaminergic vesicle content by multiphoton microscopy. We utilized FFN206, which was designed as a fluorescent VMAT2 substrate and has an apparent transport affinity ($K_m = 1.16 \pm 0.10 \mu M$) similar to that of dopamine ($K_m = 0.92 \pm 0.05 \mu M$) (ref. 8). In WT brains continuously bathed in FFN206, we observed a dense pattern of ~1 μm fluorescent puncta that varied through the neuropil, consistent with the accumulation of the probe into synaptic vesicles. To test whether FFN206 stains monoaminergic nerve terminals, we restricted FFN206 expression to different monoaminergic neuron populations using the vesicular monoamine transporter (VMAT)-GAL4 expression driver23 (VMAT Rescue, Fig. 2c). FFN206 was similarly targeted to dopamine (DA), serotonin (5HT) and octopamine (OA) neurons, consistent with the specificity of FFN206 for monoaminergic nerve terminals.
**Figure 2 | dVMAT expression in presynaptic dopamine neurons mediates amphetamine-induced Drosophila larval hyperlocomotion.** (a) Wildtype (WT) wCS10 larvae fed amphetamine (60 mM) exhibited hyperlocomotion compared with vehicle (yeast alone; Δ mean velocity = 0.16 mm s⁻¹, P = 0.002, vehicle: N = 20; amphetamine: N = 21). dVMAT null larvae exhibited significantly diminished baseline crawling velocity relative to the WT controls (5.3-fold difference, P < 0.001) with a much smaller increase in locomotion velocity to amphetamine (Δ = 0.03 mm s⁻¹, t = 4.36, P < 0.001; vehicle: N = 51; amphetamine: N = 46). dVMAT null larvae with selective rescue of dVMAT expression in presynaptic dopamine neurons (TH Rescue) exhibited blunted basal locomotion, similar to the dVMAT null, but displayed a robust amphetamine response (Δ = 0.12 mm s⁻¹, P < 0.001; vehicle: N = 44; amphetamine: N = 49). Selective dVMAT rescue in OA/TA neurons (Tdc Rescue) restored basal locomotion to WT levels but not amphetamine-stimulated hyperlocomotion (Δ = 0.01 mm s⁻¹, P > 0.05; N = 44). See Supplementary Fig. 3 showing that expression drivers alone had no effect. Every condition represents the mean ± s.e.m. with all experiments conducted on ≥2 separate occasions. (b) Schematic illustration of monoamine neurotransmitter-containing vesicles in flies of different genetic backgrounds. dVMAT expression (indicated in red) confers the ability to accumulate dopamine, OA, TA, serotonin (5-HT) and histamine (HA) in synaptic vesicles but is absent in the dVMAT null genetic background. TH Rescue or Tdc Rescue selectively restores dVMAT function in dopamine or OA/TA neurons, respectively.

**Figure 3 | FFN206 accumulation is dependent on dVMAT and selectively labels presynaptic DA nerve terminals in adult fly TH Rescue brain.** (a) Multiphoton microscopy of FFN206-loaded adult WT brain revealed extensive presynaptic monoaminergic nerve terminal labelling in the central brain neuropil, accumulating in puncta <1 µm diameter. Labelled regions include the suboesophageal ganglion (SOG), antennal lobe (AL) and the protocerebrum (Proto). (b) Absence of dVMAT expression in brains of dVMAT null mutants abolished virtually all neuropil FFN206 labelling. Residual signal was primarily autofluorescence from tracheal structures (triangle)⁷⁴ as well as some variable staining outside the neuropil. (c) Schematic illustration of the expected monoamine content of FFN206-loaded vesicles (blue fill) in WT, dVMAT null and TH Rescue brains. (d) FFN206 selectively labelled presynaptic dopamine nerve terminals in adult TH Rescue brain with particular enrichment in MB-MV1 neurons (arrow and white box). (e) Single-plane image of MB-MV1 as labelled by FFN206 in a TH Rescue brain. (f) Reserpine pretreatment of an adult TH Rescue brain prevented FFN206 labelling, similar to dVMAT null brain (b). (g) FFN206 labelling of adult TH Rescue brain was similarly blocked by pretreatment with (+)-CYY477 (1 µM, 20 min, 25 °C) (compare with f). String-like structures in e–g are autofluorescent trachea. Comparable results were obtained from N ≥ 3 independent experiments. Images are from projected Z series of coronal sections acquired with similar settings and of comparable depth; all scale bars are 25µm, except for e (Scale bar, 5µm). λ_ex = 820 nm, λ_em = 460/50 nm FWHM.
monooaminergic nerve terminals (Fig. 3a). Labelling was observed in the antennal lobes, suboesophageal ganglion and the protocerebrum (Fig. 3a). Steady-state labelling with 300 nM FFN206 yielded a high signal to background ratio of 60.2 ± 4.7. Neuronal labelling was dVMAT-specific as it was virtually absent from dVMAT null mutant flies (Fig. 3b), establishing FFN206 as an excellent tool for labelling monoaminergic vesicles in the fly.

Selective labelling of dopamine terminals with FFN206. Since FFN206 labels multiple monoaminergic populations that express dVMAT in WT brain, we used the TH Rescue genetic background to drive dVMAT expression selectively in dopamine neurons (Fig. 3c schematic). We observed strong FFN206 labelling of TH Rescue brains in a dense field of dopamine nerve terminals belonging to the MB-MV1 neurons that project to the mushroom bodies and have recently been implicated in associative learning26,27 (Fig. 3d,e). FFN206 also labelled presynaptic dopamine terminals in the suboesophageal ganglion, antennal lobes, mushroom bodies (Fig. 3d) and the central complex (data not shown). Pretreatment with the VMAT inhibitors reserpine (1 µM, Fig. 3f) or (+)-CYT477 (1 µM, Fig. 3g) completely blocked neuronal labelling by subsequent application of FFN206 in TH Rescue brains. These data are consistent with the necessary role of VMAT for FFN206 labelling, also shown in the genetic dVMAT null mutants (Fig. 3b). Therefore, by using FFN206 to label TH Rescue brains, we can specifically image dopamine terminals to examine the neurochemical mechanisms that regulate the content of dopamine vesicles in response to both exocytic and non-exocytic releasing stimuli.

To study the kinetics of stimulated release in presynaptic dopamine nerve terminals, we first measured exocytic vesicle release from FFN206-labelled dopamine terminals in TH Rescue brains. KCl-induced depolarization led to pronounced FFN206 staining pattern in TH Rescue brain (compare with Fig. 3d). The destaining rate is comparable to the rate of imagery chamber and for solution exchange (Supplementary Fig. 5). The FFN206 signal to levels comparable to destaining was not blocked by inhibiting voltage-gated Na\(^{+}\) stimuli at the larval neuromuscular junction (Fig. 4). The destaining rate is comparable to the rate of imaging chamber and for solution exchange (Supplementary Fig. 5). The destaining rate is comparable to the rate of imaging chamber and for solution exchange (Supplementary Fig. 5).

Characterization of dVMAT-pHluorin, a vesicular pH biosensor. We characterized a genetically encoded fluorescent reporter of intraluminal pH to examine changes in monoamine vesicle pH. This biosensor (termed dVMAT-pHluorin) was created by inserting pHluorin, a supereliptic, pH-sensitive GFP, into the dVMAT polypeptide’s first luminal loop. dVMAT-pHluorin was trafficked appropriately to synaptic vesicles as indicated by co-localization with synaptotagmin 1 (svt1), an established vesicle marker (Supplementary Fig. 5a–c). When driven by the TH promoter, dVMAT-pHluorin’s expression pattern in presynaptic dopamine nerve terminals (Supplementary Fig. 5g) appeared identical to FFN206’s punctate staining pattern in TH Rescue brain (compare with Fig. 3d). dVMAT-pHluorin’s fluorescence intensity was dependent on pH, with a high signal to noise ratio (162.6 ± 27.2) and broad dynamic range (Supplementary Fig. 6). Both electrical stimulation (Supplementary Fig. 5d–f) and KCl-induced depolarization significantly enhanced dVMAT-pHluorin’s fluorescence intensity by shifting the pH sensor moiety from the acidic vesicle environment to the neutral extracellular space on vesicle exocytosis (Supplementary Fig. 6a). Under basal conditions, the percentage of dVMAT-pHluorin on the cell surface in our ex vivo whole fly brain preparation (14.5% ± 2.5%) is consistent with that described previously for vesicular pH biosensors heterologously expressed in cultured cells31–33. dVMAT-pHluorin’s pK\(_{a}\) (7.5 ± 0.2; Supplementary Fig. 6b) was similar to those of synaptopHluorin (7.1–7.3) and supereliptic pHluorin (7.2)31,34. Furthermore, dVMAT-pHluorin is a functional transporter as expression of the UAS-dVMAT-pHluorin transgene using the Tdc2-GAL4 expression driver in dVMAT null background flies rescued basal locomotion to 71% of WT levels (P<0.001) (Supplementary Fig. 3b).

Amphetamine needs DAT and VMAT to alkalize dopamine vesicles. We expressed the dVMAT-pHluorin biosensor in presynaptic dopamine nerve terminals to directly monitor amphetamine-induced pH changes in dopamine synaptic vesicles in a whole brain for the first time. As a standard for comparison, we used chloroquine (100 µM) to disrupt the vesicular H\(^{+}\) gradient and thus to alkalize the lumen relative to the acidic starting pH. Amphetamine (1 µM) also caused significant concentration-dependent alkalization, that is, decacidification, of the vesicle (F(3, 16) = 29.96, P < 0.001; Fig. 5a). This amphetamine-induced rise in vesicle pH was sustained during continuous application of the drug by bath superfusion (Supplementary Fig. 7a). Methamphetamine (10 µM) also caused significant vesicular alkalization (F(3, 20) = 9.03, P = 0.001; Supplementary Fig. 8a). In contrast, treatment with the psycho-stimulant methylphenidate, a non-substrate DAT inhibitor41 that is also a lipophilic weak base (calculated pK\(_{a}\) = 9.5 with LogD = 0.24, pH 7.4 (ref. 35)), did not lead to intraluminal vesicular pH changes (P > 0.05) (Fig. 5c) even at 100 µM, a concentration far higher than that needed to block DAT36,37.

By expressing dVMAT-pHluorin in the Drosophila DAT (dDAT) null fumin mutant background38, we were able to examine the role of dDAT in transporting amphetamines into cells by using vesicle alkalization as a readout. Specifically, we asked whether passive diffusion of amphetamines across the plasma membrane supplies sufficient intracellular amphetamine to produce vesicular alkalization or whether it must be imported by the concentrative transporter dDAT. We found that in the absence of functional dDAT, amphetamine did not significantly
raise vesicle pH even at concentrations up to 700-fold greater than the 1 μM concentration that caused significant vesicle alkalization in the WT background (P > 0.05; Fig. 5d). Methamphetamine-induced vesicle alkalization was also blocked in the dDAT null mutant background (Supplementary Fig. 8b). In contrast, subsequent treatment of the same dDAT null brains with chloroquine (100 μM) still produced significant vesicle alkalization (Fig. 5d, Supplementary Fig. 8b). This is consistent with chloroquine’s ability to lipophilically diffuse across membranes and also demonstrates that the synaptic vesicles in dDAT null brains are capable of intraluminal alkalization.

Next, we used a pharmacological strategy to block dVMAT to determine whether its function at the vesicle membrane is necessary for amphetamine-induced vesicle alkalization. Pretreatment with the inhibitor reserpine (1 μM; dVMAT IC_{50} = 258 nM (ref. 39)) prevented amphetamine- and methamphetamine-induced vesicle alkalization (Fig. 5e, Supplementary Fig. 8c), even with extended incubations of increasing amphetamine concentrations (100 nM–10 μM; Supplementary Fig. 7a). Similarly, the VMAT inhibitor (+)-CYY477 blocked amphetamine-induced synaptic vesicle alkalization (Supplementary Fig. 9). In contrast, subsequent alkalization by chloroquine in the same brains (P < 0.001) was unaffected by reserpine blockade of VMAT (Fig. 5e, Supplementary Figs 7b and 8c), highlighting the ability of chloroquine to diffuse across the vesicle membrane independently of VMAT, just as it diffuses across the plasma membrane independently of DAT (Fig. 5d).

Substrate-coupled H^+ antiport by VMAT alkalizes vesicles.

The experiments above clearly demonstrated that amphetamines cause vesicle alkalization in dopamine terminals in an ex vivo whole-brain preparation. At low micromolar concentrations of amphetamines, this alkalization requires concentrative transporters at both the plasma membrane (DAT) and the synaptic vesicle membrane (VMAT). Notably, VMAT functions by stoichiometrically exporting 2H^+ from the vesicle lumen for each cationic monoamine substrate it imports. We hypothesized that if amphetamines are truly VMAT substrates, then their transport should diminish the vesicular ΔpH through the substrate-coupled H^+ antiport mechanism. We therefore tested whether proven VMAT substrates also alkalize vesicles. The endogenous substrate dopamine caused significant concentration-dependent vesicular alkalization (F(5, 21) = 6.74, P < 0.001; Fig. 6a), like amphetamine and methamphetamine. Similarly, FFN206, which is also a reserpine-sensitive VMAT substrate (Fig. 3), led to significant vesicle alkalization at 10 μM (F(3, 12) = 4.10, P = 0.032; Fig. 6b). However, unlike amphetamine or methamphetamine, 10 μM FFN206 still alkalized vesicles in presynaptic nerve terminals in vivo.
chloroquine (100 μM) treatment produced significant alcalization of synaptic vesicles as indicated by dVMAT-pHluorin brightening in the MB-MV1 region. Images are from projected Z-series (Scale bar, 10 μm; false colour, arbitrary fluorescence intensity units). (b) Increasing doses of amphetamine caused concentration-dependent dVMAT-pHluorin brightening compared with vehicle (AHL) (F(3, 16) = 29.96, P < 0.001). (c) Escalating doses of methylphenidate (1–100 μM), a DAT blocker, did not alter vesicular pH (P > 0.05). (d) The dDATfmin (dDATfmin) null genetic background prevented escalating doses of amphetamine up to 700 μM from causing significant dVMAT-pHluorin brightening. Comparison with the WT genetic background (b) shows the vast shift in amphetamine potency due to the absence of functional dDAT in dDATfmin mutant brains. (e) dVMAT blockade by the continuous presence of reserpine (1 μM) prevented dVMAT-pHluorin brightening by amphetamine (10 μM) but not by subsequent treatment of the same brains with chloroquine (100 μM) (F(4,10) = 70.47, P < 0.001; reserpine 1 μM + chloroquine 100 μM, P < 0.001), demonstrating that functional VMAT is necessary for amphetamine-induced vesicular alcalization. dVMAT-pHluorin intensity changes were measured at λex = 920 nm, λem = 525/50 nm FWHM. Pooled concentration-response data for (b–d) show fluorescence changes normalized to that evoked by subsequent chloroquine (100 μM) treatment in each brain, and those in (e) were normalized to initial fluorescence intensity. Drugs were sequentially applied to the brain preparation by bath superfusion. Fluorescence was measured before treatment and after a 10 min drug equilibration period (25 °C). Data are represented as mean fluorescence intensities ± s.e.m. in the MB-MV1 region from N ≥ 3 experiments.

Figure 6 | VMAT substrates dopamine and FFN206 alkalize synaptic vesicles in dopamine nerve terminals. (a) Dopamine caused concentration-dependent alcalization as indicated by dVMAT-pHluorin brightening (F(3, 21) = 6.74, P < 0.001; 10 μM dopamine, P = 0.006; 100 μM dopamine, P = 0.002). (b) FFN206 significantly alkalized synaptic vesicles at 10 μM (F(3, 12) = 4.10, P = 0.032; 10 μM FFN206, P = 0.03). (c) In the dDAT null background flies, FFN206 produced brightening of dVMAT-pHluorin (P = 0.035), indicating that it does not require functional DAT to attain a sufficient concentration in vesicles. Increases in fluorescence in MB-MV1 regions (compared with vehicle, AHL) after 10-min drug treatment were normalized to final chloroquine (100 μM) changes (ΔFmax). Drugs were sequentially applied to the brain preparation by bath superfusion. Fluorescence was measured before treatment and after a 10-min drug equilibration period (25 °C). Data are represented as mean intensities ± s.e.m. in the MB-MV1 region from N > 3 experiments.

the dDAT null mutant expressing dVMAT-pHluorin, demonstrating that FFN206 requires VMAT but not dDAT for vesicle entry (Figs 3b,e,f and 6c). Notably, significant vesicular pH changes were not observed with FFN206 at 1 μM or at the 300 nM concentration we used as a monoamine tracer (Fig. 6b). That amphetamine and methamphetamine alkalize vesicles with similar concentration dependence as established VMAT.
substrates, and even more importantly, that alkalization by amphetamines can be blocked by VMAT inhibitors, together support our hypothesis that substrate-coupled H⁺-antiport accounts for the observed alkalization. Nevertheless, given that amphetamine, methamphetamine, dopamine, and FFN206 are weak bases, it is conceivable that if they were transported in the neutral state, then the neutral species could bind protons from the acidic vesicle lumen, eventually dissipating the vesicular ΔpH. To determine whether substrate-coupled H⁺-antiport by VMAT is sufficient to alkalize vesicles, it was necessary to test a VMAT substrate that is not a weak base. Thus, we used the known DAT and VMAT substrate, 1-methyl-4-phenylpyridinium (MPP⁺), which cannot buffer luminal H⁺ due to the fixed positive charge of its methyl-pyridinium nitrogen 41,42 (Fig. 7a).

MPP⁺ confirms the mechanism of vesicle alkalization by VMAT. Bath application of 100 μM MPP⁺ significantly alkalized synaptic vesicles in presynaptic dopamine nerve terminals (P = 0.028; Fig. 7b). In contrast, there was no significant dVMAT-pHluorin brightening in presynaptic dopamine nerve terminals following MPP⁺ treatment in either the dDAT null mutant (Fig. 7d) or in WT brains pretreated with reserpine (P > 0.05; Fig. 7e), consistent with MPP⁺’s dependence on both dDAT and dVMAT. Moreover, MPP⁺ at the concentration that produced dVMAT-pHluorin brightening (100 μM, Fig. 7b) also destained FFN206 in dopamine terminals of TH Rescue brains even in the continuous presence of FFN206. Destaining showed monoexponential kinetics similar to amphetamine (t₁/₂ = 83.96 ± 15.98 s, R² = 0.96 ± 0.01; Fig. 7f), suggesting that, like amphetamines, MPP⁺ can also cause redistribution of dopaminergic vesicle contents through vesicle alkalization. The relatively low potency of MPP⁺ compared with amphetamine for vesicular alkalization and FFN206 destaining is consistent with its low affinity for VMAT2 (Kᵢ = 92 ± 14 μM) (ref. 43). Therefore, we also tested 3'-OHMPP⁺, a MPP⁺ derivative with a higher affinity for VMAT2 (Kᵢ = 2.4 ± 0.1 μM) (ref. 43). Indeed, 3'-OHMPP⁺ caused significant vesicular alkalization (P < 0.001) at concentrations 10-fold lower (10 μM) than MPP⁺ (compare Fig. 7b,c). Thus, despite the fact that they are not weak bases, MPP⁺ and 3'-OHMPP⁺ can still alkalize the vesicle lumen like amphetamine, methamphetamine, dopamine, and FFN206, which are weak bases. Critically, the property shared by these diverse compounds is their ability to be transported by VMAT. This finding indicates that transport-dependent H⁺ antiport by VMAT is sufficient to diminish intraluminal H⁺.

Figure 7 | MPP⁺ and 3’-OHMPP⁺ alkalize synaptic vesicles and MPP⁺ discharges FFN206 from vesicles in dopamine nerve terminals. (a) Structural formulae of MPP⁺ and 3’-OHMPP⁺. (b) MPP⁺ (100 μM) significantly alkalized synaptic vesicles as indicated by dVMAT-pHluorin brightening (F(3,16) = 137.93, P < 0.001). (c) Escalating doses of 3'-OHMPP⁺ significantly raised vesicle intraluminal pH (P < 0.001) in a concentration-dependent manner. 3'-OHMPP⁺ was effective at a 10-fold lower concentration than MPP⁺. (d) In dDAT null mutants, MPP⁺ (100 μM) did not significantly raise synaptic vesicle pH (P > 0.05). (e) Reserpine (1 μM) blocked MPP⁺ (100 μM) but not chloroquine (100 μM)-induced vesicle alkalization (F(2, 12) = 18.02, P < 0.001; 1 μM reserpine + 100 μM MPP⁺, P > 0.05; 1 μM reserpine + 100 μM chloroquine, P > 0.05). (f) MPP⁺ (100 μM) caused FFN206 destaining in presynaptic dopamine nerve terminals of adult TH Rescue brain. A representative plot was best fit to an initial plateau followed by a monoexponential decay (black; for N = 4 experiments, t₁/₂ = 83.96 ± 15.98 s; R² = 0.96). Arrow indicates drug addition and circle represents the point at which 50% of drug is in the imaging chamber. (b-d) Mean change in fluorescence intensities ± s.e.m. in the MB-MV1 region from n ≥ 3 experiments, normalized to fluorescence evoked by subsequent chloroquine (100 μM) treatment in the same brains. (e) Mean change in fluorescence intensities ± s.e.m. in the MB-MV1 region from n ≥ 3 experiments, normalized to initial fluorescence. (b-e) Drugs were sequentially applied to the brain preparation by bath superfusion. Fluorescence was measured before treatment and after a 10-min drug equilibration period (25°C). (f) Fluorescence decay of MB-MV1 region normalized to fluorescence after steady-state loading with FFN206 (300 nM).
This proposed antiport mechanism for substrate-induced vesicular alkalization is not dependent on vesicle exocytosis. An alternative explanation for dVMAT-pHluorin brightening could be that vesicle exocytosis shifts the sensor from the acidic vesicle lumen to the neutral extracellular milieu. To differentiate these mechanisms, we again took advantage of MPP⁺ ‘s properties as a non-protonatable VMAT substrate that cannot buffer H⁺. KCl or MPP⁺ were applied to brains expressing TH-driven dVMAT-pHluorin as well as a genetically encoded Tetanus toxin light chain (TeTxLC), a known blocker of exocytosis44. Given the relatively fast kinetics of exocytosis, images were acquired at higher frequency to detect rapid vesicular pH changes. Brief pressure ejection of KCl led to a prolonged, intense pH change (Fig. 8a) that was abrogated in flies expressing TeTxLC (Fig. 8b). In contrast, brief application of MPP⁺ caused rapid, transient intraluminal alkalization (Fig. 8c) that was not affected by TeTxLC expression (Fig. 8d). We also used the same genetic background of TeTxLC co-expression with dVMAT-pHluorin to construct a vesicle intraluminal pH calibration curve using a cocktail of ionophores across a broad pH range (see Methods); the TeTxLC was required to avoid the potential confound of vesicle exocytosis during calibration. By interpolating from this curve, we determined that the mean peak change in fluorescence intensity induced by transient application of MPP⁺ in these TeTxLC experiments (ΔF/F ≈ 0.31 ± 0.07) corresponded to a 0.4 pH unit change from the baseline pH of ~5.8 (95% confidence interval: 5.48–6.08) (Supplementary Fig. 6c). These results confirmed that MPP⁺ changes vesicle pH by a mechanism independent of both exocytosis and H⁺ buffering but dependent on VMAT. Taken together, our results point to the intrinsic substrate:H⁺ antiport function of VMAT as the mechanism by which amphetamines, or, more generally, any known VMAT substrate, can elevate the pH within intact synaptic vesicles if it is present in the cytoplasm at an adequate concentration.

**Discussion**

Although there is consensus that amphetamines produce behavioural effects by raising extracellular dopamine levels, diverse and often contradictory inferences have been drawn to explain the molecular basis of this effect. In order to reveal amphetamine’s actions at the synaptic vesicle level, we have combined a number of novel, complementary genetic, optical and pharmacological approaches to address these questions. In both locomotor and self-administration behavioural paradigms in rodents, acute preadministration of the novel VMAT2 inhibitor (+)-CYY477 abolished the effects of

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**Figure 8** | **MPP⁺-induced dopamine synaptic vesicle alkalization in dopamine nerve terminals is reversible and independent of exocytosis.**

(a) Pressure ejection of KCl (10 s, arrow) onto an ex vivo WT whole fly brain preparation led to a delayed brightening of TH-driven dVMAT-pHluorin with a slow decay. (b) The fluorescence response to KCl application (10 s, arrow) was abolished in fly brains expressing both dVMAT-pHluorin and tetanus toxin light chain (TeTxLC), a known blocker of exocytosis, in dopamine nerve terminals. (c) MPP⁺ application (1 s, arrow) caused a more rapid dVMAT-pHluorin brightening in WT brain with a rapid return to baseline. (d) TeTxLC co-expression with dVMAT-pHluorin did not, however, diminish the transient fluorescence response to MPP⁺ application (1 s). All traces show single-plane fluorescence intensity measured at 100-ms intervals, integrated over the MB-MV1 region, and normalized to initial values. Each trace shows 20 s of extended baseline prior to drug application and is representative of N≥3 experiments.
amphetamine and methamphetamine, indicating that VMAT2 function is obligatory for amphetamines to produce their acute behavioural effects. These data are consistent with work by Dwoiskin and colleagues who have developed inhibitors of methamphetamine behavioural actions using lobeline analogues. During their structure-driven pre-clinical studies, this group recognized that the drugs shared the common property of being VMAT blockers.\(^{45}\) Comparable behavioural effects against methamphetamine were also observed with the VMAT2 blocker tetrabenazine, but this drug shows only modest selectivity for VMAT2 over DAT.\(^{46}\) We tried to improve on the selectivity and duration of action of tetrabenazine by synthesizing \((\pm)-CYY477\), a derivative of the high-affinity analogue dihydrodibenzenazine. We found that \((\pm)-CYY477\) indeed has high VMAT2 affinity but negligible affinity for DAT (Supplementary Table 1). Selectivity for VMAT2 over DAT is especially important for discriminating between these transporters as sites of action for amphetamines, which act at both. Taken together, our data demonstrating \((\pm)-CYY477\)’s ability to block vesicular dopamine uptake in vitro and to induce catalepsy at high doses in vivo are consistent with other established VMAT inhibitors like tetrabenazine and suggest that, like its parent compound, \((\pm)-CYY477\) is a potent and effective VMAT2 blocker. The ability of \((\pm)-CYY477\) and other VMAT inhibitors\(^{46–48}\) to block amphetamines’ behavioural actions rules out two widely touted mechanisms for the in vivo action of these psychostimulants at the doses we studied: (1) It has been proposed that amphetamines elevate cytoplasmic dopamine by blocking sequestration of endogenous dopamine into vesicles by VMAT,\(^4\) thus making more dopamine available for efflux by DAT. If this were the case, then VMAT inhibitors would mimic or enhance the behavioural effects of amphetamines. Instead, we found that \((\pm)-CYY477\) antagonized the effects of amphetamines. (2) It has been proposed that, like cocaine, amphetamines elevate extracellular dopamine by competing for dopamine uptake by DAT.\(^{49,50}\) If this were so at pharmacologically relevant amphetamine concentrations, then the selective VMAT2 inhibitor \((\pm)-CYY477\), which has negligible affinity for DAT, should not abolish the acute actions of amphetamines at DAT. Instead, we found that \((\pm)-CYY477\) antagonized the effects of amphetamines, although it did indeed spare cocaine, consistent with cocaine’s competition with dopamine at DAT. That cocaine-induced behaviours are spared by \((\pm)-CYY477\) also indicates that dopamine stores and neurotransmission remain functionally intact after acute pretreatment with the VMAT2 inhibitor.

The profound and selective antagonism of amphetamine-induced behaviours by \((\pm)-CYY477\) points to a critical role for VMAT function in the acute actions of amphetamines upstream of, and in addition to, their established function to facilitate efflux of cytoplasmic dopamine through DAT.

We used a fly model system to elucidate the mechanism of amphetamines’ actions at synaptic vesicles in vivo. We recently introduced FFNs to enable rapid imaging of monoamine storage and release dynamics in vertebrate brain slice\(^{30,51}\). A new FFN, FFN206 (ref. 17) has proved to be an excellent marker of monoaminergic nerve terminals in fly brain. To focus specifically on dopaminergic terminals, we used flies genetically engineered to express dVMAT only in TH-expressing dopamine neurons. FFN206 labelling was reserpine-sensitive and was dissipated by KCl-induced depolarization, consistent with the accumulation of FFN206 in synaptic vesicles by dVMAT. Thus, this probe serves as a sensitive and selective surrogate marker for dopamine content in small synaptic and large dense core vesicles\(^{32}\). Treatment with 1 mM amphetamine diminished FFN206 signal to levels comparable to surrounding neuropil background. While similar kinetics of amphetamine-induced destaining of previous generation FFN molecules loaded into brain slices has been shown\(^{51}\), this is the first report of amphetamine-induced dopamine vesicle content release in whole brain.

Since vesicular pH plays an important role in storage and release of vesicle contents, we used the recently developed vesicular pH biosensor, dVMAT-pHluorin, as a tool to elucidate amphetamines’ effects on intraluminal pH within intact vesicles in our whole-brain preparation. This approach differs from earlier uses of pHluorin biosensors to monitor vesicle dynamics during exo- and endocytosis\(^{31,53}\). With dVMAT-pHluorin we observed that a clinically relevant amphetamine concentration (1 mM) alkalized dopamine vesicles within minutes in a VMAT-dependent manner.

The concentrations of amphetamines that alkalize fly brain dopaminergic vesicles and discharge their content are well within the range of behaviourally active amphetamine or methamphetamine levels found in human and rodent plasma \((~0.7 \mu \text{M})\) (refs 54,55) and close to target plasma levels of \((\pm\text{-})\)amphetamine in therapeutic treatment of children with attention deficit hyperactivity disorder \((0.5–1.1 \mu \text{M})\) \((\text{Ref. 56})\) as well as in chronic methamphetamine users \((1–3 \mu \text{M})\) \((\text{Ref. 57})\). Similarly, in rodents and non-human primates, behaviourally active doses of amphetamines yield brain concentrations \((1–10 \mu \text{M})\) striatal microdialysate \((\text{Ref. 58})\) that are in the range of those causing VMAT-dependent vesicular alkalization in fly brain, although lower concentrations have also been reported\(^{59}\).

Lipophilic weak bases like chloroquine cause vesicle alkalization and readily redistribute vesicle contents as we showed in whole brain with FFN206. However, two lines of evidence demonstrate that, at relevant concentrations, amphetamines do not work by the same mechanisms as chloroquine. (1) Our data show that disruption of DAT or VMAT function through genetic or pharmacological manipulation profoundly reduced amphetamine’s potency to alkalize the vesicle lumen. The requirement for functional transporters at both the plasma membrane and the vesicular membrane for amphetamine action challenges the common assumption that lipophilic diffusion alone can deliver enough amphetamine to vesicles to alkalize vesicles, even at high micromolar concentrations of the drug. By contrast, neither deletion of DAT nor blockade of VMAT hindered chloroquine-induced alkalization. (2) That FFN206 is accumulated in puncta in a VMAT-dependent manner gives optical proof that mildly lipophilic compounds can be concentrated and that the vesicular membrane provides a sufficient barrier to their egress. Since amphetamine \((\text{Log}D = –0.79 \text{ at pH 7.4 (ref. 15)})\) is less lipophilic than FFN206 \((\text{Log}D = –0.54 \text{ at pH 7.4 (ref. 17)})\), it should be even less capable of passive diffusion into or out of vesicles. Altogether, these data provide the most direct evidence to date that the amphetamines, like dopamine and FFN206, are bona fide substrates of VMAT and not merely inhibitors.

We next aimed to distinguish between two possible mechanisms for this alkalization: (1) Alkalization of the acidic lumen could occur by net export of \(2\text{H}^+\) via VMAT for every amphetamine molecule transported into the vesicle (substrate:2\(\text{H}^+\) antiport)\(^{61}\) or (2) substrates including amphetamines that are weak bases could buffer luminal \(\text{H}^+\) following their VMAT-dependent transport into the lumen if their neutral, unprotonated species were transported\(^2\). Since protonated amphetamine species \((\text{pK}_a = 9.9)\) outnumber neutral species by \(~300:1\) in the cytosol at physiological pH, it seems unlikely that sufficient neutral species would be transported into the vesicle lumen to significantly buffer the acidic environment\(^15\). Instead, our data are more consistent with the cationic substrate:2\(\text{H}^+\) antiport mechanism, especially since...
the permanently charged VMAT substrates MPP⁺ and 3'-OHMPP⁺ also alkalinize vesicles even though they are not weak bases. Thus, we infer that the H⁺ antiport-driven process of substrate translocation by VMAT is sufficient to account for the vesicular alkalization produced not only by MPP⁺ and 3'-OHMPP⁺, but which cannot be protonated, but also by the VMAT substrates dopamine, amphetamine, methamphetamine and FFN206, which have basic amino groups that are predominantly protonated at physiological pH.

Measurements of synaptic vesicle pH are typically made in cells or in isolated vesicles, and to our knowledge this study represents the first attempt to measure the intraluminal pH of synaptic vesicles in a whole living brain. Ionic manipulations used for calibrating pH measurements can lead to exocytosis, confounding interpretation of the data. To prevent exocytosis, we used flies with the TcTxC genetic background to generate a pH calibration curve and found baseline intraluminal pH of dopamine vesicles in the fly brain to be ~5.8, comparable to that reported for mammalian synaptic vesicles (pH 5.7) (ref. 62). This value is competitive non-substrate inhibitor of DAT20,21. These results support that amphetamine but not for the actions of methylphenidate, a known DAT inhibitor.

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Our data lead to a model for how pharmacologically relevant concentrations of amphetamines increase extracellular dopamine: (1) DAT imports and concentrates amphetamines in the cytoplasm. (2) Cytoplasmic amphetamines (and endogenous dopamine) are accumulated into vesicles by VMAT in a H⁺-antiport process that diminishes vesicular H⁺ concentration. (3) Diminished vesicular ΔpH promotes dopamine redistribution from vesicles into the cytoplasm through a mechanism that is still unclear. Vesicle deacidification alters the protonation state of luminal dopamine, which might be sufficient to increase its diffusion across the membrane. However, this mechanism does not readily explain protonophore-induced efflux of MPP⁺ from vesicles24, since this compound is a quaternary ammonium and thus pH cannot alter MPP⁺’s protonation state. Whether VMAT itself might be a route of dopamine release from vesicles11,65 requires further study. (4) The redistributed cytoplasmic dopamine subsequently effluxes out of the cell through DAT via amphetamine-stimulated reverse transport. Previous work demonstrated that phosphorylation of DAT is essential for dopamine efflux66 and for locomotor behaviour induced by amphetamine but not for the actions of methylphenidate, a competitive non-substrate inhibitor of DAT20,21. These results are consistent with the inference from our rodent behavioural data that competitive inhibition at DAT by amphetamines is not sufficient to produce behavioural effects at the concentrations tested. By the same logic, stimulation of dopamine synthesis by TH and/or inhibition of dopamine catabolism by monoamine oxidases, both of which can elevate cytoplasmic dopamine concentrations5, are insufficient in themselves to produce amphetamines’ acute behavioural effects. These data highlight the functional coupling of DAT and VMAT as critical to amphetamines’ actions in vivo. Because amphetamines are also substrates of norepinephrine transporter and serotonin transporter, and VMAT is present in adrenergic and serotonergic neurons5, the tandem actions of plasma membrane transporters and VMAT are likely important for amphetamine-induced release of other monoamines as well. Furthermore, our results demonstrate the first application of a novel experimental system that can be used to develop important new insights into the physiology of intact monoaminergic vesicles.

Methods

Fly strains. D. melanogaster strains include the WT strain, w1118CS10 (wCS10), which is w1118 outcropped into Canton-S for 10 generations. The dDAT null dDATfmn (gift of Dr S. Birman, Université Aix-Marseille II-III)22 and Tdc2-GAL424 which were previously shown to drive expression in DA and TA/OA neurons, respectively22,24. To detect pH changes in the intravesicular lumen of monoaminergic synaptic vesicles, we constructed a new fly strain with the novel transgene, UAS-dVMAT-pHluorin in both WT dDAT and dDATfmn genetic backgrounds. All fly strains were grown and maintained on standard cornmeal-molasses media at 25°C under a 12-h light dark schedule.

Construction of transgenic fly strains. The dVMAT-pHluorin fly strain was created by injection of the dVMAT-pHluorin probe sequence into WT fly embryos. The probe was generated by insertion of the pH-sensitive pHluorin DNA into the intracellular luminal H⁺ of dopamine vesicles in the fly brain to be ~5.8, comparable to that reported for mammalian synaptic vesicles (pH 5.7) (ref. 62). This value is competitive non-substrate inhibitor of DAT20,21. These results support that amphetamine but not for the actions of methylphenidate, a known DAT inhibitor.

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Imaging. An isolated, ex vivo whole adult fly brain preparation was obtained by rapid removal and microdissection of the brain from decapitated flies as previously described. A significant advantage of this preparation is that following removal of head cuticle and connective tissues, drugs are applied directly to brain tissue at known concentrations. This whole-brain preparation was imaged with continuous flow on a frozen laser scanning microscope (Prairie Technologies Bruker Corp., Middleton, WI). Fluorescent emission was collected using a 460 nm/50 nm FHWM bandpass filter for dVMAT-pHluorin.

Compounds. The drugs used in the present study including their respective salt and enantiomeric forms were as follows and purchased from Sigma-Aldrich (St Louis, MO) unless indicated otherwise: d-aminophenylamine hemisulfate, d-methamphetamine HCl, cocaine HCl (Merck, Whitehouse Station, NJ), methylphenidate HCl (BDH, England), benserazide HCl (Sigma-Aldrich, St Louis, MO), 3-hydroxytryptamine HCl (dopamine), 2-(N-morpholinophenoxy)ethanesulfonic acid (MES), 1-methyl-4-phenylpyridinium iodide (MPP⁺), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), monosodium A salt sodium (monensin), nigericin sodium salt (nigericin), N-methyl-N-propargylbenzylamine hydrochloride (N,N,N′,N′-2-propyltetrayanobenzene (±)-CY477) N59% yield. Optical resolution of (±)-CY477 using (±)-2,3-dibenzoyl tartaric acid ((±)-DBT) in ethanol yielded optically pure (±)-CY477 (ee >99%) according to our modifications to previously described methods (see Supplementary Note 1, Supplementary Figs 10–13).

Preparation of dVMAT-pHluorin. Preparation of (±)-CY477 is described in detail in the Supplementary Information. Briefly, treatment of commercially available 7-hydroxy-6-methoxy-3,4-dihydroisouquinoline with 2-acetyl-N,N,N′,N′-tetra-methylpentan-1-aminium iodide salt in refluxing ethanol provided racemic trans-10-desmethyltetrahydronorharman (±)-CY477 in 59% yield. Optical resolution of (±)-CY477 using (±)-2,3-dibenzoyl tartaric acid ((±)-DBT) in ethanol yielded optically pure (±)-CY477 (ee >99%) according to our modifications to previously described methods. Assay of Dopamine Uptake by VMAT2. 293-rV2 cells, HEK293 cells stably transfected with rat VMAT2, were obtained from Dr R.H. Edwards and have been previously described methods (see Supplementary Note 1, Supplementary Figs 10–13). Specifically, we subjected whole, ex vivo brain preparations expressing dVMAT-pHluorin in presynaptic DA nerve terminals to a brief acid wash (100 s, 25 °C, pH 5.5) to quench fluorescence from cell-surface expressed biosensor, followed by recovery of fluorescence after wash-out (25 °C, pH 7.5). To confirm these values, we used complementary NH4Cl alkaline (50 mM, 25 °C, pH 7.5, 60 s) to visualize both intracellular and extracellular dVMAT-pHluorin pool combined with KC treatment (40 mM, 25 °C, pH 7.5, 60 s) to visualize both intracellular and externalized cell surface dVMAT-pHluorin fluorescence. Using the brief acid wash method, we found that the percentage of dVMAT-pHluorin on the cell surface under basal conditions was 14.5% ± 2.5%, comparable to 17.7% ± 5.4% determined with the complementary NH4Cl alkalization method.

Imaging. An isolated, ex vivo whole adult fly brain preparation was obtained by rapid removal and microdissection of the brain from decapitated flies as previously described. This whole brain preparation was placed in a recording chamber (JG-23, Warner Instruments, Hamden, CT) with continuous flow of AAL. This experimental system affords facile manipulation of drug concentrations. The timewidth and drug solubility limits in the imaging buffer were determined by flowing an auto-fluorescent green dye dissolved in PBS buffer (1:100 dilution) under conditions identical to those experimentally used to deliver drugs to fly brains. Brain preparations were imaged on an Ultima multimorphon laser scanning microscope (Prairie Technologies Bruker Corporation, Middleton, WI) using the laser line 568 nm, a 63× (0.9 NA) water immersion objective lens (Carl Zeiss Microscopy LLC, Thornwood, NY). The illumination source was a Coherent Chameleon Vision ll Ti: Sapphire laser (Coherent, Inc., Santa Clara, CA) and we typically used <5 mW mean power at the sample. Fluorescent emission was collected using a 460 nm/50 nm FHWM bandpass filter for FFN206 (λex = 820 nm) and 525/50 nm FHWM bandpass filter for dVMAT-pHluorin (λex = 920 nm). When measuring the effect of FFN206 on dVMAT-pHluorin brightening, there was no cross-talk of the FFN206 signal into the dVMAT-pHluorin 525 nm/50 nm imaging channel when using λex = 920 nm. We tested for spectral bleed-through and found that even a 1000 mW solution of FFN206 generated no detectable fluorescence when using λex = 920 nm. We used a spectral bandpass filter for dVMAT-pHluorin excitation parameters (λex = 920 nm, 1.5 mW mean power at sample). R3846 multi-alkali photomultiplier-tube detectors (Hamamatsu
subsequently normalized to initial predrug treatment MB-MV1 fluorescence (cessation of stimulation.

Image processing and analysis

For experiments involving FFN206 or vMAT-P-HuLin imaging in adult fly central brain, maximum-intensity z-projections were generated using Fiji (ImageJ) (ImageJ). Time-lapse imaging was performed using a Zeiss LSM5 Pascal Laser Scanning confocal microscope equipped with a Zeiss 63 × Neofluor, 1.3 NA oil immersion objective lens. Live imaging of these larval fillets was conducted using a Zeiss Axio Observer Z1 microscope equipped with a cooled back-illuminated electron multiplying CCD camera (Andor iXon 3897, Andor, South Windsor, CT) and DG4 light source (Sutter Instrument, Novato, CA) with a GFP Brightline Filter Set (Semrock, Rochester, NY) and Zeiss Axiochroplan 100 × (1.0 NA) water-immersion objective lens.

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**Statistical analyses**

Statistical significance for the larval behavioural data were determined by one-way analysis of variance (ANOVA; \( p = 0.05 \)) followed by Bonferroni post hoc t-tests to compare between-group differences. For analysis of the rodent behavioural data, statistical sig-
rificance was assessed by one- or two-way repeated-measures ANOVA (\( p = 0.05 \)) followed by post hoc Bonferroni t-tests for all pairwise comparisons. IC50 values were computed using a nonlinear, least-squares regression analysis using GraphPad Prism. Affinities (Kv) were calculated using the Cheng–Prusoff equation (2).

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**Additional information**

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