



Striatal dopamine neurotransmission: Regulation of release and uptake



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ABSTRACT

Dopamine (DA) transmission is governed by processes that regulate release from axonal boutons in the forebrain and the somatodendritic compartment in midbrain, and by clearance by the DA transporter, diffusion, and extracellular metabolism. We review how axonal DA release is regulated by neuronal activity and by autoreceptors and heteroreceptors, and address how quantal release events are regulated in size and frequency. In brain regions densely innervated by DA axons, DA clearance is due predominantly to uptake by the DA transporter, whereas in cortex, midbrain, and other regions with relatively sparse DA inputs, the norepinephrine transporter and diffusion are involved. We discuss the role of DA uptake in restricting the sphere of influence of DA and in temporal accumulation of extracellular DA levels upon successive action potentials. The tonic discharge activity of DA neurons may be translated into a tonic extracellular DA level, whereas their bursting activity can generate discrete extracellular DA transients.

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1. Regulation of dopamine release

Dopamine (DA) neurotransmission is generally initiated by the fusion of synaptic vesicles in axonal boutons, with the exceptions of release by amphetamine-like drugs that can release DA *via* reverse transport through the DA uptake transporter (DAT) [1,2], and DA release from dendrites, which is widely suspected to occur *via* fusion of specialized secretory organelles [3]. This process is regulated at many levels, including DA synthesis, uptake and vesicular transport, as well as by Ca^{2+} homeostasis and regulatory exocytotic proteins. In addition, neurotransmitter receptors on DA neurons, axons, and dendrites provide feedback, regulate DA release, and in some conditions locally drive DA release.

1.1. Dopamine release by synaptic vesicle fusion

1.1.1. Quantal release and detection

In 1950, Bernard Katz and Paul Fatt published recordings of random electrical “noise” consisting of spontaneous small “action potentials” (a term now used quite differently) at frog neuromuscular junction they compared to “fluctuations in the number of light quanta which strike the [photo]receptor cells” [4]. These “miniature end plate potentials” required extracellular Ca^{2+} and were exacerbated by high osmolarity [5]. The events fit a Poisson distribution, which simulates the probability of random occurrences of multiple basic events [6], indicating that neurotransmission occurs in multiples of a “quantal” unit. They conjectured that “the apparatus for the release of acetylcholine (ACh) at a junction is subdivided into a large number of units (at least 100), each of which is able to operate independently of the rest” [7].

In contrast to ionotropic ACh receptors, DA primarily activates G protein-coupled receptors that do not produce small rapid currents, and so a means to detect quantal DA release was elusive. An effective approach was provided by electrochemical catecholamine detection, introduced by Ralph Adams and colleagues [8–10]. These methods were improved by the introduction of carbon-fiber microelectrodes for detection of catecholamines by Francois Gonon [11] and Michael Armstrong-James and Julian Millar [12,13].

Armstrong-James and Millar [14] also developed the method of fast-scan cyclic voltammetry (FCV) with carbon-fiber electrodes, which is widely used for monitoring DA release and uptake *in vivo* and in *ex vivo* brain slices.

The first analysis of quantal release of catecholamines used large secretory vesicles from the adrenal gland. Extracts from adrenal cells provided the original evidence for secretory transmission [15]. In 1990, Mark Wightman and colleagues [16] used amperometry to detect quantal catecholamine release from adrenal cells. In contrast to postsynaptic recording, amperometric recording indicates directly the number of molecules released and the duration of a quantal release event, which in adrenal chromaffin cells is about $\sim 10^6$ molecules over the course of $\sim 10^{-1}$ s.

Amperometric recording was then adapted to record from axonal terminals of cultured midbrain DA neurons. Synaptic vesicles in these axons are ~ 40 nm in diameter, with a volume that is ~ 1000 -fold smaller than that of adrenal chromaffin granules, with proportionally smaller quantal events that are of shorter duration than those from adrenal cells [17,18]. The released catecholamine was identified as DA based on: (1) blockade by reserpine, a vesicular monoamine transporter type 2 (VMAT2) inhibitor; (2) colocalization with tyrosine hydroxylase (TH); (3) the potential required for DA oxidation; (4) absence of detection from neurons that lack DA; and (5) elevation of quantal size following exposure to L-DOPA, a DA precursor, or increased VMAT2 expression. The shape of the majority of quantal DA events in neurons closely fit a simulation of transmitter diffusion through a pore [19], but some release events that deviate from such simple shapes (see Section 1.1.3).

In cultured DA neurons, quantal events have been recorded from boutons in axons, and from acutely dissociated DA somata [20], which may represent quantal somatodendritic release events or release of synaptic vesicle precursors that would have been trafficked to axons. Release events have also been found in acute midbrain slices, although it is difficult to exclude release from nearby DA or serotonin terminals [21]. It seems likely that occasional DA secretion occurs at cell bodies, as VMAT2 transfection of hippocampal neurons can produce quantal

release events from the cell body after exposure to L-DOPA [22]. Electron microscope (EM) studies show few obvious synaptic vesicle-like organelles in DA dendrites, but rather that VMAT2 is found primarily in tubular structures [3]. Quantal DA release from synaptic vesicles has also been recorded by amperometry from retinal bipolar cells [23] and invertebrate neuronal cell bodies [24,25].

Spontaneous DA release events, some of which may represent individual quanta, have been recorded postsynaptically after lentiviral-mediated expression of an anion channel, LGC-53, from *C. elegans* in striatal medium spiny neurons [26]. Another new technique for DA detection is the use of cell-based neurotransmitter fluorescent engineered reporters or “CNiFERS” which after transfection can produce fluorescent responses to a variety of transmitters that activate G-protein coupled receptors, including DA [27]. Another recent optical technique is provided by fluorescent false neurotransmitters that are substrates for VMAT2 and in some cases DAT, and which can be accumulated and released from DA synaptic vesicles [28,29]. Recent evidence suggests that the majority of sites with many synaptic vesicles in striatal DA axons may not exhibit fusion, even when accompanied

by depolarization-induced increase in intracellular Ca^{2+} concentration (Pereira et al. [30]).

1.1.2. The synaptic vesicle cycle

The fundamental difference between quantal release of catecholamines from secretory glands and central DA neurons is the secretory vesicles involved. In adrenal medullary cells, the large (150–300 nm diameter) “chromaffin granules” [31] that accumulate catecholamines [32,33] fuse with the plasma membrane to exocytose transmitter, but do not recycle locally to produce new storage vesicles.

In contrast, the synaptic vesicle “cycle” leads to a range of means to regulate synaptic transmission. Soon after early EM images of synapses revealed small (~40–50 nm diameter) “synaptic vesicles” in axon terminals [34,35], Sanford Palay made a link to Fatt’s conjecture from quantal recording, writing “The heretofore unrecognized structure demanded by these physiological data may be the small vesicles which crowd the axon terminals, cluster at the junctional surface, and open onto the intrasynaptic space” [36]. Indeed, catecholamine synaptic vesicles played an important role in confirming the synaptic vesicle hypothesis, as they accumulate

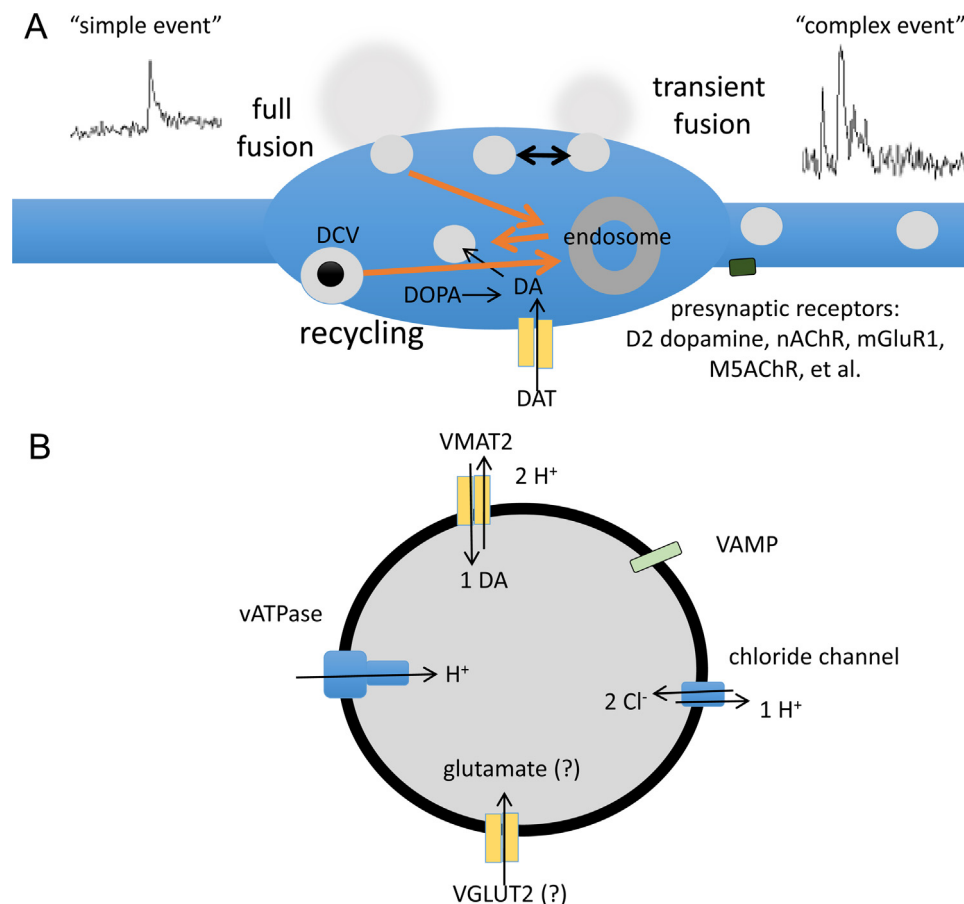


Fig. 1. Features of synaptic vesicle activity in dopamine axons.

(A) Fates of synaptic vesicle membrane. Striatal dopamine (DA) release sites are *en passant* structures on long and highly branched axons, with many small synaptic vesicles in both axons and boutons and a smaller number of dense core vesicles (DCV) that are thought to both undergo exocytosis and deliver small synaptic vesicle membrane components following membrane recycling from endosomes. The source of vesicular DA can be from synthesis via tyrosine hydroxylase (TH) action on L-DOPA or from uptake of extracellular DA by the DA transporter (DAT), with subsequent accumulation in vesicle by the vesicular monoamine transporter, VMAT2. Synaptic DA vesicles can undergo fusion in a flickering mode (“complex event”) that involves transient and reversible exocytosis [17] or can undergo full fusion (“simple event”). After full fusion, vesicle membrane is recycled via an endosomal intermediate, via either “bulk endocytosis” or clathrin-coated intermediates. The endosomes may fuse with autophagosomes for retrograde transport and lysosomal degradation [69]. Recent evidence suggests that the majority of sites with many synaptic vesicles may not exhibit fusion [30].

(B) A vastly simplified DA synaptic vesicle showing some features discussed in this article. These include the DA transporter VMAT2 which is thought to provide net antiport of two protons for each DA molecule, the vacuolar vesicular ATPase (vATPase) that pumps protons to the vesicle interior, an anion channel (the predominant chloride channel in synaptic vesicles is likely CIC-3) that provides net counterions (probably with proton efflux) to decrease the free energy required for the proton gradient DA uptake, and a v-SNARE protein, VAMP, a.k.a. synaptobrevin, involved in synaptic vesicle fusion via formation of a SNARE complex with plasma membrane t-SNAREs. Some DA synaptic vesicles might also possess the glutamate vesicular transporter, VGLUT2.

osmophilic catecholamine reaction products [37] and the osmophilic false transmitter 5-hydroxy-DA [38].

Eric Holtzman [39] and subsequently Bruno Ceccarelli [40] showed that fluid phase endocytic tracers such as horseradish peroxidase are accumulated by synaptic vesicles during stimulation, and that after the tracer is removed, further stimulation eliminates the label. Thus, synaptic vesicle membrane is endocytosed from the plasma membrane following full fusion and synaptic vesicles are re-formed (*i.e.*, recycled) and then undergo further bouts of fusion to release the tracer (Fig. 1A). Early studies indicated that some vesicles may fuse transiently *via* a fusion pore without full fusion [41–43], a process typically known as “kiss-and-run” fusion, and although established for large dense core vesicles [44], this phenomenon has long remained controversial for small synaptic vesicles. In the case of DA axons, amperometric recordings of quantal release are consistent with “flickering” transient fusion pore formation. The recycling of synaptic vesicles can be modeled as a series of kinetic steps, each of which can be regulated to alter neurotransmitter release (Fig. 1B). These include uptake of neurotransmitter by specific transporters that utilize an energy gradient formed by an ATP-driven proton pump, trafficking of vesicles to a presynaptic release site, a “docking” step that tethers the vesicle to its eventual site of fusion with the plasma membrane, a “priming” step during which the docked vesicle is placed in a fusion-ready state, a fusion step which may proceed *via* full fusion with the membrane or transient fusion [17], and a series of recycling steps leading to vesicle reformation.

1.1.3. DA synaptic vesicle fusion structure

Amperometric recordings from chromaffin and mast cells suggest that the fusion pore during large dense-core vesicle fusion can exist in at least two states, a “foot” that represents a small (~3 nm diameter) reversible fusion pore, and a full event that is often interpreted to indicate full fusion [45–48]. Such findings suggest that fusion pore modulation is capable of affecting the amount and kinetics of transmitter release.

The study of vesicle fusion modes in axons of DA neurons has been hampered by their small size. Only one study has addressed this issue, using amperometric recordings of quantal release to midbrain DA neurons in culture [17]. Such recordings at high time resolution (~50 μ s) show that although most DA release events are “simple”, with a single classical spike, 15–20% consists of a rapid succession of spikes that gradually decreases in amplitude from first to last. These “complex” events are consistent with flickering of the vesicle fusion pore, with each “flicker” releasing 25–30% of vesicular DA. The “simple” events had a similar quantal size as the first “flicker” of a “complex” event suggesting that these two types of events are variants of kiss-and-run [17]. The extent of fusion-pore flickering would impact the fraction of vesicular DA that is released per fusion event (Fig. 1A). Complex events may allow neurons to reuse vesicles rapidly without undergoing comparatively slow recycling. As complex events produce a higher quantal size, complex events could regulate transmitter spillover.

Transient flickering of the fusion pore occurs in adrenal chromaffin and other large dense core vesicles [47,49] although DA synaptic vesicle flickers are considerably shorter (100–150 μ s vs. 10,000–500,000 μ s respectively) and occur at a much higher frequency than in dense-core vesicles (4000 Hz vs. 170 Hz) [49] with a far greater fraction of transmitter released (25–30% vs. <1%) [49]. Fusion pore flickering is also seen at glutamatergic synapses [50].

Andrew Ewing and colleagues developed a microfluidic-based platform for catecholamine detection in isolated PC12 cell vesicles and found that each vesicle released ~40% of its total catecholamine load [51], consistent with kiss-and-run in these cells.

The type of synaptic vesicle secretory event may be regulated by protein kinase C (PKC) activity [17]. Drugs that enhance PKC activity increase the number of events per stimulus but decrease the fraction of complex events, whereas staurosporine, a broad spectrum kinase inhibitor, decreases the number of events but enhances the fraction of complex events. Most studies suggest a negative regulation of kiss-and-run by PKC, including evidence that pharmacological activation of PKC accelerates fusion pore expansion and induces kiss-and-run to full fusion conversion [52,53]. Conversely, PKC inhibition decreases fusion-pore size and induces a switch from full fusion to kiss-and-run with higher frequency stimulation [52]. PKC also contributes to full-fusion exocytosis downstream of muscarinic ACh receptor (mAChR) activation in chromaffin cells [54]. Interestingly, knockout or inhibition of atypical PKC θ , but not the related atypical PKC δ , decreases catecholamine quantal size [55], although whether this is *via* vesicle fusion regulation remains unknown.

Fulop and Smith [52] suggest that increased firing rates enhance intracellular Ca^{2+} levels sufficient to activate PKC, which promotes fusion-pore dilation converting kiss-and-run events to full vesicle collapse. Myosin II and the myristoylated alanine-rich C-kinase substrate (MARCKS) are downstream of PKC in this mechanism; PKC-induced phosphorylation of these proteins is necessary to disrupt the actin cytoskeleton with high stimulation, which appears to be crucial for the switch from kiss-and-run to full fusion [56,57]. Myosin inhibition and the disruption of actin polymerization can also slow fusion-pore expansion and increase fusion-pore lifetime without affecting quantal size, suggesting facilitation of catecholamine release from the vesicle by actin and myosin [58].

In addition to flickering modes and kiss-and-run mechanisms, recycling of synaptic vesicle membrane may occur *via* “bulk endocytosis” of the membranes of many synaptic vesicles [59] or *via* smaller clathrin coated endosomal intermediates [60,61]. The involvement of dynamin on vesicle retrieval following kiss-and-run exocytosis has been demonstrated by several groups [62]. While kiss-and-run and full fusion both require dynamin for vesicle retrieval, kiss-and-run appears to be independent of clathrin-mediated endocytosis, as disruption of clathrin function inhibits the slow endocytosis characteristic of full fusion, but not kiss-and-run-associated rapid endocytosis [63,64]. Confirmation of a direct role for dynamin in kiss-and-run was obtained using fluorescence imaging of PC12 cell plasma-membrane lawns, in which the retrieval of fusing granules by kiss-and-run was monitored by uptake of fluid phase markers [65]. Retrieved granules were preferentially associated with dynamin, and granule recapturing was inhibited by dynamin function disruption, which may involve a dynamin-synaptophysin interaction that is promoted by elevated Ca^{2+} [66,67]. A model of “ultra-fast” synaptic vesicle membrane endocytic recycling independent of clathrin has also been reported [68].

When synaptic vesicle membrane is ultimately sent for degradation, this may occur by fusion of endosomal compartments to autophagosomes, although small synaptic vesicles also appear to sometimes be engulfed by presynaptic autophagosomes [69].

1.2. How much DA is in a synaptic vesicle?

The term “quantal size” for monoamine systems, which mostly exert their effects *via* G-protein-coupled receptors, has come to mean the number of molecules released during a quantal event [19]. This is a different meaning than that of Katz *et al.*, in which quantal size is indicated by the ionotropic-receptor currents that result from transmitter released during fusion of a single vesicle. With the newly introduced approach of electrochemical cytometry, however, DA synaptic vesicles can be isolated directly from

brain and the quantity of DA molecules measured by an electrode as the membrane is lysed [70]. In axonal DA vesicles from healthy mice, transmitter content is $\sim 30,000$ DA molecules, which can be modulated by amphetamine and L-DOPA (see Sections 1.2.2 and 1.2.5).

A variety of means to modify the quantal size of DA have been identified (for review, see [19,71]). A long-standing parallel effort by William Van der Kloot has elucidated presynaptic mechanisms that regulate quantal size at the neuromuscular junction, and his reviews, which predate use of amperometric quantal detection in CNS cells, are highly recommended [72,73].

1.2.1. Altered free energy for vesicular DA sequestration

Secretory vesicles maintain high levels of monoamines against a large concentration gradient [74,75]. In isolated chromaffin granules, monoamines (A) distribute according to the electrochemical gradient composed of the voltage gradient ($\Delta\psi$) and pH gradient as

$$\log\left(\frac{[A]_{in}}{[A]_{out}}\right) = \Delta\psi FRT + 2\Delta pH$$

In chromaffin granules, granule pH is often estimated to be ~ 5.6 , the cytosolic pH ~ 7.2 , and $\Delta\psi \sim +80$ mV. Assuming $RT/F = 59$ mV, this indicates an equilibrium transvesicular catecholamine gradient of $\sim 36,000:1$.

This relationship hints at multiple interventions that might alter quantal size (Fig. 1B). In the next section, we will first discuss effects on the *right* hand side of the equation, the pH and electrical gradients. Then, we will discuss effects of the *left* side of the equation, the DA concentration gradient across the membrane.

1.2.2. Roles of pH, electrical gradients, and amphetamines on vesicular dopamine uptake

The pH gradient of synaptic and secretory vesicles is provided by the vacuolar H^+ -ATPase, which consists of V0 and V1 subunits [76,77]. [Some data implicate the V0 domain in the process of vesicle fusion as well [78,79].]

Determination of pH in small synaptic vesicles is not only challenging, but an elusive concept: the compartment is so small, typical values such as pH and voltage gradients do not clearly describe their state. A synaptic vesicle in a DA neuron of diameter of ~ 40 nm has a volume of

$$\left(\frac{4}{3}\right)\pi(2 \times 10^{-6}\text{cm})^3 = 3.4 \times 10^{-17}\text{cm}^3$$

corresponding to 3×10^{19} synaptic vesicles/L. If each synaptic vesicle had only a single free proton, the concentration of protons would be

$$\frac{3.4 \times 10^{19}}{6.02 \times 10^{23}} = 5 \times 10^{-5}\text{moles/L} = 50 \mu\text{M}$$

This would provide a $\text{pH} = -\log(H^+) = 4.3$! Even odder, as the log of zero is undefined, a synaptic vesicle with no free proton has no pH value. Thus, it would seem that small synaptic vesicles shuttle between no pH value and a value of 4.3 or smaller.

One means to estimate synaptic vesicle pH has been to examine quenching of “synaptobluorin”, originally an “ecliptic” fluorescent pH sensitive (pK_a 7.1) green fluorescent protein (GFP) mutant added to the luminal domain of synaptobrevin [80], and subsequently to a luminal domain of VMAT2 [81]. The fluorescence is quenched by proton binding to the residue Tyr-66, which eliminates absorption of 488 nm wavelength light. When a membrane-permeable weak base like ammonia is applied, the pH gradient collapses so that all pH values are “clamped” to that of the extracellular milieu. If extracellular pH is more basic than the

synaptic vesicle, ammonia-collapse of the acidic synaptic-vesicle gradient would enhance the fluorescent signal: thus, titration of signal by lowering extracellular pH in the presence of ammonia should indicate synaptic vesicle pH from the point at which it ceases to cause brightening.

A more useful approach is to expose synaptobluorin-expressing neuronal cultures to pH 7.4 media, and then add saturating levels of ammonia. The fraction of neutral synaptobluorin (i.e., fluorescent) moiety at a pH value is calculated from a version of the Henderson-Hasselbach equation, where at pH 7.4

$$Q^+ = \frac{1}{(1 + 10^{\text{pK}_a - \text{pH}})} = \frac{1}{(1 + 10^{7.1 - 7.4})} = 0.67$$

The level of fluorescence increase, ΔF , due to ammonia indicates the amount of synaptobluorin that has been neutralized (ΔQ^+), and can indicate the charge of the vesicle synaptobluorin (Q_i^+) prior to alkalization.

$$\Delta Q^+ = \Delta F = \frac{(Q_{7.4}^+ - Q_i^+)}{Q_i^+}$$

or

$$Q_i^+ = \frac{Q_{7.4}^+}{(\Delta F + 1)}$$

For ΔF of 9, $Q_i^+ = 0.67/(10) = 0.067$, indicating that 6.7% of vesicular synaptobluorin was neutral/fluorescent prior to alkalization. In a DA bouton, which has ~ 80 synaptic vesicles (Daniel Garton, Ciara Torres, D. Sulzer, unpublished EM data), this likely represents signal from ~ 6 vesicles. Placing this value into the Henderson-Hasselbach equation for a Bronsted acid yields

$$\text{pK}_a + \log_{10}\left(\frac{\text{base}}{\text{acid}}\right) = 7.1 + \log_{10}^{(0.067/1 - 0.067)} = \text{pH } 6.0$$

There are a variety of issues that complicate the use of synaptobluorin to estimate synaptic vesicle pH. In some cases, a high level of synaptobluorin can be present on the plasma membrane [82], although this is less of an issue with the use of VMAT2-pHluorin, introduced by Robert Edwards and colleagues [81], which has far less plasma membrane localization. Another challenge is that pHluorin probes are most sensitive near their pK which is near 7, so that a change from pH 5.0 to 5.5, typical of large dense core catecholamine secretory vesicles [83,84], would yield a very small change in signal. Nevertheless, this approach has been used to determine a synaptic vesicle pH of 5.6 in cultured DA neurons [85] and a pH of 5.8 in DA neurons in *Drosophila* brain [86]. As already noted, current data seem to indicate that *in situ* most synaptic vesicles have an undefineable pH value with no free proton, while a minority contain one or very few protons with a pH of ~ 4.3 .

In principle, ratiometric pH probes could provide more accurate pH measurements. There are ratiometric pHluorins [80] as well as a new class of pH-sensitive ratiometric fluorescent false neurotransmitters that are VMAT substrates and have preferable pK_a values in the 5–6 range. Ratiometric fluorescent false neurotransmitters have been used to study exocytosis from DA axons, as they become brighter when released to the neutral extracellular medium [28]. However, they have been used to monitor pH changes in large dense-core vesicles, such as those in PC12 cells, which have a large enough volume to monitor the pH of an individual vesicle optically. Interestingly, PC12 vesicles display a broad pH distribution even within an individual cell, which centers around pH 5.9 [87].

Despite the challenges in determining synaptic vesicle pH, the proton gradient across the vesicle membrane is of fundamental importance for its function. Exposure of isolated catecholamine

vesicles to protonophores collapses the pH gradient and rapidly redistributes transmitter from inside to outside the vesicle [74,88]. The importance of the vesicular proton gradient in determining monoaminergic quantal size was demonstrated in chromaffin cells using bafilomycin, a H^+ -ATPase inhibitor, which decreases catecholamine quantal size [84,89].

Lipophilic weak bases such as chloroquine are distributed across membranes according to the pH gradient [90]. As their concentration becomes sufficiently high, they exceed the buffering capacity of the vesicle interior and collapse the pH gradient, leading to decreases in quantal size [25].

Amphetamine and its derivatives like methamphetamine are weak base compounds that are the only widely used class of drugs known to elicit transmitter release by a non-exocytic mechanism [2]. As substrates for both DAT and VMAT [91,92], amphetamines can be taken up to the cytosol and then sequestered in vesicles, where they act to collapse the vesicular pH gradient [88]. In PC12 cells, where the pH of individual large secretory vesicles can be estimated, pH-responsive fluorescent false neurotransmitters confirmed that high methamphetamine (100 μ M for 4 min) rapidly alkalinized the mean vesicular pH from 5.9 to 6.4 [87].

Due to this ability to collapse vesicular pH gradients, amphetamine provided a first instance of pharmacological manipulation of DA quantal size, as measured in PC12 cells [25]. Subsequent work showed that two classes of DA vesicles present in the giant DA neuron of *Planorbis corneus* were differentially depleted by amphetamine [93]. In dopamine axons of the striatum, electrochemical recordings in acute striatal slices suggest similar actions of amphetamine in intact tissue [94,95], although contradictory findings have yet to be resolved. For example, Paul Garris and colleagues have shown that amphetamines indeed can enhance synaptic vesicle fusion in DA neurons *in vivo*, perhaps via a circuit mechanism [96]. Moreover, some of the decrease in evoked DA release may also be due to activation of a striatal circuit in the striatum (Lizardi-Ortiz *et al.*, ms. in preparation).

New work confirms that while amphetamines indeed collapse acidic gradients in DA synaptic vesicles in mammalian striatum and in *Drosophila* brain, the net reverse transport of protons through VMAT during vesicular amphetamine uptake plays a larger role in the collapse than does a “weak base” action, in which protons are buffered by intravesicular amphetamine [86,97]. In striatal brain slices, 20 μ M para-chloroamphetamine can release a fluorescent antipsychotic compound from synaptic vesicles; however, this effect is blocked by a VMAT2 inhibitor, reserpine, indicating a requirement for VMAT mediated-proton efflux. Reserpine does not block such redistribution by a higher concentration of para-chloroamphetamine (100 μ M), however, indicating that amphetamine concentration plays a role in these mechanisms [97]. In *Drosophila*, reserpine prevents methamphetamine-induced loss of protons, while chloroquine, a non-VMAT substrate weak base, is not prevented by reserpine. The toxin MPP+ and its derivatives that are non-protonable VMAT substrates also collapse the pH in a reserpine-sensitive manner [86]. Together, these findings are consistent with a requirement for a role for VMAT uptake and net proton antiport in the amphetamine-mediated collapse of proton gradients of small synaptic vesicles, at least at lower drug concentrations. This may contrast with amphetamine actions at isolated large dense-core catecholamine vesicles, in which VMAT blockade by reserpine was ineffective in blocking the pH collapse by amphetamine and several other drugs throughout the ranges examined [88].

An unexpected effect of prolonged amphetamine or weak base exposure, at least in adrenal chromaffin catecholamine vesicles, is a delayed rebound hyperacidification that eventually leads to an enhanced quantal size [83]. Likewise, extensive depolarization also acidifies chromaffin vesicles [84] and increases quantal size

[84,98], in tandem with a greater proportion of larger “active” vesicles that contain a halo around the dense core [84,99] [see also [89,100–102]]. The mechanisms underlying enhanced acidification with prolonged weak base exposure or stimulation are unknown, but may involve Ca^{2+} -dependent PKA and PKC effects on quantal size and exocytosis [55,103] or roles for counterions including Cl^- and glutamate [104] (see following section).

Antipsychotic drugs are also weak base compounds [105] that can accumulate in dopaminergic and other synaptic vesicles [106], although they are not thought to act as VMAT substrates [97]: as they would be released during synaptic vesicle exocytosis, such loading and release could be important for their temporal and spatial effects on synaptic transmission. Their effects on quantal size may be complex, as while they would compete with DA for protons, they would also act on D2 receptors.

1.2.3. Roles of Cl^- , glutamate, and GABA

The vesicular Cl^- channel regulates the vesicular pH gradient since the entry of Cl^- dissipates the developing electrical potential, enabling the H^+ -ATPase to generate a larger H^+ concentration gradient [74]. Regulation of ionic conductances by Cl^- [107], and TRP channels [108] across the synaptic vesicle also control net accumulation of transmitter, by regulating the electrical gradient [84,109,110]. The predominant chloride channel on synaptic vesicles, ClC-3, has been suggested to operate as a Cl^-/H^+ antiporter rather than as a classical Cl^- channel [111]. Since the stoichiometry of ionic exchange for these carriers was proposed to involve the transport of two Cl^- ions per each H^+ , and therefore the net movement of 3 positive charges out of the vesicles, this would promote a greater dissipation of the electrical gradient than of the H^+ concentration gradient, prompting the H^+ -ATPase to pump more protons in (Fig. 1B). A Cl^- channel inhibitor caused a small but significant inhibitory effect on quantal size [84], while ClC-3 knockout mice display a drastic reduction in catecholamine quantal size [112].

Glutamate may also act as a synaptic vesicle counterion for at least some DA neurons that express the vesicular glutamate transporter (VGLUT2) [113], leading to co-release of glutamate and DA [114–118]. It has been suggested that glutamate might diminish the voltage component of the free energy gradient allowing for greater DA packaging [104] (Fig. 1B). Glutamate acidifies vesicular pH in VGLUT2-containing vesicles of the ventral striatum, even in the presence of high physiological concentrations of Cl^- , suggesting that Cl^- and glutamate play distinct roles in regulating vesicular proton gradients [104], although this is controversial [118].

Recently, GABA, which is electroneutral, has also emerged as a potential co-transmitter in DA neurons [119–121], with loading of GABA into DA vesicles in a VMAT2-dependent manner [119] although evidence for this in isolated DA synaptic vesicles is presently lacking. If there is indeed significant accumulation of GABA by VMAT2, GABA might compete with DA and thus decrease DA packaging, in addition to any effects that occur on exocytosis due to GABA corelease.

1.2.4. Intravesicular storage capacity

Accumulation of high DA concentrations inside synaptic or secretory vesicles also depends on the storage capacity of the vesicular lumen. DA-containing vesicles, particularly large dense-core vesicles, contain a condensed matrix of transmitters, proteins and other small molecules. Small synaptic vesicles that released soluble contents during previous fusion still have strongly charged residues, and display a condensed matrix when exposed to 5-hydroxy-DA as a false transmitter. Components of this intravesicular matrix in dense-core vesicles are granins: water-soluble glycoproteins including chromogranin A and B and secretogranins. DA

binding to these proteins keeps vesicular osmolarity low, preventing excessive vesicle swelling while allowing more transmitter storage [122]. Granins are found in endocrine and neuroendocrine cells from which they are co-released with monoamines during exocytosis [123,124]. In addition to regulating vesicular levels of transmitters, these proteins (or their proteolytic cleavage products) are implicated in dense-core vesicle formation, vesicular sequestration of Ca^{2+} and modulation of exocytosis [123–126].

A role for chromogranins in maintaining high catecholamine levels in secretory vesicles was shown using chromaffin cells from chromogranin A and B single- and double-knockout mice [127,128]. Although cytosolic catecholamine levels increased in chromogranin single-knockout cells incubated with L-DOPA, catecholamine secretion per vesicle did not increase, suggesting saturation of vesicular catecholamine accumulation.

Upon exocytosis, DA and other monoamines dissociate from the intravesicular matrix for release into the extracellular space, likely via ion exchange [129]. Temperature, osmolarity, extracellular pH and cations can regulate quantal size in chromaffin cells, probably by modulating catecholamine degranulation, although the mechanisms involved are poorly understood [19,130–132].

1.2.5. L-DOPA and dopamine synthesis

Typically, synthesis of L-DOPA from tyrosine via TH provides the rate-limiting step in catecholamine synthesis. Regulation of TH appears to underlie the decreased quantal size mediated by D2 DA autoreceptors in PC12 cells [133]: quinpirole, a D2 receptor agonist, inhibits TH activity and decreases quantal size by 40–50%. L-DOPA increases DA synthesis and quantal size in PC12 cells independently of TH, and prevents the effect of quinpirole on DA quantal size. The effect of D2 receptor activation to decrease quantal size is likely mediated by decreasing TH affinity for its cofactor tetrahydropyridine (BH_4) by blocking a cAMP-dependent pathway that mediates TH phosphorylation [134–139].

More broadly, regulation of TH expression by transcription, RNA stability, and translation is influenced by many factors [140–142]. In midbrain DA neurons, cAMP can induce translation of TH mRNA, increasing TH protein and activity without altering TH mRNA levels [143]. Post-translational modification plays a key role in the regulation of TH activity: TH phosphorylation, which involves many steps [142], influences the affinity of TH for BH_4 and for interactions with other regulatory proteins, each of which could regulate quantal size.

As TH is rate-limiting, administration of its product, L-DOPA, proved to be a successful clinical intervention for Parkinson's disease [144], with effects at the synaptic level [145]. The effects of L-DOPA on DA release have been studied extensively in cultured cells using amperometry. In primary cultures of murine substantia nigra pars compacta (SNc) neurons, L-DOPA increases quantal size by 300% (from 3,000 to 10,000 DA molecules) in only 30 min [17,18], with a comparable increase in acutely dissociated DA somata [20], retinal DA neurons [23], chromaffin cells [146] and PC12 cells [84,147,148].

These effects on quantal size are consistent with *in vivo* FCV data from the nucleus accumbens (NAc), in which L-DOPA leads to a rapid increase in evoked DA release [149]. The ability of L-DOPA to increase DA loading into synaptic vesicles in living animals was shown by electrochemical cytometry [70]. In healthy mice, L-DOPA (50 mg/kg administered 2 h prior to sacrifice) increased vesicular DA storage by 240% (from 30,000 to 71,000 molecules). Although absolute numbers vary with experimental paradigm L-DOPA consistently increases vesicular DA content.

A surprising consequence of L-DOPA-mediated increase in vesicular DA content is that it is accompanied by increased vesicle volume [148], which helps compensate for the change in free energy. Later capacitance recordings in cultured cells [146] and

measurements of vesicles isolated from animals treated with L-DOPA [51], suggest that increased vesicle volume results from the fusion of additional membrane with the vesicles.

1.2.6. VMAT2 activity

VMAT expression levels further regulate DA accumulation. In chromaffin vesicles, inhibition of the peripheral vesicular transporter, VMAT1, with reserpine decreases catecholamine content [150,151], which is reflected in decreased quantal size detected with amperometry [152]. Surprisingly, however, VMAT1 inhibition by reserpine decreases the volume of dense-core vesicles (the converse of the increase seen with L-DOPA), although the resulting catecholamine concentration remains constant [84,99,146].

Expression of the CNS transporter isoform, VMAT2, in hippocampal neurons facilitates secretion of presumed DA from these normally non-DA cells in the presence of L-DOPA [22]. In cultured DA neurons, overexpression of VMAT2 markedly increases quantal size, and also increases the number of events per stimulus, likely by revealing events that were otherwise buried in the noise [153]. Interestingly, synaptic-vesicle recycling in DA axons in VMAT2 knockout mice appears normal, suggesting that recycling is independent of the rate of transmitter accumulation [154].

It has been suggested that DA production and vesicular loading are carried out by a complex of linked proteins [155–157] including a direct interaction between the DA synthesis machinery and VMAT [156], as TH and aromatic amino acid decarboxylase (AADC), which converts L-DOPA to DA, co-immunoprecipitate with VMAT from rat striatal lysates and PC12 cells. Uptake of DA into striatal synaptic vesicles is decreased when interaction between VMAT2 with TH and AADC are disrupted [156]. Whether this also decreases quantal size is unknown.

An essential role for vesicular catecholamine storage is demonstrated by VMAT2 knockout mice, which die within days after birth [158]. FCV recording in striatal slices from 2- to 4-day old mice showed that DA release evoked by electrical stimulation can be detected in wildtype but not VMAT2 knockout littermates [157]. VMAT2-deficient mice expressing only 5% of wildtype VMAT2 levels, however, survive well into adulthood [159,160]. Consistent with the expected decrease vesicular DA stores, single pulse-evoked DA release in slices of dorsolateral striatum from these mice is only ~30% of control [161].

VMAT is regulated at the levels of protein expression and post-translational modification. In chromaffin cells, an increase in VMAT2 mRNA and protein occurs with long-term depolarization in a Ca^{2+} -dependent manner [162–164]. Among potential VMAT regulators are G-proteins that inhibit VMAT-mediated uptake in a variety of systems [71]. The G(o2) alpha protein, for example, can associate with secretory vesicles, and inhibit the activity of VMAT1 and VMAT2 [71,165,166]. G(q) alpha inhibition of VMAT2 in mouse platelet granules depends on luminal transmitter levels as it does not occur in monoamine-depleted granules, and is restored upon vesicle refilling [167].

Pharmacological regulation of VMAT *in vivo* by DAT inhibitors and amphetamine analogues also occurs through a mechanism that involves D2 receptor activation [168]. DAT inhibition by cocaine or methylphenidate *in vivo* induces a rapid increase in vesicular DA uptake and in VMAT2 levels in purified striatal vesicles from treated rats [169,170], apparently from redistribution of VMAT2 within synaptic terminals [169] downstream of D2 activation [170–172]. In contrast to DAT inhibitors, methamphetamine and other amphetamine analogues decrease vesicular DA uptake and VMAT2 levels in striatal vesicles, an effect that is inhibited by D2 receptor antagonism [168,173].

Exposure to glial-derived neurotrophic factor (GDNF) increases neuronal DA quantal size [18], although the mechanism remains unknown. Emerging evidence suggests that brain-derived neurotrophic factor (BDNF) also regulates DA release. In transfected hippocampal neurons, BDNF co-localizes with VMAT2 [22]. This association appears to have functional consequences, with an age-related decrease in VMAT2 and DAT activity in mice with partial BDNF deletion (BDNF^{+/-}). An age-related decrease in evoked striatal DA release and in motor performance is also seen in BDNF^{+/-} mice [174,175], with partial restoration of electrically evoked DA release in slices by exogenous BDNF [174,175].

1.3. Regulation of the number of synaptic vesicles that fuse

1.3.1. Ca²⁺ dependence of dopamine release

As introduced by Fatt and Katz [5] at the neuromuscular junction, extracellular Ca²⁺ regulates quantal DA release from axons and dendrites [176,177]. The voltage-gated Ca²⁺ channels that regulate striatal DA release have recently been re-characterized without the confounding influence of Ca²⁺ channels on cholinergic interneurons (ChIs; see Section 1.4.1 and 1.4.4) that were unavoidably included in previous studies. These include N, P/Q, T and L-type Ca²⁺ channels [178], whose roles can vary with firing rate, due to Ca²⁺ effects on short-term plasticity. The relative dominance of these Ca²⁺ channels also differs between dorsal striatum (caudate-putamen, CPu) and ventral striatum (NAc), with evidence for different Ca²⁺ microdomains and buffering between these regions. A variety of heteroreceptors and the D2 autoreceptor can also regulate Ca²⁺ currents in DA axons [179] (see Section 1.4.1). The specific Ca²⁺ channels required for somatodendritic DA release remain uncertain [180], given that the release can be triggered in very low extracellular Ca²⁺ concentrations [181]; contributing factors, including intracellular Ca²⁺ stores [182], are discussed elsewhere [183].

1.3.2. Regulation of fusion modes by synaptic proteins

Mechanisms that might “overrule” control of fusion events by Ca²⁺ include presynaptic proteins can block synaptic vesicle fusion, like tomosyn [184], which has a synaptobrevin-like motif that may compete with binding of synaptic vesicles via SNARE proteins [185,186]. Through a completely different mechanism, fusion of chromaffin dense-core vesicles is regulated by an adaptor protein, AP-3 [187,188], with a decrease in quantal size associated with over-expression of the neuronal isoform, and an increase in quantal size with proportional changes in vesicle volume associated with AMP-3 deficiency. Although AP-2 and AP-3 are involved in synaptic vesicle recycling [189], effects on DA quantal size are unreported.

Examination of evoked DA release in the striatum *in vivo* using FCV in synapsin I/II/III triple knock-out mice suggests that these proteins regulate DA synaptic vesicle “reserve pools” [190], and that synapsin III might be particularly important for DA release [191]. Another form of regulation is presynaptic macroautophagy. Striatal DA boutons can form autophagic vacuoles that can sequester and degrade synaptic vesicles, resulting in decreased evoked DA release [69], for example when mTOR is inhibited in the presynaptic bouton.

The most studied endogenous regulator of quantal DA release is alpha-synuclein, a synaptic protein aggregated in Parkinson's disease that can inhibit DA release [192]. Inhibition of axonal DA release by wild-type alpha-synuclein can be attenuated by activity and elevated Ca²⁺ [192–195], possibly as Ca²⁺ elevation redistributes alpha-synuclein away from synaptic vesicles [196]. In chromaffin cells, this inhibitory effect occurs at a late pre-fusion “priming” step [197]. Animals deficient in combinations of alpha-, beta- and/or gamma synuclein show more obvious effects on DA

release than single knockouts [198]. Conversely, transgenic rodent lines overexpressing human wild-type alpha-synuclein, at levels that approximate those following gene locus multiplication in PD, show deficits in DA transmission in dorsal but not ventral striatum [199]. The A30P or A53T mutations appear to inhibit DA release, consistent with gain-of-function mutations [193,194,200,201].

1.4. Regulation of axonal dopamine release by autoreceptors and heteroreceptors

Modulation of axonal DA transmission can occur at the level of midbrain DA cell bodies and locally at presynaptic axons. Here we focus on regulation at the axonal level (see also [202,203]); description of consequences of DA neuron activity on striatal release can be found elsewhere [204–207]. A key regulator of axonal DA release are D2 autoreceptors, which were identified on DA axons using immuno-EM [208]. This method has also been invaluable for identifying heteroreceptors on DA axons, including GDNF receptors [209], β_2 -nicotinic ACh receptors (β_2 -nAChRs) [210], delta and kappa opioid receptors [211,212], metabotropic mGluR1 [213], and possibly GABA_B receptors [214]. Although the presence of other presynaptic receptors in DA axons remains to be confirmed, studies using synaptosome preparations, microdialysis, and FCV data from *in vivo* and *ex vivo* slices suggest important roles for heteroreceptor modulation on DA release, albeit with some conflicting results.

1.4.1. Dopamine autoreceptors

There is a long history of examining the role of autoregulation of DA neuron activity and DA release via DA receptors presumably expressed on DA neurons and axonal release sites. Activation of autoreceptors decreases release by mechanisms that include inhibiting DA synthesis, enhancing DA uptake by DAT, and regulating VMAT expression [215]. Here we focus on effects of autoreceptor activation on release probability. Most of this work is based on the assumption that the effects of DA on subsequent DA release are mediated by autoreceptors on DA axons, but this has been somewhat upended by studies suggesting that DA receptors on other neurons, including medium spiny neurons and ChIs, also regulate DA release [216,217].

D2 receptors are expressed along the somatodendritic extent of midbrain DA neurons [208]. Roles for D2 autoreceptors in presynaptic regulation are well established, but a role for D3 autoreceptors has been controversial. D3 immunoreactivity is found in most midbrain DA neurons, but is undetectable in axonal regions [218]. While it has been suggested that both D2 and D3 receptors function as autoreceptors [219–222], no deficits in autoreceptor function are apparent in D3-receptor knockout mice, although extracellular DA concentration ([DA]_o) in NAc is elevated [223]. Conversely, D2-receptor knockout mice show no detectable autoreceptor response to D2-family agonists on firing rate, DA release or DA synthesis. These results implicate the D2 receptor as the primary functional autoreceptor [224–227].

Nevertheless, biphasic suppression of DA release monitored using FCV in striatal slices is seen with a D3-selective agonist, 7-OH-DPAT, which provides evidence for D3- as well as D2-dependent DA release regulation [228]. This is supported by a study in striatal slices from D3-KO mice that suggested some role for D3 in regulation of secretion, but not synthesis [229]. D3 receptors can couple to the same G protein-activated inwardly rectifying potassium channels (GIRKs) as D2 receptors [230–232], and can thereby affect DA release [233], however, even in acutely dissociated midbrain neurons from D2-knockout mice, D3 receptor-activation of GIRK currents is absent [234].

In summary, DA autoreceptor function is predominantly carried out by D2 receptors. It has been suggested that of two isoforms of

the D2 receptor generated by alternative splicing, the D2S isoform serves presynaptic autoreceptor functions regulating DA release while D2L acts mainly at postsynaptic sites [235–237].

Autoreceptor regulation of DA release was initially studied *in vitro* with neurochemical approaches [238], and shown to inhibit axonal [239–245] and somatodendritic DA release [241]. The molecular mechanism underlying DA release inhibition through axonal D2 autoreceptors is unresolved. The simplest explanation is D2 receptor-dependent GIRK activation, with inhibition of DA release caused by membrane hyperpolarization and current shunting, as seen in DA neurons [246]. Consistent with this mechanism, pulse-train evoked DA release in guinea-pig CPu is enhanced by tertipapin, a GIRK channel blocker [247]. Caveats, however, are that tertipapin has limited efficacy at GIRK2 channels, the primary subtype in mouse DA neurons [248], and additional effects on cholinergic interneurons were not considered (see Section 4). Adding further doubt to GIRK channel involvement in D2-autoreceptor regulation of axonal DA release, FCV studies in striatal slices suggest involvement of a very different class of K⁺ channels, specifically Kv1.2, 1.3 and 1.6 [Martel et al. [464]], which are voltage-dependent rather than G-protein coupled channels.

Additionally, D2 autoreceptor activation can inhibit voltage-gated Ca²⁺ channel opening: patch clamp studies using dissociated midbrain DA neurons show that D2 receptor activation decreases Ca²⁺ currents primarily via N- and P-type Ca²⁺ channels [179]. As axonal DA release is supported by at least N- and P/Q-type Ca²⁺ channels [177,178,180,249], inhibition of Ca²⁺ channels could contribute to DA release inhibition. Direct D2 regulation of Ca²⁺ channels has not been proven, although K⁺ channel activation would decrease voltage-dependent Ca²⁺ currents. Further muddying the waters, autapses (synapses that a neuron makes on itself) of cultured midbrain neurons show no evidence of D2-autoreceptor regulation of Ca²⁺ influx [250].

Regulation of DA release by autoreceptors *in vivo* was first shown using microdialysis. Striatal [DA]_o increases with systemic or intra-striatal administration of D2 antagonists, and decreases with intra-striatal infusion of a D2 agonist [251]. *In vivo* electrochemical studies indicate that basal [DA]_o is sufficient for tonic stimulation of D2 autoreceptors, and inhibition of action potential-dependent DA release [252,253]. Mice lacking D2 receptors allowed evaluation of dynamic autoreceptor regulation [224]. As in similar studies in rodents and primates

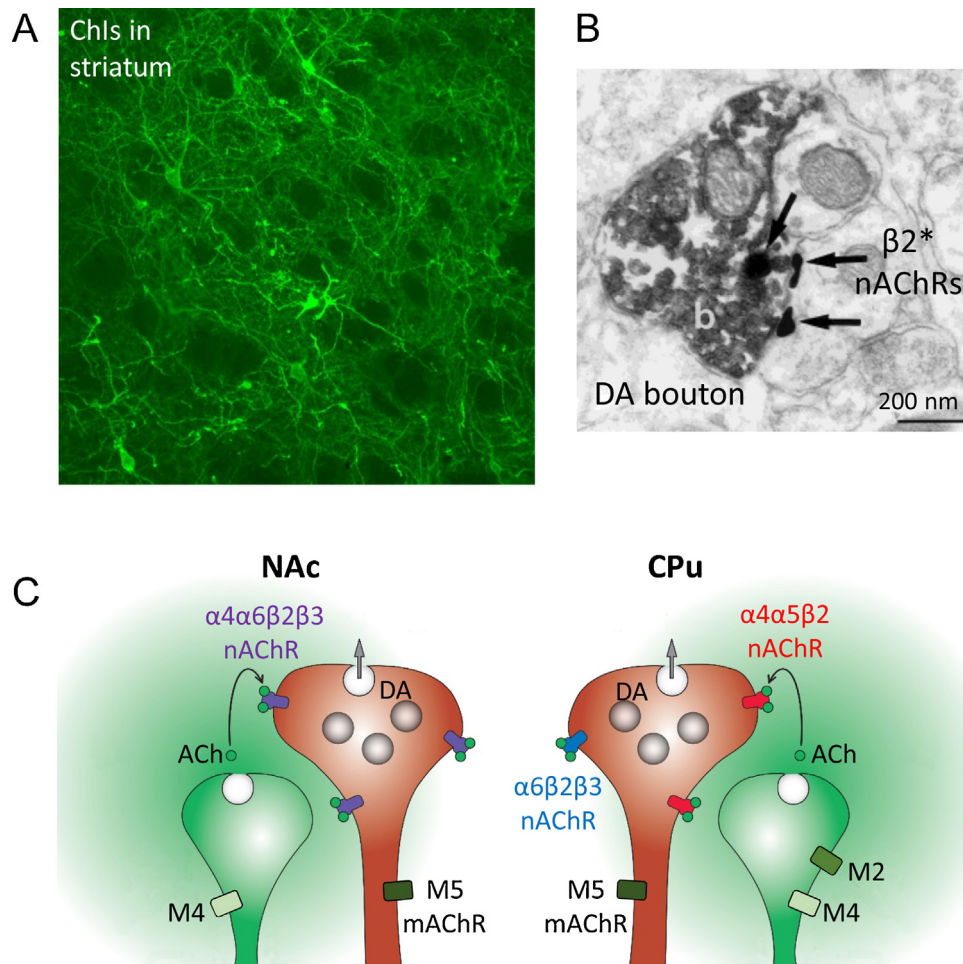


Fig. 2. Striatal cholinergic innervation of dopamine axons and the cholinergic receptor subtypes involved.

(A) The striatum receives dense innervation from intrinsic cholinergic interneurons (ChIs), shown by green fluorescence after injection of eYFP-containing constructs in ChAT-Cre mice (courtesy of KA Jennings and SJ Cragg).

(B) Direct localization of β2-subunit-containing (β2*) nicotinic cholinergic receptors (nAChRs) (arrows) on dopamine (DA) axons, reproduced from [210] with permission.

(C) Cartoon of the range of subtypes of striatal nAChRs and muscarinic cholinergic receptors (mAChRs) and their locations on ChIs or DA axons that appear to govern the control of DA release by acetylcholine (ACh) in nucleus accumbens (NAc, left) and caudate-putamen (CPu, right). A broader range of nAChR and mAChR types (color-coded) appear to operate in CPu than in NAc. M2 and M4 mAChRs regulate dopamine by regulating ChIs, whereas M5 mAChRs regulate DA release directly.

[227,254,255], a conditioning electrical stimulus was given, and the effect on a test pulse assessed at varying times afterwards. In control mice, the onset of the D2-mediated inhibition of DA release is 50–100 ms, with a maximum effect 150–300 ms after the conditioning stimulus, and termination by 800 ms; this regulation was lost in D2 knockout mice [224]. Similar kinetics occur in *ex vivo* slices, with a maximum ~500 ms and a duration <5 s. The slightly longer time course in slice studies may reflect larger evoked DA concentrations and/or lower temperatures used.

1.4.2. Glutamate

Although microdialysis studies suggest a stimulatory effect of ionotropic glutamate receptor activation on striatal DA release, FCV recordings show an inhibitory role [256–258]. The effects of ionotropic glutamate-receptor activation on DA release are most likely indirect given that DA terminals are generally thought to lack these receptors [259,260]. Studies by Margaret Rice's group suggest that glutamatergic regulation of DA release in CPu is indirect and mediated by AMPA receptors on striatal medium spiny neurons, as reviewed elsewhere [183,261]. Briefly, AMPA receptor-

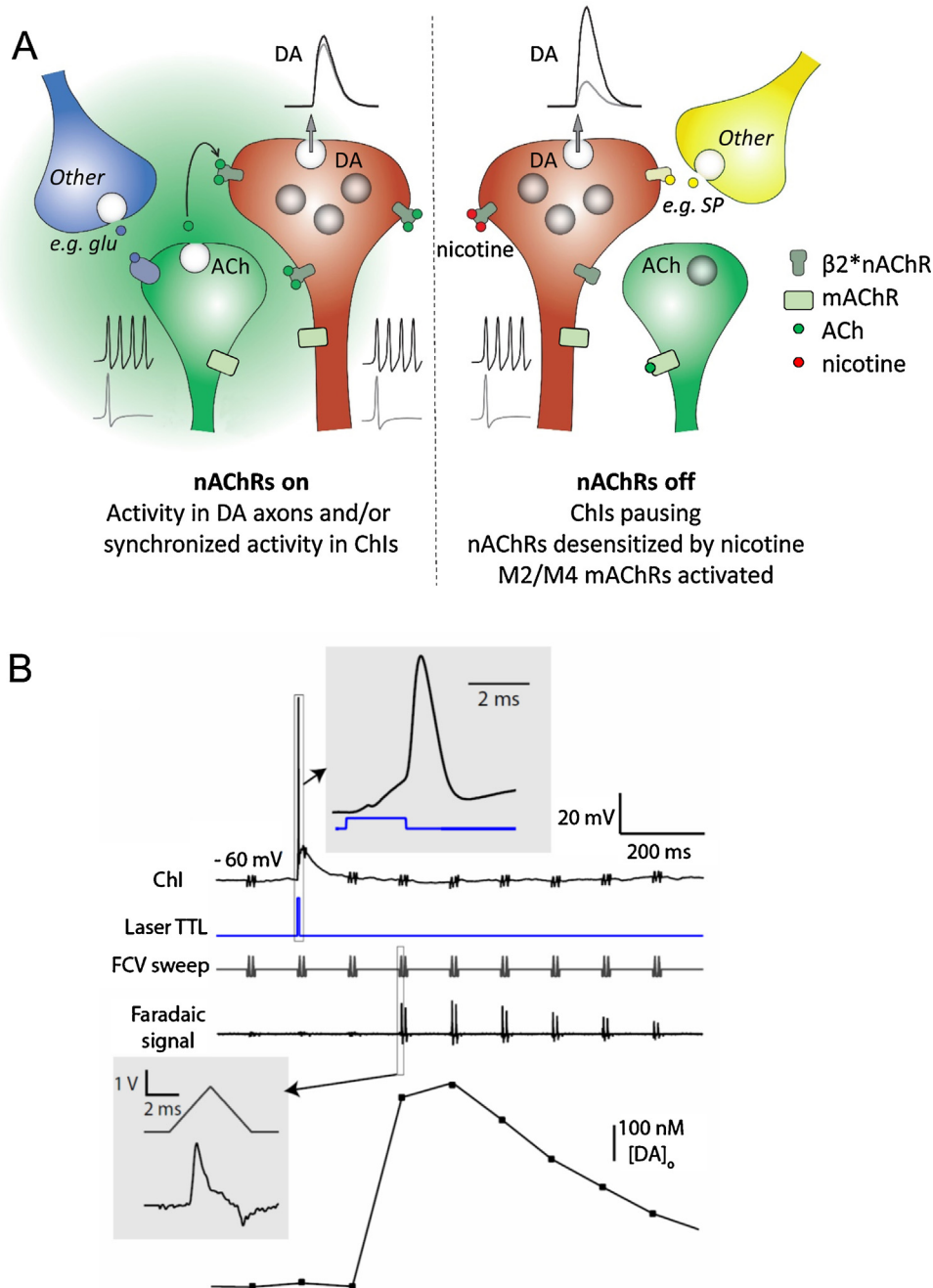


Fig. 3. Acetylcholine filters and drives dopamine transmission.

(A) Cartoon of the effects of cholinergic interneuron (ChI) activity on dopamine (DA) release. *Left of dashed line:* When nicotinic cholinergic receptors (nAChRs) are “on” (activated), DA release level only weakly reflects frequency of activity in DA neurons or ChIs. Other inputs can regulate DA release by regulating ACh release. *Right of dashed line:* When nAChRs are “off”, e.g., during ChI pause or after desensitization by nicotine, DA release better reflects frequency of activity in DA neurons. Note that the effects of other inputs could be mediated via ChIs (e.g., left) or be direct on DA axons (e.g., right), but in either case could depend on activity in ChIs.

(B) Light-activation of ChR2-expressing ChIs in striatum generates single action potentials in individual ChIs and directly drives large DA release events. Reproduced with permission from [290].

dependent activation of striatal spiny neurons enhances mitochondrial H_2O_2 generation [262]. Membrane permeable H_2O_2 then leaves the generating cell to inhibit DA release from adjacent DA axons [247,263]. Inhibition of DA release by H_2O_2 is mediated by ATP-sensitive K^+ (K_{ATP}) channels on DA axons [247,264]. Regulation of axonal DA release by H_2O_2 is rapid and transient, like that of D2 autoreceptor-dependent inhibition, with significant suppression 500–1000 ms after stimulation [264]. Notably, most studies of AMPA receptor/ H_2O_2 modulation of DA release were conducted in guinea-pig striatal slices using FCV. Although endogenous H_2O_2 also suppresses evoked DA release in rat striatum *in vivo* [265], this modulation may differ in other species, including mice [183]. Another indirect means of glutamatergic regulation of DA release in striatum is *via* effects on ChIs, which powerfully regulate DA release (see Section 1.4.4).

In contrast to ionotropic glutamate receptors, a metabotropic glutamate receptor, mGluR1, has been detected by immuno-EM on striatal DA axons [266], and evoked DA release can be inhibited *via* mGluR1 [267].

1.4.3. GABA

Microdialysis data support an influence of GABA on DA release *via* presynaptic GABA_B receptors in rats [268], consistent with some limited ultrastructural evidence in monkey for GABA_B receptors on DA-like axons [214]. In FCV recordings in mouse striatal slices, GABA_B receptor agonists inhibit single-pulse evoked release in CPU and NAc with kinetic parameters similar to those of D2 autoreceptors [227,269]. In guinea pig or mouse striatal slices, however, GABA_B receptor antagonism has no effect on single-pulse or pulse-train evoked DA release [256,270].

Modulation by GABA_A receptors seem to be postsynaptic and mediated by striatal interneurons [268]. Muscimol, a GABA_A agonist, inhibits evoked DA release in striatal synaptosomes [271] and also inhibits evoked DA release by single-pulse stimulation in striatal slices (Zhang and Sulzer, unpublished). The circuitry responsible remains unresolved, but could for example involve ACh (see Section 4). Conversely, pulse-train-evoked DA release in slices of guinea pig CPU is suppressed when GABA_A receptors are blocked, implying a normally enhancing role for endogenous GABA [256]. This is mediated by the inhibitory intermediate, H_2O_2 generated downstream from AMPA receptor activation: the effect of GABA_A antagonism is lost in the presence of catalase, an H_2O_2 metabolizing enzyme, or when AMPA receptors are blocked (see [261]).

1.4.4. Acetylcholine

ACh plays a dominant role in shaping DA release probability and its dynamic short-term plasticity through action at nAChRs and mAChRs. nAChRs are expressed densely on DA axons throughout the dorsal and ventral striatum [272,273]. Rodent SNc and ventral tegmental area (VTA) DA neurons express mRNAs for nAChR subunits $\alpha 3$ –7 and $\beta 2$ –4 [274] and DA axons have diverse subtypes of heteropentameric $\beta 2$ -subunit-containing ($\beta 2^*$) nAChRs [275–278]. FCV studies using an $\alpha 6$ -specific antagonist and subunit-specific knockout mice indicate that the nAChRs subtypes regulating DA release are $\alpha 4\alpha 5\beta 2$ and $\alpha 6\beta 2\beta 3$ in CPU but $\alpha 4\alpha 6\beta 2\beta 3$ in NAc [277–281] (Fig. 2). Non- $\beta 2^*$ -nAChRs, *i.e.*, homomeric $\alpha 7$ -nAChRs, have not been identified on DA axons; however, $\alpha 7$ -nAChRs can regulate evoked striatal [^3H]-DA release indirectly *via* nAChRs on glutamate terminals [282].

Recent slice FCV studies have revised the view of presynaptic nAChRs on DA axons. First, these nAChRs can modulate or filter how action potentials in DA neurons are translated into DA release (Fig. 3). When nAChRs are antagonized, DA release evoked by local single-pulse or low-frequency pulse-train stimulation is suppressed [283–285] indicating that local ACh acting at nAChRs is

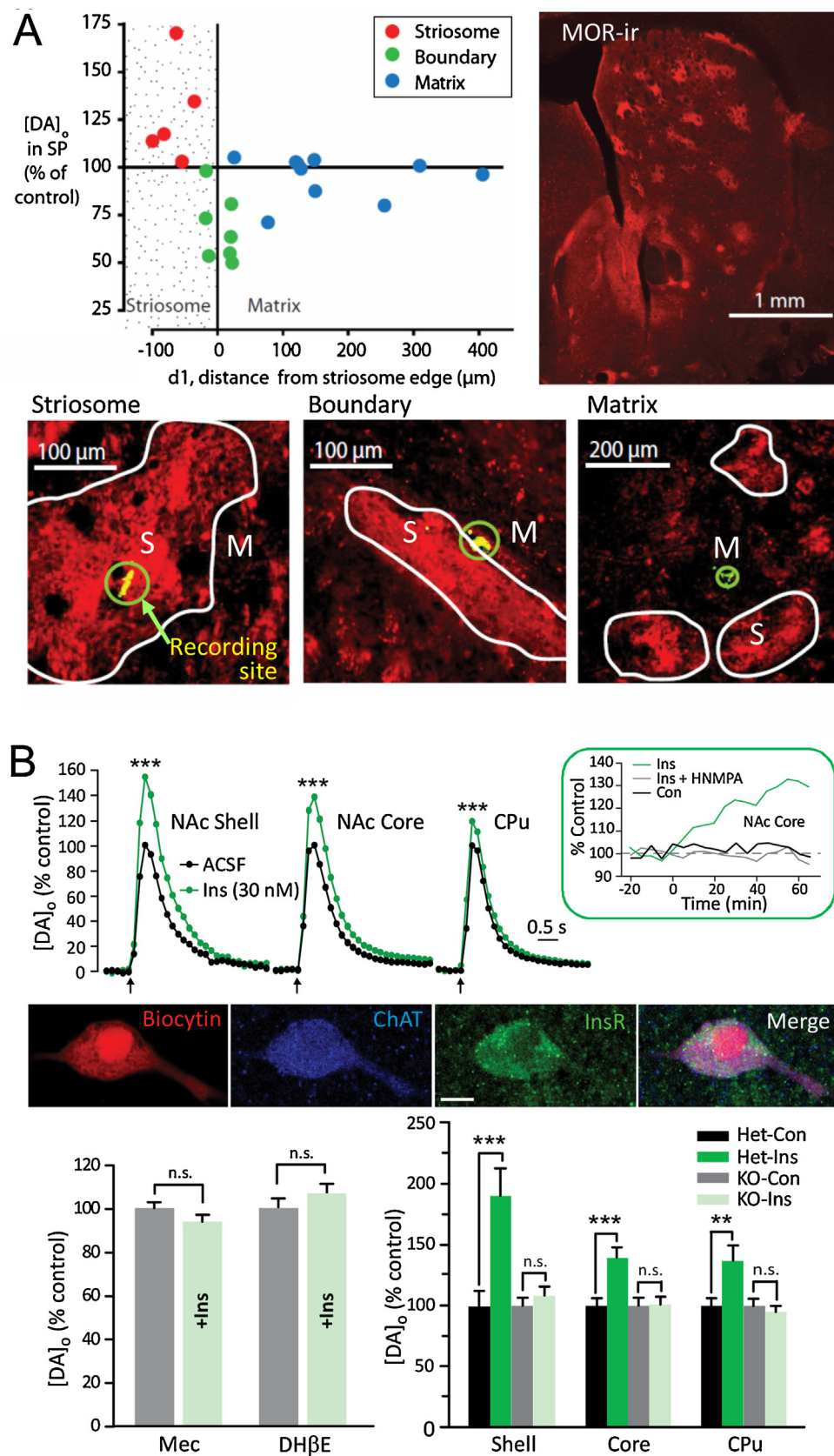
excitatory. Without nAChR activation, however, the short-term depression that normally limits subsequent release of DA is relieved, facilitating release; moreover, the shorter the inter-pulse interval in a train (higher frequency), the greater the short-term facilitation of release. Release by high-frequency activity when nAChRs are off can exceed that when on. Changes in frequency dependence are also seen after knockout of specific nAChRs or deletion of forebrain ACh from striatal ChIs [286]. Thus, pauses in ChIs may allow more faithful translation of action potentials into DA release, and enhance signal gain. Nicotine, a nAChR agonist, rapidly desensitizes striatal nAChRs, and has the same outcome as turning nAChRs off by an antagonist [283–285]. *In vivo*, systemic nicotine enhances $[\text{DA}]_o$ detected by microdialysis [287] and, like cocaine and alcohol, increases the frequency of DA transients in NAc [288]. These powerful effects of ACh on DA release could participate in the effects of many other striatal modulators that act on striatal ChIs (Fig. 3A, left), or could influence whether or not effects of other modulators acting directly (Fig. 3A, right) can be seen (Brimblecombe and Cragg, unpublished observations).

The combination of FCV with optogenetics and patch-clamp recordings indicates that synchronized action potentials in a small network of striatal ChIs activates nAChRs and drives axonal DA release in *ex vivo* slices and *in vivo* [289,290], bypassing action potential generation in midbrain (Fig. 3B). DA release can be driven by nAChRs in response to direct ChI activation or indirect ChI activation by optogenetic stimulation of glutamatergic inputs (*e.g.*, from thalamus) [290]. Amperometric studies with millisecond time resolution [291], as well as electrophysiological studies using expression of a DA-activated ion channel [26], indicate that local electrical stimulation first drives a DA release event by direct depolarization of DA axons, which summates with a second DA release event several milliseconds later driven by ACh release from ChIs. This contributes to nAChR-dependent modulation of DA release and its subsequent depression. Nicotine turns off ChI-driven DA release [290,291], which may contribute to the enhancement of reinforcement by nicotine.

The striatum shows dense expression of mAChRs, with evidence for tonic DA release inhibition by mAChRs in human imaging studies [292]. Pharmacological studies of mAChR effects on DA release in rodents have been contradictory, however, in part because of the limited subtype selectivity of available ligands [293–301]. DA neurons express only M5 mAChRs [302,303], but the use of mice lacking M1–M5 mAChRs indicates that different subtypes mediate facilitation (M4/5) versus inhibition (M3) of DA release, with some receptors acting *via* modulation of striatal GABA at GABA_A receptors [304], presumably indirectly given the apparent absence of GABA_A receptors on DA axons.

Key controversies in this area have been resolved using FCV in striatal slices [270], which showed that a key action of mAChRs is to regulate ACh release, which then acts at nAChRs on DA axons to modulate DA release (see Fig. 2). The broad-spectrum mAChR agonist, oxotremorine, decreases single-pulse or low-frequency evoked DA release, but relieves short-term depression, allowing enhanced release by longer or higher-frequency trains. This effect was identical to, substituted for, and was prevented by prior application of nAChR antagonists. Thus, activation of mAChRs on ChIs, which inhibits their firing [305], inhibits ACh release and nAChR activation. Experiments in subtype-specific knockout mice implicated M4-mAChRs in NAc, but M2- and M4-mAChRs in CPU [270] (Fig. 2). This regional distinction was surprising, but broadly consistent with the expression of M2/M4-family by cholinergic interneurons [306,307].

The role of M5-mAChRs has been refined further: M5-mAChRs are expressed by DA neurons but have not been localized on DA axons [308]. M5 knockout mice (M5-KO) show decreased DA release [309], but the effects of mAChR agonists on frequency



dependence are intact [270], consistent with a direct facilitatory role for M5-mAChRs on DA axons [304] (Fig. 2). Recent studies in mice with conditional M5-mAChR deletion in DA neurons confirm that M5Rs on DA axons promote DA release [310].

1.4.5. Opioids

Kappa-opioid and delta receptors are located on DA axons [211] whereas mu-opioid receptors are not [311]. Schlosser *et al.* [312] first demonstrated that mu, delta, and kappa receptors inhibit DA release in striatal slices. The key role of ACh in regulating DA release also explains the finding that activation of mu-opioid receptors in NAc shell, which can inhibit ChIs, decreases DA release driven by single-pulse but not pulse-train stimulation [313]. Thus, the effect of kappa opioid receptors on DA overflow is likely to be direct, whereas the influence of mu opioid receptors is indirect, mediated by an inhibition of ChIs [211,313]. Whether the effect of delta opioid receptors on DA release is direct remains unclear.

1.4.6. Neuropeptides: Substance P, insulin, growth factors, and CRF

Neurokinin-1 receptors (NK1Rs) for substance P (SP) are expressed by DA neurons [314], although whether they traffic to axons is unresolved. In striatum, SP is released by D1R-expressing neurons and is enriched within striatal compartments termed striosomes. Slice FCV studies show that SP acting at NK1Rs modulates DA release in a manner that varies with striosome-matrix location [249]: SP enhances DA transmission within striosomes at the expense of DA transmission at striosomes-matrix borders, with no effect in the surrounding matrix (Fig. 4A). These findings resolve previous contradictory reports about SP, and reveal that mechanisms that regulate DA can vary between the striosome and matrix compartments. The extent of DA release regulation by striosomes-matrix compartmental enrichment of other modulators and receptors [315], including mu-opioid receptors, is unknown but may provide an important process in the regulation of DA release and basal ganglia function. The recent generation of new transgenic animals providing identification of striosomes versus matrix will aid future work in this area.

Insulin receptors (InsRs) are expressed widely in the brain, including the striatum, with particularly high levels in the NAc [316] and on DA neurons in the VTA and SNc [317]. In contrast to SP and other neuroactive peptides discussed in this section, brain insulin is derived primarily from pancreatic β -cells in the periphery, rather than arising locally. Insulin acting at InsRs can promote an increase in DA uptake *via* the DAT in a PI3 kinase-dependent manner in the striatum [318,319] and in the VTA [320]. In the VTA, increased uptake is the predominant effect, so that the net effect of insulin is to decrease evoked $[DA]_o$ monitored using FCV in midbrain slices. This acute effect of insulin is augmented by the induction of long-term depression of VTA neurons *via* endocannabinoids [321].

In striking contrast, FCV studies in striatal slices show that the net effect of insulin throughout the striatum is to increase evoked $[DA]_o$ (Fig. 4B), despite a simultaneous increase in DAT-dependent uptake, indicating a potent enhancement of DA release [319]. This insulin-dependent enhancement is InsR and PI3-kinase dependent, and occurs at physiological insulin concentrations (low nM) (Fig. 4B). Companion immunohistochemical studies demonstrate the presence of InsRs not only on striatal DA axons, but also on ChIs (Fig. 4B). Consistent with the dominant role of ACh in shaping DA

release dynamics, the enhancing effect of insulin on evoked $[DA]_o$ was shown to require nAChRs and ACh: the effect of insulin is blocked by nAChR antagonists and is absent in choline acetyltransferase (ChAT) knockout mice (Fig. 4B). These data suggest that in addition to the established role of insulin as a satiety signal, insulin may also signal reward. Indeed, behavioral tests using a flavor preference paradigm in behaving animals indicate that insulin in the NAc shell influences food preference [319]. This not only implicates insulin in food-related learning, but also confirms its role as a reward signal.

The trophic factor BDNF acts at TrkB (and P75) receptors. Paradoxically, genetic deletion of brain BDNF leads to an increase in striatal DA content, but lower evoked $[DA]_o$ in striatal slices that is independent of changes in DA uptake [322]. The same pattern of increased DA content and decreased DA release is seen in heterozygous BDNF mutant mice (BDNF^{-/+}) [175,323]. These data imply a role for BDNF in dynamic regulation of DA release. In line with this hypothesis, exogenous BDNF enhances depolarization evoked $[^3H]DA$ overflow from rat striatal tissue [324], and can partially restore suppressed electrically evoked $[DA]_o$ in BDNF^{-/+} mice [175].

The trophic factor GDNF, which acts at its canonical receptor Ret, can also regulate striatal DA release and uptake. Through its downstream signaling pathway, GDNF plays a key role in the development, maintenance, and regeneration of the mesostriatal DA system (for review, see [325]). *In vivo* electrochemical studies in rhesus monkeys show an increase in K⁺-evoked DA release in the CPu, three weeks after a single, ipsilateral GDNF injection in the SNc [326]. Subsequent studies in rodents showed that intrastriatal infusion of GDNF causes a long lasting increase in TH phosphorylation and presumed DA synthesis in striatum and SNc [327]. As discussed in Section 1.2.6, GDNF increases quantal size for DA release from axonal varicosities of midbrain DA neurons in culture, and is the only growth factor that does so [18]. Recent studies using genetic amplification of GDNF levels with spatially unchanged localization [328] show that GDNF increases DA neuron number in midbrain and terminals in striatum, and increases DA content; unsurprisingly, striatal DA release and uptake are increased in these mice [328]. The role of GDNF and Ret signaling does not end there, as GDNF acting *via* Ret also regulates DAT surface expression through a signaling cascade that involves the Rho-family guanine nucleotide exchange factor protein, Vav2 [329]. Mice deficient in either Vav2 or Ret have *elevated* DAT activity in the NAc, suggesting that GDNF is a key determinant of DAT trafficking *in vivo* and contributes to DA homeostasis [329].

Corticotropin-releasing factor (CRF), a neuropeptide released in response to acute stressors, acts at the CRF receptors CRFR1 and CRFR2 and is associated with physiological and behavioral responses to stress. Both acute and chronic antagonism of CRFR1 decrease cocaine-stimulated DA overflow in the NAc; this is correlated with an attenuation of cocaine-induced inhibition of DA neuron activity from D2 autoreceptor activation by elevated $[DA]_o$ [330]. Such data provide insight into the observation that CRFR1 antagonists decrease cocaine self-administration and inhibit stress-induced reinstatement of cocaine-seeking behavior. *Ex vivo* slice studies show that application of exogenous CRF (10–1000 nM) to the NAc increases DA release through co-activation of CRFR1 and CRFR2 [331]. Severe stress *in vivo* abolishes the enhancing effect of CRF on NAc DA release in slices for at least

*** $p < 0.001$. Upper right, representative recordings of peak evoked $[DA]_o$ vs. time at a single site in NAc core in the absence of drug application (Con), during application of insulin (30 nM), or when insulin was applied in the presence of an InsR inhibitor, HNMPA (5 μ M). Middle, striatal ChI filled with biocytin, then immunolabeled for choline acetyltransferase (ChAT), and insulin receptor (InsR); merged shows InsR expression on ChIs; scale bar is 10 μ m. Lower left, Single-pulse evoked $[DA]_o$ in rat NAc core before and after insulin (30 nM) in the presence of an nAChR antagonist, mecamylamine (Mec; 5 μ M) or DH β E (1 μ M), normalized to 100% peak control ($p > 0.05$ vs. control). Lower right, Single-pulse evoked $[DA]_o$ in *ex vivo* slices from heterozygous control (Het) and ChAT KO mice before and after insulin (30 nM). Insulin increases evoked $[DA]_o$ in Hets (** $p < 0.01$, *** $p < 0.001$ vs. control), but had no effect on evoked $[DA]_o$ in any striatal subregion of ChAT KO mice ($P > 0.1$). Adapted with permission [319].

90 days. This may indicate how both traumatic and chronic stress can promote the onset of major depressive disorder in which an acute stressor is no longer perceived as motivational, but rather seen as an insurmountable impediment. Interestingly, loss of CRF regulation of DA release is accompanied by a switch in the response to CRF from appetitive to aversive [331], indicating the crucial role of both CRF and DA in the emotional response to acute stressors.

1.4.7. Adenosine

Evidence for presynaptic localization of A1 receptors on DA axons is indirect [332–334], with confirmed absence of A2A receptors [335,336]. Nevertheless, both regulate striatal DA release, likely indirectly, with inhibition by A1-receptor activation [337,338] and enhancement by A2A-receptor activation [339].

1.4.8. Cannabinoids and others modulators

FCV studies in CPu indicate a lack of direct DA release regulation by cannabinoid receptors (CB1Rs) [267,340,341], consistent with anatomical evidence for the absence of CB1Rs on DA axons [342–344]. Nevertheless, cannabinoids do influence striatal DA release (for review see [345]). In rat striatum *in vivo*, CB1 agonist administration decreases evoked DA release, while increasing the frequency of spontaneous DA transients that presumably reflect effects on neuronal firing [346]. In striatal slices, CB1R activation suppresses pulse-train evoked DA release, but has no effect on single-pulse evoked release, suggesting an indirect effect [341]. Additional evidence supports a role for local circuit interactions involving inhibition of GABA release, consequently enhanced generation of H₂O₂, and activation of K_{ATP} channels to inhibit DA release [341]. Additionally, midbrain VTA DA neurons produce cannabinoids [347–349], that activate local receptors that regulate DA neuron activity and shape NAc reward encoding [350].

Other candidate neuromodulators in striatum might modulate DA release via heteroreceptors on DA axons or their inputs, as well as without classic plasma membrane receptors. For example, nitric oxide (NO) can act directly at DA axons to enhance DA signals, and indirectly via local circuits particularly ChIs to modify frequency dependence [351]. *In vivo* evidence suggests that NO can also enhance DA levels by inhibiting the DAT [352].

1.5. Relationship between firing and vesicular release

1.5.1. Frequency dependent modulation of dopamine release

The studies summarized in Sections 1.4.2–1.4.8 demonstrate heteroreceptor regulation of DA release, despite some inconsistencies that reflect differences in stimulation and recording paradigms, preparations, and species. Some conflicting results have been reconciled following identification of the key role of ACh from ChIs, which can have bidirectional effects on DA release, depending on the frequency of activation. Decreased ACh input can decrease DA release probability, leading to low release evoked by a single stimulus or low-frequency train; this leaves more DA for release by subsequent pulses, especially at higher frequencies, thus amplifying the frequency dependence of release [284,285]. The outcome of heteroreceptor modulation of DA release will therefore depend on the protocol used to drive DA release.

There are known differences between striatal DA release in slices, in which local stimulation produces paired-pulse depression, and *in vivo*, in which medial forebrain bundle-evoked DA release shows no depression. A key difference is that local electrical stimulation not only generates action potential dependent DA release, but also recruits ChIs [290], which profoundly increases DA release probability and subsequent depression of release, as already discussed. In addition, neither *in vivo* or *ex vivo* slice experiments can exclude wider circuit effects on other striatal

circuitry, or additive effects on inputs to DA somata and dendrites *in vivo*.

A good example of complex effects is seen with systemic nicotine administration. Nicotine at levels experienced by smokers (~250–300 nM) desensitizes nAChRs, which potently inhibits striatal DA release evoked by single-pulse or low-frequency stimulation, but enhances the frequency dependence of DA release [284,285] (Fig. 2). In concert with increases in DA-neuron firing frequency and burst-firing from activation of nAChRs in VTA and SNc [204,280,353], this leads to enhanced phasic vs. tonic DA signals, and also a net increase in striatal [DA]_o, monitored by microdialysis [287]. Nicotine thus enhances the contrast of DA signals associated with reward-related cues processed by DA neurons [284,285]. These effects also explain previous reports that dismissed striatal nAChRs as a site of nicotine action because intra-striatal nAChR antagonist administration did not prevent effects of systemic nicotine on striatal DA levels [354,355].

1.5.2. Silent dopamine release sites?

In the sympathetic nervous system, release of noradrenaline exhibits low release probability per site (about 1%) [356,357]. An initial study using fluorescent false neurotransmitters (FFNs), which are fluorescent VMAT2 substrates, indicates that the fraction of total presynaptic DA released per action potential in striatum is even less than 1% and is regulated by the frequency of activity [29]. Recent data are consistent with a high fraction of presynaptically silent release sites that contain synaptic vesicles, even with high levels of Ca²⁺ entry as shown by simultaneous imaging with a GCaMP Ca²⁺ indicator [30].

2. Dopamine uptake

During the 1960s it was shown that DA neurons express DAT, which was later confirmed to be densely and specifically localized on DA cell bodies and axons [358,359]. Given this discrete localization, it was hypothesized that the DAT might control the intensity and duration of DA transmission [360]. Microdialysis and voltammetric techniques enabled monitoring [DA]_o *in vivo*. Both approaches showed that under resting conditions, [DA]_o is low (10–20 nM) and that DA uptake strongly contributes to DA clearance after release. Indeed, pharmacological inhibition of DA uptake by drugs like cocaine potently enhance [DA]_o [361,362] in an activity-dependent manner, indicated by the loss of effect when impulse-flow dependent DA release is blocked [363].

Early voltammetric and microdialysis methods could not provide an accurate description of the kinetics of DA clearance because they could not distinguish between increased release and decreased DA clearance, either of which would increase [DA]_o. This limitation was resolved with improvements in voltammetric techniques, and new methods to fit evoked increases in [DA]_o that allowed independent extraction of uptake and release terms [364,365].

The development of mice lacking DAT (DAT^{−/−}) [366] allowed the relative contribution of DA uptake to DA clearance vs. extracellular degradation, non-neuronal uptake, and diffusion to be elucidated. In DAT^{−/−} mice, striatal DA content and TH levels are decreased by 90%; paradoxically, however, DA synthesis rate by TH is doubled, with a further decrease in DA content when TH is inhibited [367]. Peak evoked [DA]_o in striatal slices from DAT^{−/−} mice is decreased by 75%, despite the absence of competing DA clearance by uptake [367]. Similarly, evoked [DA]_o *in vivo* is decreased by >90% in the dorsal striatum and by >80% in NAc in DAT^{−/−} vs. DAT^{+/+}, with a rapid and near complete loss of DA release in DAT^{−/−} after inhibition of DA synthesis by TH [368]. These observations show that in striatum, recycling of released DA

by uptake plays a major role in determining the releasable pool of DA.

2.1. Kinetics of dopamine uptake

Electrical stimulation of DA axons induces brief DA overflow that can be detected with rapid electrochemical techniques *in vitro* [95] and *in vivo* [149,365]. The decay phase of evoked DA overflow reflects the clearance of released DA, with an assumption of little diffusional component because of the relatively uniform increase in $[DA]_o$ in a given volume of stimulated striatal tissue. DA uptake inhibitors slow the clearance rate by one order of magnitude [95,149,369]. However, in DAT^{−/−} mice the decay phase is slowed by two orders of magnitude, both *in vitro* [366,367] and *in vivo* [368]. We and others have developed mathematical means to determine the K_m and V_{max} values of DAT from the decay phase, using modified versions of random walks [95] [tutorials on conducting these analyses are at sulzerlab.org]

Inhibition of enzymatic DA degradation does not slow the decay phase in the striatum of DAT^{−/−} mice *in vitro*. Inhibition of monoamine oxidase *in vivo*, however, slows the decay phase in DAT^{−/−}, but not WT mice [367,368]. Therefore, in the intact striatum, DA uptake represents the primary mechanism responsible for the clearance of uniformly released DA, with comparatively negligible roles for extracellular DA degradation and non-neuronal uptake. In DAT^{−/−} mice, DA clearance is mainly due to DA diffusion [367,368]. Diffusion also plays a role in decreasing $[DA]_o$ immediately near a release site under normal conditions [370,371].

In brain structures with weak DA innervation, including amygdala, globus pallidus, prefrontal cortex and cingulate cortex, DA clearance rates are much slower than in striatum, with half-lives for evoked $[DA]_o$ of ~2 s in these structures vs. 60 ms in striatum [372]. In these regions, DAT inhibitors are less effective in slowing DA clearance than in striatum [373,374]. Notably, in prefrontal and cingulate cortex, hippocampus, and VTA, DA is also cleared by the norepinephrine transporter [374–376].

Regulation of DA release and uptake is altered in parkinsonian striatum [145]. Interestingly, the parallel loss of DA release and uptake sites can allow for a similar net steady-state levels of evoked $[DA]_o$ *in vivo* across a range of levels of DA depletion induced by 6-OHDA, and also in striatum after DA axon reinstatement by neuronal transplantation [377], which led Bergstrom and Garris to propose the notion of passive stabilization [378]. Nevertheless, the overall decrease in DAT activity in lesioned striatum increases reliance on DA synthesis (as in DAT^{−/−} mice), which contributes to the efficacy of DA replacement by L-DOPA [145], along with changes in DA release probability [379].

Following exocytosis, DA clearance from the extracellular space is achieved by DAT. In addition to being important for shaping the time frame, spatial constraints and the signal-to-noise ratio of DA neurotransmission [19,215,371], recycling DA back into DA axons provides a synthesis-independent mechanism to refill vesicles. DAT can therefore play an important role in regulating quantal size by affecting presynaptic DA levels during neuronal activity. Indeed, DAT inhibition by cocaine in PC12 cells causes a concentration-dependent decrease DA released per quantal event [380]. Evoked striatal DA release can also be decreased by sufficiently high concentrations of cocaine and methylphenidate [381]. In PC12 cells, this is not due to an inhibitory effect of D2 autoreceptor activation on quantal size from elevated $[DA]_o$, as the effect of cocaine is unaltered by sulpiride, a D2-receptor antagonist [382]. A possible weak base effect of cocaine on the pH gradient of synaptic vesicles was also ruled out since amfonelic acid, a DAT inhibitor that is not a weak base, mimicked cocaine's inhibitory effect on quantal size [133].

A role for DAT in the regulation of DA quantal size was confirmed in DAT^{−/−} mice. As discussed, evoked striatal DA release is markedly decreased in *in vivo* and in acute slices [367,368]. Given the absence of anatomical abnormalities in striatal DA release sites in these animals [383], the decreased release is likely linked to the dramatic decrease in intracellular DA storage. These studies, together with PC12 cell data, suggest that the vesicular DA stores are regulated by DAT functionality.

A further role for DAT has also been proposed in the regulation of mobility of vesicle pools for release. Cocaine and other DAT inhibitors can increase DA release in a DAT- and synapsin-dependent manner [190,191] consistent with mobilization of the vesicle pool, and with a normal function for DAT in restricting pool mobility and/or releasability.

2.2. DAT regulation

DAT expression can be affected by many factors, including DAT substrates and inhibitors, and D2 receptor agonists [215,384–387]. In particular, the trafficking of DAT to and from the cell surface may provide the predominant mechanism of acute DAT regulation [388,389]. Since the subject of DAT regulation has been extensively covered [388–390], we will only briefly summarize some of the key mechanisms here.

2.2.1. DAT regulation by psychostimulants

Considerable evidence supports a role for DAT substrates, like amphetamine, in inducing DAT internalization *in vivo* and *in vitro*, thus decreasing DA uptake [391–396]. Interestingly, the effect of DAT substrates is biphasic with a rapid and transient upregulation in surface DAT levels followed by a more lasting downregulation [397,398]. Regional differences have also been reported [395,399,400]. Studies on DAT regulation by DAT blockers have focused on the effects of cocaine, which blocks DAT internalization induced by DAT substrates [392,393,397,398]. On the other hand, cocaine's action on DAT trafficking *per se* is controversial [393,401–403].

While several kinases are involved in the regulation of DAT, PKC is by far the most thoroughly investigated [389,404]. PKC activation induces DAT internalization [393,405–407], although the mechanism of PKC activation by DAT substrates remains largely unknown [389,390,404,408,409].

2.2.2. DAT regulation by D2 receptors

Activation of several GPCRs can also regulate DAT function [388,389]. Studies using acute administration of agonists and antagonists of the D2 receptor have established a positive regulation of DAT activity and surface expression by D2 DA autoreceptor activation *in vivo* and *in vitro* [410–416]. D2 receptor-dependent upregulation of DAT activity may involve G-proteins, ERK1/2 and PKA [411,415,417], with inhibition of ERK1/2 leading to a decrease in DAT function and its cell-surface localization [418].

There is also evidence, albeit inconsistent, for co-localization and direct interaction of DAT and D2 receptors. Association with D2 receptors appears to promote DAT recruitment to the plasma membrane [417]. Consistent with this result, Dickinson *et al.* [419] found a decrease in DAT function in D2-deficient mice, based on a decreased rate of clearance of exogenously applied DA [419]. However, Benoit-Marand *et al.* [224] detected no effect on the DA clearance of electrically evoked DA release *in vivo* in response to electrical stimulation in D2-receptor knockout mice [224]. DA uptake in striatal slices from D2-knockout mice showed increased DAT function, and an enhanced effect of DAT inhibition [227]. Note that pharmacological studies that addressed these interactions typically involve acute inhibition or activation of D2 receptors,

whereas genetic ablation of D2 receptors may lead to changes in DAT function due to long-term or developmental effects of D2 receptor deficiency.

2.3. Uptake limits the radius of DA diffusion in the extracellular space

EM studies of the striatum show that DA axons can form small symmetrical synaptic contacts on the neck of dendritic spines of medium spiny neurons [420]. The majority of postsynaptic DA receptors appear to be extrasynaptic along dendritic membranes, and often distant from DA synapses [420–423], although D1 and D2 receptors are also found in the perisynaptic zone of asymmetric synapses formed by glutamate terminals on the heads of dendritic spines. This anatomy suggests that it is necessary for DA to spillover from release sites to activate its receptors, and therefore that DA transmission is mainly extrasynaptic [423]. Consistent with this mode of action, DA uptake sites are rarely observed on the presynaptic membrane and rather may be distributed evenly along the membrane of DA fibers [359,424,425].

In the striatum, the density of DA release sites is very high and the average distance between two release sites is $\sim 4 \mu\text{m}$ [426]. It should be noted that estimates of the fraction of purported striatal DA release sites that includes a postsynaptic specialization may be as low as 30% [427], so that DA “varicosities” or “boutons” are more accurate terms than “synapse” or “terminal” to describe these.

The maximal amplitude of the DA overflow evoked by a single stimulation pulse in the rodent striatum *in vivo* and detected using a carbon-fiber microelectrode is in the range of 100–400 nM [149,365,368,428]. This value may underestimate the change in intact tissue; models of electrochemically detected changes in $[\text{DA}]_o$ assume that there is a pool extending 6–8 μm between intact tissue and the electrode surface, *i.e.*, the dimensions of the electrode tip diameter [95,428,429]. This estimate is consistent with EM studies of the extent of tissue damage caused by a carbon-fiber implanted for 4 h in the striatum of anesthetized rats [430]. Therefore, the DA overflow recorded by a carbon-fiber microelectrode may be much larger than that typically reported in electrochemical studies.

In striatum, D1 receptors appear to be in a low affinity state (1 μM) whereas D2 receptors are in the high affinity state (10 nM) [431], although *in vivo* evidence for this is still lacking. It has been estimated that the basal $[\text{DA}]_o$ resulting from tonic DA neuron activity is in the range of 10–20 nM, which would be high enough to exert a tonic stimulation of presynaptic [361] and postsynaptic [432] D2 receptors.

There have been several models suggested to estimate DA overflow in the striatum. In a model of diffusion of DA in the extracellular space following a quantal release event [370], at short distance (1 and 2 μm), diffusion entirely governs the DA overflow, whereas at increasing distance from release site (5 to 20 μm), DA uptake increasingly limits the DA overflow both in term of maximal amplitude and duration. At the latter distance range, the maximal amplitude of the DA overflow is below 100 nM. This model therefore suggests that DA transmission mediated by D1 receptors, assuming they are in the low affinity state, occurs after DA release from a single vesicle to a distance of 2 μm and is largely unaffected by DA uptake. In contrast, DA transmission mediated by D2 receptors might be effective at a distance of 7 μm from release sites. This distance as well as the duration of effective D2 stimulation (*i.e.*, time during which the extracellular DA concentration exceeds 10 nM) is limited by DA uptake [370].

A similar approach [19] estimates that in the striatum, basal DA release would bind to $\sim 0.1\%$ of DA receptors, but within 5 ms after a quantal event, $\sim 60\text{--}100\%$ of DA receptors in a perisynaptic zone of $\sim 22 \mu\text{m}$ distance would be bound for nearly 1 s, depending on quantal size. This system is immensely plastic: the “sphere of

influence” for which DA levels are $>10 \text{ nM}$ with a quantal size of 3000 molecules has a radius of 12 μm , but in the case of decreased DAT, as in Parkinson’s disease, after L-DOPA can reach 32 μm , corresponding to a 19-fold increase in striatal volume [145]. This helps explain the remarkable efficacy of L-DOPA in Parkinson’s as long as there is some surviving SNc input to the dorsal striatum.

The view that released DA escapes release sites and its diffusion is not strongly affected by uptake in the immediate vicinity (*i.e.*, $<5 \mu\text{m}$) is supported by electrochemical measurements of evoked DA overflow. Indeed, DAT inhibition strongly slows DA clearance after single-pulse evoked release, but only moderately enhances the maximal $[\text{DA}]_o$ amplitude [95,372,433].

In summary, the rising phase of the evoked $[\text{DA}]_o$ is rapid and reflects mainly DA release, whereas the decay phase is slower and reflects DA uptake. The kinetics of release and uptake can be obtained by simulation, taking into account DA diffusion from the intact tissue to the electrode surface. Simulations show that the half-life of released DA in striatum *in vivo* is $\sim 30 \text{ ms}$ [95,372,428,429], which allows for diffusion of $\sim 7 \mu\text{m}$. Of course, given the dense innervation of the striatum, DA overflow from each release site will contribute to spatial and temporal summation to lead to larger, longer-lasting DA transients that will be more spatially constrained by DA uptake than are individual release events [371].

In brain structures with lower DA innervation and consequently lower DAT, including prefrontal and cingulate cortices, globus pallidus, subthalamic nucleus, SNc and VTA, diffusion should play a larger role in DA clearance of released DA. Even in sparsely innervated regions, however, DAT inhibition may delay DA clearance [374,375,434].

3. Relationship between DA neuron firing and extracellular DA

DA neurons exhibit two main activity patterns: regularly spaced spikes at 2–5 Hz, and brief higher-frequency bursts of 2–6 action potentials [435,436]. Under resting conditions and during sleep, most DA neurons show regular, tonic activity; however, administration of a reward (*e.g.*, food) or a sensory stimulus that predicts a reward can trigger a burst of action potentials in most midbrain DA neurons in monkeys [437,438] and rats [439]. In rats, the intraburst frequency is 15–30 Hz, but can be higher [435,436]. Grace and Bunney [435] hypothesized that bursting would be more potent than tonic activity to induce DA release in distant striatal regions. Consistently, electrical stimulation mimicking bursting is twice as effective in increasing $[\text{DA}]_o$ *in vivo* as regularly spaced tonic stimuli with the same duration or pulse number [440]. The difference in efficacy of tonic and burst firing involves the timecourse of DAT-mediated uptake.

3.1. Tonic $[\text{DA}]_o$

With tonic DA neuron firing, the pause between successive action potentials exceeds 200 ms, so that DA released by one action potential can be cleared by before the next action potential. The role of uptake in maintaining $[\text{DA}]_o$ was demonstrated *in vivo* with MFB stimulation at 4 Hz [441]. Synchronous activity can occur between pairs of adjacent DA neurons recorded with the same electrode [442,443], but not between pairs of distant neurons recorded with two electrodes [442]. Moreover, synchrony of near-neighbor firing is more prominent during bursting activity [443]. Thus, given the very high density of striatal DA release sites [424,426,427], and the extensively overlapping DA axonal arbors [444], tonic activity induces a steady-state $[\text{DA}]_o$, in the range of 10–30 nM, which is stable with time and spatially homogenous

[428]. This steady-state is firing-rate and DAT dependent [432,445]. Indeed, pharmacological DAT inhibition uptake increases $[DA]_o$ by 300% [363,428,445], consistent with the 500% higher $[DA]_o$ seen in DAT $^{-/-}$ vs. DAT $^{+/+}$ mice [367].

3.2. Phasic changes in $[DA]_o$

Burst-like stimuli are more effective than tonic in evoking DA overflow, in part because the shorter interpulse interval of bursts allows less time for DAT-mediated clearance than with tonic firing. In addition to this consequence of DAT-mediated uptake, other factors include short-term plasticity in DA release probability that depend on firing frequency. One confounding factor that contributes to apparent short-term plasticity is presynaptic nAChR activation that promotes short-term depression (Fig. 2). Even without the effects of nAChRs, presynaptic Ca^{2+} -dependent mechanisms govern an inverse relationship between initial and subsequent DA release probability [178] reflecting classic Ca^{2+} -dependent regulation of short-term plasticity [446]. Briefly, when depolarization results in initially low DA release probability, high frequencies can promote short-term facilitation of release [178], presumably through summation of intracellular Ca^{2+} at sufficiently short interpulse intervals.

In behaving rats, brief, spontaneous changes in $[DA]_o$, termed DA transients, occur with similar kinetics and amplitude to $[DA]_o$ evoked by brief electrical stimulation [447,448]. DAT inhibition increases the maximal amplitude and duration of spontaneous transients [449]. In NAc, resolvable DA transients can result from bursting activity of VTA DA neurons: NMDA-mediated excitatory inputs to VTA DA neurons generate bursts [450–452]. Correspondingly, VTA injections of NMDA and of NMDA antagonists in freely moving rats alter the frequency of DA transients [453].

Although midbrain DA neurons initially respond to unpredicted reward with a burst of activity, after intense training they respond to a cue predicting a reward but no longer to the expected reward in monkeys [437,438] and rats [454]. Electrochemical studies have shown the same relationship between DA transients and reward or cue predicting a reward in NAc [455]. DA neurons burst with appetitive stimuli and are inhibited by aversive stimuli in monkeys [438] and rats [454,456], and consistently, appetitive stimuli trigger NAc DA transients, whereas aversive stimuli briefly decrease $[DA]_o$ [457], although these responses may arise from different DA neuron subpopulations, particularly in VTA [458–460]. For example, intense stress induced by social defeat enhances burst firing in a distinct subpopulation of VTA DA neurons in freely moving rats, and is associated with increased frequency of DA transients in NAc [461]. In summary, FCV recording of DA transients *in vivo* generally supports the encoding of reward-prediction related information revealed by studies of DA neuron firing rate. However, unlike the relatively standardized responses of midbrain DA neurons [462], phasic DA transients that report unpredicted reward and reward-predictive cues are highly specific to striatal subregions: DA transients seen in NAc are not typically detected in CPu [463].

4. Conclusions

The net $[DA]_o$ at any point in time results from a dynamic equilibrium between release and uptake. Both are regulated by mechanisms that range from DA neuron firing to local intracellular and intercellular regulatory signals within target regions. Tonic activity of DA neurons translates into tonic $[DA]_o$, whereas burst activity apparently translates into transiently enhanced $[DA]_o$, which is also locally regulated. Overall, the moment-to-moment interplay among firing, release, uptake, and auto- and hetero-

receptor-dependent modulation is not only dynamic, but state- and region-dependent. A challenge of future studies will be to determine the relative contributions of these factors in specific DA-dependent behaviors.

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