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# Mechanisms of dopamine quantal size regulation

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## 1. ABSTRACT

The study of dopamine (DA) quantal size, or the amount of transmitter released per vesicle fusion event, has been enabled by subsecond resolution amperometric recordings. These methods, together with other electrophysiology techniques, novel optical approaches and classical molecular biology and biochemistry methodologies, have advanced our understanding of quantal size regulation in dopaminergic and other catecholaminergic systems. The presynaptic mechanisms that determine DA quantal size regulate two features: the amount of transmitter stored in each vesicle and the fraction of vesicular contents that are released per fusion event. The amount of vesicular DA is dependent on DA synthesis, DA vesicular loading and storage and on DA reuptake from the extracellular space upon exocytosis. The mode of vesicle fusion and the related fusion pore dynamics control the fraction of DA released per fusion event. We will summarize current understanding on the regulation of these steps by endogenous and exogenous factors, including drugs of abuse and DA itself.

## 2. INTRODUCTION

Dopamine (DA) neurotransmission plays a critical role in a number of brain functions that include habit and motor learning and reward-seeking behaviors (1). It is therefore not surprising that a considerable amount of work has been devoted to understanding the mechanisms underlying DA release modulation (2). DA and other catecholamines are stored in small synaptic vesicles in neurons and in small and large dense core vesicles in both neurons and neuroendocrine cells. Stimuli that increase the intracellular $Ca^{2+}$, such as the arrival of a depolarizing action potential to the nerve terminal, promote the fusion of these vesicles with the plasma membrane, releasing transmitter into the extracellular space. Modulation of the exocytotic release of catecholamines and other neurotransmitters can be achieved by altering the number of vesicles that fuse upon a given stimulus or the amount of transmitter that is released per vesicle. In this review we

focus on the current knowledge on the mechanisms that regulate the amount of DA released per fusion event.

The term "quantal size" was initially used by Bernard Katz and associates to refer to small alterations of postsynaptic current or voltage that appeared to be the basic units of synaptic transmission (3, 4). They soon drew the conclusion that this could be due to the secretion of neurotransmitter associated with synaptic vesicle exocytosis. In the 1990s, following the introduction of single cell carbon fiber amperometric recordings of quantal release events by Mark Wightman and colleagues (5, 6), our laboratory, together with Andrew Ewing's, appropriated the term for presynaptic use (7). Regardless of the wisdom of that decision, "quantal size" is now widely used to also mean the amount of transmitter that is released per vesicle fusion event.

The modulation of presynaptic quantal size has important functional consequences for neurotransmission, especially in the social dopaminergic synapse where DA "spills" beyond the synaptic cleft. This is particularly important if we consider that both the dopamine transporter (DAT) responsible for DA reuptake and most DA receptors, including the D2 receptor, are located extrasynaptically (8-11). Changes in quantal size will therefore alter the temporal and spatial pattern of DA efflux and, consequently, the number of DA receptors activated (reviewed in detail in 12).

Diffusion of DA beyond the synapse is also useful from a practical perspective of studying these synapses since it allows the detection of DA by cyclic voltammetry in the extracellular space, following electrical stimulation (13). This technique relies on the application of a triangular voltage wave to a carbon fiber electrode that alternately oxidizes and reduces molecules producing a voltammogram that discriminates between compounds based on their characteristic oxidation and reduction peaks (12). While cyclic voltammetry has proved extremely helpful to monitor DA release with high specificity in acute slices and *in vivo* (14-16), the temporal resolution of this method is limited by the electrode's response to the oxidation-reduction wave and it is not optimal for monitoring rapid quantal release events. Amperometry entails the application of a constant oxidation potential at the carbon fiber electrode providing far better temporal resolution (12). Quantal recordings have been successfully obtained by amperometry from both small synaptic vesicles from midbrain DA neurons in culture and dense core vesicles from neuroendocrine cells such as the PC12 and chromaffin cell systems.

Quantal size is dependent on multiple cell intrinsic factors that regulate two crucial presynaptic features: i) the amount of transmitter stored within secretory vesicles and ii) the fraction of transmitter that is released per vesicle upon fusion with the plasma membrane. In the dopaminergic system, the former step is regulated by a wide range of intracellular players involved in DA synthesis, vesicular uptake, intravesicular storage and reuptake following exocytosis. The fraction of DA released per vesicle is controlled by the mode of vesicle fusion. In the following sections we will discuss how quantal size is modulated by each of these factors and describe other factors whose mode of action is still poorly understood.

## 3. QUANTAL SIZE REGULATION BY FACTORS THAT AFFECT DA VESICULAR CONTENTS

### 3.1. DA synthesis

The amount of DA stored in synaptic vesicles or secretory granules is largely dependent on DA levels available in the cytoplasm, which are controlled in great part by DA synthesis. The biosynthetic pathway responsible for DA formation involves the conversion of L-tyrosine into L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH) and the subsequent conversion of L-DOPA to DA by the aromatic amino acid decarboxylase (AADC). A direct correlation between increased DA synthesis and quantal size has been established by measuring quantal DA release by carbon fiber amperometry in PC12 cells (17), chromaffin cells (18) and midbrain DA neurons in culture (19). When cells were incubated with exogenous L-DOPA, an increase in DA synthesis was confirmed by the elevated cytosolic DA levels (20) and the effect on quantal size was demonstrated by the 2- to 3-fold increase in the amount of DA released by individual vesicles (17-19). The reaction catalyzed by TH is typically the rate limiting step in the DA biosynthetic pathway and bypassing TH with L-DOPA administration in these experiments was crucial to induce a significant increase in DA synthesis. On the other hand, this positions TH as a primary player in determining intracellular and vesicular DA levels. It is therefore not surprising that TH is under stringent control by a variety of enzyme activity modulators and mechanisms regulating protein expression (21-23), as detailed below.

#### 3.1.1. Regulation of TH expression

The regulation of TH protein levels at the level of transcription and RNA stability and translation has been extensively studied. Several stimuli and conditions are able to increase TH expression *in vivo* and *in vitro*. These include cold or immobilization stress, hypoxia, glucocorticoids, growth factors, and several drugs such as cocaine (a blocker of monoamine uptake into nerve terminals), nicotine (an acetylcholine receptor agonist) and reserpine (a monoamine vesicular uptake inhibitor that depletes catecholamine vesicular stores) (21, 24). The variety of signaling pathways involved in the regulation of TH transcription is evident upon molecular dissection of the TH promoter. The transcription factor binding sites identified in this regulatory region include two cAMP-responsive elements (CRE) (25-28), an AP-1 sequence (the binding site for the transcription factor complex comprising of c-Fos, c-Jun and JunD, which is downstream of protein kinase C [PKC]) (29) and a glucocorticoid regulatory element (GRE) (21, 30). Many of the factors that stimulate TH transcription also increase the stability of TH mRNA. This has been described for cAMP and glucocorticoids (31), PKC activation by phorbol esters (32), nicotinic receptor activation (33) and hypoxia (34). Note, however,

that while increases in TH transcription might be expected to lead to increased TH protein levels and activity, there are many examples for which changes in TH mRNA levels are not reflected at the protein level (21). Conversely, increases in TH protein expression may occur at the level of translation without a concomitant change in TH mRNA. For example, in midbrain DA neurons, cAMP induces the translation of TH mRNA, increasing TH protein and activity, without altering TH mRNA levels (35).

### 3.1.2. Regulation of TH activity by phosphorylation and other post-translational modifications

TH catalyzes the conversion of L-tyrosine to L-DOPA using $O_2$, $Fe^{2+}$ and the cofactor tetrahydrobiopterin ($BH_4$) to hydroxylate tyrosine. DA, and to a lesser extent L-DOPA, inhibit this reaction by competing with $BH_4$ for binding to TH and by stabilizing the enzyme in a less active form (21, 22). This end-product inhibition acts as a sensor for the local concentration of DA and other catechols and is a target for the modulation of TH activity.

Phosphorylation is the most thoroughly studied post-translational modification involved in the activation of TH. The regulatory domain of this enzyme is phosphorylated *in vivo* in response to various stimuli at four serine residues, ser8, -19, -31 and -40, in the case of rat TH (22, 23). The most compelling evidence for TH activation in response to phosphorylation involves the ser40 residue. *In vitro* phosphorylation at ser40 increases $V_{max}$ and TH affinity for $BH_4$ and decreases the affinity of TH towards catechols, which dramatically reduces end-product inhibition (23). Ser40 is mainly phosphorylated by the cAMP-dependent protein kinase (PKA) (36, 37), which increases TH activity and catecholamine synthesis in PC12 cells (38), bovine adrenal chromaffin cells (39, 40), rat striatal slices (41, 42), and the rat striatum *in vivo* (43). Indeed, the first suggestion that TH could be activated by phosphorylation was provided by studies that reported increases in TH activation in response to cAMP analogues (44, 45), but only under conditions that activated PKA (46, 47).

Several other kinases, including PKC, calcium/calmodulin-dependent kinase II (CaMKII), cGMP-dependent kinase (PKG), extracellular-signal regulated kinase 1 and 2 (ERK1/2), cyclin-dependent kinase 5 (cdk5) and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2), are able to phosphorylate TH at either ser40 and/or one of the other 3 serine residues mentioned above (22, 23). An impact of TH phosphorylation on TH activity and catecholamine production *in situ* or *in vivo*, however, has not been convincingly demonstrated for some of these kinases, while two of the phosphorylated sites, ser8 and -19, appear to have little or no role in TH activation (22, 23). This is not the case for Ser31, which has long been established as a phosphorylation target of ERK1/2 in several cell systems, in response to growth factors, G-protein coupled receptors (GPCRs) and $Ca^{2+}$ influx-inducing stimuli. Phosphorylation of Ser31 by ERK1/2 increases enzyme activity and catecholamine synthesis by increasing TH affinity for $BH_4$ (38, 48-52). In contrast to the multitude of protein kinases involved in TH phosphorylation, only two phosphatases, protein phosphatase 2A (PP2A) and, to a lesser extent protein phosphatase 2C (PP2C), are known to dephosphorylate the four serine residues of the TH regulatory domain in chromaffin cells and in the rat striatum (37, 53-56). Interestingly, PP2A containing the brain-specific subunit B'beta appears to be particularly efficient at dephosphorylating TH. Moreover, the restricted location of this subunit to the soma of dopamine neurons in the rat substantia nigra may underlie the observed enrichment of phosphorylated TH in dopaminergic processes and striatal terminals (57). This suggests a compartmentalization of TH activity to regions where DA synthesis is needed to load synaptic vesicles, which may in part be achieved through selective subcellular localization of factors that regulate TH phosphorylation.

In addition to phosphorylation, other post-translational modifications are emerging as regulators of TH activity such as nitration and thiolation, although evidence for the physiological relevance of these events is still lacking (23). Allosteric regulation, on the other hand, has been known for quite some time to affect TH activity. Heparin, phospholipids and other polyanions have all been shown to increase TH activity by a reversible allosteric interaction that increases TH affinity for its cofactor $BH_4$ (21, 58-60). However, with the exception of phospholipids that may contribute to TH interaction with membrane compartments, most polyanions are not expected to play a significant role in TH regulation due to their segregation to intracellular compartments that do not contain TH (21).

### 3.1.3. Regulation of TH activity by protein interactions

The interaction of TH with other proteins is crucial for the regulation of its intracellular location and function. One of the earliest reported examples is the interaction of TH with 14-3-3 proteins. This is a ubiquitous family of proteins that act as molecular scaffolds to modulate the function of a wide variety of binding partners by inducing conformational changes, occluding localization sequences or promoting functional interactions (61). TH binding to 14-3-3 proteins has been described to increase TH activity (62-64), although other studies have failed to observe this effect (22). Interestingly, phosphorylation at ser19 is needed for 14-3-3 proteins to interact with TH and increase its activity, which may underlie the physiological relevance of phosphorylated ser19 that is not believed to increase TH activity on its own (22, 23).

A series of studies have analyzed the interaction between TH and alpha-synuclein (65, 66), a small synaptic protein implicated in Parkinson's disease (PD) that co-immunoprecipitates with TH in rat brain homogenates. Alpha-synuclein is the major component of PD's neuropathological hallmark, the Lewy body, and mutations or multiple copies of the alpha-synuclein gene are associated with both familial and sporadic PD (67). While alpha-synuclein's role in PD is not clearly understood, the interaction of this protein with DA may exacerbate the neurodegenerative effects of high cytosolic DA levels (67-69). It is therefore interesting that alpha-synuclein has been described to decrease DA levels by inhibiting TH activity,

which could provide a countervailing neuroprotective effect. Alpha-synuclein overexpression decreases TH phosphorylation and concomitantly reduces TH activity and DA synthesis in catecholaminergic cells in culture and in dopaminergic tissues of transgenic mice (65, 66, 70, 71). Conversely, an increase in TH activity and DA production is observed in the striatum of alpha-synuclein -/- mice and in a dopaminergic cell line with knocked down alpha-synuclein levels (70, 72). While it is not clear if the observed interactions between TH and alpha-synuclein are direct in nature, or if their interaction and physical proximity (65) play a role in alpha-synuclein modulation of TH, it has been reported that alpha-synuclein relies on PP2A activation to dephosphorylate and inhibit TH (66, 70). On the other hand, alpha-synuclein has also been suggested to negatively regulate TH protein expression at the level of transcription (73-75), and a correlation between increased alpha-synuclein and decreased TH levels at the single cell level was observed in PD patients (76, 77). At this time, several studies support a negative regulation of both TH expression and activity by alpha-synuclein suggesting that increased levels of this protein may lead to a decrease in DA quantal size. We note however that while several studies have uncovered a role for alpha-synuclein in the regulation of exocytotic release of DA and other neurotransmitters, most suggest an effect at the level of vesicle pool dynamics or at a late step in exocytosis (67, 78-80). The only study addressing a role for alpha-synuclein on quantal size demonstrated that DA quantal size was not affected in chromaffin cells derived from either alpha-synuclein knockout or overexpressing mice (78), arguing against a correlation between alpha-synuclein levels and quantal size in these cells. It is of course possible that quantal size regulation by alpha-synuclein occurs in genuine dopaminergic neurons.

To maximize the efficiency of DA synthesis and subsequent vesicular uptake, it would seem advantageous for proteins involved in DA synthesis and vesicular transport to be in close association. Recent reports support this idea by suggesting that DA production and vesicular upload are carried out by a complex of proteins that are physically and functionally linked (81-83). TH and AADC can directly interact *in vitro* with each other and also with DJ-1, a transcriptional regulator associated with familial PD and involved in the response to oxidative stress (84). DJ-1, TH and AADC are pulled down as a protein complex from a human dopaminergic cell line and DJ-1 positively regulates TH and AADC activity in a manner dependent on DJ-1's oxidation state (81). Drosophila TH was also found to interact *in vitro* and *in vivo* with GTP cyclohydrolase (GTPCH), the rate-limiting enzyme of $BH_4$ synthesis (83). In addition to providing TH with a ready supply of this cofactor for L-DOPA synthesis, this interaction was suggested to reciprocally enhance the activation of the two enzymes (83). An interesting report by Cartier *et al.* demonstrates the physical connection between the DA synthesis machinery and the vesicular monoamine transporter (VMAT) that accumulates DA into the synaptic vesicle lumen (82). While the localization of TH in close proximity to synaptic vesicles has been previously described (65), Cartier and colleagues showed that TH and AADC co-immunoprecipitate with VMAT from rat striatal lysates and PC12 cells and that both enzymes were able to directly bind VMAT *in vitro*. Moreover, TH and AADC co-fractionated with VMAT and synaptic vesicle markers and were also pulled down by VMAT from a purified synaptic vesicle fraction. The functional relevance of this association was suggested in an *in vitro* assay, using an enriched striatal synaptic vesicle preparation, that showed a reduction of vesicular DA uptake when the interaction between VMAT2 with TH and AADC was disrupted (82). It would be of great interest to verify whether interfering with the TH/AADC/VMAT complex *in situ* or *in vivo* would also result in reduced DA quantal size, as this report suggests.

### 3.1.4. Regulation of TH activity by D2 dopamine receptor activation

DA binding to D2 autoreceptors is believed to negatively regulate DA release by inhibiting $Ca^{2+}$ currents and by activating a hyperpolarizing G protein-coupled inwardly rectifying $K^+$ (GIRK) current that slows the firing rate of dopaminergic neurons (85). In addition to decreasing exocytosis through the modulation of ion channel activity, D2 receptor activation also decreases DA release by inhibiting DA synthesis (85). Acute inhibition of D2 receptor function *in vivo*, either by axotomy of nigrostriatal DA fibers or injection of D2 antagonists, has long been known to potentiate DA synthesis by a mechanism that relies on TH activation by increasing its affinity for the cofactor $BH_4$ (86-91). The inhibitory effects of D2 receptors on TH activity have been confirmed *in vitro*, using striatal slices, striatal synaptosomes and dopaminergic cell lines (90, 92-94).

The mechanism whereby D2 receptor activation inhibits TH activity involves the blockade of a cAMP-dependent pathway that mediates phosphorylation of TH, as evident from the observation that D2 receptor agonists inhibit TH phosphorylation and activation induced by the adenylyl cyclase activator, forskolin (93, 95-97). Also, D2 receptor activation induces TH dephosphorylation primarily at Ser40, the residue targeted by the cAMP-dependent PKA (43, 97). As D2 receptors are negatively coupled to adenylyl cyclase activity through inhibitory G-proteins (98, 99), D2 receptor agonists probably inhibit DA synthesis by decreasing cAMP levels via G-protein inhibition of adenylyl cyclase, preventing PKA activation and phosphorylation of TH at ser40. This model is supported by the inhibitory effect of pertussis toxin, a G-protein inhibitor, on D2 receptor-induced reduction of TH activity and by the lack of effect of D2 receptor agonists on TH activation by cAMP analogues, which obviate the requirement for adenylyl cyclase (95, 97).

A link between D2 receptor-mediated decrease in DA synthesis and quantal size has been directly demonstrated in PC12 cells. Quinpirole, a D2 receptor agonist, reduced both TH activity and the quantal size of release events stimulated by KCl depolarization by about 40-50% (100). Incubating PC12 cells with L-DOPA increased DA synthesis and quantal size independently of TH and prevented the inhibitory effect of quinpirole on DA

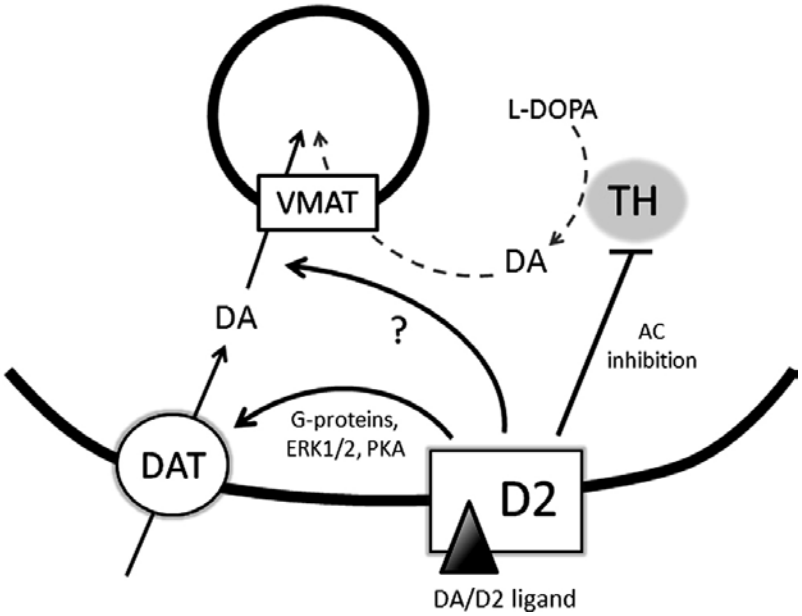


**Figure 1.** Mechanisms of quantal size regulation by dopamine D2 receptors. D2 receptor activation inhibits TH activity by a mechanism that involves G-protein inhibition of adenylyl cyclase (AC) and the consequent reduction of TH phosphorylation. The decrease in DA synthesis that follows has been linked to D2 receptor inhibition of quantal size. D2 receptor activation has also been shown to increase DA reuptake at the plasma membrane by augmenting surface DAT levels. G-proteins, ERK1/2 and PKA have been implicated in D2 modulation of DAT function. While an increase in DA reuptake by D2 receptor activation could lead to an increase in quantal size, this has not been directly addressed. An increased quantal size would also be expected from enhanced DA vesicular uptake in response to *in vivo* D2 agonist administration. While the contribution of each of these three D2 receptor actions for quantal size regulation is unclear, the overall effect of D2 autoreceptor activation on DA release appears to be inhibitory (see text).

quantal size (100), confirming the connection between D2 receptor activation, TH inactivation and the decrease in DA levels available to upload secretory vesicles. Nevertheless, the effect of D2 receptor activation on DA quantal size is probably not restricted to the inhibition of DA synthesis, as we will discuss in upcoming sections (Figure 1).

### 3.2. DA vesicular uptake and storage

Following synthesis in the cytoplasm, DA molecules are transported by VMAT into the intravesicular lumen. This transporter uses the proton gradient that exists across the vesicle membrane as a driving force to transport DA into vesicles while shuttling protons out (12, 101). The amount of DA stored in vesicles is therefore dependent on vesicular pH, VMAT expression and activity and on the capacity of the intravesicular matrix to store DA.

#### 3.2.1. Vesicular pH gradient

The vesicular proton gradient is the driving force behind the accumulation of high levels of monoamines within synaptic or secretory vesicles against a large concentration gradient. The vesicular pH achieved by the concerted effects of the several players involved has been estimated to be about 5.6, both in chromaffin granules (102) and in DA synaptic vesicles of dopaminergic ventral midbrain neurons in culture (103). This pH gradient is driven by the vacuolar $H^+$-ATPase that uses the energy released by ATP hydrolysis to produce a proton electrochemical gradient comprising of a $H^+$ concentration gradient and of an electrical gradient of positive charges across the vesicle membrane, as reviewed in detail elsewhere (12, 101).

The vesicular chloride channel is important to establish the vesicular pH gradient since the entry of chloride anions dissipates the developing electrical potential enabling the $H^+$-ATPase to generate a larger $H^+$ concentration gradient (102). Interestingly, the predominant chloride channel on synaptic vesicles, ClC-3, has recently been suggested to operate as a $Cl^-/H^+$ antiporter rather than as a classical chloride channel (104), in similarity to chloride channel family members present in other acidic organelles (105, 106). The stoichiometry of ionic exchange for these carriers was proposed to involve the transport of two $Cl^-$ ions per each $H^+$, with 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 accumulate more protons in the vesicle lumen.

A surprising role for the vesicular glutamate transporter 2 (VGLUT2) in the acidification of dopaminergic synaptic vesicles has recently been proposed (107). VGLUT2 is expressed by a subset of mouse ventral tegmental area (VTA) DA neurons that project to the ventral striatum (107, 108), where it is appears to colocalize with VMAT2 in the same synaptic vesicles (107). Glutamate was able to decrease vesicular pH in

VGLUT2-containing synaptic vesicles of the ventral striatum even in the presence of high physiological concentrations of chloride, suggesting that chloride and glutamate play distinct roles in the regulation of vesicular proton gradients (107).

A role for the vesicular proton gradient in determining monoaminergic quantal size has been directly investigated in chromaffin cells using pharmacological agents to target the vesicular $H^+$-ATPase and the chloride channel. Bafilomycin, a $H^+$-ATPase inhibitor, was shown to decrease catecholamine quantal size in two independent studies (18, 109). On the other hand, the effects of the chloride channel inhibitor 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) were less clear, as one group found a small but significant inhibitory effect on quantal size (18), while another group found no effect (109). Remarkably, amperometric measurements obtained from ClC-3 knockout mice found a drastic reduction in catecholamine quantal size (110). However, this was deemed to be an indirect action of ClC-3 channels since the same study failed to detect ClC-3 in large dense core vesicles while it clearly detected it in endosomes and synaptic-like microvesicles, in wildtype chromaffin cells (110). The regulation of vesicular pH by VGLUT2, on the other hand, appears to affect DA quantal size (107). Indeed, VGLUT2 transfection in a heterologous system increased monoamine vesicular uptake while a DA neuron-specific VGLUT2 knockout decreased the DA tissue content and single pulse-evoked DA release in the ventral striatum. It was therefore suggested that glutamate promotes DA vesicular storage by increasing the pH gradient that drives vesicular monoamine transport (107).

The importance of an intact $H^+$ gradient across synaptic vesicles for monoamine vesicular storage is reiterated by the effect of weak bases on quantal size. Weak bases are compounds that are uncharged, and thus, membrane-permeable, at neutral pH but become protonated once inside the vesicle acidic lumen. When their concentration becomes sufficiently high, they exceed the vesicular buffering capacity and collapse the proton gradient (12). The amphetamine-like psychostimulants amphetamine, methamphetamine, fenfluramine and methylenedioxymethamphetamine (MDMA, also known as "Ecstasy") are examples of weak base drugs. These compounds are recognized and transported by both DAT and VMAT (111-113), causing the displacement of DA from vesicles to the cytosol after dissipating the vesicular pH gradient (7, 114). This leads to an increase in cytosolic DA promoting DA release by DAT through reverse transport (7, 20, 115, 116). The effect of amphetamine on DA quantal size was directly demonstrated in PC12 cells where, at a concentration of 10 μm for 10 min, it decreased the amount of DA released per vesicle by about 50% (7, 117). Cyclic voltammetry and amperometry recordings in acute striatal slices, revealed an amphetamine-induced increase in DA overflow, due to decreased DA uptake, and a sharp decrease in the amount of DA released per stimulation pulse (118), suggesting that this psychostimulant has similar actions *in vivo*.

A paradoxical long term effect of membrane-permeable weak bases in cultured chromaffin cells was recently reported (119): exposure to physiologically relevant concentrations (5-10 μM) of methamphetamine for 6 to 48 hours induced a rebound hyperacidification of secretory vesicles. Concomitantly, these prolonged methamphetamine incubations increased quantal size and reinstated catecholamine release in chromaffin cells (119). The mechanism whereby methamphetamine and other weak bases induce such rebound hyperacidification is currently unknown.

The regulation of vesicular pH gradient and monoamine quantal size was also suggested to occur under physiological circumstances. Stimulation of adrenal chromaffin cells or enteric nerve terminals by different secretagogues increased vesicle acidification (18). The decrease in chromaffin vesicle pH in response to depolarization was accompanied by an increase in vesicular volume and in the quantal size of secreted catecholamines, and required $Ca^{2+}$ influx (18). The mechanisms responsible for activity-dependent acidification remain otherwise poorly understood.

### 3.2.2. VMAT

Two homologous but distinct genes code for the known isoforms of vesicular monoamine transporters, VMAT1 and VMAT2. Although the distribution of the two isoforms varies somewhat across species, VMAT1 is found in the neuroendocrine system including the adrenal gland, while VMAT2 is mostly expressed in the brain (120-123). Different degrees of VMAT efficiency, due to changes in protein expression or transporter activity, would be expected to have an impact on quantal size, particularly in vesicles that exhibit transmitter leakage following its concentration gradient. Small synaptic vesicles present in monoaminergic nerve terminals in the brain appear to be especially "leaky", as it was found that purified vesicle preparations lose tritiated DA exponentially *in vitro* with a halftime of 4 min (124). Chromaffin granules, on the other hand, are able to retain transmitter over longer periods of time due to a pH-dependent kinetic barrier to amine efflux (125). Interestingly, the capacity of these different types of vesicles to retain catecholamines appears to be inversely correlated with the amount of time needed for them to be filled with transmitter. By determining the molecular turnover of VMAT and the ratio of the amount of transmitter per VMAT molecules in different tissues, monoamine vesicular accumulation has been calculated to take a few minutes in brain synaptic vesicles and several hours in adrenal chromaffin granules (126). This suggests that "leaky" vesicles can be quickly reloaded while vesicles that take a long time to be filled will be able to hold a stable amount of transmitter for longer periods. It also indicates that small synaptic vesicles in brain nerve terminals are more susceptible to the modulation of quantal size by changes in VMAT efficiency.

The importance of sustained VMAT activity in maintaining vesicle quantal size was clearly demonstrated by amperometry experiments that explored the effects of VMAT pharmacological inhibition on the release of

catecholamines. The VMAT inhibitors reserpine and tetrabenazine reduce quantal size to about 25 to 45% of control in bovine chromaffin cells (127, 128), depending on concentration and incubation time, and to even lower levels in PC12 cells (129). In support of a marked dependence of small synaptic vesicles on continuous VMAT activity, reserpine dramatically reduced quantal DA release from midbrain DA neurons to levels below detection (19). A decrease in the frequency of quantal release events in response to both VMAT inhibitors was also observed in all systems and this is believed to represent exocytosis of "empty" vesicles (127-129). Indeed, this was demonstrated by cell attached patch amperometry, where simultaneous capacitance measurements of membrane area and quantal release recordings by amperometry clearly show a reserpine-induced increase in the number of catecholamine-free vesicles undergoing exocytosis, in chromaffin cells (128). Interestingly, this and other studies also suggest that either decreasing or increasing the amount of catecholamines inside vesicles with reserpine or L-DOPA, respectively, does not significantly change vesicular catecholamine concentration due to a concomitant change in vesicular volume (18, 128-130).

The pharmacological data on the role of VMAT in maintaining vesicular DA levels is supported by studies using genetic approaches to alter VMAT expression. VMAT2 knockout mice die within a few days after birth (131, 132), complicating the study of catecholamine exocytosis in VMAT2-null tissues. Nevertheless, cyclic voltammetry recordings in striatal slices from 2- to 4-day old mice showed that DA release evoked by electrical stimulation could be detected in wildtype but not VMAT2 knockout littermates (131). On the other hand, a different set of VMAT2-deficient mice expressing about 5% of wildtype VMAT2 levels survive well into adulthood (132, 133). As expected, these animals exhibit a sharp reduction in single pulse-evoked DA release in the dorsolateral striatum to about one third of control release, as measured by cyclic voltammetry (134), suggesting decreased DA vesicular stores. Serendipitously, these VMAT2-deficient mice were unintentionally created on an alpha-synuclein null background. This was useful for studying the role of alpha-synuclein interactions with high cytosolic DA in PD, especially since VMAT2-deficient mice on a wildtype alpha-synuclein background were later generated (135, 136). As for the study of DA quantal size, since alpha-synuclein can decrease DA synthesis (see previous section), it would be interesting to assess whether the presence of regular alpha-synuclein levels would further decrease striatal DA release in VMAT2-deficient mice and to determine how DA quantal size is affected. Finally, overexpressing VMAT2 induced an increase in quantal size in PC12 cells and in both quantal size and exocytotic event frequency in midbrain DA neurons in culture (137). The effect on quantal event frequency in these cells is consistent with the recruitment of synaptic vesicles that do not normally release DA or that could otherwise contain DA levels below the detection limit. In conclusion, VMAT limits the rate of transmitter accumulation and changes in VMAT protein levels and/or activity can alter quantal size and synaptic strength.

#### 3.2.2.1. Regulation of VMAT expression

Similarly to TH, VMAT regulation is described to occur at the protein expression and post-translational levels. In bovine chromaffin cells, an increase in VMAT2 mRNA and protein expression has been observed in response to long-term depolarization in a $Ca^{2+}$-dependent manner (120, 138, 139). This effect may involve PKC and cAMP since phorbol esters and forskolin also increased VMAT expression (138). Studies on the VMAT2 promoter in gastric epithelial cells have demonstrated a crucial role for a cAMP-responsive element (CRE) and an AP2/SP1 consensus sequence on the stimulation of VMAT2 transcription by gastrin. PKC and ERK were also found to mediate gastrin's effect on the VMAT2 promoter in this system, possibly by acting upstream of the AP2/SP1 site (140).

#### 3.2.2.2. Regulation of VMAT activity by G-proteins and other signaling factors

Among the potential regulators of VMAT activity that have so far been described, heterotrimeric G-proteins have been convincingly demonstrated as inhibitors of vesicular monoamine uptake in a variety of systems (101). The G(o2)alpha protein, for example, has been shown to associate with secretory vesicles and inhibit both VMAT1 and VMAT2 activity (101, 141, 142). Although the mechanism of VMAT inhibition by G-proteins is not clear, G(q)alpha inhibition of VMAT2 in mouse platelet granules has been shown to depend on luminal transmitter levels since it does not occur in monoamine-depleted granules and is restored upon vesicular refilling (143). However, this effect of luminal transmitter depends on the type of monoamine involved and it appears to be different for VMAT1 and VMAT2 (144). On the other hand, both transporters rely on the integrity of their first vesicular loop for their susceptibility to G-protein inhibition (144). The $Ca^{2+}$-dependent activator proteins for secretion (CAPS) 1 and 2 have been suggested to mediate the regulation of catecholamine vesicular uptake by G-proteins by an unresolved mechanism that may involve modulation of CAPS levels by G(o2)alpha (145). CAPS 1 and 2 promote vesicular uptake by VMAT in synaptic vesicles from adult mouse brain (145), while chromaffin cells derived from CAPS 1 knockout or CAPS 1/2 double knockout mice show a decrease in the frequency of quantal release events (146, 147). Notably, CAPS deficiency has a much smaller effect (or no effect whatsoever, in the case of the CAPS 1 knockout) on the number of vesicle fusion events assessed by capacitance measurements or by total internal reflection fluorescence microscopy (TIRFM). This suggests that in the absence of CAPS 1/2 there is an increase in the number of "empty" vesicles that fuse with the plasma membrane, in agreement with CAPS's suggested role in monoamine VMAT uptake (145). Paradoxically, the quantal size of the remaining release events detected by amperometry in CAPS 1/2-deficient chromaffin cells was not different from control in these studies (146, 147), while an independent report has found a dramatic decrease in chromaffin granule quantal size, as well as event frequency, in response to intracellular administration of anti-CAPS antibodies (148).

Other factors involved in VMAT activity may include phosphorylation and glycosylation, which have been suggested to affect VMAT trafficking to the appropriate vesicular compartments (149). The most compelling case for regulation of VMAT by phosphorylation is that described for casein kinase II, which phosphorylates VMAT2 on two serine residues present in a cluster of acidic residues that is crucial for VMAT2 localization to large dense core vesicles (150, 151). Phosphorylation of the serine residues appears to induce the removal of VMAT2 from these vesicles (150). PKA also seems to be required for the correct localization of VMAT 1 and 2 to large dense core vesicles in PC12 cells, although it does not directly phosphorylate VMAT *in vitro* (152).

**3.2.2.3. Regulation of VMAT activity by psychostimulants and D2 receptors**

VMAT is regulated *in vivo* by DAT inhibitors and amphetamine analogues through a mechanism that involves D2 receptor activation (153). DAT inhibition by cocaine or methylphenidate administration *in vivo* induces a rapid and reversible increase in vesicular DA uptake and in VMAT2 levels in purified synaptic vesicles obtained from the striatum of treated rats (154, 155). This increase in VMAT2 levels appears to be due to a redistribution of VMAT2 within synaptic terminals since no changes in VMAT2 immunoreactivity were found in whole synaptosomal fractions (154).

A role for D2 dopamine receptor activation is suggested by the inhibitory action of the D2 receptor antagonist eticlopride on the effects of cocaine and methylphenidate (154, 155). Also, D2 activation *in vivo* with quinpirole or the mixed D1/D2 agonist apomorphine mimics the effect of DAT inhibitors on both vesicular DA uptake and VMAT2 redistribution (155-157). These results seem to suggest that the increase in extracellular DA induced by DAT inhibition would increase D2 receptor activity that would then promote VMAT2 relocalization and stimulate vesicular DA uptake. This would be a paradoxical mechanism considering the negative actions of D2 receptor activation on DA synthesis and neuronal excitability that result in decreased DA release, as reviewed above.

In contrast to DAT inhibitors, *in vivo* administration of methamphetamine and other amphetamine analogues was found to cause a decrease in vesicular DA uptake and VMAT2 levels in purified striatal synaptic vesicles, an effect that was inhibited by the D2 receptor antagonist eticlopride (153, 158). While it is not surprising that amphetamine analogues and DAT inhibitors produce different results, due to the multiple actions of amphetamines described in the previous section (collapse of vesicular pH gradients, increase of cytosolic DA and reverse transport), it is puzzling that D2 receptors appear to mediate opposing effects of these two classes of compounds on VMAT2 redistribution and vesicular DA uptake. Whereas D2 receptors are likely to operate through a complex mechanism, this may be partly explained by technical considerations since the purified vesicle fraction used in the majority of these studies contains only a subset of synaptic vesicles that may consist of a particular pool not representative of the entire pool of vesicles of striatal terminals (153). On the other hand, since the D2 antagonists are applied *in vivo*, these apparently controversial results may reflect an involvement of D2 receptors present on different cells (and not only the D2 autoreceptors on DA terminals), which could have an overall opposing effect in response to DAT inhibitors or amphetamine analogues. Additional studies will be required to clarify the role of D2 receptors in VMAT2 distribution and DA uptake.

**3.2.3. Intravesicular storage capacity**

The accumulation of high concentrations of DA inside synaptic or secretory vesicles is not only dependent on the vesicular pH gradient and on VMAT function but also on the storage capacity of the vesicular lumen. The interior of DA-containing vesicles possesses a condensed matrix of transmitters, anionic proteins and other small molecules. The major components of the intravesicular matrix of dense core vesicles are the granins, a family of water-soluble glycoproteins that includes chromogranin A and B and the secretogranins. DA binding to these proteins is responsible for keeping the osmolarity low, preventing vesicles from swelling excessively while allowing the storage of higher amounts of transmitter (159). The granins are found in endocrine and neuroendocrine cells and also in neurons from the peripheral and central nervous system from where they can be co-released with monoamines during exocytosis (160, 161). In addition to regulating the vesicular levels of transmitter, these proteins or their proteolytic cleavage products have been proposed to be involved in large dense core vesicle formation, in the vesicular sequestration of $Ca^{2+}$ and in the modulation of exocytosis (160-163). Small synaptic vesicles are also thought to possess an intravesicular matrix comprised of glycoconjugated membrane lipids and proteins, such as gangliosides and proteoglycans. The hydrophylic sulfonated carbohydrate residues of the proteoglycans, for example, are directed towards the interior of the vesicle and have been proposed to help package the transmitter (164, 165). The transmembrane versus luminal location of the intravesicular matrix components highlights another important feature of small synaptic vesicles that dense core vesicles don't share: the ability of undergoing recycling by clathrin-dependent endocytosis. In principle, small synaptic vesicles that completely fuse with the plasma membrane can recycle their intravesicular matrix by endocytosis since it is mostly composed of membrane proteins and lipids. Dense core vesicles, on the other hand, will lose their soluble matrix components upon full fusion with the plasma membrane hampering effective recycling by endocytosis.

The role for chromogranins in maintaining high levels of catecholamines in secretory vesicles has been recently demonstrated in chromogranin A and B single and double knockout mice. Amperometric recordings of chromaffin cells derived from either chromogranin A or B single knockouts have shown a decrease in evoked catecholamine release due to a ~30% decrease in quantal

size (166, 167). Importantly, unlike wildtype cells, incubation of the chromogranin single knockout chromaffin cells with L-DOPA did not increase the amount of catecholamines secreted per vesicle. Since L-DOPA was still capable of increasing cytosolic catecholamine levels in these cells, it was concluded that the mechanism for catecholamine vesicular accumulation was saturated in the absence of either chromogranin (166, 167). No changes in vesicle size or fusion pore kinetics were found in chromogranin A -/- cells by cell-attached patch amperometry (166). The chromogranin A and B double knockout chromaffin cells exhibited a slightly higher reduction in quantal size, of about 40%, while their large dense core vesicles had an atypical appearance and a larger size (168). These morphological vesicular changes, together with the decreased frequency of release events observed in the double knockout cells, may reflect the previously suggested role of chromogranins in large dense core vesicle biogenesis (168).

Upon exocytosis, DA and other monoamines need to dissociate from the intravesicular matrix to be released into the extracellular space. In mast cell granules, that contain the monoamine serotonin, ion exchange was suggested to mediate the dissociation of the transmitter from the matrix, also known as degranulation (169). Temperature, osmolarity, extracellular pH and cation species have all been shown to regulate quantal size in adrenal chromaffin cells, probably by modulating the degranulation of catecholamines (12, 170-172). While these studies were important to understanding basic mechanisms of exocytosis, major changes in the extracellular osmolarity, pH and cationic composition are not expected to occur under physiological conditions. On the other hand, intracellular messengers such as PKA and PKC, which are regulated by several physiological stimuli, have been shown to regulate quantal size and single-vesicle release kinetics in a manner that could be explained by effects on degranulation (12, 173, 174). Importantly, the effects of PKA activation on quantal size did not seem to be due to increased TH activity since the total catecholamine cell content did not increase significantly (174). The putative mechanisms involved in second messenger regulation of degranulation are poorly understood and further studies are needed to clarify how cytoplasmic factors can modulate the release of monoamines from the intravesicular matrix.

### 3.3. DA reuptake through DAT

Following exocytosis, DA clearance from the extracellular space is achieved by DAT, the DA plasma membrane transporter that is highly enriched in striatal DA terminals (10, 11). In addition to being important for shaping the time frame, spatial constrains and the signal to noise ratio of DA neurotransmission (12, 85, 175), recycling released DA back into the nerve terminal provides a synthesis-independent mechanism to refill synaptic vesicles. DAT can therefore play an important role on regulating quantal size by affecting presynaptic DA levels during neuronal activity. Consistently, cocaine decreased the amount of DA released per quantal event in a concentration-dependent manner, when applied to PC12 cells for 30 min (117). This was not due to an inhibitory effect of D2 receptor activation on quantal size, in response to increased extracellular DA, since the D2 receptor inhibitor sulpiride had no effect on cocaine's ability to reduce quantal size (117). 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 (117). Therefore, the reduction in DA quantal size induced by cocaine was consistent with a decrease in cytosolic DA available to stock up synaptic vesicles.

The development of DAT knockout mice provided confirmation for a role of DAT in the regulation of DA quantal size. A drastic decrease in DA release in response to a single electrical pulse was observed in the striatum of DAT knockout mice *in vivo* and in acute slices, while the rate of DA clearance was, as expected, greatly reduced (176, 177). These animals exhibited a 5-fold increase in extracellular DA striatal levels whereas their tissue DA content in the striatum was less than 5% of that found in controls (176). Since no major anatomical abnormality was found for striatal DA terminals in the DAT knockouts (178), this indicates that DA intracellular stores are dramatically decreased in these animals. Conversely, it was found that DA release is far more dependent on DA synthesis in DAT knockouts than in wildtype mice, as was demonstrated by the rapid and extensive inhibitory effect of alpha-methyl-para-tyrosine, a TH inhibitor, on DA release in DAT knockout striatum in conditions that had no effect in wildtype striatum (177). These studies, together with the study on PC12 cells described above, suggest that the vesicular DA stores are acutely dependent on DAT-mediated DA reuptake.

#### 3.3.1. General considerations on DAT regulation

The regulation of DAT function has been the subject of intense investigation. While DAT expression can be affected by several factors, such as DAT blockers and substrates and D2 receptor agonists (85, 179-182), DAT synthesis is a slow process as indicated by the 2 to 3 day half-life of striatal DAT proteins (183). Therefore, post-translational regulatory strategies are likely responsible for most of the rapid changes in DAT function in response to endogenous and exogenous stimuli. In particular, the trafficking of DAT to and from the cell surface appears to be the predominant mechanism of acute DAT regulation (184, 185). Multiple factors regulate DAT activity, including DAT blockers and substrates, such as cocaine and amphetamine, and ligands of GPCRs, such as the D2 dopamine, nicotinic acetylcholine**,** metabotropic glutamate and the gamma-aminobutyric acid (GABA) B receptors (184). These factors usually converge at either the insertion or removal of DAT from the plasma membrane following the onset of protein-protein interactions and/or the activation of intracellular second-messenger pathways that often involve protein phosphorylation (185). Since the subject of DAT regulation has been extensively covered elsewhere (184-186), we will just briefly summarize the effects of some of the most established DAT regulators.

#### 3.3.2. Regulation of DAT by psychostimulants

Many of the studies on the regulation of DAT activity focused on the role of psychostimulants. A considerable amount of work supports a role for DAT

substrates, such as amphetamine, methamphetamine and even DA itself, in inducing DAT internalization *in vivo* and *in vitro*, thus reducing DA reuptake (187-192). Interestingly, the effect of DAT substrates appears to be biphasic with a rapid and transient upregulation in surface DAT levels followed by a more lasting downregulation (193, 194). Regional differences have also been reported, since the effects of DAT substrates on DAT trafficking observed in the striatum have not been consistently replicated in the nucleus accumbens (187, 191, 195). Studies on DAT regulation by DAT blockers have focused on the effects of cocaine, which has been shown to block DAT internalization induced by DAT substrates (188, 189, 193, 194). On the other hand, cocaine's action on DAT trafficking *per se* is less clear, with some studies indicating an increase in DAT surface expression (196, 197) and others finding no effect (189, 198).

While several kinases have been involved in the regulation of DAT function and trafficking, including PKC, ERK1/2 and phosphatidylinositol 3-kinase (PI3K), the role of PKC in these events is by far the most thoroughly investigated (185, 199). PKC activation induces DAT internalization (190, 200-202) and it appears to be involved in the similar effect of DAT substrates on DAT trafficking (186, 191). However, the mechanism of PKC activation by DAT substrates remains largely unknown (185). The means by which PKC regulates DAT internalization are also not understood and a straightforward mechanism based on direct phosphorylation of DAT by PKC does not seem to be involved (185, 186, 199, 203, 204). In contrast to DAT substrates, cocaine and other DAT inhibitors do not seem to significantly affect DAT phosphorylation (198).

Interestingly, the inhibitory effect of amphetamine analogues on DA vesicular uptake and their disruption of the vesicular pH gradient, discussed in the previous section, should have a similar effect on DA quantal size as their negative effect on DA reuptake. Therefore, the concerted actions of amphetamine-like molecules on vesicular pH, VMAT and DAT should all induce a decrease in DA quantal size. Cocaine's effect on DAT trafficking remains elusive and while *in vivo* administration of this DAT blocker appears to increase vesicular DA uptake, it was shown to decrease quantal size in chromaffin cells. Further studies will be needed to clarify the full plethora of effects of DAT blockers on DA quantal size.

### 3.3.3. Regulation of DAT by D2 receptors

Several GPCRs have been known to regulate DAT function (184, 185). We will focus on D2 receptors as the most established GPCR regulators of DAT activity. Most of the studies using acute administration of agonists and antagonists of the D2 receptor have demonstrated a positive regulation of DAT activity and surface expression by D2 receptors *in vivo* and *in vitro* (205-211). The mechanism responsible for D2 receptor upregulation of DAT activity has been suggested to involve G-proteins, ERK1/2 and PKA (207, 210, 212). Interestingly, ERK1/2 inhibition has been shown to decrease DAT function and its localization at the cell surface (213). Co-localization and direct interaction of DAT and D2 receptors have also been reported and the association with D2 receptors appears to promote the recruitment of DAT to the plasma membrane (212).

Studies with D2-deficient mice, however, have yielded contradictory results on D2 receptor regulation of DAT activity. Dickinson *et al.* have shown a decrease in DAT function in D2-deficient mice by *in vivo* electrochemistry, as the clearance of exogenously applied DA was decreased in these animals (214). Benoit-Marand and colleagues, on the other hand, detected no difference in the clearance of endogenous DA released *in vivo* in response to electrical stimulation, in D2 receptor knockout mice (215). Nevertheless, the D2-deficient mouse line used by this group was reported to have a higher density of DAT-immunoreactive terminals in the dorsal striatum (216), which could suggest decreased DAT activity per DA terminal in order to obtain similar DA clearance between D2 knockout and wildtype animals. Finally, one study demonstrated increased DA uptake in striatal slices of D2 knockout mice, based on the half-life of evoked DA overflow measured by cyclic voltammetry and on the effect of DAT blockers (16). The different results obtained by these three groups may be due to differences pertaining to the mouse models (different DAT-deficient mouse lines were used in all three studies) and to the methodology used (exogenous vs. endogenous DA clearance; *in vivo* vs. *in situ*). Most of the pharmacological studies mentioned above involved acute inhibition or activation of D2 receptors, while the genetic ablation of D2 receptors may reflect changes in DAT function due to either long term or developmental effects of D2 receptor deficiency.

Recapitulating the putative mechanisms whereby D2 receptor activation may affect quantal size, we would expect conflicting effects between the inhibition of TH synthesis, which is known to reduce DA quantal size, and the increase in both DA reuptake through DAT and vesicular DA uptake, which is expected to lead to an increase in DA quantal size (Figure 1). While it's not clear how all these factors converge downstream of D2 receptors to modulate the amount of DA per vesicle, the overall effect of D2 autoreceptor activation on DA release appears to be consistently inhibitory (85).

## 4. QUANTAL SIZE MODULATION BY MODES OF VESICLE FUSION

### 4.1. Vesicle fusion modes: kiss-and-run versus full fusion

The fate of secretory vesicles upon fusion with the plasma membrane is a much debated topic in the field of exocytosis. Efficient recycling of the lipid and protein components of the vesicle membrane is essential not only to ensure vesicle availability for sustained transmission but also to maintain the integrity of the plasma membrane that would otherwise swell excessively, jeopardizing its functional architecture. Two general modes of vesicle fusion have been suggested that entail very different mechanisms of vesicle retrieval. The classic mode, which is commonly referred to as full fusion, proposes that vesicles

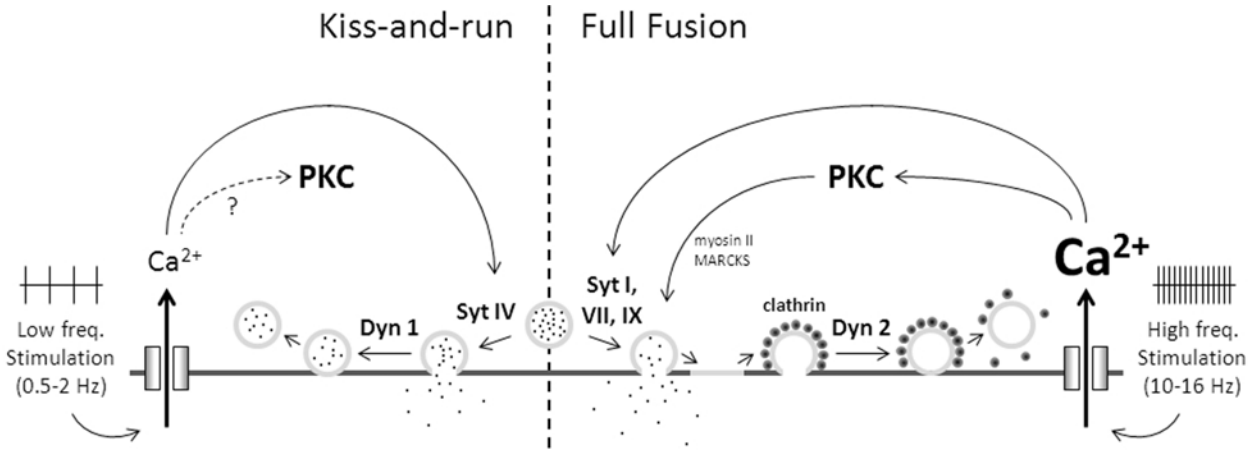


**Figure 2.** Putative mechanisms regulating kiss-and-run and full fusion. Upon $Ca^{2+}$ influx into synaptic terminals, vesicles may undergo complete fusion with the plasma membrane, releasing all of their transmitter content (full fusion), or they may fuse transiently, with full or partial release of their contents before the fusion pore reseals to recycle the vesicle (kiss-and-run). For simplicity, only the case of partial vesicular content loss during kiss-and-run is depicted in this figure. While the mechanisms that regulate vesicle fusion mode are not well understood, several studies performed mostly in chromaffin cells support an increased incidence of full fusion during high frequency stimulation and the ensuing high intracellular $Ca^{2+}$ levels and PKC activation. Myosin II and MARCKS have been suggested to act downstream of PKC to promote full fusion. In contrast, kiss-and-run appears to be favored by low frequency stimulation, smaller increases in intracellular $Ca^{2+}$ and inhibition of PKC. Pharmacological activation of PKC switches the mode of vesicle fusion from kiss-and-run to full fusion at low frequencies of stimulation, suggesting that under these stimulation conditions PKC is either not activated or activated below the threshold needed to promote full fusion. Other studies have disputed the above described effects of PKC activation and high $Ca^{2+}$ levels on kiss-and-run vs. full fusion regulation (see text). Several synaptic proteins have been suggested to influence quantal size and the mode of vesicle fusion and most of the evidence to date has been reported for synaptotagmins, with isoform IV favoring kiss-and-run and isoforms I, VII and IX promoting full fusion. Interestingly, both forms of vesicle fusion appear to be dependent on dynamin, while only full fusion requires clathrin, as it is dependent on clathrin-mediated endocytosis for vesicle recycling. Dynamin 1 and 2 have been suggested to mediate kiss-and-run and full fusion, respectively, but this apparent selectivity is not yet clearly established (see text for details). Syt – synaptotagmin; Dyn – dynamin.

fuse with the plasma membrane by fully collapsing into it, leading to a complete release of vesicle contents and to presumed lipid continuity between the two membranes (Figure 2). Vesicles would then be recycled by clathrin-dependent endocytosis. Although some variations on this model have been suggested over time (217), the basics of full fusion have been proposed by Heuser and Reese in the early seventies, based on electron microscopy studies of horseradish peroxidase-stained endocytic organelles at the frog neuromuscular junction (218). An alternative mode of vesicle fusion, later termed kiss-and-run (219), was suggested by Ceccarelli and colleagues, also grounded on anatomical observations at the frog neuromuscular junction (220). This model proposed that vesicles fuse transiently with the plasma membrane, releasing their contents through a fusion pore that would then reseal to retrieve the vesicle into the cytoplasm without complete loss of identity (Figure 2). The terminology used to denominate these transient fusion events has been diverse including "transient fusion", "fuse-pinch-and-linger" and "porocytosis", among others (217). We will mostly refer to them as "kiss-and-run" events as this appears to be the most commonly used term. As will be discussed below, a kiss-and-run event may or may not lead to the release of the full vesicle content. Therefore, the mode of vesicle fusion and fusion pore dynamics are of critical importance to the amount of transmitter released per fusion event.

While full fusion and clathrin-dependent retrieval of vesicles have been established over the years as an essential mode of exo-endocytosis, the prevalence of kiss-and-run as an alternative mode of vesicle fusion has been widely controversial (217, 221-224). This is particularly the case for small central nervous system (CNS) presynaptic terminals that due to their diminutive size have not allowed for membrane capacitance measurements which have greatly contributed to the vindication of kiss-and-run in other systems. In these small terminals, the study of vesicle fusion modes has relied mostly on optical approaches that have produced indirect yet compelling evidence for kiss-and-run (217, 223). In this section we will mostly focus on studies of vesicle fusion in DA and catecholaminergic systems, where direct evidence exists in favor of kiss-and-run, and discuss how that influences quantal size.

Early quantal recordings by amperometry from the Wightman and Neher groups allowed for the detection of quantal release events as amperometric spikes that are often preceded by a smaller "foot" signal (6, 225). The foot corresponds to the efflux of catecholamines through a narrow low conductance pore before the pore dilates for complete exocytosis identified as a full amperometric spike (225). The first direct demonstration of kiss-and-run was

enabled by the powerful combination of patch clamp capacitance measurements and amperometry that simultaneously monitor the opening of individual fusion pores and the kinetics of catecholamine release from the same vesicle (226, 227). These studies, by the groups of Fernandez and Lindau, clearly showed transmitter release from secretory vesicles that did not undergo complete fusion, in both mast cells and chromaffin cells. Moreover, the low conductivity and the narrow size of the transient fusion pore were similar to the characteristics of the fusion pore during the foot (227), suggesting that kiss-and-run occurs when the narrow foot fusion pore reseals before expanding to produce a full fusion event. Interestingly, while in mast cells only a small percentage of vesicular serotonin was released during these transient kiss-and-run fusion events (226), most of the catecholamine contents of chromaffin granules seemed to be released (227). Evidence for the occurrence of kiss-and-run, as well as for the preservation of vesicle shape during this fusion mode, has also been provided in mast cells and neuroendocrine PC12 cells by optical approaches, as the large size of secretory vesicles in these cells allows them to be resolved at the single vesicle level by light microscopy. Williams and Webb have combined the use of plasma membrane and extracellular aqueous dyes to demonstrate that vesicles can either retain their shape or fully collapse with the plasma membrane in mast cells, since fusion pore opening allows dyes to access the vesicle membrane and lumen (228). Almers and colleagues have shown that a small fluorescently-tagged luminal vesicle marker is lost in less than a second upon exocytosis in PC12 cells, while a tagged transmembrane vesicle protein present in the same vesicle can be retained for more than a minute. Also, combining the use of a vesicular pH-sensor for exocytosis with the diffusible cytosolic marker cyan fluorescent protein (CFP), which allowed the visualization of the vesicles as dark spots that excluded the dye, demonstrated that individual vesicle fusion events could occur without collapse into the plasma membrane. This was evident by the persistence of the CFP-excluding vesicle shape even as individual vesicles were undergoing exocytosis, as reported by the vesicular pH-sensor (229).

The study of vesicle fusion modes in dopaminergic neuronal synapses has been hampered by their small size. Only one study has so far addressed this problem by applying amperometric recordings of quantal release to ventral midbrain dopamine neurons in culture (230). While most amperometric events recorded were "simple", as they consisted of a single traditional spike, 15 to 20% of events were comprised of a rapid succession of spikes that gradually decreased in amplitude. These "complex" events were consistent with flickering of the synaptic vesicle fusion pore, with each "flicker" releasing 25-30% of vesicular dopamine. These results remain the most direct evidence for kiss-and-run exocytosis in small CNS dopamine neurons.

### 4.2. Kiss-and-run fusion: prevalence and impact on quantal release

The prevalence of kiss-and-run in neuroendocrine cells and DA neurons has not been a consensual matter varying from about 10 to 90% of all fusion events according to different studies, even if we just consider reports using the chromaffin cell model (227, 231-233). Some of these discrepancies may be due to the use of different stimulation conditions, since, as discussed below, this affects the percentage of fusion events that occur by kiss-and-run. Indeed, the lowest fractions of kiss-and-run have been described by patch amperometry (227, 233), where the exocytosis-inducing stimulus is the actual sealing of the patch pipette onto the cell membrane, which is described as sufficient to stimulate exocytosis of all releasable granules in the patch (227). Higher fractions of kiss-and-run were suggested by studies where amperometric or capacitance recordings were used on their own or in combination with optical techniques to detect vesicle fusion induced by electrical stimulation (231, 232, 234). While the simultaneous capacitance and amperometric measurements produced by patch amperometry are still the most reliable method to detect kiss-and-run, it is possible that the fraction of different fusion events induced by the sealing of the patch pipette may not reflect the actual prevalence of kiss-and-run under more physiological stimuli. Reports using this technique also suggest that the full vesicular content is released during kiss-and-run (227, 233), while other studies favor the partial release of vesicular transmitter during these events and the modulation of the fraction of release per event by several factors (231, 232, 235), as reviewed below. Recently, a microfluidic-based platform for amperometric detection of catecholamines in isolated individual PC12 cell vesicles was developed to bypass the fusion process in measuring the total vesicular transmitter content. A direct comparison of amperometric data from *in situ* release experiments in PC12 cells and from the single vesicle cell free system indicated that vesicles release, on average, only 40% of their total catecholamine load (236). This provides evidence not only for the high prevalence of kiss-and-run in these cells but also for the fractional release of transmitter during transient fusion events. In midbrain DA neurons, the flickering fusion that originates "complex" events was determined to release about a quarter of vesicular DA per "flicker", as explained above. Moreover, the "simple" events observed in these neurons have 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 (230). Therefore, not only is kiss-and-run release fractional in midbrain DA neurons but the extent of fusion pore flickering impacts the fraction of DA that is released per fusion event.

### 4.3. Stimulation frequency, $Ca^{2+}$ and PKC as regulators of vesicle fusion modes

Several studies demonstrate that stimulation frequency, $Ca^{2+}$ concentration and PKC activity regulate the prevalence of one fusion mode versus the other. These three factors probably converge on the same pathway to affect vesicle fusion modes (Figure 2). It is well established that moderate stimulation frequencies (0.5-2 Hz) predominantly induce kiss-and-run exocytosis in chromaffin cells (231, 232, 235, 237-240). Increasing the frequency of stimulation to 10-16 Hz increases quantal size and decreases the rapid endocytosis associated to kiss-and-

run to produce a higher percentage of full fusion events, as determined by capacitance measurements, amperometry or a combination of these methods with optical approaches (231, 232, 235, 237-240).

Increased buildup in intracellular $Ca^{2+}$ was suggested to mediate the conversion of kiss-and-run to full fusion at high stimulation frequencies (237). Indeed, increasing intracellular $Ca^{2+}$ levels by manipulating the $Ca^{2+}$ concentration either directly in the patch pipette or in the extracellular buffer favored the occurrence of full fusion in chromaffin cells (231, 237) and accelerated the fusion pore expansion rate in horse eosinophils (241). Moreover, augmenting the extracellular $Ca^{2+}$ concentration from 2.8 to 10 mM switched the chromaffin vesicle fusion mode from kiss-and-run to full fusion at the low stimulation frequency of 0.5 Hz (237). In contrast, another study reported an increase in the proportion of release events that occur by kiss-and-run upon increasing intracellular $Ca^{2+}$ levels (233). The $Ca^{2+}$ concentrations used in this report were, however, quite high (5 to 90 mM) raising concerns about the physiological relevance of these results.

PKC activation may be a predominant mechanism involved in the control of vesicle fusion by calcium. Most studies propose a negative regulation of kiss-and-run by PKC since pharmacological activation of this kinase accelerates fusion pore expansion in horse eosinophils and induces kiss-and-run to full fusion conversion and fusion pore dilation in chromaffin cells, in response to 0.5 Hz stimulation (237, 241). Conversely, PKC inhibition decreases fusion pore size and induces a switch from full fusion to kiss-and-run at 15 Hz (237). Moreover, PKC has also been suggested to favor full fusion exocytosis downstream of muscarinic acetylcholine receptor activation in chromaffin cells (242). The role of PKC was also studied in midbrain DA neurons in culture where PKC activation promoted the conversion of "complex" fusion events, exhibiting successive fusion pore flickers, to "simple" events (230). However, it is not clear whether these results support positive regulation of full fusion by PKC activation since "simple" events are unlikely to represent full fusion as they exhibit similar quantal size as the first "flicker" of a "complex" event. It is possible that PKC promotes a switch between kiss-and-run variants instead.

A model that brings together stimulation frequency, $Ca^{2+}$ and PKC was put forward by Fulop *et al.* (237), proposing that increased firing rates lead to an enhanced increase in $Ca^{2+}$ levels that activates PKC, which can then promote fusion pore dilation converting kiss-and-run events to full vesicle collapse (Figure 2). Another two reports from the Smith laboratory place myosin II and the myristoylated alanine-rich C-kinase substrate (MARCKS) downstream of PKC in this mechanism. PKC-induced phosphorylation of these proteins was demonstrated to be necessary for the disruption of the actin cytoskeleton in response to high stimulation conditions that appears to be crucial for the switch from kiss-and-run to full fusion observed under those circumstances (234, 243). However, inhibition of myosin or of actin polymerization was found in another study to slow fusion pore expansion and increase fusion pore lifetime without affecting chromaffin granule quantal size (244). These results were interpreted as a facilitation of catecholamine degranulation by actin and myosin possibly through the generation of mechanical forces (244).

Additional reports seem to contradict the above mentioned model. For example, Graham *et al.* have shown a decrease in chromaffin granule quantal size in response to PKC activation that could suggest an increase in kiss-and-run (245, 246). This study, however, employs digitonin permeabilization to induce $Ca^{2+}$-dependent vesicle fusion which may differ somewhat from the exocytotic mechanisms activated under more physiologically-relevant stimuli such as electrical stimulation. Also, PKC inhibitors were found to decrease the rapid membrane retrieval that occurs in chromaffin cells in response to 0.5 Hz stimulation, which is thought to underlie kiss-and-run (240). On the other hand, $Ca^{2+}$ and PKC modulation of fusion pore acceleration rate appeared to be independent in horse eosinophils (241). These apparent discrepancies may be due to differences in cell system, stimulation protocols and in the methods used in the different studies to study kiss-and-run and fusion pore kinetics.

### 4.4. Dependence of kiss-and-run on dynamin

The involvement of dynamin on vesicle retrieval following kiss-and-run exocytosis has been demonstrated on chromaffin and PC12 cells by several groups. Artalejo and colleagues have used capacitance recordings to show that the rapid endocytosis associated to kiss-and-run events (231) is blocked by inhibiting dynamin function, either by removing GTP, applying the non-hydrolysable GTP analogue GTPgammaS (that blocks dynamin GTPase activity) or using an anti-dynamin IgG (247). While kiss-and-run and full fusion share a dependence on dynamin for vesicle retrieval, the former appears to be independent of clathrin-mediated endocytosis since disruption of clathrin function inhibits the slow endocytosis characteristic of full fusion but not kiss-and-run-associated rapid endocytosis (240, 247, 248). Concomitantly, inhibition of calcineurin, a phosphatase involved in clathrin-dependent membrane retrieval, decreased full fusion-associated endocytosis but did not affect rapid kiss-and-run endocytosis (239, 240). Interestingly, the rapid vesicle retrieval following kiss-and-run is dependent on dynamin 1 while endocytosis after full fusion relies on dynamin 2 (Figure 2; 248). However, this distinction in the effects of different dynamin isoforms may not be as clear cut since disruption of dynamin 1 function has recently been shown to limit fusion pore dilation during full vesicle collapse, without preventing subsequent membrane internalization (249). Amperometric recordings in chromaffin cells are in agreement with the capacitance measurement studies mentioned thus far, showing increased quantal size and slower quantal release upon inhibition of dynamin function (235, 246, 249). Confirmation of a direct role for dynamin in kiss-and-run was obtained in a fluorescence imaging study of PC12 cell plasma membrane lawns, where the retrieval of fusing granules by kiss-and-run was monitored by uptake of fluid phase markers (250). Retrieved granules were

preferentially associated with dynamin and granule recapturing was inhibited by dynamin function disruption. Finally, a dynamin-synaptophysin interaction, which is promoted by high $Ca^{2+}$ concentrations, was suggested to be necessary for a clathrin-independent mechanism of synaptic vesicle endocytosis at the squid giant axon by a combination of electrophysiology and electron microscopy techniques (251, 252). This may indicate a role for the synaptic vesicle protein synaptophysin in kiss-and-run-like vesicle recycling by recruiting dynamin to vesicles. Taken together, these studies suggest that kiss-and-run is dependent on dynamin but independent of clathrin (Figure 2), probably due to the preservation of vesicle shape that is inherent to this fusion mode.

### 4.5. Regulation of fusion modes by synaptic proteins

The synaptic vesicle protein synaptotagmin, a widely accepted $Ca^{2+}$ sensor for exocytosis (253), is by far the most studied synaptic protein in the context of vesicle fusion mode regulation. There are several isoforms of synaptotagmin that appear to regulate vesicle fusion and fusion pore dynamics in distinct ways (Figure 2). It was demonstrated in PC12 cells that augmenting synaptotagmin IV levels, either by transfection or by forskolin treatment, increases the frequency and duration of kiss-and-run events, as detected by amperometry as "stand-alone" feet (254). Moreover, a mutation in a $Ca^{2+}$ ligand in the C2B domain of synaptotagmin IV inhibited kiss-and-run while mutating a homologous $Ca^{2+}$ ligand in the C2A domain of the synaptotagmin I isoform inhibited full fusion (254). The interaction of synaptotagmin I with SNAP-25 (synaptosomal-associated protein of 25 kDa) and the membrane phospholipid phosphatidylserine was also suggested to be important for fusion pore stability (255, 256). One should consider, however, that kiss-and-run events may not be fully described by stand-alone feet (small amperometric events that resemble the prespike foot but are not followed by a full scale spike), since it has been shown by patch amperometry that full amperometric spikes also occur during kiss-and-run (233). On the other hand, these findings have been supported by results obtained by optical methods. Transfection of PC12 cells with fusion proteins consisting of different synaptophysin isoforms coupled to a pH-dependent fluorophore enabled the monitoring of secretory vesicle exocytosis by TIRFM. This technique allows the visualization of single vesicle fusion by detecting fluorescent signals on a very superficial region of the cell just below the plasma membrane (257). PC12 cell vesicles containing synaptotagmin I or IV showed the highest percentage of kiss-and-run events (~40%), while the ones containing synaptotagmin IX or VII exhibited much lower kiss-and-run frequency (about 15 and 7%, respectively) (258). Also, in agreement with the amperometry study mentioned above, increasing synaptotagmin IV levels by forskolin treatment increased the prevalence of kiss-and-run events of vesicles containing synaptotagmin I, although it did not affect the vesicle fusion mode of synaptotagmin VII vesicles (258). An independent TIRFM study using a fluorescent vesicle lumen marker to detect exocytosis in PC12 cells reported an increase in kiss-and-run upon downregulation of the levels of synaptotagmin I or IX (259). Finally, mutations that abolish $Ca^{2+}$-binding to the C2B domain of synaptotagmin VII cause the fusion pore to become unstable and dramatically increase kiss-and-run fusion events, as determined by patch amperometry in chromaffin cells (260). Overall, these studies seem to indicate that synaptophysin IV promotes kiss-and-run exocytosis while $Ca^{2+}$-binding to the other isoforms favors full fusion (Figure 2).

Other synaptic proteins have been suggested to modulate vesicle fusion modes. Munc18, a key protein involved in the last steps of vesicle fusion (261), has been suggested to modulate quantal size and fusion pore dynamics in chromaffin cells. Expression of Munc18 mutants that are not able to bind syntaxin induced a decrease in quantal size and faster quantal release kinetics that were interpreted as an acceleration of fusion pore expansion (262, 263). Syntaxin-independent effects of Munc18 on quantal size and fusion pore dynamics, probably involving other synaptic proteins such as Mint, were also suggested (264). However, these studies relied solely on amperometry to investigate effects of Munc18 on exocytosis and complementary studies with other techniques are needed to confirm the role of Munc18 on fusion pore and vesicle mode regulation. Recently, a patch-clamp capacitance study in lactotrophs implicated the interaction between Munc18 and syntaxin, Rab3A or Mint proteins in either fusion pore dwell-time or stability (265). Complexin II, another crucial synaptic protein that regulates SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein complexes (261), was also suggested by amperometric studies to affect quantal size and quantal release kinetics but a definite link to vesicle fusion mode regulation is still lacking (266). The same could be said about cdk5, since a dominant negative form of cdk5 was shown to increase quantal size and affect the prespike foot by Munc18-dependent and -independent mechanisms, in adrenal chromaffin cells (267). Understanding the role of these and other synaptic proteins in the regulation of vesicle fusion modes will undoubtedly provide great insight on the mechanisms whereby secretory vesicles at the brink of fusion commit to kiss-and-run or fully collapse into the plasma membrane.

## 5. OTHER FACTORS AFFECTING QUANTAL SIZE

### 5.1. Intracellular factors

In addition to all the intracellular factors described above, there are several other proteins that are known to affect quantal size by mechanisms that are not yet understood. One example is the $Ca^{2+}$-binding protein calbindin-$D_{28K}$. Chromaffin cells from calbindin-$D_{28K}$ knockout mice exhibited reduced quantal size and frequency of quantal events (268). The absence of this protein did not produce significant effects on intracellular $Ca^{2+}$ dynamics in these cells, suggesting $Ca^{2+}$-independent roles for this protein on synaptic function (268). Adaptor protein 3 (AP3) is a vesicle coating protein that decreased quantal size when overexpressed in mouse chromaffin cells (269). Conversely, deletion of AP3 produced a dramatic increase in quantal size that correlated with an increase in the size of chromaffin granules. These results, together with

the localization of AP3 at the trans-Golgi network and immature vesicles, suggest a role for AP3 in the formation of neurosecretory vesicles and therefore in the modulation of quantal size through the regulation of vesicular volume (269).

At least three proteins that have been linked to PD have also been suggested to affect quantal size. Parkin and PINK1 (phosphatase and tensin homolog-induced putative kinase 1) loss-of-function mutations are associated with early-onset familial PD (270). Mice deficient in either one of these proteins exhibited reduced quantal size in chromaffin cells and a concomitant decrease in evoked striatal DA release (271, 272). The levels of DA receptors were unchanged in either mouse model while striatal DA levels and the number of substantia nigra DA neurons were reported to be normal in the PINK1 knockout (271, 272). It is not currently understood how parkin, a ubiquitin E3 ligase, and PINK1, a mitochondria-associated kinase, affect the amount of transmitter that is released per fusion event. Leucine-rich repeat kinase 2 (LRRK2) is a large multidomain protein thought to mediate protein-protein and/or protein-membrane interactions and whose mutations are associated to familial PD (270). Amperometry recordings in chromaffin cells obtained from knockin mice with a PD-linked missense mutation in LRRK2 (R1441C) have revealed a decrease in the frequency and quantal size of vesicle fusion events (273). While an impairment in D2 receptor function has been detected in these mice, no change has been found in striatal DA levels, and so a D2-mediated decrease in DA vesicle content seems unlikely (273). The mechanisms used by each of these proteins to modulate quantal size in the dopaminergic system remain unclear, although it is possible that the effects are due to downstream consequences on cellular health.

### 5.2. Extracellular factors

Glucocorticoids and growth factors are known to affect quantal size, and while their mode of action is unknown, it may involve increased DA synthesis. The glucocorticoid dexamethasone, for example, increased the frequency and quantal size of single release events when applied for several days to PC12 cells (274). This could involve an increase in DA synthesis since dexamethasone was reported to increase TH activity, DA stores and DA release in the same cell system (275).

Several growth factors are known to regulate neurotransmitter release and, in some cases, a clear modulation of the quantal size of release events has been demonstrated (12). Glial cell-derived neurotrophic factor (GDNF) is, however, the only trophic factor known to regulate quantal size in the dopaminergic system. Incubation of midbrain DA neurons in culture with GDNF for three to six weeks increased the amount of DA released by quantal events by about fourfold in comparison to control cultures (19). A likely explanation for this effect is increased DA synthesis since GDNF is known to increase TH activity in dopamine neurons *in vivo* (276). It would be interesting to know whether other growth factors known to enhance evoked DA release from striatal DA terminals, such as brain-derived neurotrophic factor (BDNF) (277), possess the capability of modulating quantal DA release.

## 6. SUMMARY AND PERSPECTIVE

Since our previous review on this topic, about ten years ago, the idea of quantal size modulation has evolved from an emerging concept to an established property of synaptic terminals and endocrine cells. The major question is no longer whether alterations in quantal size are physiologically relevant, but rather to determine the cellular and molecular underpinnings that regulate a property once widely thought to be immutable. Much progress has been made on this front, particularly in the dopaminergic system where quantal size can be measured directly by electrochemistry techniques. New players have emerged in the modulation of the amount of dopamine and other catecholamines that can be stored in vesicles, either at the synthesis, vesicular storage or reuptake steps. The modes of action of additional players, including DA itself, are now better understood although there are still plenty of unsolved issues.

One area that has greatly evolved during the last decade has been the regulation of quantal size by different modes of vesicle fusion. Although still quite controversial, especially at small CNS synapses, the study of kiss-and-run fusion has been approached by multiple new methodologies. Thanks to these efforts, the field has identified $Ca^{2+}$, PKC, dynamin and synaptophysin as key mediators of the last choice a vesicle has to make upon exocytosis: whether to “kiss” or to “collapse”. It will be important to translate the study of vesicle fusion modes, which has mostly been advanced in neuroendocrine cells, to DA neurons *in vitro* and *in vivo*. To strive towards that goal, the development of new technical methods needs to progress. The development of new fluorescent probes to specifically study exocytosis at monoamine synapses (278, 279) and the novel super-resolution fluorescence microscopy techniques (280) may assist this endeavor. We look forward for new insights over the next decade on the regulation of quantal exocytosis.

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**Abbreviations:** AADC: aromatic amino acid decarboxylase; AP3: adaptor protein 3; BDNF: brain-derived neurotrophic factor; $BH_4$: tetrahydrobiopterin; CaMKII: calcium/calmodulin-dependent kinase II; CAPS: $Ca^{2+}$-dependent activator protein for secretion; cdk5: cyclin-dependent kinase 5; CFP: cyan fluorescent protein; CNS: central nervous system; CRE: cAMP-responsive element; DA: dopamine; DAT: dopamine transporter; L-DOPA: L-dihydroxyphenylalanine; ERK1/2: extracellular-signal regulated kinase 1 and 2; GABA: gamma-aminobutyric acid; GDNF: Glial cell-derived neurotrophic factor; GIRK: G protein-coupled inwardly rectifying K+; GPCR: G-protein coupled receptor; GRE: glucocorticoid regulatory element; GTPCH: GTP cyclohydrolase; LRRK2: leucine-rich repeat kinase 2; MAPKAPK2: mitogen-activated protein kinase-activated protein kinase 2; MARCKS: myristoylated alanine-rich C-kinase substrate; MDMA: methylenedioxymethamphetamine; PD: Parkinson's disease; PI3K: phosphatidylinositol 3-kinase; PINK1: phosphatase and tensin homolog-induced putative kinase 1; PKA: cAMP-dependent protein kinase; PKC: protein kinase C; PKG: cGMP-dependent kinase; PP2A: protein phosphatase 2A; PP2C: protein phosphatase 2C; SNAP-25: synaptosomal-associated protein of 25 kDa; SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TH: tyrosine hydroxylase; TIRFM: total internal reflection fluorescence microscopy; VGLUT2: vesicular glutamate transporter 2;VMAT: vesicular monoamine transporter; VTA: ventral tegmental area

**Key Words:** Dopamine, Quantal size, Exocytosis, Tyrosine hydroxylase, VMAT, DAT, Kiss-and-run, Full fusion, Review

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