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Presynaptic effects of L-DOPA and their possible role in dyskinesia

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Abstract

L-DOPA replacement therapy has long provided the most effective treatment for Parkinson's disease. We review how this dopamine precursor enhances dopaminergic transmission by providing a greater sphere of neurotransmitter influence, due to the confluence of increased quantal size and decreased dopamine reuptake, as well as loading dopamine as a false transmitter into surviving serotonin neuron synaptic vesicles. We further review literature on how presynaptic dysregulation of DA release following L-DOPA might trigger dyskinesias in Parkinson patients.

Since its introduction [1,2], L-DOPA has provided effective treatment for Parkinson's disease (PD) by replacing dopamine (DA) neurotransmission following the death of substantia nigra (SN) neurons. This therapy however leads to motor side effects, L-DOPA-induced dyskinesias (LIDs), limiting the utility of the drug. Here we review literature on DA neurotransmission in healthy and DA depleted striata and on presynaptic mechanisms that may underlie the development of LIDs.

Dopamine neurotransmission

In SN neurons, DA is normally synthesized from tyrosine in a two step enzymatic reaction (Fig 1A). First, tyrosine hydroxylase (TH) attaches a hydroxyl group to tyrosine using oxygen, tetrahydrobiopterin and Fe^{2+} as cofactors [3] to produce L-DOPA. Next, aromatic L-amino acid decarboxylase (AADC) converts L-DOPA to DA using pyridoxal phosphate as a cofactor. Both TH and AADC are regulated by DA D2 autoreceptor-mediated second messenger systems, with enzyme activities increased by receptor antagonists and decreased by agonists [4-6]. Together, these homeostatic responses decrease DA synthesis when extracellular DA is increased.

The cytosolic DA is loaded into synaptic vesicles to provide neurotransmission. The neuronal synaptic vesicle monoamine transporter (VMAT2) [7-9] translocates DA using energy generated by the vesicular proton pump, V-type H^+-ATPase, exchanging two intravesicular protons for each cytoplasmic DA molecule. The acidic vesicular lumen

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prevents DA auto-oxidation and degradation by cytosolic enzymes, allowing accumulation of very high (up to molar) intravesicular transmitter concentrations for extrasynaptic release by stimulation-dependent exocytosis [10,11]. VMAT2 translocates many compounds, and in addition to native transmitters - DA, norepinephrine, epinephrine, serotonin and histamine - can transport a diverse range of synthetic substrates [10,12].

An important attribute of DA and other monoamine neurotransmitter synapses is that the neurotransmitter overflows far past its release site to interact with multiple synapses, a form of volume transmission [13] labeled *social* neurotransmission [14]. That social nature of striatal DA transmission is evident from electrochemical measurements – the probes used for microdialysis and voltammetry are thousands of times too large to be within synapses and must be measuring extrasynaptic DA.

In striatum, action of DA is terminated by diffusion and reuptake by the dopamine uptake transporter (DAT), which is fairly evenly distributed on the membrane surface of DA fibers rather than at release sites [15,16]. DAT is very efficient at removing DA from the extracellular milieu, as demonstrated in striatal slices prepared from DAT-deficient mice where stimulation-evoked DA peaks exhibit a 300-fold longer duration [17]; similar responses are measured following treatment with DAT antagonists, such as cocaine [18].

Midbrain DA neurons are tonically active and demonstrate autonomous pacemaking at ~4Hz frequency. In response to stimuli, which coincides with primary reinforcers such as food or environmental cues predicting reward, DA neurons respond with bursts of action potentials (*phasic* activity) in which the firing frequency reaches ~15Hz. Most striatal DA release sites are positioned near the neck of dendritic spines of the medium spiny neurons [19]. Phasic activity, in part by saturating DAT reuptake, provides DA levels sufficient to activate pre- and post-synaptic DA receptors on a broad range of cells, including medium spiny neurons, cholinergic and GABAergic interneurons and corticostriatal synapses, [20]; the majority of these receptors is found in perisynaptic zones distant from the DA release site.

Effects of L-DOPA on quantal size in DA neurons

Exogenous L-DOPA provided as a drug is accumulated by the L-amino acid transporter (LAAT) into neuronal cytosol [21]. As AADC is typically not saturated, L-DOPA is efficiently converted to DA. In cultured SN neurons and the PC12 dopaminergic cell line, L-DOPA treatment rapidly increases DA levels in the cytosol by >100-fold [22,23].

The effects of L-DOPA on dopaminergic neurotransmission was extensively studied in cultured cells using amperometric recordings to provide a direct measurement of DA molecules released during single synaptic vesicle fusion [24], a parameter known as “quantal size” [14]. In primary cultures of murine SNc neurons, L-DOPA produces a ~300% increase in quantal size (from 3,000 to 10,000 DA molecules) in only 30 minutes [25,26]. Similar increase in quantal size occurs in large dense cored vesicles in chromaffin cells [27] and PC12 cells [28,29].

The ability of L-DOPA to increase the loading of DA into synaptic vesicles in living animals was recently confirmed using a technique known as electrochemical cytometry, in which

synaptic vesicles are isolated from brain and the quantity of DA molecules is measured inside individual synaptic vesicles [30]. In healthy mice, L-DOPA (50 mg/kg injected intraperitoneally 2 hours prior to sacrifice) increases vesicular DA storage by 240% (from 30,000 to 71,000 molecules). Thus, even in intact DA axons, L-DOPA can be transported into cytosol and converted to DA that is accumulated into synaptic vesicles. A surprising consequence of L-DOPA-mediated increase in vesicular DA content first noted by Andrew Ewing and colleagues is that it is accompanied by an increase in the volume of secretory vesicles [29]. While this was initially controversial, as it was not clear that vesicle membrane would have sufficient elasticity, it has been confirmed by capacitance recordings in cultured cells [27] and optical measurements of vesicles isolated from animals treated with L-DOPA [31]; it is possible that the increased volume is due to the fusion of additional membrane with the vesicles.

How does L-DOPA normalize basal ganglia function in PD?

These findings beg a central issue in L-DOPA therapy: as striatal DA axons degenerate in PD and only a small number of DA release sites remain that can be refilled, how can L-DOPA correct basal ganglia function? The answer stems in large part from the properties of social neurotransmission.

The motor symptoms of PD appear when ~30% of the pigmented neurons of the SNc has degenerated, and striatal terminal density is decreased by 50-70% (reviewed by [32]). Similarly, in 6-OHDA lesion rat model of PD, motor function and striatal DA release is unchanged until >60% of the DAT immunoreactivity is lost and tissue DA levels are decreased by >80% [33,34]. Thus, the degenerating DA system is able to maintain sufficient DA transmission to preserve motor functions until relatively late stages of the disease. This remarkable property is mostly due to two adaptive changes in presynaptic function of the spared DA terminals. First, diminished extracellular DA and reduced activation of DA autoreceptors stimulates DA synthesis [35] which may enhance DA release from the remaining terminals. Second, the degeneration of DA axons also produce a comparable loss of DAT activity, which results in a larger volume of transmission, so that extrasynaptic DA receptors are activated further from release sites [20].

These effects of L-DOPA and DAT depletion on striatal DA neurotansmission can be roughly estimated with diffusion models. Calculations by Margaret Rice and Stephanie Cragg indicate that an increase in quantal size corresponds to a commensurate increase in the sphere of influence of transmitter response, and so the increased quantal size from L-DOPA alone would roughly increase the volume that a DA release event affects by 3 or 4-fold [36]. Consistently, Figure 2A shows a random walk simulation of the diffusional profile of DA following single vesicle release within the striatum [14,26]. An increase in quantal size from 3,000 to 10,000 molecules exerts proportional effect on the amplitude of extrasynaptic DA peak and increases the distance at which transmitter concentration is above 10 nM - the EC_{50} of the activation of D2 DA receptors - from 12 to 18 μm, which corresponds to a ~3.5-fold increase of the sphere of DA influence, from 7,200 μm^3 to 24,400 μm^3.

In the PD patient, however, DA overflow is greatly enhanced due to the decreased DAT levels. The maximal velocity of DAT in normal striatum in mice is ~ 5 μM/sec [37] and would decrease by 80% following degeneration of striatal DA terminals in symptomatic PD. A random walk simulation indicates that such diminished DAT activity dramatically increases both the amplitude and the duration of extrasynaptic DA (Figure 2B). In pre-symptomatic PD, this may account for the ability of the remaining more sparsely distributed DA terminals to "cover" larger areas of the striatum, compensating for the loss of a large portion of SNc axons. In later stages of PD, the levels of DA overflow following L-DOPA treatment exceed those in unperturbed striatum and the overall increase in the sphere of influence due to the combination of increased quantal size and decreased DAT levels may be 20-fold (to 137,300 μm^3).

The simulations above do not take into account the presence of other transport mechanisms, including SERT, OCT-3, PMAT and others [41-43] that may in part compensate for the loss of striatal DAT leading to DA accumulation in non-catecholaminergic cells. L-DOPA-mediated neurotransmission will moreover be affected differently in healthy and PD brains by the presence of D2-mediated auto-inhibition of DA terminals [44]. As detailed below, in later PD it appears that the majority of L-DOPA conversion to DA, vesicle uptake, and release are likely occurring in striatal serotonin (5-HT) axons. D2-receptor mediated regulation effectively inhibits DA release from intact DA axons, but not from 5-HT terminals that lack D2 receptors. Thus, D2 autoinhibition would limit the effect of L-DOPA in a normal striatum, but would have little effect in the DA-depleted brain (Figure 1).

Overall, these changes in the volume of neurotransmission may help explain why L-DOPA has minimal effect on striatal extracellular DA levels in normal animals but increases it dramatically in animals with severe DA lesion [33,38,39]. Similarly, L-DOPA has little or no effect in healthy people and is not abused as are other drugs that enhance extracellular DA in intact striatum, but is sometimes consumed compulsively by PD patients [40].

Neuronal stress due to increased cytosolic DA

There has long been a controversy on whether L-DOPA treatment may enhance neurodegeneration. Metabolic turnover of DA is tightly regulated by its synthesis, degradation and compartmentalization and high cytosolic DA levels following L-DOPA treatment have been shown to induce the formation of neuromelanin [47], a pigment derived from the oxidized metabolites of DA and localized to autophagic vacuoles [48]. L-DOPA also promotes alpha-synuclein-dependent neurodegeneration [22] and antigen presentation by SN neurons in culture [49]. We recently found that AADC activity is dramatically enhanced by Ca^{2+} [22], creating much higher cytosolic DA levels and oxidative stress in L-DOPA-treated neurons that rely on Ca^{2+} - driven pacemaking, *i.e.*, the SNc and LC neurons [50,51], which are compromised in PD. Additionally, non-catecholaminergic cells, including serotonergic and glutamatergic neurons, exposed to L-DOPA accumulate cytosolic DA and undergo degeneration as a result [22,52]. These studies, however, have been performed on cultured DA neurons with high L-DOPA exposures intended to greatly increase cytosolic DA, and there is no clear evidence at this time that similar neurotoxicity occurs with doses of L-DOPA used in patients.

More broadly, the selective vulnerability of neuromelanin-containing neurons and the ability of cytosolic DA to produce oxidative stress and protein damage have long suggested that a dysregulation of DA homeostasis may play a role in PD [37,53-58]. Following cleavage by MAO, DA generates the highly reactive compound DOPAL [59] the presence of which has been demonstrated both *in vitro* and *in vivo* [60,61]. The cytosolic catechols, DA, L-DOPA and DOPAC, can auto-oxidize and further react with cysteine residues of proteins and glutathione. 5-S-cysteine conjugated catechol derivatives are often used as markers of excess cytosolic DA and oxidative stress *in vivo* [62,63]. Increased loading of DA from cytosol to vesicles following overexpression of VMAT2 can provide neuroprotection from L-DOPA [22] and other interventions that increase cytosolic DA, such as exposure to MPTP or methamphetamine [58,64,65]. A recent report suggests that PD patients have low levels of VMAT2 on striatal DA neuron synaptic vesicles [57], although as these patients were almost certainly treated with L-DOPA, a decrease in VMAT expression could be a compensatory response rather than a cause of PD.

L-DOPA-induced dyskinesia (LIDs)

By the 5th year of L-DOPA treatment, more than 50% of patients develop debilitating motor side-effects, collectively defined here as L-DOPA induced dyskinesia (LID) [66,67]. Several forms of LID have been characterized. “Peak-dose” LID is associated with high plasma L-DOPA concentrations and is expressed as involuntary spasmodic twitching or jerking in muscle (choreic movements). “Diphasic on/off” dyskinesias coincide with rising and decreasing plasma concentrations of L-DOPA and may include both chorea and dystonia (uncontrollable repetitive or twisting movements). “Off” dyskinesia presents during unmedicated states with low L-DOPA and is characterized by an often painful dystonic posture. There is a gradual increase in incidence and severity of LIDs during prolonged therapy, and by the 10th year of L-DOPA medication, LID is experienced by ~ 80% of the patients [68]. The risk factors for LID include the age of PD onset, duration of L-DOPA treatment and dose, indicating that both the progressive loss of DA neurons and L-DOPA exposure play roles in the establishment of LID [69-71]. Consistently, several studies report decreased LIDs with continuous rather than intermittent exposure to L-DOPA (reviewed in [72]), and a recent clinical study reported that continuous delivery of L-DOPA using an intestinal gel increased on-time without dyskinesia more than immediate-release oral L-DOPA [73].

Several animal models of “peak-dose” LID have been developed based on lesion of SNc DA neurons by MPTP or 6-OHDA followed by repeated administration of L-DOPA or synthetic DA receptor agonists [74]. These studies suggest a wide spectrum of metabolic and neuroadaptive changes at multiple synaptic connections of the basal ganglia [75,76]. Hypotheses on the cause of LID can be divided into two non-exclusive categories [69]. *Postsynaptic hypotheses* ascribe LID to the changes in DA receptor sensitivity in denervated striatum, which may occur in virtually all striatal neurons. These alterations entail a dysregulation of signaling cascades activated by DA receptors, and consequent effects on gene transcription, protein translation, and post-translational modifications in striatal neurons that are beyond the scope of this review [see [77] for a recent review]. A new study by Paul Greengard and colleagues demonstrates changes in several hundred genes in the

striatum following DA depletion and L-DOPA reinstatement creating an extraordinarily high number of potential molecular candidates [78].

Presynaptic hypotheses of LID are directly relevant to the effects of L-DOPA on DA neurotransmission, as they attempt to explain LID in terms of dysregulated presynaptic control of vesicular loading, uncontrolled release and decreased reuptake of DA [69,79]. A presynaptic cause of LIDs is supported by imaging studies in PD that demonstrate a positive correlation between the ability of L-DOPA to increase synaptic DA levels and the severity of "peak-dose" LID [80-82]. Similarly, L-DOPA-induced increase in striatal extracellular DA in animals with severe DA lesion is higher than non-lesioned animals [33,38,39], and still higher in lesioned animals with LID [83,84]. Because L-DOPA mediated DA efflux is particularly prominent in advanced PD patients and animals with severe DA lesions, it has been suggested that LID is linked to an inability of the striatum to maintain presynaptic control of DA released following L-DOPA administration.

Strong evidence supporting a presynaptic locus for LID comes from a rat model where severe striatal DA deficit was produced by silencing TH using short-hairpin RNA (shRNA), resulting in a reduction of DA levels comparable to a 6-OHDA lesion [85]. Repeated L-DOPA did not produce LID in these animals, apparently because the DA terminals and synaptic vesicles were preserved and DA produced by L-DOPA could be released by normal SN neuronal activity. We note, however, that patients carrying genetic TH deficiency still develop severe LIDs in response to L-DOPA [86].

DA release from striatal 5-HT terminals

In advanced PD, most of the L-DOPA conversion to DA, vesicular uptake, and release appear to occur within striatal 5-HT axons, which are low in number compared to DA innervation, but survive longer in the disease [87], and their density may be substantially increased due to sprouting in advanced stages of PD [88]. The initial evidence that L-DOPA can be decarboxylated to DA in 5-HT neurons was obtained with the Falck-Hillarp histochemistry technique, which provides visualization of catecholaminergic neurotransmitters [89]; administration of L-DOPA to rats increased the fluorescent signal in both DA and 5-HT neurons. Consistently, in lesioned mice, L-DOPA's increase in extracellular DA levels is substantially reduced following destruction of 5-HT neurons with the neurotoxin 5,7 dihyroxytryptamine [90-92], confirming 5-HT neurons as a source of L-DOPA-mediated DA overflow. DA efflux in 6-OHDA lesioned animals treated with L-DOPA is also abolished by the sodium channel blocker tetrodotoxin, which inhibits neuronal activity, or the VMAT inhibitor reserpine [38,93] which indicates that DA released by 5-HT terminals requires neuronal activity and accumulation of DA into monoamine synaptic vesicles. Conversely, in DA lesioned striatum, inhibition of DAT or activation of D2 receptors has no effect on L-DOPA-mediated DA release [38,94], consistent with the loss of presynaptic regulatory control of DA neurotransmission.

Serotonergic neurons use AADC for the biosynthesis of 5-HT [90,91,95,96] [see however [97]]. Both 5-HT and DA neurons use VMAT2 for the accumulation of neurotransmitters into synaptic vesicles (Figure 1), enabling vesicle loading and stimulation-dependent release of

DA from 5-HT axons [98,99]. Furthermore, high doses of L-DOPA reduce brain 5-HT levels in mice, confirming that DA displaces endogenous 5-HT from synaptic vesicles [89,100]. Thus, L-DOPA therapy may cause changes in 5-HT transmission [101], not only in striatum but also in SN pars reticulata, prefrontal cortex and the hippocampus [102], as well as in histamine neurons [103].

A role for DA release from 5-HT neurons in causing “peak-dose” LID has been suggested because neurotransmission would be dependent on the availability of exogenous L-DOPA rather than DA neuronal activity [96,104]. In support of this, lesion of the neurons with 5,7 dihyroxytryptamine virtually abolished LID in 6-OHDA lesioned rats, while still providing effective L-DOPA treatment [105,106]. Agonists of inhibitory 5-HT1A and 5-HT1B auto-receptors on 5-HT terminals and cell bodies moreover decreased L-DOPA mediated increase in DA efflux, and inhibited LID in rodents [84,99,105,107,108]. It is possible that such agonists may provide future therapy.

DA release from systemically administered L-DOPA does not occur exclusively in the striatum, and the brain regions that receive serotonergic innervation, including prefrontal cortex, hippocampus and SNr, display increased extrasynaptic DA following L-DOPA treatment in both healthy and DA lesioned brains [45]. It is possible that extrastriatal DA release or the action of L-DOPA itself [46] contribute to the development and manifestation of LIDs.

DA derived from L-DOPA may also be released from other cells, as behavioral effects and DA efflux following very high doses of L-DOPA (100 mg/kg) in 6-OHDA lesioned animals were not affected by 5-HT lesions [92,97]. A possible source for L-DOPA induced DA release may include histamine neurons as well as striatal interneurons and glia cells that may have AADC expression [97,109-111], although this is controversial [112,113]. Thus, multiple neuronal and non-neuronal cells may be candidates for DA release following L-DOPA, particularly under conditions where cytosolic DA levels are so high that means of release occur that are independent of VMAT expression.

Concluding remarks

L-DOPA in early stages of PD provides an elegant therapy, as the “social” nature of DA transmission and decreased DAT-mediated uptake allow fewer DA release sites to engage distant receptors and provide replacement of normal DA signaling. In the later stages of PD, however, DA denervation with subsequent L-DOPA treatment leads to unregulated DA release that produces larger and a longer-lasting diffusional cloud of the transmitter.

Understanding more precisely how L-DOPA reinstatement leads to over activity of basal ganglia circuitry is of central importance in developing PD therapies that avoid LID. If the loss of controlled DA release in more advanced PD genuinely causes LID, elucidating the steps involved will be important even if replacement of DA by transplantation becomes a standard treatment, as these cells will also not participate in normal circuitry to drive their activity. It seems most likely, however, that a relatively complex combination of presynaptic and postsynaptic alterations are involved, in which case there may be multiple possible therapeutic avenues that could interrupt the circuits that lead to LID.

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Abbreviations

AADC	aromatic L-amino acid decarboxylase
AD	aldehyde dehydrogenase
DA	dopamine
DAcyt	cytosolic DA
DAT	DA uptake transporter
DAR	DA receptors
D2	DA autoreceptor 2
DA-Cys	5-S-cysteinyl-DA
DA-Q	DA-o-quinone
DOPAC	3,4-dihydroxyphenylacetate
DOPAL	3,4-dihydroxyphenylacetaldehyde
5-HIAA	5-hydroxyindole acetic acid
5-HIAAL	5-hydroxyindole acetaldehyde
5-HT	5-hydroxytryptophan (serotonin)
5-HT1A	serotonin autoreceptor 1A
5-HTR	serotonin receptors
LAAT	L-amino acid transporter
LC	locus coeruleus
L-DOPA	L-3,4-dihydroxyphenylalanine (levodopa)
MAO	monoamine oxidase
MPP^+	1-methyl-4-phenylpyridinium
MSN	medium spiny neurons
NM	neuromelanin
PD	Parkinson’s disease
SNc	substantia nigra pars compacta
ROS	reactive oxygen species
SERT	serotonin uptake transporter
TH	tyrosine hydroxylase
TPH2	tryptophan hydroxylase 2
Trp	tryptophan

VTA	ventral tegmental area
VMAT	vesicular monoamine transporter

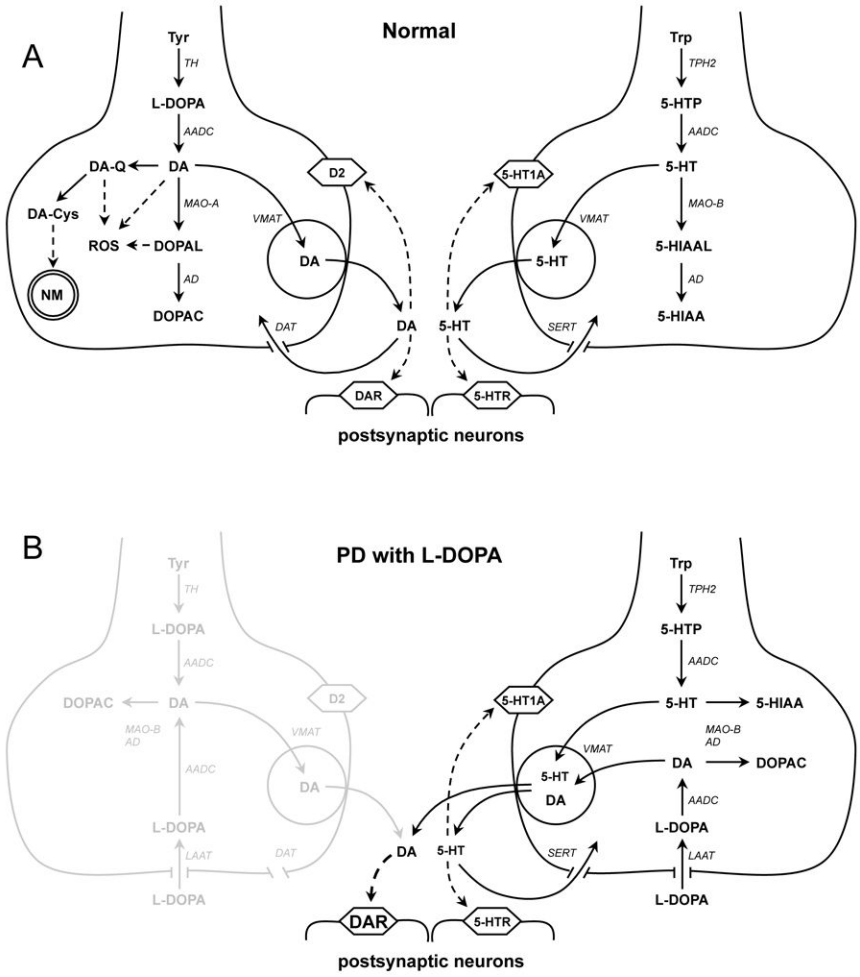

Figure 1.

DA and 5-HT homeostasis in normal conditions (A) and Parkinson's disease (B). (A) Schematics of DA (left) and 5-HT (right) synthesis, degradation and neurotransmission. There are substantial similarities between DA and 5-HT metabolism, including the presence of AADC, MAO and AD in the cytosol and VMAT2 on the synaptic vesicle membrane. Note that some metabolic pathways, such as neuromelanin synthesis, are more prominent in the cell bodies than in the terminals of DA neurons. (B) Exogenous L-DOPA can be taken up by the L-amino acid transporter (LAAT) and metabolized to DA in both types of neurons. In a healthy brain, very high levels of L-DOPA are required to induce dyskinesia [114]. In earlier stages of PD, DA release from surviving dopaminergic terminals is augmented by exogenous L-DOPA, which provides the therapeutic benefits of the drug, while decreased DAT activity provides a larger sphere of influence. It may be that at high doses, the system is flooded with DA released from 5-HT neurons, triggering dyskinesias. A dose of L-DOPA that gives symptomatic relief without causing motor side effects provides a "therapeutic window" of DA replacement therapy. In later stages of PD, severe loss of dopaminergic terminals reduces the therapeutic window and striatal DA levels become less dependent on the activity of DA neurons and more on the availability of exogenous L-DOPA.

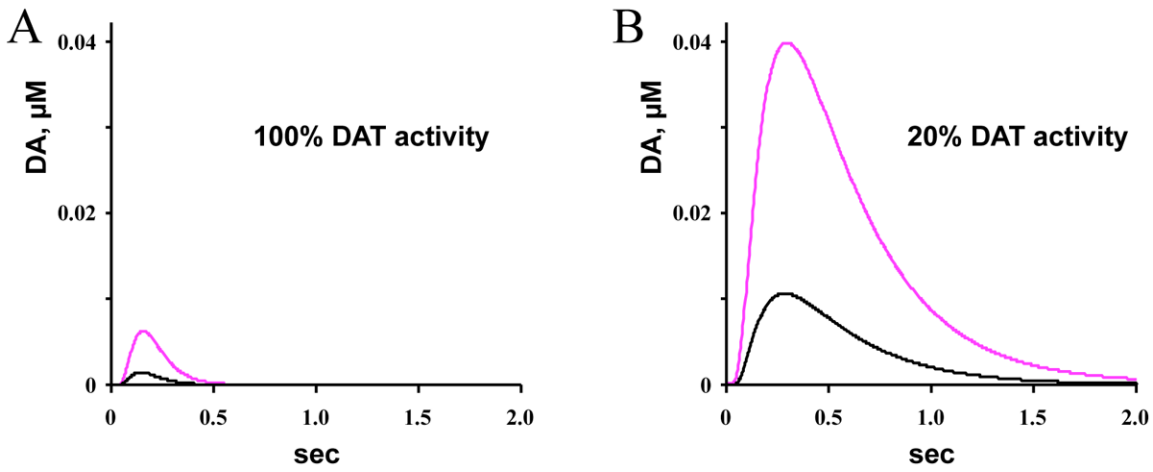

Figure 2.

Random walk simulations of DA overflow from a quantal release event under normal (5 μM/sec) and PD-like (1 μM/sec) levels of DAT activity. The traces represent diffusional profiles of DA concentrations 20 μm from the presynaptic release site following transmitter exocytosis from a synaptic vesicle containing 3,000 (black) or 10,000 molecules (magenta), corresponding to L-DOPA's increase of quantal size in cultured midbrain neurons [25,26]. The "spheres of influence", *i.e.*, the volume around the release sites where DA concentrations are > 10 nM, were as follows. **A:** 3,000 molecules, r = 12 μm, 7,200 μm^3 (100 %); 10,000 molecules, r = 18 μm, 24,400 μm^3 (338 %). **B:** 3,000 molecules, r = 21 μm, 38,800 μm^3 (536 %); 10,000 molecules, r = 32 μm, 137,300 μm^3 (1896 %). Tutorials on this random walk analysis and spreadsheets with the simulation can be downloaded from http://sulzerlab.org/download.html.