Activity-Dependent Vesicular Monoamine Transporter-Mediated Depletion of the Nucleus Supports Somatic Release by Serotonin Neurons

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Packaging by the vesicular monoamine transporter (VMAT) is essential for mood-controlling serotonin transmission but has not been assayed during activity. Here, two-photon imaging of the fluorescent serotonin analog 5,7-dihydroxytryptamine and three-photon imaging of endogenous serotonin were used to study vesicular packaging as it supports release from the soma of serotonin neurons. Glutamate receptor activation in dorsal raphe brain slice evoked somatic release that was mediated solely by vesicle exocytosis. This release was accompanied by VMAT-mediated serotonin depletion from the nucleus, a large compartment free of monoaminergic degradation pathways that has not been implicated in neurotransmission previously. Finally, while some monoamine packaged at rest was held in reserve, monoamine packaged during stimulation was released completely. Hence, somatic vesicles loaded by VMAT during activity rapidly undergo exocytosis. In the absence of active zones and with limited neurotransmitter reuptake, somatic release by serotonin neurons is supported by recruitment from a large pool of extravesicular serotonin in the nucleus and cytoplasm, and preferential release of the newly packaged transmitter.

Introduction

Dorsal raphe nucleus (DR) serotonin neurons project widely throughout the forebrain to control mood and behavior. In addition to synaptic release from nerve terminals, DR neurons also release serotonin (5-hydroxytryptamine) extrasynaptically from somatodendritic compartments in a paracrine manner to regulate activity (Bunin and Wightman, 1998; Adell et al., 2002; De-Miguel and Trueta, 2005). Similar to synaptic release, somatodendritic release in serotonin neurons is mediated by calcium-induced vesicle exocytosis (de Kock et al., 2006), suggesting a critical role of the transport of serotonin by the vesicular monoamine transporter (VMAT). In fact, VMAT alterations in the DR have been implicated in the pathology and pharmacotherapy of psychiatric disorders (Cordeiro et al., 2002; Schwartz et al., 2003). However, VMAT-dependent packaging during release has not been assayed directly *in vivo*.

In principle, optical assays of neurotransmitter dynamics could be used to study activity-dependent monoamine packaging and release in DR serotonin neurons. 5,7-Dihydroxytryptamine (dHT) is a pH-sensitive, fluorescent serotonin analog (Schlossberger, 1978; Vaney, 1986; Kim et al., 2000) that accumulates in serotonin neurons through the serotonin transporter (SERT) (Björklund et al., 1974) and can be detected in serotonin neuron vesicles (Gershon and Sherman, 1982). Prolonged exposure to high concentrations of dHT is toxic, but this effect is inhibited by

preventing its chemical and enzymatic oxidation (Björklund et al., 1975; Silva et al., 1988). Indeed, acute dHT uptake identifies viable monoaminergic neurons without altering neuronal morphology or electrical properties (Silva et al., 1988; Hahn et al., 2003, 2006). Endogenous serotonin also has innate fluorescence that has been detected with three-photon microscopy in the cell bodies of DR serotonin neurons in the brain slice (Kaushalya et al., 2008a,b). Furthermore, depolarization of these neurons leads to a decrease in serotonin fluorescence consistent with stimulation-dependent release (Kaushalya et al., 2008b). Therefore, the fluorescence of dHT and serotonin led us to explore whether multiphoton microscopy could be used to study VMAT and somatic release.

Here, two-photon imaging, photobleaching, and vesicular pH collapse experiments reveal activity-dependent vesicular transport of dHT from the nucleus into somatic vesicles of DR serotonin neurons. The releasable and reserve vesicular pools are quantified and the acute contribution of stimulated VMAT activity to somatic release is established. Finally, three-photon imaging of serotonin is used to corroborate these findings with the native transmitter.

Materials and Methods

PC12 cell experiments

PC12 cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. For imaging, cells were seeded on poly-D-lysine-coated coverslips (#1.5, VWR International) and transfected via Tfx50 (Promega) with SERT and/or eGFP. When indicated, PC12 cells were differentiated by application of 50 ng/ml 2.5 S NGF for 36 h. Five days after transfection, to allow for expression and trafficking of SERT, cells were incubated with 20 μ M dHT creatine sulfate (Regis Technologies), and inhibitors of monoamine oxidation (568 μ M ascorbate and 100 μ M pargyline) in culture medium for 1 h or overnight. Overnight loading

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was followed by a 1 h wash in culture medium at 37°C to allow cytoplasmic dHT to clear. Cells stably expressing ANF-emerald GFP (Han et al., 1999b) were transfected with SERT, differentiated, and loaded with dHT as described above. When cells were loaded in the presence of fluoxetine (10 μ M) or reserpine (100 nM), drug was added along with dHT. For imaging, cells were bathed in normal saline (containing, in mM: 5.4 KCl, 140 NaCl, 2 CaCl₂, 0.8 MgCl₂, 10 mM Na-HEPES, 10 mM glucose, pH 7.4) and excited by epifluorescence or two-photon microscopy as described. For pH collapse experiments, monensin (1 μ M) was bath applied during imaging. PC12 cells were stimulated by exchange of normal saline for high K⁺ saline (containing, in mM: 100 KCl, 45 NaCl, 5 BaCl₂, 0.8 MgCl₂, 10 Na-HEPES, 10 glucose, pH 7.4).

Slice experiments

All experiments were conducted in accordance with protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague Dawley rats p14–p21 (Hilltop Labs) were anesthetized with isoflurane and decapitated. Brains were removed and bathed in 95% O₂ and 5% CO₂-saturated, ice-cold, sucrose-based artificial CSF (s-aCSF) (containing, in mM: 87 NaCl, 75 sucrose, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 0.5 CaCl₂, 7.0 MgSO₄, 25 glucose, 0.15 ascorbic acid, 1 kynurenic acid, pH 7.4). Coronal brain slices (250 μ m) containing the DR were cut with a vibratome (The Vibratome Company). Slices loaded with dHT were incubated in 20 μ M dHT, 568 μ M ascorbate, and 100 μ M pargyline in s-aCSF for 3 h at 37°C followed by a brief wash in normal aCSF (containing, in mM: 124 NaCl, 4 KCl, 25.7 NaHCO₃, 1.25 NaH₂PO₄, 2.45 CaCl₂, 1.2 MgSO₄, 11 glucose, 0.15 ascorbic acid, pH 7.4) before imaging. For slices loaded in the presence of fluoxetine (10 μ M) or reserpine (500 nM), drugs were applied 15 min before addition of dHT. Three-photon imaging of endogenous serotonin was done on unloaded slices in normal aCSF after 1 h at 37°C in sucrose aCSF. Stimulation of slices was induced by a bath exchange with aCSF supplemented with 10 μ M AMPA. For stimulation with AMPA in zero calcium, CaCl₂ in normal aCSF was replaced with MgSO₄ and 1 mM EGTA was added. When indicated, nuclei were stained by a 30–45 min incubation of loaded slices with 1.6 mM Hoechst 33342 at the end of the experiment (i.e., after AMPA stimulation). For experiments requiring acute inhibition of VMAT, reserpine (500 nM) was added to slices 5 min before stimulation with AMPA. Finally, for pH collapse experiments, 50 mM NH₄Cl replaced 50 mM NaCl in normal aCSF. NH₄⁺ aCSF was applied to slices at rest or after AMPA stimulation as indicated.

Optical setups

Wide-field epifluorescence experiments were done on an Olympus IX71 inverted microscope equipped with a 340UV 40 $\times$ 1.35 numerical aperture (NA) oil-immersion objective and a xenon arc lamp. dHT epifluorescence used a 360/40 excitation filter and 420 nm long-pass emission filter, which was collected by a cooled CCD camera (Hamamatsu Orca ER). Multiphoton imaging experiments were done on an Olympus Fluoview FV1000 upright confocal scanning microscope. Excitation illumination from a Coherent chameleon ultra titanium sapphire laser was attenuated with an acoustical optical modulator and expanded with a motorized telescope (LSM Technologies) before being focused by a 60 $\times$, 1.1 NA water-immersion objective. All optical measurements were performed at room temperature. For dHT imaging, excitation with 725 nm light induced fluorescence that was collected by a 400–480 nm bandpass filter (Chroma Technology) and quantified with a non-descanned detector (LSM Technologies), containing a cooled Hamamatsu photomultiplier tube. Stacks of consecutive, 2.5 μ m spaced images were taken through dHT loaded neurons of the DR. Endogenous serotonin could not be detected using the optical parameters for dHT imaging. For photobleaching studies, a small region of a proximal dendrite was repetitively scanned in a single optical section at normal imaging laser intensity to achieve $\sim$ 18% bleach. For three-photon imaging of serotonin, slices were excited with 740 nm light at $\sim$ 1.67 times the amount of power used with dHT imaging. Emission light was collected through a 2 mm BGG22 filter (Chroma Technology). Optical sections through stacks were taken at 1 μ m intervals.

Image analysis

Analysis was done with ImageJ (NIH) software. Images were contrast enhanced or pseudo-colored to aid figure presentation without altering the primary data used for quantification. When necessary, series of images were aligned with the ImageJ plug-ins StackReg or Align 3TP. Mean fluorescence intensity was measured in regions of interest (ROIs) in single images or summed z-projections of image stacks and subtracted by background values (i.e., unloaded cells or tissue background fluorescence). Percentage change in fluorescence ($\Delta F(\%) = (1 - (F/F_0)) \times 100$) was normalized to control experiments to allow for comparisons across experimental manipulations. n represents the number of cells from which data were collected. All data were compiled from at least three independent experiments. Error bars represent SEM. Statistical tests are as indicated in text or figure legends.

Arithmetic analysis

v is defined as the fraction of dHT in vesicles and c as the fraction of extravascular dHT. The subscript “0” defines the variable in resting cells (i.e., before experimental manipulation). In all cases c and v are expressed in terms of fraction of total content at rest. Hence, $v_0 + c_0 = 1$. The fractional fluorescence of extravascular dHT is denoted as F_c and equals the fraction in extravascular content “ c ”. The fractional fluorescence of vesicular dHT is denoted as F_v and equals $0.505v$ due to the pH-dependent quenching of dHT fluorescence in acidic vesicles. Hence, total somatic fluorescence can be written in terms of the fractional dHT content in the vesicular and extravascular pools ($F_0 = F_{c_0} + F_{v_0} = c_0 + 0.505v_0$).

Equation 1. Photobleaching of dHT fluorescence in a small dendritic region led to a fractional loss of fluorescence in the summed soma. This measurement (S) can be written as the fractional change of the sum of the vesicular and extravascular fluorescence due to photobleaching. ($S = (F_{c_B} + F_{v_B})/(F_{c_0} + F_{v_0})$). The fluorescence of the extravascular content after photobleaching (F_{c_B}) is equal to the fractional fluorescence loss measured in the nucleus after photobleaching (B) times the original content (c_0) ($F_{c_B} = B \times c_0$). The fluorescence of the somatic vesicular content after photobleaching (F_{v_B}) should not be affected by photobleaching in the dendrite and is therefore equal to the vesicular fluorescence before bleaching ($F_{v_B} = F_{v_0} = 0.505v_0$).

$$S = (F_{c_B} + F_{v_B})/(F_{c_0} + F_{v_0}),$$

$$S = (B \times c_0 + 0.505v_0)/(c_0 + 0.505v_0); \text{ substituting } (1 - v_0) \text{ for } c_0,$$

$$S = (B \times (1 - v_0) + 0.505v_0)/(1 - v_0 + 0.505v_0). \quad (1)$$

Paired measurements of B and S were used to solve for v_0 in each experiment. The average vesicular content equals 26%.

Equation 2. The fraction of dHT in vesicles is calculated based on the measured fractional fluorescence change of total dHT, T , in loaded slices after exposure to ammonium chloride (at rest, $T = F_{\text{NH}_4}/F = 1.28$ [total]; after AMPA, $T = 1.18$ [total]) and the ammonia-induced fractional increase of extravascular dHT, E , measured in slices loaded in the presence of reserpine (at rest, $E = F_{\text{NH}_4\text{Res}}/F_{\text{Res}} = 1.15$ [extravascular]; after AMPA, $E = 1.12$ [extravascular]). The fluorescence of dHT in vesicles after ammonia equals the vesicular dHT content as its fluorescence is unquenched ($F_{v_{\text{NH}_4}} = v$).

Therefore,

$$\begin{aligned} T &= (F_{c_{\text{NH}_4}} + F_{v_{\text{NH}_4}})/(F_c + F_v), \\ &= (E \times F_c + v)/(F_c + F_v), \\ &= (E \times c + v)/(c + 0.505v); \text{ substituting } c = 1 - v, \\ &= (E \times (1 - v) + v)/(1 - v + 0.505v). \end{aligned} \quad (2)$$

Hence, the fraction in vesicles at the time of measurement (v) is:

$$v = 0.26 \text{ at rest and } 0.12 \text{ after AMPA.}$$

Equation 3. Ammonium chloride experiments done after AMPA stimulation demonstrated that 12% of the somatic content after stimula-

tion is in vesicles. To express the vesicular content after stimulation in terms of fraction of total content at rest, the following calculation is performed.

$$\begin{aligned}v_{\text{AMPA}} &= 0.12 (c_{\text{AMPA}} + v_{\text{AMPA}}); c_{\text{AMPA}} = 0.65. \\v_{\text{AMPA}} &= 0.12 (0.65 + v_{\text{AMPA}}). \\v_{\text{AMPA}} &= 0.089 = 9\%.\end{aligned}\quad (3)$$

Equation 4. A prediction of the AMPA-induced change in fluorescence can be made from measurements of vesicular and extravesicular content before and after stimulation. This prediction can be compared with actual AMPA-induced fluorescence changes measured independently ($18 \pm 2.4\%$).

$$\begin{aligned}\Delta F_{\text{pred}} (\%) &= (1 - (F_{\text{AMPA}}/F_0)) * 100, \\&= (1 - (F_{c_{\text{AMPA}}} + F_{v_{\text{AMPA}}})/(F_{c_0} + F_{v_0})) * 100, \\&= (1 - (c_{\text{AMPA}} + 0.505v_{\text{AMPA}})/(c_0 + 0.505v_0)) * 100, \\&= (1 - (0.65 + 0.505(0.089))/(0.74 + 0.505(0.26))) * 100, \\&= 20\%.\end{aligned}\quad (4)$$

Equation 5. Predictions of the change in fluorescence while acutely inhibiting VMAT during stimulation can be made for each model. Without AMPA-induced packaging of extravesicular dHT, the extravesicular pool is not depleted. Hence, $c_{\text{AMPA}} = c_0 = 0.74$, $v_0 = 0.26$.

$$\begin{aligned}\Delta F_{\text{pred}} (\%) &= (1 - (F_{\text{AMPA}}/F_0)) * 100, \\&= (1 - (F_{c_{\text{AMPA}}} + F_{v_{\text{AMPA}}})/(F_{c_0} + F_{v_0})) * 100, \\&= (1 - (c_0 + 0.505v_{\text{AMPA}})/(c_0 + 0.505v_0)) * 100, \\&= (1 - (0.74 + 0.505v_{\text{AMPA}})/(0.74 + 0.505 * (0.26))) * 100.\end{aligned}\quad (5)$$

In model i, all preloaded vesicular transmitter is released and then vesicles are refilled by VMAT. With acute VMAT inhibition, vesicles cannot be refilled. Therefore, no transmitter remains in vesicles after stimulation ($v_{\text{AMPA}} = 0.0$).

$$\begin{aligned}\Delta F_{\text{pred.i}} (\%) &= (1 - (0.74 + 0.505v_{\text{AMPA}})/(0.74 + 0.505v_0)) * 100, \\&= (1 - (0.74 + 0.505 * (0.0)) \\&\quad / (0.74 + 0.505 * (0.26))) * 100, \\&= 15\%.\end{aligned}$$

In model ii only a portion of preloaded transmitter is released, while the rest is held in reserve and accounts for the vesicular content after AMPA stimulation. Because VMAT activity during the stimulus does not contribute to the amount remaining in vesicles after stimulation in this model, acute reserpine does not alter v_{AMPA} . Hence, $v_{\text{AMPA}} = 0.09$.

$$\begin{aligned}\Delta F_{\text{pred.ii}} (\%) &= (1 - (0.74 + 0.505 * v_{\text{AMPA}})/(0.74 + 0.505v_0)) * 100, \\&= (1 - (0.74 + 0.505 * (0.09)) \\&\quad / (0.74 + 0.505 * (0.26))) * 100, \\&= 9.9\%.\end{aligned}$$

Results

Two-photon excitation and pH sensitivity of dHT

The additional hydroxyl group of the serotonin analog dHT (Fig. 1A) shifts its fluorescence emission into the visible range and renders broad pH dependence to its absorption (Schlossberger, 1978; Silva et al., 1988). Therefore, to test whether dHT fluorescence is affected by the acidity found inside secretory vesicles,

Figure 1. Two-photon characteristics of dHT. **A**, Structures of serotonin (5-HT) and dHT. **B**, Empirical two-photon excitation spectrum of dHT. Background-subtracted collected fluorescence of a 5 mM dHT solution was excited at indicated wavelengths. This empirical plot does not correct for the increase in laser output associated with increasing wavelength. **C**, Two-photon absorption of dHT. Dorsal raphe slices loaded with dHT (see Materials and Methods) were excited with increasing levels of power measured at the specimen. Symbols represent collected data and lines represent linear regression from individual cells ($n = 6$). Mean slope = 2.0 ± 0.01 .

images of 500 μM dHT solutions buffered to pH 5.5 (the pH inside secretory vesicles) and pH 7.4 were collected. Fluorescence was 1.98-fold greater at pH 7.4 than at pH 5.5. Thus, unlike serotonin (Maiti et al., 1997), dHT fluorescence should be affected markedly by vesicular pH, a property that can be used to distinguish between vesicular and extravesicular monoamine independently of optical resolution of vesicles.

dHT fluorescence can be evoked by two-photon excitation. A solution of dHT in ascorbate was exposed to increasing wavelengths of infrared light and emitted fluorescence was collected as described in the Materials and Methods. Mean dHT fluorescence, after subtraction of the ascorbate signal, was maximal around 720 nm (Fig. 1B), which is twice the wavelength for maximal single-photon excitation (Vaney, 1986). Furthermore, fluorescence excited at 725 nm was proportional to the square of the excitation power (i.e., the slope of a log (fluorescence) versus log (power) plot equals 2.0 ± 0.01) (Fig. 1C), a property of two-photon excitation.

SERT-mediated uptake, VMAT-mediated packaging and activity-dependent release of dHT

To demonstrate specific uptake of dHT, PC12 cells transfected with SERT and GFP were incubated with 20 μM dHT. Cells expressing SERT, but not a control vector, accumulated dHT within an hour (Fig. 2A). This accumulation was inhibited by preincubation of cells with the specific SERT inhibitor fluoxetine.

Figure 2. SERT-mediated uptake and VMAT-mediated loading of dHT into release competent secretory vesicles. **A**, Two-photon dHT fluorescence images of representative undifferentiated PC12 cells transfected with GFP and either control (Con) or SERT DNA, and incubated for 1 h with 20 μ M dHT or a combination of dHT and 10 μ M fluoxetine. Scale bar, 10 μ m. GFP fluorescence was used to mark transfected cells. **B**, Representative two-photon images of NGF-differentiated PC12 cells loaded with control solution (Con) or dHT for 1 h or overnight (o/n) as indicated. o/n loading was followed by a 1 h wash. Scale bar, 10 μ m. **C**, Representative images of differentiated, dHT-loaded PC12 cells before (Con) and 5 min after application of 1 μ M monensin. Monensin induced a doubling of dHT fluorescence in growth cones. Scale bar, 10 μ m. **D**, Representative images of differentiated PC12 cells loaded o/n with dHT (Con) or 100 nM reserpine and dHT. Reserpine prevented the localization of dHT fluorescence to growth cones. Scale bar, 10 μ m. **E**, Colocalization in growth cones of epifluorescence from dHT and the neuropeptide secretory granule cargo ANF-GFP. PC12 cells stably expressing ANF-GFP were transfected with SERT and then loaded with dHT o/n. Scale bar, 20 μ m. **F**, K^+ -induced depolarization (STIM) induces parallel decreases in dHT and ANF-GFP signals from growth cones ($n = 6$).

Thus, two-photon microscopy detected SERT-mediated dHT uptake into cells.

Several experiments demonstrated that dHT is packaged by VMAT into secretory vesicles and released upon stimulation. First, SERT-transfected, NGF-differentiated PC12 cells were incubated overnight in dHT, washed for 1 h and then imaged (Fig. 2*B*). In contrast to the distributed fluorescence pattern produced with the 1 h incubation, this longer protocol resulted in fluorescence localized to growth cones, the location of secretory vesicles in differentiated PC12 cells. Second, application of 1 μ M monensin, a cation ionophore that neutralizes the acidic pH in PC12 cell secretory vesicles (Han et al., 1999a), doubled the fluorescence in the growth cone (Fig. 2*C*; control = 1.0 ± 0.02 , monensin = 2.0 ± 0.14 , $n = 4$; $p < 0.001$, t test). The sensitivity to a collapse of the vesicular pH gradient, along with the pH-sensitive fluorescence of dHT, is consistent with efficient packaging of dHT in PC12 cell secretory vesicles. Third, incubation of SERT-transfected cells with 100 nM reserpine, a specific VMAT inhibitor, before and during the dHT incubation prevented the preferential accumulation of fluorescence in growth cones (Fig. 2*D*). This was quantified by comparing the ratio of dHT fluorescence in growth cones to cell bodies ($F_{GC}:F_{CB}$) when loaded normally or in re-

serpine (control $F_{GC}:F_{CB} = 4.0 \pm 0.50$, reserpine $F_{GC}:F_{CB} = 0.3 \pm 0.07$, $n \geq 3$, $p = 0.0015$, t test). Finally, induction of exocytosis by K^+ -induced depolarization for 10 min evoked a $50 \pm 3\%$ loss of dHT fluorescence ($n = 5$, $p < 0.0001$, t test). In PC12 cells, VMAT-containing secretory vesicles also contain neuropeptides. Therefore, to confirm that this response is due to vesicular release, PC12 cells that express a GFP-tagged neuropeptide, atrial natriuretic peptide (ANF-GFP) (Burke et al., 1997; Han et al., 1999b) were transfected with SERT and then loaded with dHT overnight. The fluorescence signals from dHT and the neuropeptide colocalized in growth cones (Fig. 2*E*). Furthermore, depolarization-induced decreases in both dHT and ANF-GFP fluorescence signals that were similar in magnitude and kinetics (Fig. 2*F*), demonstrating that dHT fluorescence loss paralleled release of secretory vesicle content. Thus, multiple independent criteria show that dHT is taken up by SERT and packaged by VMAT into acidic, release competent secretory vesicles.

Somatic distribution of monoamine in raphe neurons

Having established vesicular packaging and release of dHT in a cell line, monoamine dynamics were explored in neurons of the DR. Coronal, DR-containing brain slices were incubated in dHT for 3 h before imaging. This duration was chosen because previous studies in neurons indicated that dHT is detected in synaptic vesicles within 2–4 h (Gershon and Sherman, 1982). Furthermore, endogenous serotonin in acute brain slices decreases markedly over hours (Liu et al., 2005), reducing potential competition for the vesicular transporter by native transmitter. Stacks of two-photon images showed that dHT fluorescence accumulated in neuronal cell bodies and processes of the DR with morphology expected for serotonin neurons (Fig. 3*A*) (Descarries et al., 1982). However, fluorescence was never seen in unloaded slices or slices incubated in the SERT inhibitor fluoxetine before and during dHT loading (Fig. 3*A*). Moreover, dHT-labeled neurons contained serotonin, which was detected by three-photon imaging of endogenous transmitter (data not shown). Hence, dHT accumulates through SERT into serotonin neurons in the slice.

Unlike the strong localization of dHT fluorescence to growth cones in PC12 cells, the resting fluorescence of somatic dHT in DR neurons was not punctate. Moreover, examination of single optical sections of image stacks revealed that dHT was present throughout the neuron, including regions that must be within the nucleus. As nuclear neurotransmitter had not been previously described, the presence of monoamine within the nucleus was further investigated. First, nuclei in dHT-loaded slices were marked with the nuclear stain Hoechst 33342 to explicitly demonstrate that dHT is present in the nucleus (Fig. 3*B*). Then, photobleaching experiments were conducted to test whether nu-

clear monoamine could diffuse throughout the soma. For each experiment, a small region in the proximal dendrite of one optical section of a dHT-loaded neuron was photobleached by continuous scanning (Fig. 3C). This led to a drop in fluorescence in the nucleus ($\Delta F = 18.1 \pm 0.5\%$, $n = 3$), showing that monoamine must diffuse between the nucleus and cytoplasm. Interestingly, the average fluorescence drop in the entire soma was smaller than the average drop in the nucleus ($\Delta F = 15.2 \pm 1.5\%$, $n = 3$), suggesting that a portion of the dHT outside the nucleus is sequestered. Based on this difference in the loss of fluorescence, it was calculated that 26.3% of total somatic content must be sequestered (Eq. 1).

The presence of a sequestered fraction of dHT is consistent with VMAT-dependent packaging of dHT into secretory vesicles in the soma. This vesicular packaging could be measured by taking advantage of the pH sensitivity of dHT fluorescence, which is quenched inside of acidic secretory vesicles. For this purpose, ammonium chloride, pH 7.4, was used to equilibrate pH across all cellular compartments (Fig. 3D). As a control experiment, vesicular packaging was prevented by reserpine before this treatment. Because the latter slices contained no vesicular dHT, the fractional increase in fluorescence observed was used as a baseline measurement of the broad pH dependence of extravesicular dHT to ammonium application (Fig. 3E, Extravesicular). Ammonium was then applied to slices loaded normally (i.e., in the absence of reserpine). In these slices, ammonium induced a 13% larger fractional increase in fluorescence (Fig. 3E, Total) than in control experiments. Together with the *in vitro* measured, twofold pH sensitivity of dHT, this result indicates that 26% of total somatic content is packaged into VMAT-containing secretory vesicles at rest (Eq. 2). This quantification is in agreement with the measurement of the sequestered fraction of dHT in photobleaching experiments. Furthermore, this measurement, which was verified by the quantification of the vesicular packaging of endogenous serotonin through three-photon imaging (see below), shows that in the soma, where the nucleus accounts for much of the somatic volume, most transmitter (i.e., 74%) is extravesicular, despite the high concentration of monoamine in secretory vesicles.

Somatic release is exclusively vesicular

To study release, somatic fluorescence was compared from stacks of images through DR neurons acquired before and 1 min after bath application of control solution (Con) or AMPA (Fig. 4A), a

Figure 3. Nuclear and vesicular dHT distribution in soma of DR serotonin neurons. **A**, Maximum Z-projection of 16 consecutive, 2.5 μm -spaced two-photon images through the DR of rat brain slice loaded with dHT or dHT and fluoxetine for 3 h. Scale bar, 20 μm . **B**, dHT is present within the nucleus. Single optical section through the center of a dHT (green) loaded neuron stained with Hoechst 33342 (blue) to mark the nucleus. Three-dimensional XY and YZ projections of the same neuron are shown. Scale bars, 10 μm . **C**, Extravesicular monoamine diffuses between the cytoplasm and the nucleus. Summed image stacks of a dHT loaded neuron before and after photobleaching a small region of a proximal dendrite in a single optical section. Scale bar, 20 μm . **D**, Summed dHT fluorescence before (Con) and after 30 s application of 50 mM ammonium chloride (NH_4^+). Scale bar, 20 μm . **E**, Quantification of extravesicular (black bar, measured in slices loaded in the presence of reserpine) and total (gray bar) fractional fluorescence changes after NH_4^+ application. The difference, indicated with a bracket, represents the NH_4^+ -induced fractional fluorescence change in vesicular dHT ($n \geq 13$). NH_4^+ experiments were done on slices at rest and after AMPA-induced stimulation.

glutamate receptor agonist shown to increase the firing rate of DR serotonin neurons (Gartside et al., 2007). AMPA (10 μM), but not the control solution, induced a significant drop in the somatic dHT signal. This fluorescence change [$\Delta F = 1 - (F/F_0)$] required extracellular Ca^{2+} (Fig. 4B). Furthermore, the AMPA response was inhibited by incubating slices in reserpine before and during dHT exposure to prevent VMAT-mediated loading of vesicles (Fig. 4B). Thus, AMPA-evoked release by the serotonin neuron

Figure 4. AMPA induces somatic exocytosis from DR serotonin neurons. **A**, Pseudo-colored summed stack of dHT loaded DR neurons before (Con) and after 1 min of stimulation with 10 μM AMPA. Scale bar, 20 μm . **B**, Quantification of the decrease in somatic dHT fluorescence upon 1 min incubation with control solution (white bar), AMPA (black bar), and AMPA in zero calcium (blue bar). The AMPA-induced fluorescence responses are also shown for slices loaded in the presence of reserpine (red bar). $n \geq 16$ for each condition; $*p < 0.001$ compared with Con, Dunnett's multiple-comparisons test after one-way ANOVA.

soma is exclusively mediated by Ca^{2+} -dependent exocytosis of VMAT-containing vesicles.

Activity-dependent vesicular transport depletes nuclear monoamine

Surprisingly, AMPA-induced dHT depletion was not limited to the periphery, but occurred throughout the entire soma. This raised the possibility that the nucleus, a compartment that has not previously been implicated in vesicular neurotransmitter release, could be relevant for vesicular release at the soma. To test this hypothesis, dHT fluorescence was measured in the nucleus before and after stimulation (Fig. 5A). The nuclear region was defined in dHT-loaded cells either by Hoechst stain (Fig. 5A) or by selecting a small region in the center of the soma (Fig. 5B), which always fell within the nucleus. Upon application of AMPA, both measurements revealed a comparable depletion of nuclear dHT content (Fig. 5C, AMPA_H and AMPA_C, 12%). This 12% decrease of extraventricular dHT (which was 74% of the total monoamine) corresponds to 9% of total somatic content during AMPA stimulation.

The observed decrease of nuclear monoamine content could be due to a stimulation-induced redistribution of extraventricular dHT into vesicles. Therefore, to test whether this depletion was mediated by VMAT, slices were incubated in reserpine for 5 min before and during AMPA stimulation. The 5 min reserpine pre-incubation did not evoke any change in dHT fluorescence, showing that vesicular content was maintained. However, the presence of reserpine during stimulation completely eliminated the AMPA-induced depletion of nuclear dHT (Fig. 5B,C, AMPA_C + reserpine). Therefore, the depletion of nuclear monoamine is due to stimulation-induced vesicular transport. Hence, AMPA induces acute VMAT-dependent packaging that draws on nuclear monoamine in the soma of DR serotonin neurons.

Quantification of activity-induced vesicular transport and somatic release

The above detection of the activity-dependent vesicular packaging reveals that the total somatic AMPA fluorescence response

Figure 5. Activity-dependent vesicular transport depletes nuclear monoamine. **A**, Single pseudo-colored equatorial section of dHT-loaded neuron before (Con) and after AMPA stimulation reveals dHT loss in the nucleus. The nucleus as marked by Hoechst stain is outlined. Scale bar, 10 μm . **B**, Single pseudo-colored equatorial sections of a dHT-loaded neuron exposed acutely to reserpine are shown before (Con) and after AMPA stimulation. Reserpine incubation during stimulation prevented a drop in nuclear fluorescence as measured in a small region in the center of the neuron (outlined). **C**, Quantification of the AMPA-induced decrease in nuclear fluorescence measured within the Hoechst defined nuclear border (AMPA_H, black bar; $n = 6$) or the center of the neuron (AMPA_C, gray bar; $n = 7$). Acute application of reserpine just before and during stimulation blocked the AMPA-induced dHT depletion in the nucleus (AMPA_C + reserpine, white bar; $n = 7$; $*p < 0.01$ Tukey's multiple-comparison test after one-way ANOVA). **D**, Summary of stimulation-induced monoamine dynamics deduced from Figures 3E and 5C. AMPA induces VMAT-dependent vesicular packaging and vesicle exocytosis to release the equivalent of the entire preloaded vesicular pool. The predicted ΔF (%) of these dynamics (20%) is within the error of the measured AMPA-induced fluorescence change (Fig. 4B).

reflects both exocytotic release from vesicles and the pH-induced quenching that accompanies dHT transport into acidic VMAT vesicles. Having quantified the amount of monoamine packaged during stimulation, the amount of transmitter released could be

determined by measuring the vesicular content remaining after AMPA. Specifically, ammonium experiments, as previously described, were repeated on slices after AMPA stimulation and the pH-dependent increase in vesicular fluorescence monitored (Fig. 3*E*, After AMPA). The observed 6% fractional increase of vesicular dHT fluorescence, together with the twofold pH sensitivity of dHT, indicated that 12% of the somatic content remaining after stimulation was packaged in vesicles (Eq. 2). This 12% is equivalent to 9% of total dHT content before stimulation (Eq. 3). Thus, interestingly, the content that remains in vesicles after stimulation equals the amount that was packaged during stimulation (i.e., 9%), as revealed by nuclear depletion experiments. This finding also demonstrates that 26% of total somatic content, which is equivalent to the entire preloaded vesicular pool, was released during AMPA stimulation (Fig. 5*D*).

Having measured vesicular sequestration before and after AMPA, activity-dependent vesicular packaging and somatic release (Figs. 3*E*, 5*C*), a prediction of the total somatic change in fluorescence induced by these dynamics (Fig. 5*D*) was calculated ($\Delta F_{\text{pred}} = 20\%$, Eq. 4). This prediction is within the error of the independently measured AMPA-induced somatic fluorescence response (Fig. 4*B*, $18 \pm 2.4\%$), showing that results from diverse experimental designs all confirm the same stimulation-induced dynamics.

Efficient release of monoamine loaded during stimulation

The above analysis, however, cannot distinguish whether activity-evoked monoamine packaging lags after release to restore a depleted vesicular pool or acutely contributes to release. For example, the above results are compatible with a simple model in which all somatic vesicles undergo exocytosis only once to completely release their preloaded content and then are refilled by VMAT (Fig. 6*Ai*). According to this hypothesis, VMAT activity during the stimulation partially replenishes emptied vesicular stores, but does not contribute acutely to somatic release. However, an alternative hypothesis is that AMPA evokes release of only a portion of preloaded vesicular content. In this case, the rest of the release derives from the efficient release of monoamine packaged by VMAT during stimulation (Fig. 6*Aii*). Preloaded vesicular content held in reserve would then account for the vesicular content detected after AMPA. Hence, the two models diverge with respect to the contribution of acute, activity-dependent vesicular transport to release.

Therefore, the two models can be distinguished by the effect of acute inhibition of VMAT on AMPA-evoked release. Specifically, with the first hypothesis, blocking VMAT acutely during stimulation would not affect release because all release derives from monoamine that was packaged before AMPA application (Fig. 6*Ai*). In contrast, the second hypothesis predicts that acute inhibition of VMAT during stimulation would reduce release because monoamine packaged in the presence of AMPA contributes to release (Fig. 6*Aii*). In fact, AMPA-induced fluorescence changes during acute inhibition of VMAT can be calculated for each hypothesis based on the results presented thus far (Eq. 5). If release is supported exclusively by preloaded vesicles (Fig. 6*Ai*), then AMPA stimulation after acute inhibition of VMAT should evoke a 15% decrease in fluorescence (Fig. 6*B*, model i). On the other hand, if all transmitter packaged during stimulation is released (Fig. 6*Aii*), then acute inhibition of VMAT would reduce release to the fraction supported by preloaded vesicles. This would correspond to a 9.9% decrease in fluorescence (Fig. 6*B*, model ii).

To inhibit VMAT during stimulation, reserpine was applied acutely (i.e., just before and during AMPA application) as described earlier (Fig. 5*B,C*). AMPA stimulation after acute reser-

Figure 6. Contribution of activity-dependent vesicular transport to somatic release. **A**, Models of neurotransmitter packaging and release. Transmitter already packaged in vesicles at rest (i.e., prepackaged) is indicated in blue. Extravesicular transmitter before stimulation is indicated in red. In model i, all prepackaged transmitter is released. Extravesicular transmitter is then packaged into vesicles. In model ii, a portion of the prepackaged transmitter and all transmitter packaged during stimulation is released. Note that the activity-dependent contribution of VMAT to release is different in the two models: model ii relies on VMAT during the stimulus to contribute to released transmitter, while released transmitter in model i is independent of VMAT function during AMPA stimulation. **B**, Quantification of somatic ΔF (%) in response to 1 min of AMPA stimulation in slices acutely exposed to reserpine ($n \geq 14$). Open and gray bars show calculated AMPA responses for model i and model ii respectively (Eq. 5). The experimental result (AMPA + reserpine) is comparable to the prediction of model ii, but significantly different from model i ($p = 0.01$, one-sample t test compared with prediction of model i).

pine application evoked a $9.2 \pm 2.0\%$ decrease in somatic fluorescence (Fig. 6*B*). This result agrees with the predicted response of the second model (Fig. 6*Aii*), and is significantly different from the first model ($p < 0.01$). Hence, while some monoamine transported into somatic vesicles at rest is held in reserve, monoamine transported by VMAT during AMPA stimulation is released efficiently. These experiments show that activity-dependent vesicular transport, which depletes the nucleus, is efficiently coupled to somatic release.

Endogenous serotonin is depleted from the nucleus by VMAT to acutely contribute to somatic release

The pH-dependent, two-photon-based fluorescence of dHT allowed measurements of activity-dependent VMAT function and monoamine dynamics in living cells for the first time, revealing the contribution of acute packaging to somatic release. To determine whether conclusions from the analog apply to serotonin, three-photon excitation of serotonin was

used to examine vesicular transport and somatic release of the native transmitter.

First, the percentage of serotonin in vesicles was determined. Although the serotonin signal is somewhat punctate, single serotonin vesicles cannot necessarily be resolved by three-photon microscopy. Therefore, a resolution-independent approach was required to determine vesicular sequestration. However, pH could not be used because serotonin fluorescence is relatively insensitive to the acidic pH inside secretory vesicles (Maiti et al., 1997). Hence, another approach was taken. Because the nucleus excludes vesicles, but is in equilibrium with any extravesicular monoamine (Fig. 3C), the mean nuclear fluorescence was subtracted from the raw image stack to reveal the signal from sequestered serotonin (Fig. 7A, Vesicular). This signal was then measured as a fraction of the background-subtracted total signal throughout the soma (Fig. 7A, Total). Given that serotonin is highly concentrated in VMAT vesicles, the resulting measurement represents the percentage vesicular serotonin. This measurement, $28 \pm 2.2\%$ ($n = 7$), was comparable to the 26% of dHT that was sequestered (Fig. 3C,E) showing that the resting distribution of dHT and serotonin are similar.

In addition to the similar resting distribution of dHT and native transmitter, stimulation with AMPA induced the release of serotonin as measured in the total soma (Fig. 7B, Summed Projection). Moreover, examination of single optical sections revealed that stimulation-induced depletion of serotonin in the nucleus (Fig. 7B, Single Section) that was inhibited by reserpine (data not shown, $n = 19$, $p = 0.04$, t test). Thus, AMPA induces VMAT-dependent redistribution of endogenous nuclear serotonin to vesicles. Finally, acute application of reserpine did not alter the vesicular distribution of serotonin ($n = 5$) and significantly decreased stimulation-induced somatic release (Fig. 7C). Thus, direct detection of native transmitter corroborates that in the soma of DR serotonin neurons, stimulation induces VMAT-dependent vesicular loading that draws on extravesicular (including nuclear) serotonin, which is then rapidly released.

Discussion

Multiphoton monoamine imaging has revealed fundamental insights into serotonin packaging and somatic release in the DR. First, detection of monoamine in the nucleus, cytoplasm and vesicles enabled measurements of the relative size of reserve and releasable pools, the content of vesicles before and after release, and the dynamics of activity-evoked packaging by VMAT. Furthermore, the contribution of vesicular packaging before and during stimulation to release was determined. Together, these findings show that in the absence of active zone specializations and with limited serotonin reuptake in the soma of DR neurons, somatic release is supported by recruitment of nuclear serotonin and the rapid and preferential release of this newly packaged transmitter.

Nuclear transmitter supports somatic release

Previously, the role of the nucleus in neurotransmitter release had not been considered because nuclear transmitter content had never been detected. In retrospect, this is not surprising because the rapid equilibration of extravesicular monoamine between the cytoplasm and the nucleus (Fig. 3C), which is consistent with the known permeability of the nuclear envelope (Gerace and Burke, 1988), would prevent serotonin retention during isolation of nuclei or nuclear extracts. However, in the context of intact neurons, this same permeability allows monoamines to

Figure 7. Three-photon imaging of serotonin corroborates recruitment of nuclear monoamine and efficient release of newly packaged transmitter. **A**, Representative three-photon images of serotonin fluorescence are shown as single optical sections through the center of the nucleus and summed projections of the entire soma. Vesicular serotonin fluorescence is shown after subtracting the extravesicular serotonin (i.e., signal measured in the nucleus, Vesicular). **B**, The same neuron before and after 1 min of stimulation with $10 \mu\text{M}$ AMPA. Single optical sections through the center of the neuron reveal nuclear serotonin depletion induced by AMPA, but not control solution ($n \geq 7$, $p = 0.007$, t test), whereas summed projections demonstrate total AMPA-induced release of serotonin (**C**). **C**, Quantification of somatic ΔF (%) in response to 1 min of control solution (Con, black bar), AMPA (white bar), or AMPA in slices acutely exposed to reserpine (AMPA + Reserpine, gray bar). Reserpine significantly attenuated serotonin release ($n \geq 6$; $*p < 0.05$, Tukey's multiple-comparison test after one-way ANOVA).

enter the nucleus. This may be significant for two reasons. First, the reactive transmitter dopamine may induce nuclear damage that contributes to Parkinson's disease. Second, because the nucleus occupies a large volume (Fig. 3B) and does not contain monoamine oxidase, the enzyme responsible for intracellular catabolism of monoamine transmitters, the nucleus can serve as a large depot of diffusible transmitter for somatic vesicle loading.

The participation of the nucleus via VMAT-mediated depletion further distinguishes release at the serotonin neuron cell body, which has low levels of SERT (Tao-Cheng and Zhou, 1999), from release at terminals, which relies on reuptake.

Activity-dependent vesicular transport couples to release

In addition to a role for the nucleus in supporting neurotransmission, a functional coupling between activity-dependent vesicular transport and efficient release was demonstrated. Specifically, while only a fraction of monoamine packaged at rest is released, monoamine packaged during activity is released completely. This implies that vesicles that are filled with transmitter during stimulation rapidly undergo exocytosis.

One potential interpretation of this finding is that stimulation induces a calcium-dependent activation of VMAT specifically in a subset of vesicles that will undergo exocytosis. In this case, additional transmitter would be loaded into these vesicles before release. Interestingly, the vesicular pre-fusion protein calcium-dependent activator of protein secretion (CAPS) has been shown to increase VMAT function and vesicular storage of catecholamines (Speidel et al., 2005; Brunk et al., 2009), suggesting a potential mechanism for the unique regulation of packaging in vesicles destined for release. An alternative interpretation is that upon stimulation, a subset of vesicles undergoes exocytosis, refilling and subsequent exocytosis. In this case, a pool of VMAT vesicles must rapidly cycle in the soma despite the lack of active zones. Regardless of whether stimulation-induced vesicular transport is occurring before or after the initial fusion event, the coupling of activity-dependent vesicular transport is optimal for sustaining somatic release.

This coupling also suggests that modulation of vesicular transport may acutely regulate neurotransmission. Indeed, the experimental approaches developed here provide a means to test whether regulation of vesicular packaging defines properties of neurotransmission during activity-dependent plasticity, exposure to drugs and during neuropathology. Finally, our results suggest that the classic observation that newly synthesized (and hence newly packaged) transmitter is released preferentially (Kopin et al., 1968; Besson et al., 1969; Collier, 1969) is a consequence of the coupling of activity-induced vesicular transport to exocytosis.

Multiphoton monoamine imaging techniques

While fluorescent vesicle markers have been used to measure vesicle cycling and exocytosis, dynamic measurements of neurotransmitter packaging and release had not been made previously. The use of a closely related serotonin analog and direct detection of the endogenous transmitter allowed *in vivo* measurements of these complex processes. Whereas three-photon excitation directly reveals the native transmitter, the high energy required, rapid photobleaching and low fluorescence emission limit the detection of serotonin. Thus, while three-photon experiments yielded valuable qualitative results (Fig. 7), quantification of serotonin dynamics during stimulation was necessarily limited. Two-photon dHT imaging, however, has comparably much stronger signal-to-noise, allowing reproducible measurements that could be confirmed by multiple independent experimental approaches. In addition, the pH dependence of dHT fluorescence, although limiting the ability to optically resolve individual vesicles, facilitated resolution-independent quantification of packaged and unpackaged monoamine (Fig. 3E). Importantly, these pH-based dHT results were validated by pH-insensitive serotonin measurements. Hence, the ability to assay dHT vesic-

ular transport based on its pH sensitivity holds great potential for measuring vesicular packaging in nerve terminals, where the content in vesicles cannot be resolved optically from the cytoplasmic pool.

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