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# Quantitative Evaluation of 5-Hydroxytryptamine (Serotonin) Neuronal Release and Uptake: An Investigation of Extrasynaptic Transmission

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Whether neurotransmitters are restricted to the synaptic cleft (participating only in hard-wired neurotransmission) or diffuse to remote receptor sites (participating in what has been termed volume or paracrine transmission) depends on a number of factors. These include (1) the location of release sites with respect to the receptors, (2) the number of molecules released, (3) the diffusional rate away from the release site, determined by both the geometry near the release site as well as binding interactions, and (4) the removal of transmitter by the relevant transporter. Fast-scan cyclic voltammetry allows for the detection of extrasynaptic concentrations of many biogenic amines, permitting direct access to many of these parameters. In this study the hypothesis that 5-hydroxytryptamine (5-HT) transmission is primarily extrasynaptic in the substantia nigra reticulata, a terminal region with identified synaptic contacts, and the dorsal raphe nucleus, a somatodendritic region with rare synaptic incidence, was tested in brain slices prepared from the rat. Using carbon fiber microelectrodes, we found the concentration of 5-HT released per stimulus pulse in both regions to be identical when elicited by single pulse stimulations or trains at high frequency. 5-HT efflux elicited by a single stimulus pulse was unaffected by uptake inhibition or receptor antagonism. Thus, synaptic efflux is not restricted by binding to intrasynaptic receptors or transporters. The number of 5-HT molecules released per terminal was estimated in the substantia nigra reticulata and was considerably less than the number of 5-HT transporter and receptor sites, reinforcing the hypothesis that these sites are extrasynaptic. Furthermore, the detected extrasynaptic concentrations closely match the affinity for the predominant 5-HT receptor in each region. Although they do not disprove the existence of classical synaptic transmission, our results support the existence of paracrine neurotransmission in both serotonergic regions.

*Key words: 5-hydroxytryptamine; volume transmission; substantia nigra reticulata; dorsal raphe; fast-scan cyclic voltammetry; transporter kinetics*

Views of synaptic transmission in the CNS are based in part on the action of acetylcholine (ACh) at peripheral synapses (Cooper et al., 1991). At the neuromuscular junction, ACh is released into the synaptic cleft, where it diffuses and interacts with intrasynaptic receptors. The diffusion of ACh is restricted by rapid binding to receptor sites, a process known as "buffered diffusion" (Katz and Miledi, 1973). This buffered diffusion, in addition to the presence of postsynaptic invaginations, increases the probability of degradation of released ACh by acetylcholinesterase (Magleby and Terrar, 1975; Bartol et al., 1991). In this way chemical communication involving ACh is restricted spatially to the length of the synaptic cleft. Glutamate and GABA in the CNS have been shown to interact primarily with receptors in the synaptic cleft (Isaacson et al., 1993a; Goda and Stevens, 1994; Tong and Jahr, 1994; Borges et al., 1995; Geiger et al., 1997). In contrast, dopamine can escape the synaptic cleft (Garris et al., 1994) and interact with receptors and transporters located at more remote sites (Smiley et al., 1994; Nirenberg et al., 1996, 1997). Thus, the locations of release sites as well as the affinities, binding kinetics, and location of receptors, transporters, and degradative enzymes are important parameters that determine whether neurotransmission is restricted to the synaptic cleft (Wathey et al., 1979), promoting hard-wired communication, or can occur in the extrasynaptic space (Clements, 1996), allowing for longer range, less specific interactions.

Neurotransmitter systems that primarily use synaptic transmission in the brain share several characteristics (Clements, 1996). They have receptors that have relatively low affinity for the transmitter and are localized within the synaptic cleft. Additionally, transporter sites in the synaptic or perisynaptic region restrict the efflux of released neurotransmitter. Therefore, like acetylcholine transmission at the neuromuscular junction, such neurotransmitter systems maintain synaptic neurotransmission via buffered diffusion. By itself, however, the presence of synaptic specializations is not sufficient to prevent an extracellular mode of communication. For instance, dopamine neurons exhibit synaptic specializations but have high-affinity receptors and transporters located at sites other than the synapse (Smiley et al., 1994; Nirenberg et al., 1996, 1997). Indeed, in the presence of uptake inhibitors even GABA (Isaacson et al., 1993b) and glutamate (Barbour et al., 1994; Asztely et al., 1997) may have extrasynaptic effects.

In this work we investigate the nature of 5-HT neurotransmission, a system in which ultrastructural studies have revealed both synaptic and nonsynaptic terminals. The paradigm of nonsynaptic 5-HT neurotransmission is the supraependymal axons located inside the cerebral ventricles (Chan-Palay, 1977). Similarly, ultra-

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structural observation of synaptic 5-HT terminals is rare in the median eminence and cerebral cortex (Calas et al., 1974; Descarries et al., 1975). On the other hand, electron microscopic studies reveal that >90% of 5-HT terminals in the substantia nigra reticulata (SNr) exhibit junctional complexes (Moukhles et al., 1997). The ultrastructure of other serotonergic brain regions exhibits both junctional and nonjunctional 5-HT terminals (Beaudet and Descarries, 1981; Descarries et al., 1990; Maley et al., 1990).

A particularly complex example is the dorsal raphe nucleus (DR), the primary site of 5-HT cell bodies in the CNS. In this region 5-HT cell bodies and dendrites accumulate 5-HT and package it in vesicles, apparently in a releasable form (Hery and Ternaux, 1981; Iravani and Kruk, 1997; Bunin et al., 1998). Early studies suggested that 5-HT accumulation was restricted to cell bodies and dendrites (Fuxe, 1965; Loizou, 1972; Descarries et al., 1979, 1982; Baraban and Aghajanian, 1981), but 5-HT axon collaterals and terminals appear to exist as well (Mosko et al., 1977; Liposits et al., 1985; Chazal and Ralston, 1987). Ultrastructural studies suggest that release sites in the DR are both junctional and nonjunctional, although the latter predominate.

Although anatomical studies reveal the structural architecture of neurons, functional studies are required to establish the precise mode of chemical communication. Glutamate and GABA both rapidly affect postsynaptic cells, and in this way their synaptic actions have been revealed. Dopamine and 5-HT are both oxidized easily. Thus, their concentration in the extracellular fluid adjacent to release sites can be monitored with carbon fiber microelectrodes. To test the importance of 5-HT extrasynaptic neurotransmission in the SNr and DR, we compared the release induced by a single electrical impulse with that evoked by trains of two or more pulses delivered rapidly so that uptake sites did not have time to transport and unload their substrate and so that autoreceptors did not have time to modulate release. In the case of synaptic transmission, outward efflux of 5-HT after release induced by a single pulse must be restricted (or buffered) by binding to receptors, transporters, and other proteins within and on the perimeter of the synaptic cleft. Thus, the maximal 5-HT concentration evoked by a single stimulus pulse is expected to be near or below the detection limit of our extrasynaptic sensor (~20 nM). However, 5-HT molecules released in successive pulses would find many of the binding sites occupied by previously released molecules, and a majority of them should diffuse into the extracellular space. For this reason the concentration seen in the extracellular fluid during trains should not be directly proportional to the number of pulses in the train but should be greater than the product of the number of stimulus pulses and the maximal 5-HT concentration evoked by a single pulse. Likewise, occupancy of intrasynaptic receptors and transporters by antagonists and inhibitors should increase the extracellular 5-HT concentration evoked by a single stimulation pulse. Previously, this approach has been used to show that dopamine neurotransmission in the nucleus accumbens can be extrasynaptic (Garris et al., 1994). Our results show that under all circumstances that were tested 5-HT release is proportional to the number of stimulus pulses, indicative of paracrine neurotransmission.

## MATERIALS AND METHODS

*Animals.* Adult male Sprague Dawley rats (200–550 gm) were purchased from Charles Rivers (Wilmington, MA). Food and water were provided *ad libitum*. Animal care was in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 865-23, Bethesda, MD) and approved by the Institutional Animal Care and Use Committee. For all experiments the rats were decapitated and their brains removed in the mornings, before 12:00 P.M. Slice experiments were performed ~1 hr after brain removal.

*Slice procedures.* Coronal brain slices (400 μm thick) containing the SNr or DR were prepared from male Sprague Dawley rats (Charles River, Wilmington, MA), using a Lancer Vibratome as previously described (Kelly and Wightman, 1987). Slices containing DR were taken from a segment of brain corresponding to interaural measurements between 0.7 and 1.7, and those containing SNr were from measurements between 2.96 and 4.2, according to the atlas of Paxinos and Watson (1986). The slices were submerged in a Scottish-type chamber and perfused with buffer, preheated to 37°C, at 1 ml/min. The buffer contained (in mM): 126 NaCl, 2.5 KCl, 1.2 $NaH_2PO_4$, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 11 DL-glucose, and 20 HEPES. The buffer pH was adjusted to pH 7.4 and saturated with 95% $O_2$/5% $CO_2$. Slices were perfused for 45 min before the measurements were made.

Detection of stimulated neurotransmitter release was accomplished by a carbon fiber electrode, inserted 75 μm into the slice and 100–200 μm from the center of the stimulation electrode pair. Electrode placements were made with the aid of a stereoscopic microscope. Repetitive stimulations caused reproducible responses for >1.5 hr.

*Electrical stimulation.* Electrical stimulation was accomplished by a bipolar stainless steel electrode (Plastic Products, Roanoke, VA) placed on the slice surface. Unless stated otherwise, the stimulation consisted of biphasic square-wave (2 msec/phase), constant current (350 μA) pulses. Stimulation waveforms were computer-generated or used a waveform generator (model 33120A, Hewlett Packard, Loveland, CO). Stimuli were isolated optically (NL 800, Neurolog, Medical Systems, Great Neck, NY) from the electrochemical system. To examine the effects of the stimulation amplitude on release, we randomly applied stimulations (20 pulses, 100 Hz), using varying current amplitudes. Stimulations were separated by >2 min.

*Electrochemistry.* Microelectrodes with beveled tips were fabricated from carbon fibers ($r = 5$ μm; Thornel P-55, Amoco, Greenville, SC), as previously described (Kawagoe et al., 1993). The tips were coated with Nafion to restrict chemical interference from anions. Triggering and acquisition parameters were controlled by locally written software, using a commercial interface board (Labmaster, Scientific Solutions, Solon, OH). The voltammetric waveform was produced by a function generator (Model 5200A, Krohn-Hite, Avon, MA) and consisted of a rest potential of 0.2 V scanned to 1.0, then to −0.1, and back to 0.2 V, at a rate of 1000 V/sec. This waveform previously has been shown to provide sensitive and selective detection of 5-HT; the voltammogram obtained for 5-HT is distinct from that for DA, and the selectivity for 5-HT over DA is at least 20:1 (Jackson et al., 1995; Bunin et al., 1998). A saturated sodium calomel reference electrode (SSCE) was used in all experiments. The stainless steel tubing, through which buffer was perfused into the slice chamber, served as the auxiliary electrode. An EI-400 potentiostat (Ensman Instrumentation, Bloomington, IN), operated in three electrode mode, was used to record cyclic voltammograms every 100 msec. The output was computer-digitized, and the peak oxidation current amplitude (typically occurring between 500 and 700 mV) was plotted versus time to obtain a current profile. The current was converted to a concentration by using a postcalibration factor determined in solutions of the HEPES buffer containing 2 μM 5-HT (Jackson et al., 1995). The cyclic voltammogram was obtained by background subtraction of the nonfaradaic residual current with locally written software (Kawagoe et al., 1993). All data obtained by one- and two-pulse stimulations are presented as an average of 5–10 concentration profiles obtained from a single placement of an electrode in a single slice. All other data are unaveraged (i.e., single trace).

*Data analysis.* Where applicable, data are presented as the mean ± SEM. Pooled data correspond to $n = 4$ slices.

An uncertainty in some of the calculations is the effect of the volume fraction of the extracellular space on our measurements. In the calculations we take this value to be 1, although measured values over long distances show it to be 0.2 (Nicholson and Rice, 1986). The uncertainty arises because the space adjacent to the electrode has not been defined microscopically (Kawagoe et al., 1992). If all of the released 5-HT in the measurement region partitions into the Nafion coating, a value of 1 is appropriate. If not, the calculated values of the 5-HT turnover number, transporter binding rate, synaptic and terminal 5-HT concentrations, and number of 5-HT molecules released per terminal are overestimated. In this case the excess of receptors and transporters over released 5-HT molecules is even greater, and the conclusions of this paper are reinforced.

*Chemicals.* Drugs were dissolved in 1 ml of doubly distilled water at a concentration of 10 mM, diluted with HEPES buffer to the final concen-

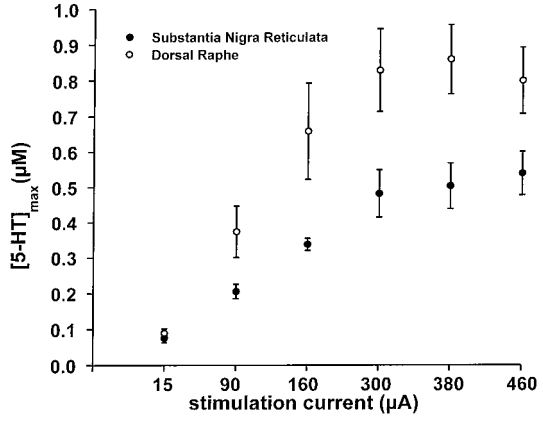


*Figure 1.* 5-HT maximal release as a function of current amplitude. Each *data point* was obtained with a 20-pulse (2 msec, biphasic pulses) stimulation at 100 Hz ($n = 4$ slices). *Open circles*, DR; *filled circles*, SNr.

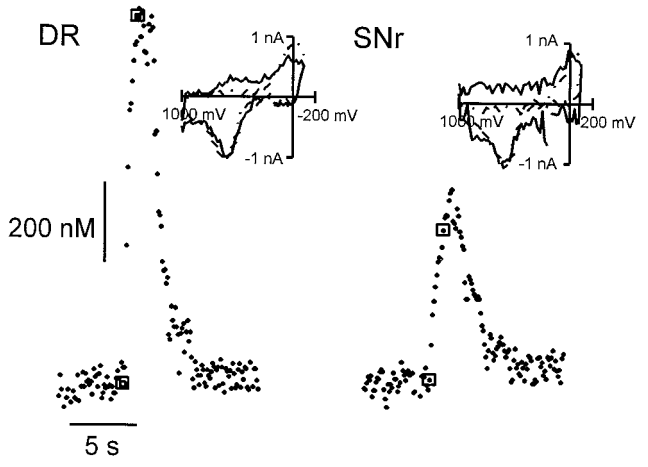


*Figure 2.* Representative 5-HT concentration profiles elicited in the DR and the SNr when 20 stimulation pulses (350 μA) are applied at 10 Hz. The *open squares* indicate the beginning and end of the stimulation. The *insets* are the cyclic voltammograms obtained at the maximal 5-HT concentrations elicited: the *solid line* is that obtained in the slice; the *broken line* is that obtained during postcalibration with 2 μM 5-HT and is normalized in amplitude.

tration (a final volume of 250 ml), and perfused for 20 min before postdrug data were collected. Fluoxetine hydrochloride was a gift from Eli Lilly and Company (Indianapolis, IN), and methiothepin mesylate was purchased from Research Biochemicals International (Natick, MA). 5-HT hydrochloride was obtained from Sigma (St. Louis, MO).

## RESULTS

### Effect of stimulation amplitude on evoked 5-HT release

The effect of the stimulus current amplitude (applied with an adjacent bipolar stimulating electrode) on the maximal concentration of 5-HT was examined in brain slices containing either the DR or the SNr with 0.2 sec, 100 Hz pulse trains (Fig. 1). Short duration trains were used to avoid the inhibition of release caused by autoreceptor activation that occurs on a time scale of 400 msec (O'Connor and Kruk, 1991; Davidson and Stamford et al., 1995). Currents <15 μA did not evoke detectable 5-HT release in either region. The maximum elicited 5-HT concentration increased as the stimulation current was increased from 15 to ~300 μA in both regions. For currents >300 μA, no additional increase in maximal 5-HT concentration was observed. This behavior is identical to that obtained for dopamine release *in vivo* during stimulation of the medial forebrain bundle (Kuhr et al., 1984; Wiedemann et al., 1992) but differs from that for dopamine release in slices (Mickelson et al., 1998). All subsequent measurements were made by using a current amplitude in the plateau region (350 μA) to ensure maximal release.

### Release of 5-HT evoked by different frequencies

Representative concentration changes of 5-HT, obtained in brain slices containing DR or SNr during 2 sec, 10 Hz (350 μA) electrical stimulations, are shown in Figure 2. The maximum concentration of 5-HT evoked in the DR is approximately twice that of the SNr. We previously have established that the rapid disappearance after stimulation is attributable to uptake (Bunin et al., 1998). The rate of disappearance obtained after high-frequency stimulations, resulting in concentrations that are at least 10 times the value of $K_m$ for uptake (170 nM; Mosko et al., 1977), is $V_{max}$. This value in the DR ($V_{max} = 1.30 \pm 0.02$ μM/sec; $n = 4$ slices) is also twice that obtained in the SNr ($V_{max} = 0.57 \pm 0.07$ μM/sec; $n = 4$ slices), as reported elsewhere (Bunin et al., 1998). Curves at frequencies from 10 to 100 Hz were simulated previously by assuming that each stimulus pulse results in a fixed increment in the 5-HT extracellular concentration, defined as $[5\text{-HT}]_p$, and that uptake follows Michaelis–Menten kinetics in the interval between and after stimulus pulses (Bunin et al., 1998). Values for release (SNr: $[5\text{-HT}]_p = 55 \pm 7$ nM; DR: $[5\text{-HT}]_p = 100 \pm 20$ nM) revealed that it is doubled in the DR also. Note, however, that the maximal concentrations with 20 pulse, 10 Hz stimulations (Fig. 2) are far less than $20 \times [5\text{-HT}]_p$. For example, in the DR $[5\text{-HT}]_{max}$ is 0.64 μM, whereas $20 \times [5\text{-HT}]_p$ is 2.0 μM. This occurs because uptake lowers the concentration in the 100 msec between stimulus pulses, and, over the 2 sec interval of the stimulation, considerable 5-HT is removed. Thus, so that values of $[5\text{-HT}]_p$ directly from the experimental data can be obtained, one-pulse stimulations or high-frequency trains of short duration are required. In the present study $[5\text{-HT}]_p$ is simply the maximal concentration of 5-HT elicited by a single stimulation pulse.

### Release of 5-HT by one and two stimulus pulses

A single stimulus pulse evokes a measurable 5-HT concentration in the DR (Fig. 3) that rapidly disappears with uptake rates identical to those obtained with stimulus trains. Importantly, the mean amplitude of the concentration change ($0.08 \pm 0.03$ μM) is not statistically different from the value of $[5\text{-HT}]_p$ obtained in this region with stimulus trains. Furthermore, two stimulus pulses delivered 10 msec apart evoked twice the maximal concentration obtained with a single pulse, the result expected for extrasynaptic transmission. In the SNr, release evoked by a single pulse was close to the detection limit. However, in all cases in which it was measurable ($n = 3$; an example is shown in Fig. 4), it also was statistically the same as $[5\text{-HT}]_p$ obtained from released concentrations evoked by stimulus trains.

As shown in Figure 4, in both the SNr and the DR for stimulus trains of up to 20 pulses delivered at 100 Hz the maximal evoked 5-HT concentration was directly proportional to the number of pulses applied (i.e., the 5-HT concentration amplitude obtained with 20 pulses was exactly 20 times that evoked with a single stimulus pulse). Thus, in both regions the concentration of 5-HT detected per stimulus pulse is independent of impulse number from 1 to 20 pulses at 100 Hz, providing evidence that the binding of 5-HT to receptors and transporters near release sites does not inhibit its efflux into the extracellular fluid (ECF).

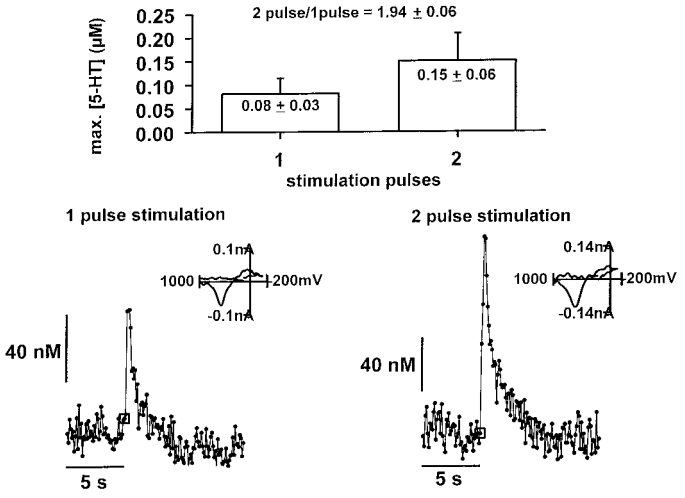


*Figure 3.* Comparison of one- and two-pulse stimulations in the DR. *Bottom left panel*, The 5-HT concentration profile obtained when a single stimulus pulse (1p) is applied. *Bottom right panel*, The 5-HT concentration profile obtained when two stimulus pulses (2p) are applied at 100 Hz. *Open squares* indicate the time of stimulation. The *insets* are the cyclic voltammograms obtained at the maximal evoked 5-HT concentration. *Top panel*, Pooled data (mean ± SEM; $n = 4$ slices) comparing the maximal 5-HT concentration elicited by a single stimulus pulse and two stimulus pulses (100 Hz).

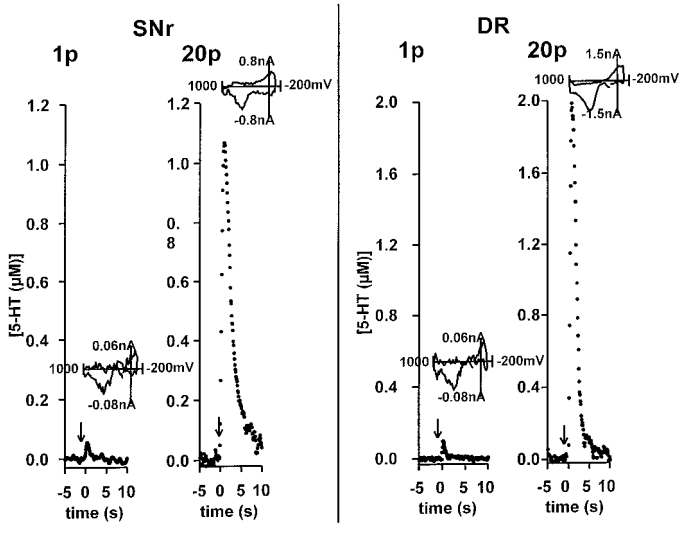


*Figure 4.* Comparison of 1- and 20-pulse stimulations (350 μA, 100 Hz) in the SNr and DR. The maximal 5-HT concentration in the SNr example is 54 nM for the single pulse (*1p*) and 1100 nM for the 20-pulse (*20p*) stimulation; in the DR example, these values are 97 and 2000 nM, respectively. The *arrows* indicate the onset of the electrical stimulation. The *insets* are the cyclic voltammograms obtained at the maximal 5-HT concentration that was evoked.

## Effects of uptake inhibition and receptor antagonists on 5-HT release

Figure 5 shows the combined effects of the selective 5-HT uptake inhibitor, fluoxetine (0.5 μM), and the nonspecific 5-$HT_1$/5-$HT_2$ antagonist, methiothepin (0.5 μM), on the 5-HT concentration profile elicited by a one-pulse electrical stimulation in the DR. When applied alone, neither of these pharmaceutical agents had any effect on 5-HT release evoked by one stimulus pulse (data not shown). Even when the brain slice was exposed to these two drugs

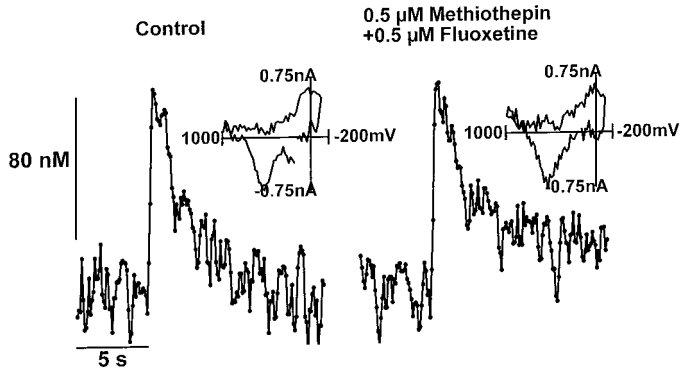


*Figure 5.* Effects of methiothepin (0.5 μM) and fluoxetine (0.5 μM) on the concentration profile obtained with a single stimulation pulse in the DR. The concentration profiles shown were obtained in a single location within the same brain slice; the *insets* are cyclic voltammograms obtained at the maximal concentration of 5-HT that was elicited. The mean maximal 5-HT concentration from similar experiments ($n = 4$ slices) revealed that the presence of these drugs did not alter release (value with drugs present was 91% ± 2 of control; $p = 0.67$; Student's $t$ test).

together, there was no change in the maximal amplitude of evoked 5-HT. However, uptake inhibition is clearly operant because there was an increase in the amount of time necessary for the clearance of 5-HT. This result shows that the blockade of transporters and receptors does not increase 5-HT efflux into the ECF.

Because of the lower concentrations of 5-HT release obtained in the SNr, the effects of uptake inhibition and autoreceptor antagonism on single pulse stimulations could not be ascertained; however, with longer stimulus trains (100 Hz, 0.2 sec) the value of $[5\text{-HT}]_p$ did not increase with the application of fluoxetine (Bunin et al., 1998).

## Failure rate of 5-hydroxytryptamine release during electrical stimulation

The 5-HT concentration curves shown in Figures 3, 4, and 5 are the average results of at least five repetitive one-pulse stimulations at single locations. Examination of individual traces allows the possibility of failures in stimulated release to be assessed. Figure 6 shows the individual responses obtained in the DR during an experiment in which single pulses were applied 10 times (120 sec apart). None of the stimulations in this region resulted in the failure of detectable 5-HT. In fact, every experiment that used a one-pulse stimulation (>80 stimulations and >8 slices) resulted in 5-HT release. Furthermore, the constancy of the maximal 5-HT concentration shows that release did not fatigue with these stimulus parameters.

## DISCUSSION

The results of this study are consistent with the hypothesis that, in the DR and the SNr, released 5-HT is not buffered by binding to receptor or transporter sites within the time scale of our measurement, but 5-HT is able to enter the extracellular space at a rate governed only by diffusion. Once there, transporter uptake restricts its long range diffusion. Indeed, recent anatomical studies show that the uptake sites are not localized synaptically but, rather, are distributed to control optimally the extracellular 5-HT neurotransmission (Tao-Cheng and Zhou, 1997; Zhou and Tao-Cheng, 1997). *In vitro* binding studies suggest an affinity of the predominant 5-HT receptor in both regions (5-$HT_1$) for the endogenous ligand to be in the low nanomolar range (Green and Maayani, 1987; Pranzatelli, 1989). In this work the maximal

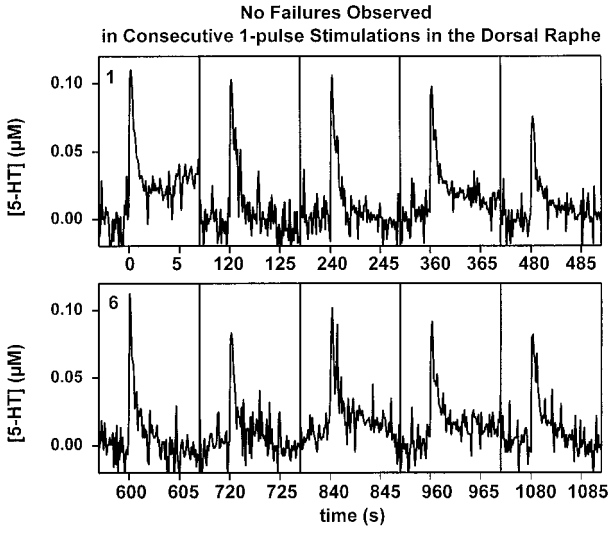


*Figure 6.* Absence of failures in successive one-pulse stimulations in the DR. Each plot is an individual response obtained in the DR during an experiment in which single pulses were applied 10 times (120 sec apart). The concentration changes resulting from the first stimulation of the series is shown in the *top left panel* (labeled *1*); the results of the last stimulation of the series is shown in the *bottom right panel*.

extracellular concentration of 5-HT evoked by a single stimulus pulse is found to be 50 nM for the SNr and 100 nM for the DR. Thus, although dilution will occur when 5-HT diffuses away from its release site (Eccles and Jaeger, 1958; Garris et al., 1994), its concentration will remain sufficient to be efficacious for some distance away. In this way 5-HT can participate in what has been termed "volume" (Fuxe and Agnati, 1991) or paracrine transmission in the two regions examined. This conclusion is consistent with the anatomical architecture of 5-HT neurons in the DR (Descarries et al., 1982; Chazal and Ralston, 1987), which exhibit little synaptic specialization. However, electron microscopic results indicate prevalent junctional organization in the SNr (Moukhles et al., 1997), yet the release results are consistent with synaptic efflux. The behavior of 5-HT is similar to dopamine in the nucleus accumbens, another region in which ultrastructural studies have identified abundant synaptic specializations (Garris et al., 1994). These systems stand in dramatic contrast to the glutamate and GABA systems, in which the preponderance of evidence implicates classical synaptic transmission (Isaacson et al., 1993a,b; Clements et al., 1996).

The effect of uptake sites on efflux is dependent not only on their anatomical location but also on their kinetic characteristics. The macroscopic rate constants for 5-HT uptake, determined with our technique, allow for the calculation of the turnover number and affinities for the 5-HT transporter. The $B_{max}$ values for [$^3$H]paroxetine binding are 0.56 and 0.22 pmol/mg tissue in the DR and SNr, respectively (Chen et al., 1992). Assuming an equivalence between 1 mg of tissue and 1 $\mu$l, the ratio of $V_{max}$ to $B_{max}$ yields the turnover number. This was found to be 2.4 $\pm$ 0.3 and 2.6 $\pm$ 0.3/sec for the DR and SNr, respectively, values that are not statistically different. These values are comparable to those describing 5-HT uptake into both platelets (Talvenheimo et al., 1979) and synaptosomes (Ross and Hall, 1983) and are remarkably similar to those measured for the dopamine transporter (Garris et al., 1994). The similarity with dopamine transport is expected, because the dopamine and 5-HT transporters are members of the same structural family with similar sequences (Amara and Kuhar, 1993; Lester et al., 1994). With this turnover number each transporter only has sufficient time to transport one 5-HT molecule on the time scale of the 100 Hz stimulations used herein. Division of the turnover number by $K_m$ gives a minimal value for the initial binding rate, $1.5 \times 10^7 \cdot M^{-1} \cdot sec^{-1}$. This large rate constant is typical for transporters (Stein, 1986). Thus, binding is rapid and could restrict diffusion.

Because of the complex nature of 5-HT release sites in the DR, its density of release sites has not been reported. However, the density of 5-HT varicosities in the SNr is known: $9 \times 10^6$ sites/mm$^3$ (Moukhles et al., 1997). If each terminal is equidistant, it would be located in a cube that has an average length of 4.8 $\mu$m. The dimensions of this cube are smaller than our sensor (10 $\mu$m diameter at the sensing tip); thus, the sensor samples from several terminals. To fill each cube with a uniform concentration of 5-HT, the released molecules must diffuse ~3 $\mu$m from the release site in all directions. If release is synchronized, as would be expected to occur with local stimulation, the secreted molecules will encounter each other at the cube boundaries, establishing a homogeneous concentration throughout the stimulated area. *In vivo*, the firing of 5-HT neurons appears synchronized (Wang and Aghajanian, 1982) and can occur spontaneously at frequencies of 100 Hz (Hajos et al., 1995, 1996). To improve the likelihood that all terminals release with the electrical stimulation, we set the stimulation amplitude at the plateau of the response curve. Using this amplitude in the DR, we never observed release failures, suggesting that the stimulation conditions ensure a maximal probability of release. However, the lack of observed failures may arise because multiple varicosities contribute to the locally measured signal.

The diffusion problem in spherical coordinates for a release volume of similar dimensions had been solved previously (Garris et al., 1994), revealing that concentration uniformity occurs within 3 msec after simultaneous release events. Thus, the amount released per site is the concentration released per stimulus pulse ([5-HT]$_p$) divided by the terminal density. This gives a value of 3500 molecules/terminal, comparable to that found for quantal release from cultured neurons with vesicles of similar size to 5-HT neurons in the CNS ($r$ = 25 nm) (Beaudet and Descarries, 1981). For example, 4000 molecules of 5-HT are released from single vesicles of Retzius neurons of the leech (Bruns and Jahn, 1998), whereas a quantal size of 1800 dopamine molecules has been found for cultured midbrain neurons (Pothos and Sulzer, 1998) that contain vesicles of similar dimensions.

The estimate of 5-HT molecules released per stimulus event allows for the comparison of the stoichiometry of released molecules and transporter sites. The number of transporter sites per terminal can be calculated from the $B_{max}$ values for [$^3$H]paroxetine binding (Chen et al., 1992) and the terminal density and is computed to be 15,000. This number compares favorably with estimates of 5-HT transporter densities in other regions of the rat brain (Dewar et al., 1991). Thus it appears that for 5-HT terminals in the SNr there is a considerable excess of transporters as compared to released molecules. If all of these transporters reside near the release site, their large number and their rapid binding rate would inhibit efflux into the ECF for at least four stimulus pulses. However, in contrast to this prediction, the concentration released per stimulus pulse is independent of pulse number. Thus, our data provide strong evidence that the majority of uptake sites is extrasynaptic, consistent with recent anatomical findings (Tao-Cheng and Zhou, 1997; Zhou and Tao-Cheng, 1997).

Similarly, the stoichiometry of released molecules and 5-HT$_1$

receptors can be computed. $B_{max}$ for [$^3$H]sumatriptan binding in the SNr is 2.7 pmol/mg protein (Pazos and Palacios, 1985). Assuming that the brain is 10% protein by weight, 18,000 receptors per terminal are calculated. Again, if these were all in the synaptic cleft and if binding were rapid, few 5-HT molecules would escape. Unfortunately, binding rates and ultrastructural localization of these receptors are not yet known. Our results predict an extrasynaptic location. The computed excess of both receptor and transporter sites relative to the number of released molecules in the SNr is surprising and suggests that this 5-HT region is designed to control optimally the large amount of 5-HT released by rapid bursts that can originate in the cell bodies of the raphe neurons (Hajos et al., 1996).

Finally, we estimate the concentrations of 5-HT in the SNr from its initial stored state until it reaches its receptor site. If the estimated 3500 molecules released per terminal were all in one vesicle, their concentration would be 90 mM, consistent with previous estimates from isolated vesicle preparations (Floor et al., 1995). When released into a space with the dimensions of an SNr synapse [0.3 $\mu$m length (Moukhles et al., 1997), 0.015 $\mu$m width, the value for dopamine synapses (Pickel et al., 1981)], the concentration would be 6 mM. This is very high as compared with the affinity of the 5-HT$_1$ receptors. When 5-HT diffuses into the extracellular compartment, its measured maximal concentration is 55 nM, a value much closer to the affinity for receptors and $K_m$ for transport. This concentration is removed from the extracellular space with a time constant given by $V_{max}/K_m$ or 3.2/sec in the SNr, resulting in a half-life of ~200 msec. This time course is four times longer than that for dopamine in the nucleus accumbens (Wightman and Zimmerman, 1990) and allows 5-HT to diffuse >20 $\mu$m, a distance sufficient to interact with many extrasynaptic elements.

The central factors that affect the time course of transmitter concentration at a synaptic cleft after release have been outlined previously (Clements, 1996). Two of these, the amount released into the extracellular space and the uptake rate, are measured directly by the cyclic voltammetry technique. A third, diffusion out of the synapse, can be deduced from the experimental measurements. Because CNS synaptic specializations are small, upon release the high vesicular concentration of neurotransmitters floods the synapse and then rapidly diffuses into the extracellular space, greatly diluting the original concentration (Eccles and Jaeger, 1958; Garris et al., 1994), unless substantial intrasynaptic binding occurs. Our results in the SNr reveal that the anatomical presence of release sites within the synaptic cleft is not sufficient for hard-wired transmission. For systems that communicate by extrasynaptic means, synapses may exist simply as points of anatomical connectivity. The more important parameter that predicts whether transmission is synaptic or otherwise appears to be the affinity of the receptors and transporters. Indeed, it is striking that for both 5-HT and dopamine the affinity of their respective transporters matches closely to the concentration released into the extracellular space by a single impulse. This adds strong credence to the belief that voltammetrically detected concentrations are those that are physiologically important.

Although these results show that paracrine neurotransmission is possible for 5-HT in the DR and SNr, they do not preclude simultaneous synaptic communication. Because glutamate and GABA receptors do not activate second messenger systems but, rather, directly activate ion channels, their synaptic effects can be studied by electrophysiological techniques. This is not the case for DA or for 5-HT, because most of their receptors are coupled to second messengers (Kandel et al., 1991). The ability to monitor their extracellular concentrations with carbon fiber microelectrodes, however, provides a unique way to probe their function, as shown here. Note that 5-HT$_3$ receptors are members of the ligand-gated ion channel family and could be associated with fast synaptic neurotransmission (Peters et al., 1992). 5-HT$_3$ receptors have a lower affinity for 5-HT than 5-HT$_1$ receptors (Hoyer, 1990) and have a high density in the area postrema, the entorhinal cortex, the amygdala, and certain brainstem nuclei (Kilpatrick et al., 1990). Thus, it is possible in these regions that 5-HT neurotransmission is restricted more spatially.

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