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 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 eflux 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; Asztyely 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-
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; Irvani and Kruk, 1997; Bunin et al., 1998). Early studies suggested that 5-HT accumulation was restricted to cell bodies and dendrites (Fuxe, 1965; Loizou, 1979; 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 extrasynaptic 5-HT concentration evoked by a single stimulus 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 g) 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 2.7 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 a raised surface. Unless stated otherwise, the stimulus waveform consisted of a 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; Thorne 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 281 (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 (Ensmann Instrumentation, Bloomington, IN), operated in three electrode mode, was used to record cyclic voltammograms every 100 msec. The output was computer-digitized, integrated, and displayed on a personal computer (typically 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 extrasynaptic 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 per 5-HT 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-
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. When 20 stimulation pulses (350 μA) were delivered at 100 Hz, the maximal concentration of 5-HT elicited by a single pulse was 0.07 ± 0.03 μM/sec; the mean amplitude of the concentration change (0.08 ± 0.02 μM/sec; n = 4 slices) is the maximal concentration of 5-HT elicited by a single stimulus pulse. Thus, in both regions the concentration of 5-HT 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 ± 0.03 μM) is not statistically different from the value of [5-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-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).
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-HT₁/5-HT₂ 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 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-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. 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-HT₁) for the endogenous ligand to be in the low nanomolar range (Green and Maayani, 1987; Pranzatelli, 1989). In this work the maximal...
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 synaptic, consistent with recent anatomical findings (Tao-Cheng and Zhou, 1997; Zhou and Tao-Cheng, 1997).

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 $B_{\text{max}}$ 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 µl, the ratio of $V_{\text{max}}$ to $B_{\text{max}}$ yields the turnover number. This was found to be 2.4 ± 0.3 and 2.6 ± 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, $1.5 \times 10^{-7}$ mol·m$^{-1}$·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 × 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 µm. The dimensions of this cube are smaller than our sensor (10 µm 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 µm 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 sec 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 (Pothis 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 transporters sites. The number of transporter sites per terminal can be calculated from the $B_{\text{max}}$ 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$_{i}$...
receptors can be computed. \( B_{\text{max}} \) 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 previously estimated 3500 molecules released per terminal were all in its initial stored state until it reaches its receptor site. If the raphe neurons (Hajos et al., 1996).


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