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Transport mechanisms governing serotonin clearance in vivo revealed by high-speed chronoamperometry

Lynette C. Daws[a,b,c,*], Sylvia Montañez[a], W. Anthony Owens[a], Georgianna G. Gould[b], Alan Frazer[b], Glenn M. Toney[c], Greg A. Gerhardt[d]

[a] *Department of Psychiatry, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA*

[b] *Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA*

[c] *Department of Physiology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA*

[d] *Departments of Anatomy and Neurobiology, Neurology, Psychiatry, Morris K. Udall Parkinson's Disease Research Center of Excellence, Center for Sensor Technology, University of Kentucky, Lexington, KY, USA*

Abstract

High-speed chronoamperometry was used to determine the kinetics of clearance of exogenously applied serotonin (5-HT) in the dorsal raphe nucleus (DRN), dentate gyrus, CA3 region of the hippocampus or corpus callosum of anesthetized rats. Maximal velocity (V_{max}) for 5-HT clearance was greatest in the DRN > dentate gyrus > CA3 > corpus callosum. Apparent affinity (K_T) of the serotonin transporter (5-HTT) was similar in DRN and CA3 but greater in dentate gyrus and corpus callosum. A 90% loss of norepinephrine transporters (NET) produced by 6-hydroxydopamine pretreatment, resulted in a two-fold reduction in V_{max} and a 30% decrease in K_T in the dentate gyrus, but no change in kinetic parameters in the CA3 region. Pretreatment with 5,7-dihydroxytryptamine that resulted in a 90% reduction in 5-HTT density, modestly reduced V_{max} in dentate gyrus but not in CA3. The same treatment had no effect on K_T in the dentate gyrus but increased K_T two-fold in the CA3. Neurotoxin treatments had no effect on 5-HT clearance in the corpus callosum. In hippocampal regions of intact rats, local application of the selective serotonin reuptake inhibitor, fluvoxamine, inhibited 5-HT clearance most robustly when the extracellular concentration of 5-HT was less than the K_T value. By contrast, the NET antagonist, desipramine, significantly inhibited 5-HT clearance when extracellular concentrations of 5-HT were greater than the K_T value. These data indicate that hippocampal uptake of 5-HT may be mediated by two processes, one with high affinity but low capacity (i.e. the 5-HTT) and the other with low affinity but a high capacity (i.e. the NET). These data show for the first time in the whole animal that 5-HT clearance in brain is regionally distinct with regard to rate and affinity.

Keywords: Serotonin transporter; Norepinephrine transporter; Chronoamperometry; Serotonin clearance rates; Hippocampus; Dorsal raphe nucleus; Rat

1. Introduction

Uptake of serotonin (5-HT) is the principle active mechanism for removal of 5-HT from extracellular fluid of brain. Because 5-HT is important in regulating many complex behaviors and physiological functions (e.g. thermoregulation, feeding, sleep and mood), understanding the kinetics of 5-HT clearance and factors that influence it has great significance. Unlike dopamine, the kinetics of 5-HT uptake in vivo have not been well characterized. Bunin and co-workers, in an elegant series of experiments, used fast scan cyclic voltammetry to evaluate 5-HT release and uptake in brain slices containing the dorsal raphe nucleus (DRN) or substantia nigra (Bunin and Wightman, 1999; Bunin et al., 1998). These data produced seminal evidence for the existence of volume or paracrine transmission as a mode of serotonergic neurotransmission, at least in these brain regions.

Active uptake by the serotonin transporter (5-HTT) as well as diffusion contribute to clearance of 5-HT away from its re-

* Corresponding author. Tel.: +1 210 567 0171; fax: +1 210 567 5381.
E-mail address: daws@uthscsa.edu (L.C. Daws).

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lease site, but evidence indicates that other factors may also participate. For example, we have shown that in the dentate gyrus the norepinephrine transporter (NET) is able to remove 5-HT from extracellular fluid in vivo (Daws et al., 1998). Evidence for 5-HT uptake by the NET and also the dopamine transporter has been provided by others (Jackson and Wightman, 1995; Shaskan and Snyder, 1970; Zhou et al., 2002) suggesting that promiscuity among transporters for the biogenic amines exists.

In this study, our aim was to determine the relationship between 5-HTT density and the kinetics of 5-HT clearance as well as conditions under which transporter "promiscuity" occurs in vivo. We used high-speed chronoamperometry to investigate clearance of exogenously applied 5-HT in four brain regions of anesthetized rats. This approach allows 5-HT clearance to be measured in the absence of any direct contributions from endogenously released 5-HT (Daws et al., 2000). The brain regions compared were the DRN (a cell body region that expresses the greatest density of 5-HTTs in brain) (Hensler et al., 1994; Montañez et al., 2002), two terminal field regions, the dentate gyrus and CA3 region of the hippocampus (which express four- to nine-fold fewer 5-HTTs than in DRN) (Hensler et al., 1994; Montañez et al., 2002; Swanson et al., 1987) and the corpus callosum, a fiber tract with comparatively little 5-HTT expression (Oleskevich and Descarries, 1990; Sur et al., 1996; Zhou et al., 1998). Our general hypothesis was that the maximal velocity (V_{max}) for 5-HT clearance and "apparent" transporter affinity (K_T) for 5-HT depend not only on the density of 5-HTTs but also on "non-selective" uptake by catecholaminergic transporters. Because studies of transporter affinity and uptake capacity in vitro (e.g. Shaskan and Snyder, 1970) report the existence of two uptake processes for 5-HT, a high affinity–low capacity process, the 5-HTT, and a low affinity–high capacity process, presumably catecholaminergic transporters, we hypothesized that in brain regions where the NET predominates (e.g. dentate gyrus), the apparent K_T value would be greater (i.e. lower affinity) than in regions where the 5-HTT predominates (e.g. CA3 region of hippocampus and DRN) (see Hensler et al., 1994; Tejani-Butt, 1992). We also hypothesized that the ability of selective uptake inhibitors of 5-HT and norepinephrine to inhibit clearance of exogenously applied 5-HT would be dependent on the extracellular fluid concentration of 5-HT at the time of their application. These studies extend our earlier work by applying high-speed chronoamperometry to calculate kinetic parameters (V_{max} and K_T) for 5-HT clearance in vivo.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA), weighing 280–380 g, were used for all experiments. They were housed in groups of three, or individually after recovering from surgery and maintained under a 12:12 h light dark cycle with food and water provided ad libitum. All animal procedures were approved by the local institutional animal care and use committee and were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize both the number of animals used and stress or discomfort to the animals during experimental procedures.

2.2. Electrochemical recordings

High-speed chronoamperometric recordings were made using the FAST-12 system (Quanteon, Nicholasville, KY). Oxidation potentials consisted of 100 ms pulses of +0.55 V. Each pulse was separated by a 900 ms interval during which the electrode potential was maintained at 0.0 V. Voltage at the active electrode was applied with respect to an Ag/AgCl reference electrode positioned in the extracellular fluid of the ipsilateral superficial cortex. Oxidation and reduction currents were digitized and averaged over the last 80 ms of each 100 ms voltage pulse.

2.2.1. Electrode preparation and in vitro calibration

Carbon fiber electrodes (active recording area 30 μm tip diameter × 300 μm length, Quanteon, Nicholasville, KY) were coated 3–6 times with Nafion® (5% solution, Aldrich Chemical Co, Milwaukee, WI), to prevent interference from anionic substances in extracellular fluid (Daws et al., 1997; Gerhardt and Hoffman, 2001; Gerhardt et al., 1984). Electrodes were dried at 200 °C between each coat. Electrodes were tested for sensitivity to the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA, 250 μM) and calibrated with 6 (0.5–3.0 μM) or 12 (1.0–12.0 μM) accumulating concentrations of 5-HT depending on the experiment. Only electrodes displaying a selectivity ratio for 5-HT over 5-HIAA greater than 500:1 and a linear response ($r^2 \geq 0.90$) to 5-HT were used. The detection limit for the measurement of 5-HT was defined as the concentration that produced a response with a signal-to-noise ratio of 3 and in these experiments averaged 43 ± 8 nM ($n = 168$).

Electrodes were calibrated prior to use in vivo. Post-calibrations were not carried out, as it is not known when and by what function electrodes lose sensitivity. In fact, sensitivity can be lost when electrodes are removed from brain, due to adsorption of blood and proteins, if they are not carefully handled. Because of this, start of experiment calibration values were used as they can be easily calculated and loss of electrode sensitivity can be easily determined during the experiment. Loss of sensitivity was defined as a greater than 15% decrease in signal amplitude for the same amount of serotonin pressure-ejected at randomized intervals throughout the experiment. In the event of this occurring, the electrode was replaced.

2.2.2. In vivo experimental protocols

As described in Daws et al. (1998) rats were anesthetized by intraperitoneal (ip) injection of chloralose (85 mg/kg)

and urethane (850 mg/kg). A tube was inserted into the trachea to facilitate breathing and the rat was placed into a stereotaxic frame. The electrode–micropipette assembly was positioned in brain according to the following stereotaxic coordinates where all anterior–posterior (AP) measures are from bregma, medial–lateral (ML) measures are from midline and dorsal–ventral (DV) measures are from dura: DRN (AP, −7.8; ML, +1.7; DV, −5.2 to −5.6; at an angle 13° to the vertical plane), the dentate gyrus (AP, −3.8; ML, +1.6; DV, −3.0 to −3.2), the CA3 region of the dorsal hippocampus (AP, −4.1; ML, +3.3; DV, −2.7 to 3.0), or the corpus callosum (AP, −4.3; ML, +1.0; DV, −2.2) according to the atlas of Paxinos and Watson (1986). Body temperature was maintained at 37 ± 1 °C by a water circulated heating pad (Seabrook, Cincinnati, OH).

The electrochemical recording assembly consisted of a Nafion-coated, single carbon fiber electrode attached to a 7-barrelled micropipette (Frederick Haer Corp. Inc. Bowdoinham, ME). The assembly was constructed using a micropositioner such that the electrode and micropipette tips were separated by 300 μm. The tip diameter of each barrel of the multi-barrelled micropipette was between 10–15 μm. Barrels were filled with either 5-HT (200 μM), the selective serotonin reuptake inhibitor (SSRI) fluvoxamine (400 μM), the norepinephrine uptake inhibitor desipramine (DMI, 400 μM) or vehicle. All drugs were dissolved in 0.1 M phosphate-buffered saline with 100 μM ascorbic acid added as an antioxidant with the exception of DMI, which was dissolved in ultra pure water with 100 μM ascorbic acid. The pH of all solutions was adjusted to 7.3–7.4.

2.2.3. Brain region dependency of 5-HT clearance

In these experiments 5-HT (200 μM) was pressure-ejected (5–25 psi for 0.25–3 s) into either the DRN, dentate gyrus, CA3 region of the hippocampus or the corpus callosum. The volume of 5-HT delivered (10–150 nl) was measured by determining the amount of fluid displaced from the micropipette using a dissection microscope fitted with an eyepiece reticule and was varied to yield signals ranging in amplitude from 0.25 to 12.0 μM. This approach was adopted based on a comprehensive study by Zahniser and colleagues (1999) who reported no difference in clearance parameters when different pmol amounts of DA were achieved by keeping DA concentration constant and varying volume ejected, versus ejecting the same volume of different barrel concentrations of DA. The sequence of 5-HT signals with different amplitudes was randomized between each subject. From these data, estimates of the maximal velocity of clearance (V_{max}) and apparent affinity of the transporter for serotonin (K_T) could be calculated and compared between brain regions. These experiments were also repeated in the CA3 region, dentate gyrus and corpus callosum of rats previously treated with either 5,7-dihydroxytryptamine (5,7-DHT) or 6-hydroxydopamine (6-OHDA) to destroy serotonergic and noradrenergic neurons, respectively. The DRN was not included in this series of experiments as it was not possible to achieve a >90% loss of [^{3}H]-cyanoimipramine binding to the 5-HTT after intracerebroventricular administration of 5,7-DHT.

2.2.4. Effect of serotonin and norepinephrine uptake inhibitors on 5-HT clearance in the CA3 region of the hippocampus

In these experiments 5-HT (200 μM) was pressure-ejected to yield signals that averaged 0.5 or 4.0 μM. For low signal amplitudes 3 ± 1 pmol (15 ± 3 nl) of 5-HT was delivered and for high signal amplitudes 20 ± 3 pmol (99 ± 12 nl) 5-HT was pressure-ejected. Once a reproducible signal was obtained (usually after 3–4 applications), vehicle or drug (fluvoxamine or DMI) was applied 2 min before the next application of 5-HT in order to allow sufficient time for diffusion of drug to areas around the recording electrode. In addition, we find that the transient decrease in signal amplitude that is sometimes observed after DMI, presumably due to its transient adsorption to the electrode (Hoffman and Gerhardt, 1998), can be avoided by delaying the post-drug application of 5-HT by 2 min. These solutions were pressure-ejected over 10–20 s to minimize disturbances to the baseline electrochemical signal. The volume of drug or vehicle ejected was 2–4 times the volume of 5-HT. Signals were allowed to return to baseline before administration of another drug or vehicle. The order of drug administration was randomized between experiments to eliminate order effects.

2.3. Histology

At the conclusion of each in vivo electrochemical recording experiment, an electrolytic lesion was made to mark the placement of the electrode tip and the rat was decapitated while still anesthetized. The brain was removed before being rapidly frozen on dry ice and stored at −80 °C until use. At this time brains were thawed to −15 °C and cut into 20 μm sections for either histological verification of electrode localization and/or autoradiographic quantitation of 5-HTT or NET density. Only data from rats where the electrode tip was confirmed to be in the desired target brain region were included in the data analyses.

2.4. 5,7-DHT and 6-OHDA lesions

Rats were intracerebroventricularly administered either 5,7-DHT or 6-OHDA. Animals lesioned with 5,7-DHT were given nomifensine (30 mg/kg, ip) 30 min before infusion of 5,7-DHT to prevent destruction of noradrenergic and dopaminergic neurons (Gershanik et al., 1979); those lesioned with 6-OHDA were given sertraline (10 mg/kg, ip) and GBR12909 (30 mg/kg, ip) 30 min prior to 6-OHDA to prevent the destruction of serotonergic and dopaminergic neurons, respectively (McCann et al., 1997; Nissbrant et al., 1991). Animals were anesthetized with chloral hydrate (400 mg/kg, ip) and placed into a stereotaxic frame. Neurotoxin was delivered bilaterally into the lateral ventricles using a 28-gauge stainless steel injector, connected to a microsy-

ringe pump (stereotaxic coordinates: AP −0.8 from bregma; ML 1.5 from midline; DV −3.7 from skull). Ten microliters of phosphate-buffered saline containing 100 μg of neurotoxin and 100 μM ascorbic acid was infused into each ventricle at a rate of 1 μl/min. The total amount of 5,7-DHT or 6-OHDA delivered was therefore 200 μg. After completion of the intracerebroventricular infusion, the injector was left in place for 5 min to allow diffusion of neurotoxin from the injector. Control rats were subjected to the same procedure but were infused with vehicle containing 100 μM ascorbic acid alone. The control rats also received pretreatment with nomifensine (5,7-DHT sham) or sertraline and GBR 12909 (6-OHDA sham) to mimic the pretreatment of the lesioned groups. In vivo electrochemical recordings were performed 2–4 weeks after the lesioning procedure. The extent of lesion was confirmed by quantitative autoradiography binding of [^{3}H]-cyanoimipramine to the 5-HTT and of [^{3}H]-nisoxetine binding to the NET.

2.5. Quantitative autoradiography

2.5.1. [^{3}H]-cyanoimipramine binding

Brain sections (20 μm) were cut in a cryostat at −15 °C, thaw-mounted onto gelatin-coated slides and dehydrated overnight at 0–4 °C. Serotonin uptake sites were measured using [^{3}H]-cyanoimipramine according to the method of Kovachich et al. (1988). Brain sections were incubated with 1 nM [^{3}H]-cyanoimipramine (80–85 Ci/mmol: American Radiolabelled Chemicals) in a buffer consisting of 50 mM Tris, pH 7.4 and 120 mM NaCl at 4 °C for 24 h. Non-specific binding was defined using 5 μM sertraline and was approximately 5% of total binding. After incubation, sections were washed in cold buffer for 60 min at 4 °C, dipped in cold distilled water and then dried on a slide warmer at 60 °C. Sections and calibrated [^{3}H] standards (American Radiolabelled Chemicals, St. Louis, MO) were exposed against Hyperfilm-[^{3}H] (CEA AB Sweden for Amersham) for 14 days to generate autoradiogram (or 7 days for DRN). Optical densities of brain images were converted to femtomoles per milligram protein using NIH-IMAGE software, which compared optical densities to images of [^{3}H] standards on the same film. Measurements were taken from plates containing the DRN, CA3, dentate gyrus or corpus callosum using the atlas of Paxinos and Watson (1986). The concentration of [^{3}H]-cyanoimipramine used is approximately 8× its K_D value (Kovachich et al., 1988) so the values obtained approximate 89% of B_{max} values.

2.5.2. [^{3}H]-nisoxetine binding

Brain sections (20 μm) were cut in a cryostat at −15 °C, thaw-mounted onto gelatin-coated slides and dehydrated overnight at 0–4 °C. Norepinephrine uptake sites were measured using [^{3}H]-nisoxetine according to the method of Tejani-Butt (1992). Brain sections were incubated with 3 nM [^{3}H]-nisoxetine (86 Ci/mmol; Amersham) in a buffer consisting of 50 mM Tris, pH 7.4, 300 mM NaCl and 5 mM KCl at 4 °C for 4 h. Non-specific binding was defined using 10 μM mazindol and was approximately 5% of total binding. After incubation, sections were washed three times for 5 min each in cold buffer, dipped in ice-cold distilled water and then dried on a slide warmer at 60 °C. Sections and calibrated [^{3}H] standards were exposed against Hyperfilm-[^{3}H] for 5 weeks to generate autoradiograms. These were quantified as described above. The concentration of [^{3}H]-nisoxetine used is approximately 4× its K_D value (Tejani-Butt, 1992) so the values obtained approximate 80% of B_{max} values.

2.6. Data analyses

Shown in Fig. 1 (upper panel) is a typical signal evoked by the exogenous application of 5-HT into the CA3 region of the hippocampus. Shown is the oxidation current converted to a micromolar value using the calibration factor derived in vitro. From this the velocity of clearance (*V*) for 5-HT was calculated by fitting the descending portion of the signal to an equation describing one-phase exponential decay. This type of analysis has been used to determine kinetic parameters for DA clearance in the striatum of freely moving rats and is described in detail in Sabeti et al. (2002). Fig. 1 (lower panel) illustrates the fit of the descending portion of the above

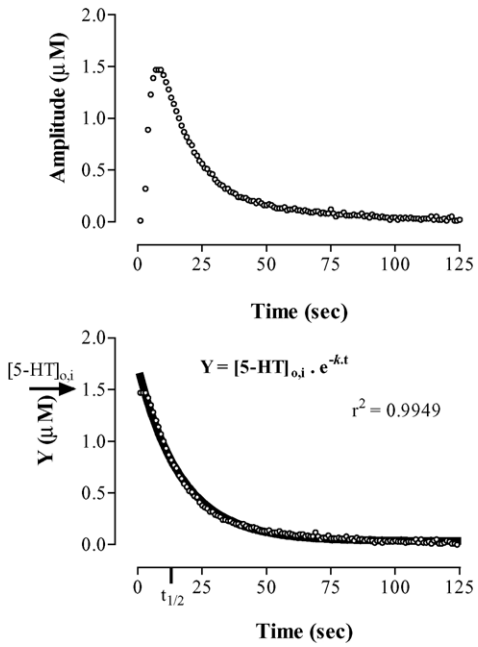

Fig. 1. Fit of the descending portion of a 5-HT signal to an equation describing one-phase exponential decay. Shown in the upper panel is the current, converted to a micromolar value using a calibration factor determined in vitro, produced by pressure-ejection of 5-HT into the CA3 region of the hippocampus. The lower panel shows the fit of the descending portion of this signal to an equation describing one-phase exponential decay, where *Y* is the concentration of 5-HT, $[5\text{-HT}]_{o,i}$ is the initial maximal extracellular concentration of 5-HT, *t* is the time, and *k* is the rate constant. The half-life of decay is 0.6932/*k*. Shown are the actual signal (open circles) and the line fitting the equation (solid line). From this equation, *k* is derived. The product of *k* and the maximal amplitude of the signal gives *V*.

oxidation current to the following equation describing a one-phase exponential decay:

$$Y = [5\text{-HT}]_{o,i}\, e^{-kt} + [5\text{-HT}]_{o,t}$$

where Y is the concentration of 5-HT (μM), $[5\text{-HT}]_{o,i}$ the initial maximal extracellular concentration of 5-HT (μM), $[5\text{-HT}]_{o,t}$ the extracellular concentration of 5-HT (μM) at baseline and k the rate constant (s^{-1}). The half-life of decay is $0.6932/k$. Note that because the concentration of 5-HT at baseline (i.e. $[5\text{-HT}]_{o,t}$) equals zero (the value given to represent the baseline electrochemical signal prior to addition of exogenous 5-HT), this component of the equation is redundant and subsequently not used in the analyses. The calculated k value from this equation is then multiplied by the amplitude of the signal to give the velocity of transport (V). These calculations are made for signals of increasing amplitude until an apparent V_{max} is reached. Data are organized according to signals of increasing amplitudes and then averaged into bins according to a pseudo-geometric progression. V_{max} and K_T (concentration of 5-HT that corresponds to $V_{max}/2$) values for group data are then determined by fitting a four-parameter logistic equation (sigmoid function with variable slope and minimum value constrained to zero) to a plot of signal amplitude versus V. Each point on the curves used for kinetic analyses of 5-HT clearance was derived from four to eight rats.

K_T values were corrected for volume fraction (α). In the dentate gyrus and CA3 region of the hippocampus this value has been calculated to range between 0.18 and 0.22. The volume fraction for the DRN has not been previously reported; however α appears to be relatively homogenous throughout brain (see Nicholson and Syková, 1998). For these studies, α was taken to be 0.20 in all regions.

In addition, the effect of uptake inhibitors on $T_{20–60}$ (the time, in seconds, for the signal to decline from 20% to 60% of the maximal amplitude) and T_{80} (the time for the signal to decline by 80% of the maximal amplitude) values were analyzed. We have previously found that SSRIs produce robust effects on the T_{80} time course parameter (e.g. Daws et al., 1997, 1998, 2000; Montañez et al., 2002, 2003a,b). However, Nafion coating has a tendency to make electrodes "adsorptive". The T_{80} parameter may therefore be affected by the presence of 5-HT and other hydroxyindoles that can form an insulating film on the electrode after electro oxidation (Jackson et al., 1995). For this reason the $T_{20–60}$ time course parameter was also evaluated. This parameter occurs earlier, in the relatively linear declining phase of the electrochemical signal produced by 5-HT and thus is less likely to be affected by adsorption of 5-HT.

Amplitude and time course data were analyzed with paired, two-tailed t-tests (pre- versus post-application of drug). The percentage change from pre-drug value for these parameters was analyzed by Mann–Whitney U-tests. One-way analysis of variance (ANOVA) with Bonferroni post-hoc comparisons were used to test for significant differences in V or K_T between brain regions or treatment groups (sham versus 5,7-DHT versus 6-OHDA). Two-way ANOVA with Bonferroni post-hoc comparisons were carried out to assess the main effect of treatment and signal amplitude on V. A two-tailed probability level of $P < 0.05$ was accepted as statistically significant for all tests. Data are expressed throughout as mean ± standard error of the mean (S.E.M.).

2.7. Drugs

Serotonin hydrochloride, 5-HIAA, desipramine hydrochloride, GBR 12909 dihydrochloride, 6-OHDA hydrobromide and 5,7-DHT creatinine sulfate were purchased from Sigma (St. Louis, MO). Fluvoxamine maleate was generously provided by Pharmacia and Upjohn (Kalamazoo, MI), nomifensine maleate was a gift from Hoechst-Roussel Pharmaceuticals (North Somerville, NJ), sertraline hydrochloride was provided by Pfizer Inc. (Groton, CT) and mazindol from the Sandoz Institute (East Hanover, NJ).

3. Results

3.1. Kinetic parameters for 5-HT clearance in different brain regions

To determine if clearance of 5-HT is dependent on brain region, we studied clearance of exogenously applied 5-HT from extracellular fluid in the DRN, dentate gyrus, CA3 region of the hippocampus and the corpus callosum. These regions were chosen in order to compare a 5-HTT rich area (DRN) to those with significantly lower 5-HTT density (dentate gyrus and CA3 regions in the hippocampus) or an area with essentially no 5-HTT (corpus callosum). The relative density of [^{3}H]-cyanoimipramine binding to 5-HTTs in these brain regions is summarized in Fig. 2. The density of 5-HTTs in the DRN is approximately 4.5-, 9- and 52-fold greater than in the CA3 region of hippocampus, dentate gyrus and corpus callosum, respectively. The density of 5-HTTs in the CA3 region of hippocampus is approximately 2- and 11.5-fold greater than in the dentate gyrus and corpus callosum, respectively. In the dentate gyrus the density of 5-HTTs is approximately six-fold greater than in the corpus callosum.

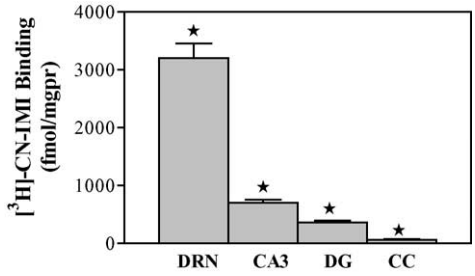

Fig. 2. Specific binding of [^{3}H]-cyanoimipramine (CN-IMI) to 5-HTTs in the dorsal raphe nucleus (DRN), dentate gyrus (DG), CA3 region of hippocampus and corpus callosum (CC). $^\star P < 0.001$ one-way ANOVA with Bonferroni post-hoc comparisons, from all other brain regions.

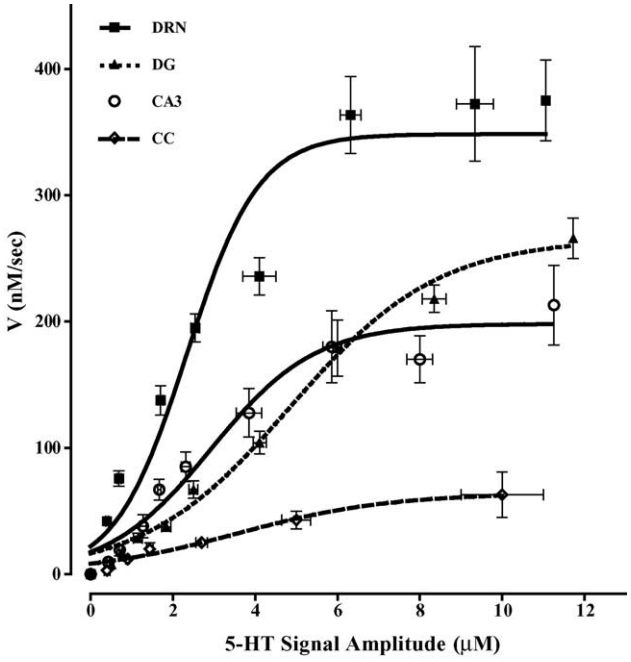

Fig. 3. Velocity of 5-HT clearance in the DRN, dentate gyrus (DG), CA3 region of hippocampus and corpus callosum (CC) as a function of increasing extracellular concentration of 5-HT. Values represent mean ± S.E.M. from four to eight rats per point. Curves represent fits of a four-parameter logistic equation to the data. This equation fits a sigmoid function with variable slope. The minimum value was constrained at zero. V_{max} and K_T values were determined from this equation and are summarized in Table 1.

Table 1
Summary of V_{max} and K_T values for 5-HT clearance in DRN, dentate gyrus (DG), CA3 region of hippocampus and corpus callosum (CC) of intact rats

Brain region	V_{max} (nM/s)	K_T (μM)	K_T (μM), corrected for α (=0.2)
DRN	$368 \pm 6^{**}$	2.74 ± 0.10	0.54 ± 0.02
DG	$266 \pm 4^{**}$	$4.86 \pm 0.10^{*}$	$0.97 \pm 0.02^{*}$
CA3	$198 \pm 4^{**}$	2.93 ± 0.11	0.58 ± 0.02
CC	$64 \pm 2^{**}$	$3.55 \pm 0.16^{*}$	$0.71 \pm 0.03^{*}$

V_{max} and K_T values were determined by fitting the data shown in Fig. 3 to a four-parameter logistic equation.

* $P < 0.01$ one-way ANOVA with Bonferroni post-hoc comparisons, from all other brain regions.

** $P < 0.001$ one-way ANOVA with Bonferroni post-hoc comparisons, from all other brain regions.

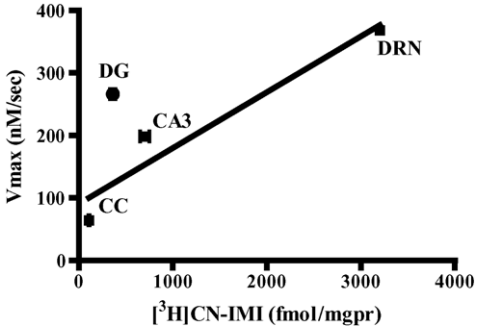

Fig. 5. Maximal velocity for serotonin clearance as a function of [^{3}H]-cyanoimipramine (CN-IMI) binding. V_{max} values are those determined from fitting the data in Fig. 3 to a four-parameter logistic equation. Regression analysis of data derived from DRN, CA3 region of hippocampus and corpus callosum (CC) revealed a strong positive correlation between V_{max} and 5-HTT density (solid line, $r^2 = 0.9268$). Inclusion of the dentate gyrus (DG) yielded a markedly weaker correlation ($r^2 = 0.6518$).

Shown in Fig. 3 are the *V* values for 5-HT clearance plotted against signal amplitude. V_{max} and K_T values were derived by fitting the data to a four-parameter logistic equation (sigmoid curve with variable slope and minimum constrained to zero). V_{max} and K_T values are summarized in Table 1. The velocity for 5-HT clearance appeared to be saturable in all brain regions with V_{max} values being greatest in the DRN > dentate gyrus > CA3 > corpus callosum (see Fig. 3 and Table 1). The V_{max} values for each brain region were significantly different from each other. Representative 5-HT signals are shown in Fig. 4. K_T values for 5-HT clearance were similar in the DRN and CA3 region of the hippocampus, but significantly greater in the dentate gyrus and corpus callosum (see Table 1).

As highlighted in Fig. 5, linear regression analysis of [^{3}H]-cyanoimipramine binding to the 5-HTT and V_{max} values for 5-HT clearance in the DRN, CA3 region of hippocampus and corpus callosum yielded a strong, positive correlation ($r^2 = 0.9268$). Thus, V_{max} for 5-HT clearance increases with

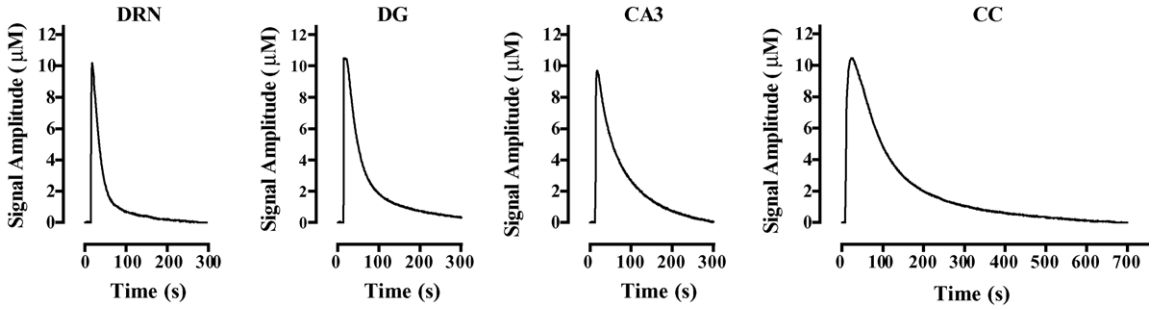

Fig. 4. Representative oxidation currents for 5-HT pressure-ejected into four different brain regions to attain maximal velocity for 5-HT clearance. The oxidation currents are converted into micromolar units using a calibration factor determined in vitro. The velocity of 5-HT clearance for each signal is representative of the V_{max} value for each brain region. The *V* values are 465, 239, 159 and 109 nM/s for the DRN, dentate gyrus (DG), CA3 region of hippocampus and corpus callosum (CC), respectively.

Table 2
Specific binding for [^{3}H]-cyanoimipramine to the 5-HTT and [^{3}H]-nisoxetine to the NET in the CA3 region of hippocampus, dentate gyrus (DG) and corpus callosum (CC) of rats treated with either 5,7-DHT or 6-OHDA or vehicle (sham)

Brain region	[^{3}H]-cyanoimipramine (fmol/mg pr)			[^{3}H]-nisoxetine (fmol/mg pr)		
	Sham	5,7-DHT	6-OHDA	Sham	5,7-DHT	6-OHDA
CA3	668 ± 14	36 ± 5	622 ± 19	428 ± 14	379 ± 28	41 ± 7
DG	381 ± 19	35 ± 5	362 ± 25	650 ± 17	618 ± 21	58 ± 18
CC	108 ± 5	31 ± 3	96 ± 10	68 ± 4	56 ± 8	19 ± 3

increasing 5-HTT density. However, when data for the dentate gyrus were included in the regression analysis the correlation coefficient was markedly reduced ($r^2 = 0.6518$).

It is important to emphasize that calibration curves for 5-HT constructed in vitro, remained linear with increasing concentrations of 5-HT to as high as 12 μM ($r^2 \geq 0.90$). Because of this, it is unlikely that the curvilinear nature of the clearance profiles is due to reduced sensitivity of the electrode. It is also important to note that electrodes were exposed to high concentrations of 5-HT for a maximum of three times in order to minimize loss of electrode sensitivity due to adsorption of electrogenerated products at the electrode surface (Jackson et al., 1995). We found that by minimizing exposure of the electrode to high 5-HT concentrations and by randomizing the order in which concentrations of 5-HT were applied, the sensitivity of the electrode did not diminish appreciably over the course of a given experiment. In the event where an electrode clearly had become less sensitive to 5-HT, the electrode/micropipette assembly was removed, the electrode discarded and a new electrode was attached to the micropipette before recordings were continued.

3.2. Effects of neurotoxic lesion on 5-HT clearance parameters in the CA3 region of the hippocampus, dentate gyrus and corpus callosum

We previously have shown that the NET is able to clear 5-HT from extracellular fluid in the dentate gyrus (Daws et al., 1998). These data, together with earlier reports that 5-HT can be taken up by at least two transport processes, a high affinity-low capacity process into serotonergic neurons and the other, a low affinity–high capacity process, occurring in catecholaminergic neurons (Lichtensteiger et al., 1967; Shaskan and Snyder, 1970), prompted us to determine the relative involvement of the 5-HTT and the NET in defining the differences in V_{max} and K_T values for 5-HT clearance between the CA3 region of the hippocampus and dentate gyrus. To this end rats were treated with either 5,7-DHT or 6-OHDA. These treatments resulted in a greater than 90% reduction in [^{3}H]-cyanoimipramine binding to the 5-HTT and [^{3}H]-nisoxetine binding to the NET, respectively (see Table 2). Treatment with 5,7-DHT did not alter [^{3}H]-nisoxetine binding and likewise, 6-OHDA treatment did not alter [^{3}H]-cyanoimipramine binding in either region (Table 2). Non-specific binding represented less than 10% of the total binding.

Studies were also carried out in the corpus callosum. In control animals total binding was very low. Because of this, non-specific binding, which did not differ from that obtained in other brain regions, comprised approximately one third of the total binding (e.g. total [^{3}H]-cyanoimipramine binding 170 fmol/mg pr versus non-specific binding 63 fmol/mg pr; total [^{3}H]-nisoxetine binding 103 fmol/mg pr versus non-specific binding 35 fmol/mg pr). Treatment with 5,7-DHT or 6-OHDA reduced binding of [^{3}H]-cyanoimipramine and [^{3}H]-nisoxetine, respectively, to levels comparable with that observed in the CA3 region of hippocampus and dentate gyrus (see Table 2). However, because of the low baseline binding, these treatments constituted only a 70% reduction in [^{3}H]-cyanoimipramine and [^{3}H]-nisoxetine binding sites, respectively. It should be noted that efforts were made to carry out comparable studies in the DRN, however, it was not possible to obtain greater than 70% loss of [^{3}H]-cyanoimipramine binding sites in the DRN via intracerebroventricular administration of 5,7-DHT. For this reason, the DRN was not included in this aspect of the study.

Shown in Fig. 6 is the consequence of treatment with 5,7-DHT or 6-OHDA on 5-HT clearance in the CA3 region of hippocampus, dentate gyrus and corpus callosum. V_{max} and K_T values for each of these groups are summarized in Table 3. In the CA3 region the most notable change in the 5-HT clearance profile occurred in 5,7-DHT-treated rats (Fig. 6, left panel). Although statistical analyses did not reveal any significant effect of treatment on either V_{max} or K_T values, the curve in 5,7-DHT treated rats was clearly shifted to the right of that for either sham or 6-OHDA-treated rats. The lack of statistical significance may be attributed to the large error associated with the K_T value derived from fitting the curve to a four-parameter logistic equation (see Table 3). Two-way ANOVA (treatment × signal amplitude) revealed significant effects of both treatment ($P < 0.0034$) and signal amplitude ($P < 0.0001$) on the velocity of 5-HT clearance. Bonferroni's post-hoc tests showed this to be due to significantly lower velocities for 5-HT clearance at intermediate signal amplitudes in 5,7-DHT treated rats (see Fig. 6, left panel). Consistent with no significant difference in V_{max} values between sham and 5,7-DHT-treated rats, the velocity of 5-HT clearance was not different between treatment groups at the highest signal amplitude tested (ca. 11 μM) (Fig. 6, left panel).

In the dentate gyrus a different pattern emerged (see Fig. 6, middle panel). Here both 5,7-DHT and 6-OHDA treatments resulted in a significant reduction in the V_{max} for 5-HT clear-

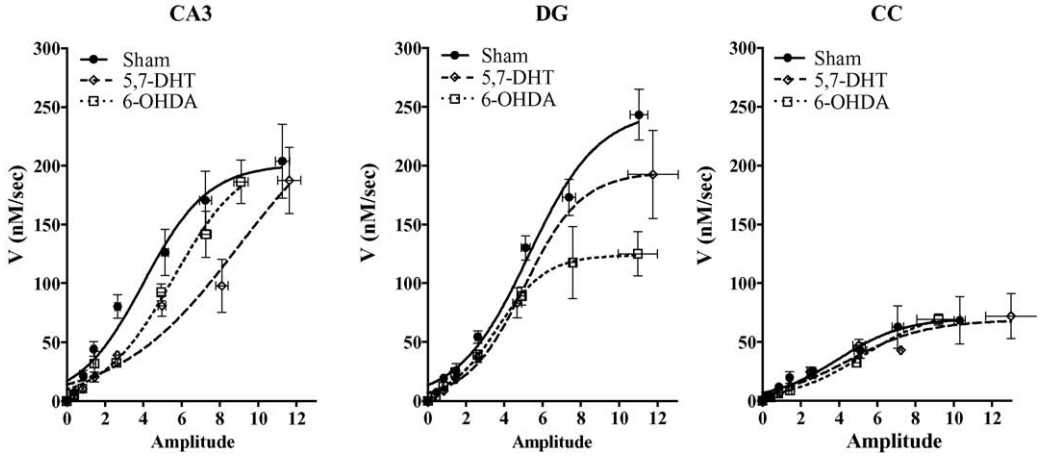

Fig. 6. Velocity of 5-HT clearance in the CA3 region of hippocampus, dentate gyrus (DG) and corpus callosum (CC) of rats treated with 5,7-DHT or 6-OHDA as a function of increasing extracellular concentration of 5-HT. Values represent mean ± S.E.M. from four to eight rats per point. Curves represent fits of a four-parameter logistic equation to the data. This equation fits a sigmoid function with variable slope. The minimum value was constrained at zero. V_{max} and K_T values were determined from this equation and are summarized in Table 3.

ance compared to control rats. Moreover, 6-OHDA treatment produced a greater reduction in V_{max} for 5-HT clearance than treatment with 5,7-DHT (see Fig. 6, middle panel and Table 3). Treatment with 5,7-DHT did not alter the K_T value. However, treatment with 6-OHDA significantly decreased the K_T value relative to control and 5,7-DHT-treated rats. Notably the K_T value in the dentate gyrus of 6-OHDA-treated rats did not differ from that for the CA3 region of hippocampus of sham-treated rats.

There was no significant effect of either 5,7-DHT or 6-OHDA lesion on V_{max} or K_T values for 5-HT clearance in the corpus callosum (Fig. 6, panel 3; Table 3).

3.3. Effect of uptake inhibitors on 5-HT clearance in the dentate gyrus and CA3 region of the hippocampus

To further explore the role of the 5-HTT and NET in removing 5-HT from the extracellular fluid in dentate gyrus and CA3 region of the hippocampus, the effect of fluvoxamine and DMI on 5-HT clearance was investigated when the 5-HT signal amplitude was ∼0.5 or ∼4.0 μM. These signal amplitudes were selected to reflect K_T values for a high affinity, low capacity uptake mechanism (i.e. the 5-HTT) and a low affinity but high capacity uptake mechanism (i.e. the NET), respectively (Shaskan and Snyder, 1970 and present data). We hypothesized that the relative ratio of 5-HTTs to NETs in a given brain region would dictate the effectiveness of each uptake inhibitor to prolong the time course for 5-HT clearance. Thus, in a brain region where the ratio of 5-HTTs to NETs is 1:2, such as the dentate gyrus, both fluvoxamine and DMI would be effective inhibitors of 5-HT clearance regardless of 5-HT concentration. However, in brain regions such as the CA3 region of the hippocampus where the ratio of 5-HTTs to NETs is reversed, fluvoxamine would inhibit clearance of 5-HT when its extracellular concentrations were ∼0.5 or ∼4.0 μM, but DMI would be effective only when extracellular 5-HT concentrations are high (∼4.0 μM).

Table 3
Summary of V_{max} and K_T values for 5-HT clearance in the CA3 region of hippocampus, dentate gyrus and corpus callosum of rats treated with 5,7-DHT or 6-OHDA

Treatment	CA3	Dentate gyrus	Corpus callosum
V_{max} (nM/s)			
Sham	201 ± 15	247 ± 16	70 ± 6
5,7-DHT	263 ± 11	194 ± 5[*]	69 ± 9
6-OHDA	209 ± 23	124 ± 4[**,+]	78 ± 12
K_T (μM) [corrected for α = 0.20]			
Sham	4.02 ± 0.50 [0.80±0.10]	5.27 ± 0.43[§] [1.05 ± 0.09]	3.94 ± 0.59 [0.68±0.12]
5,7-DHT	8.97 ± 3.00 [1.79±0.60]	5.04 ± 0.20 [1.01±0.04]	4.50 ± 0.95 [0.90±0.19]
6-OHDA	5.58 ± 0.61 [1.16±0.12]	3.65 ± 0.19[*,#] [0.70 ± 0.04][*,#]	5.33 ± 0.97 [1.07±0.19]

V_{max} and K_T values were determined by fitting the data shown in Fig. 6 to a four-parameter logistic equation.
[*] $P < 0.01$.
[**] $P < 0.001$ from sham.
[+] $P < 0.001$ from 5,7-DHT counterpart.
[#] $P < 0.05$ from 5,7-DHT counterpart.
[§] $P < 0.05$ from other brain regions; one-way ANOVA with Bonferroni post-hoc comparisons.

Table 4
Summary of baseline signal parameters for experiments assessing the effect of serotonin and norepinephrine uptake inhibitors on serotonin clearance as a function of extracellular fluid concentration of 5-HT

	Amplitude (μM)	$T_{20–60}$ (s)	T_{80} (s)
"Low" (range 0.17–0.79 μM)			
CA3 (n = 12)	0.47 ± 0.07	53 ± 7	125 ± 18
Dentate gyrus (n = 11)	0.57 ± 0.04	53 ± 10	83 ± 12
"High" (range 2.00–8.67 μM)			
CA3 (n = 12)	4.50 ± 0.60	50 ± 7	131 ± 18
Dentate gyrus (n = 9)	3.21 ± 0.57	40 ± 6	106 ± 16

No significant differences between pairs of data, t-test for independent samples.

Table 5
Summary of the effect of fluvoxamine (46 ± 6 pmol) and DMI (49 ± 3 pmol) on the $T_{20–60}$ time course parameter as a function of 5-HT signal amplitude in the CA3 region of hippocampus and dentate gyrus

5-HT signal amplitude	CA3		Dentate gyrus	
	<1.0 μM	>2.0 μM	<1.0 μM	>2.0 μM
Fluvoxamine				
Pre-	57 ± 11	49 ± 10	41 ± 12	40 ± 8
Post-	85 ± 21*	53 ± 8	72 ± 20*	49 ± 10
DMI				
Pre-	42 ± 6	55 ± 12	68 ± 15	39 ± 7
Post-	44 ± 6	73 ± 14*	70 ± 14	69 ± 14*

Paired t-test, pre- vs. post-drug $T_{20–60}$ values; n = 4–6 per group.
* $P < 0.05$.

The pre-drug values for signal amplitude, $T_{20–60}$ and T_{80} time course parameters for groups representing the "low" and "high" extracellular concentration of 5-HT are summarized in Table 4. As shown in Fig. 7, and consistent with earlier studies (Daws et al., 1997, 1998; Montañez et al., 2002), when the signal amplitude for 5-HT was less than 1 μM (i.e. extracellular fluid concentrations of 5-HT less than 1 μM) fluvoxamine prolonged the T_{80} time course parameter in both the CA3 region of hippocampus and dentate gyrus. Although not significant, there was a trend for this effect to be greater in the dentate gyrus than in the CA3 region. By contrast, at similar signal amplitudes, the norepinephrine uptake inhibitor, DMI, prolonged the T_{80} time course parameter in the dentate gyrus but not in the CA3 region of hippocampus.

When 5-HT signal amplitudes were much greater (range from 2.00 to 8.67 μM) fluvoxamine no longer exerted any significant effect on T_{80} in the CA3 region and had a diminished (although still significant) ability to prolong T_{80} in the dentate gyrus (see Fig. 7). In the dentate gyrus, the ability of DMI to prolong T_{80} was preserved when 5-HT signal amplitudes were greater than 2.0 μM. However, whereas DMI had no effect on T_{80} in the CA3 region at low 5-HT signal amplitudes, at these higher signal amplitudes DMI now significantly prolonged the T_{80} time course parameter for 5-HT clearance.

These results analyzed as a function of the $T_{20–60}$ time course parameter are summarized in Table 5 and in general reflect the results observed for the T_{80} time course parameter. In the CA3 region $T_{20–60}$ was significantly prolonged by fluvoxamine when the 5-HT signal amplitude was low but not when it exceeded 2 μM, whereas the opposite was found for DMI. That is, at high, but not low signal amplitudes, DMI significantly extended the $T_{20–60}$ time course parameter. The same pattern emerged in the dentate gyrus. Thus, because fluvoxamine and DMI significantly prolonged the T_{80} time course parameter in the dentate gyrus regardless of 5-HT signal amplitude, it appears that at least in this brain region the T_{80} time course parameter is more robustly affected by these drugs. The overriding generality from these data however, is that the ability of DMI to inhibit 5-HT clearance is most profound when extracellular 5-HT was high, whereas fluvoxamine produced greatest inhibition of 5-HT clearance when extracellular 5-HT was low.

In the majority of instances neither fluvoxamine nor DMI significantly altered signal amplitude. However, in the CA3 region under high signal amplitude conditions, a small but significant decrease in signal amplitude occurred following both fluvoxamine and DMI (data not shown). As we have previously reported (Daws et al., 1998), vehicle (phosphate-

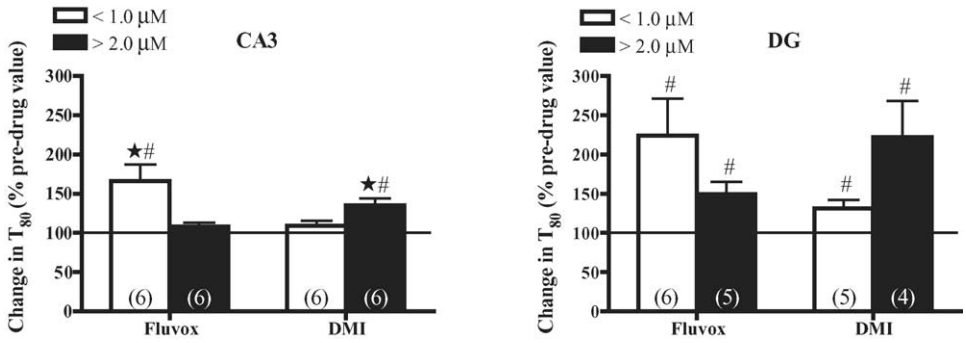

Fig. 7. Summary of the effect of fluvoxamine (46 ± 6 pmol) and DMI (49 ± 3 pmol) on the T_{80} time course parameter as a function of 5-HT signal amplitude in the CA3 region of hippocampus and dentate gyrus (DG). Serotonin was delivered so as to yield signal amplitudes of approximately 0.5 μM (range 0.17–0.79 μM) or 4.0 μM (range 2.00–8.67 μM). Once a reproducible baseline 5-HT signal was obtained, drug was pressure-ejected followed 2 min later by another ejection of 5-HT. Data are expressed as mean ± S.E.M. percent change from pre-drug baseline. Number of rats per group is shown in parentheses. $^{\star}P < 0.05$ Mann–Whitney U; $^{\#}P < 0.05$. Paired t-test, pre- vs. post-drug T_{80} values.

buffered saline or water) had no significant effects on 5-HT signal parameters (data not shown).

4. Discussion

Three major findings resulted from this study. First, that kinetics of 5-HT clearance in vivo is distinct with respect to brain region. Second that the 5-HTT as well as the NET contribute to 5-HT clearance concordant with the relative density of each transporter within a region. Third, that the ability of an SSRI or norepinephrine reuptake inhibitor to inhibit 5-HT clearance is dependent both on brain region and on extracellular levels of 5-HT. The data presented here support the idea that factors governing 5-HT clearance in vivo are in a state of dynamic flux, continually responding to changes in the extracellular milieu in order to maintain homeostasis of serotonergic neurotransmission. Moreover, these experiments demonstrate the utility of high-speed chronoamperometry for measuring clearance of exogenously applied 5-HT in vivo and for determining factors that govern its clearance.

Consistent with our earlier reports (Daws et al., 1997; Montañez et al., 2002) as well as those of others who have used synaptosomal or brain slice preparations (Bunin and Wightman, 1998; Bunin et al., 1998; Shaskan and Snyder, 1970), the rate of 5-HT clearance in vivo is dependent on brain region. Generally, rate of 5-HT clearance increases with increasing 5-HTT density. Thus, V_{max} for 5-HT clearance is highest in the DRN, where the greatest density of 5-HTTs are located, and lowest in the corpus callosum, where very low binding of [^{3}H]-cyanoimipramine to the 5-HTT occurs. Interestingly however, the V_{max} values for 5-HT clearance did not correspond precisely as would be predicted based on 5-HTT density alone. For example, 5-HTT density is approximately 4.5-fold greater in the DRN than in the CA3 region of hippocampus, and yet the V_{max} for 5-HT clearance was only two-fold greater. This apparent disparity supports the notion that multiple factors govern 5-HT clearance within a given brain region. Another notable caveat to the relationship between 5-HTT density and V_{max} occurred in the dentate gyrus. The dentate gyrus has a significantly lower density of 5-HTTs (approximately two-fold less) than in the CA3 region of hippocampus however, V_{max} for 5-HT clearance was significantly greater in the dentate gyrus than in the CA3 region of hippocampus. Moreover, the K_T value for 5-HT clearance was greater in the dentate gyrus than in either the CA3 region of hippocampus or DRN. These data suggested to us that the primary mechanism(s) governing 5-HT clearance is different in the dentate gyrus and subsequently became the focus of this study.

We have previously demonstrated that the NET can contribute to clearance of 5-HT in the dentate gyrus (Daws et al., 1998), a finding consistent with earlier observations suggesting that 5-HT can be taken up by at least two transport processes (Lichtensteiger et al., 1967; Shaskan and Snyder, 1970), one residing on serotonergic neurons and the other on catecholaminergic neurons. Shaskan and Snyder (1970) described transport of 5-HT by high and low affinity processes. The high affinity process, termed uptake-1, was found to be localized on serotonergic neurons, whereas the low affinity process, termed uptake-2, reflected uptake into catecholaminergic neurons. For 5-HT they reported a K_m value for uptake-1 of 0.1–0.2 μM and V_{max} values of 1.2 and 0.7 μmol/min/g in the striatum and hypothalamus, respectively. Uptake-2 had a K_m value of ∼8 μM but a much greater capacity to transport 5-HT than uptake-1, with V_{max} values being 12- to 15-fold greater than the corresponding values for uptake-1. The K_T values derived in vivo in the present study are remarkably similar. Although at first glance the K_T values corresponding to uptake-1 appear greater (e.g. ranging from ∼2.5 to 4.0 μM), this is likely a consequence of the prominent role diffusion plays in determining the rate of clearance of 5-HT away from the recording electrode (Near et al., 1988). When K_T is corrected for volume fraction (α) (Nicholson, 1995; Rice and Nicholson, 1991) the values derived in vivo (e.g. ∼0.5 μM) are consistent with those determined using an in vitro approach (0.01–0.5 (M) (Codd and Walker, 1987; Masson et al., 1999; Shaskan and Snyder, 1970). Similarly, the K_T values derived for 5-HT uptake by the NET are consistent with those determined using in vitro approaches (e.g. Kuhar et al., 1972; Shaskan and Snyder, 1970). The higher K_T and V_{max} values derived in the dentate gyrus suggest that clearance in this region is mediated by at least two processes as defined by Shaskan and Snyder (1970), where the 5-HTT represents uptake-1 and the NET represent uptake-2.

To investigate the contribution of the NET in determining the V_{max} for 5-HT clearance, we determined V_{max} and K_T values for 5-HT clearance in rats treated previously with 5,7-DHT or 6-OHDA. These treatments resulted in a greater than 90% loss of 5-HTTs and NETs, respectively, in the hippocampal regions. In the dentate gyrus both treatments resulted in significant reductions in the V_{max} for 5-HT clearance however, the magnitude of this reduction was markedly greater in 6-OHDA-treated rats (approximately 50% decrease in V_{max} compared to only a 20% decrease in 5,7-DHT-treated rats). Moreover, in the dentate gyrus the K_T value in rats treated with 6-OHDA was significantly lower and resembled that determined in the CA3 region of hippocampus and DRN of intact rats. By contrast, the K_T value was not significantly altered in the dentate gyrus of rats treated with 5,7-DHT. Taken together these results are consistent with 5-HT uptake in the dentate gyrus being mediated by at least two processes: a high affinity–low capacity mechanism, the 5-HTT; and a low affinity–high capacity mechanism, the NET.

This interpretation is supported further by our findings in the CA3 region of hippocampus. Here neither 6-OHDA nor 5,7-DHT treatments altered the V_{max} for 5-HT clearance. However, there was a trend for K_T values to be increased relative to sham-treated rats. This increase in K_T value (i.e. decrease in apparent transporter affinity for 5-HT) was most marked in 5,7-DHT-treated rats. While it is difficult to comment on the statistical significance of the apparent increase in

K_T values, it is clear from visual inspection of the curves in Fig. 6, left panel, that 5,7-DHT treatment resulted in a shift to the right of the concentration response curve and a relatively poor fit of the data to a four-parameter logistic equation. Consequently the error associated with the derived K_T value is large. Nonetheless, the increased K_T value together with the finding that V_{max} values were not altered in 5,7-DHT-treated rats suggests that the NET also contributes to 5-HT clearance in the CA3 region of hippocampus. Notably it appears that the NET contributes most prominently when extracellular fluid levels of 5-HT are high.

These data are in agreement with our earlier work demonstrating that in the CA3 region of hippocampus clearance of 5-HT is prolonged in rats treated with 5,7-DHT (Daws et al., 1998; Montañez et al., 2003b). However, in these initial studies 5-HT was never applied in amounts sufficient to obtain V_{max} and so a role for the NET in mediating 5-HT clearance was not revealed. According to these original data it was concluded that the 5-HTT is the primary mechanism governing 5-HT clearance in the CA3 region of hippocampus. Given this, deficits in 5-HT clearance as a consequence of 5,7-DHT treatment would be predicted to become even more apparent as extracellular fluid concentrations of 5-HT increase. The present data show that this does not occur. Instead, it appears that when 5-HT concentrations become sufficiently high, other active transport mechanisms come into play to maintain V_{max} at "normal" rates. The data presented here are consistent with that transport mechanism being the NET.

Of fundamental importance is whether uptake by catecholaminergic neurons has either physiological or pharmacological importance. Although this cannot be answered precisely, the most current estimate for synaptic concentrations of 5-HT is 6 mM (Bunin and Wightman, 1999), a value consistent with estimates for other transmitters such as glutamate and dopamine (Clements, 1996; see Cragg and Rice, 2004). Given estimates that 5-HT can diffuse >20 μm away from the cleft such that concentrations fall to a micromolar to nanomolar range (Bunin and Wightman, 1998, 1999; Clements, 1996; Cragg and Rice, 2004) the micromolar values required to reach an apparent V_{max} for 5-HT clearance in the present study fit within the current framework of our knowledge. In addition, our data also are consistent with those of Bunin and coworkers, and support paracrine (or extrasynaptic) transmission as a mode for 5-HT neurotransmission. Such data are supported by ultrastructural studies showing that the 5-HTT is located primarily extrasynaptically (on axons and dendrites) and rarely synaptically (Miner et al., 2000) and at a distance from potential target sites (e.g. 5-HT2A receptor, Huang and Pickel, 2002). Similarly, NET labeling is primarily confined to noradrenergic neuronal somata, axons and dendrites. Of particular relevance to the present data is evidence that noradrenergic neurons arborize extensively within the hippocampus (Schroeter et al., 2000). Thus, there are both anatomical and functional data supporting the involvement of multiple transport mechanisms that govern 5-HT neurotransmission.

These ideas are further supported by our studies investigating the ability of antagonists of the 5-HTT and NET to inhibit 5-HT clearance when the peak amplitudes of the electrochemical signal produced by local application of 5-HT into these hippocampal regions were less than 1 μM ("low") or greater than 2 μM ("high"). Consistent with our earlier studies, DMI did not inhibit 5-HT clearance in the CA3 region of hippocampus when extracellular fluid concentrations of 5-HT were "low". However, when 5-HT concentrations were high, 5-HT clearance was significantly inhibited by DMI. In the dentate gyrus, DMI generally inhibited 5-HT clearance regardless of 5-HT concentration. It is also noteworthy that in the CA3 region, fluvoxamine failed to inhibit 5-HT when extracellular fluid 5-HT levels were high. These data are perhaps best explained in terms of the relative ratio of 5-HTTs and NETs in these hippocampal structures and the capacity of each to transport 5-HT (see Fig. 8). In the CA3 region the density of 5-HTTs is approximately two-fold greater than the density of NETs. Under conditions of high extracellular

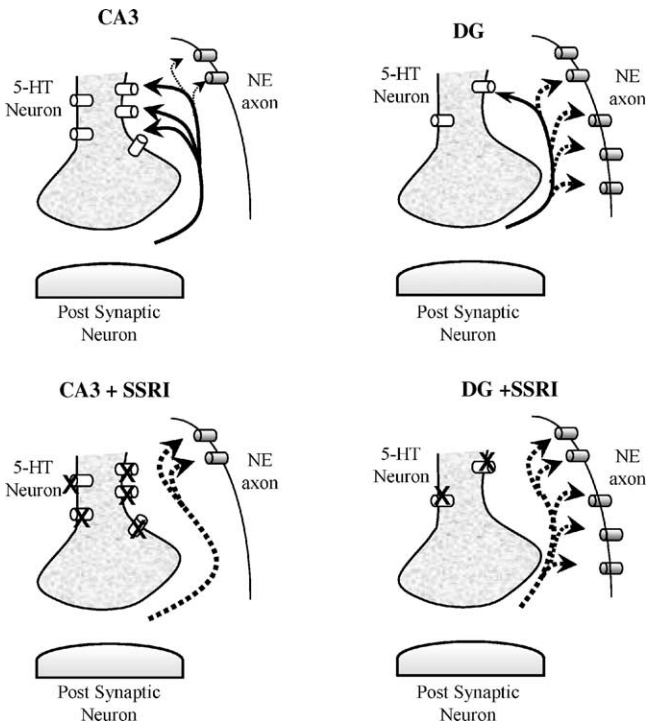

Fig. 8. Schematic representation of the contribution of 5-HTTs (open cylinders) and NETs (shaded cylinders) in mediating serotonergic neurotransmission in dentate gyrus (DG) and CA3 region of hippocampus. In the CA3 region of hippocampus the greater relative density of 5-HTTs compared to NETs precludes the NET from playing a significant role in clearance of 5-HT from extracellular fluid (top left panel). However, when the 5-HTT is inactivated by administration of an SSRI, the concentration of extracellular 5-HT climbs and the NET becomes an active player in mediating clearance of 5-HT (bottom left panel). By contrast, in the dentate gyrus the greater relative density of NETs compared to 5-HTTs makes regulation of extracellular levels of 5-HT in this region under greater control by the NET (top right panel). The involvement of the NET in regulating clearance of 5-HT in the dentate gyrus becomes even more apparent in the presence of an SSRI (bottom right panel).

5-HT, the NET is actively involved in the uptake of 5-HT. Fluvoxamine, by its action at the 5-HTT, serves to maintain high levels of 5-HT and thus its effect to inhibit 5-HT clearance is masked by the high capacity of NETs to take up 5-HT. When concentrations of 5-HT are low at the time fluvoxamine is administered, 5-HT concentrations do not climb sufficiently high for NETs to significantly influence uptake, at least under the conditions used here, and so inhibition of clearance is apparent. In the dentate gyrus the ratio of 5-HTTs to NETs is approximately 1:2. Because NETs are more numerous in the dentate gyrus, determination of extracellular fluid 5-HT concentration is more likely to be under their control. Consistent with this idea, we found that DMI inhibited 5-HT clearance regardless of whether 5-HT signals were "high" or "low". Fluvoxamine was also able to inhibit 5-HT clearance at both "low" and "high" 5-HT signal amplitudes however, consistent with the idea put forth above, the ability of fluvoxamine to inhibit clearance was less robust when the extracellular fluid concentration of 5-HT was "high" at the time drug was administered. The model proposed here is typical of cooperative binding interactions that occur in many biological systems (e.g. binding of ligand to an oligomer). Specifically we propose that the data presented here are analogous to a model of positive cooperativity, with the caveat that a conformational change in the protein is not required. That is, the binding of one ligand (fluvoxamine) at site 1 (5-HTT) enhances the binding of a second ligand (5-HT) to site 2 (NET). This idea is illustrated in Fig. 8. Indeed there is evidence that similar dynamics occur between other transport systems. For example, 5-HT can also be taken up by the dopamine transporter (Jackson and Wightman, 1995) and dopamine can be taken up by the NET (Cass and Gerhardt, 1995).

It is important to note that only rats where a greater than 90% loss of 5-HTT or NET was attained were included in the analysis. Great care was taken to ensure this criteria was fulfilled as previous studies from our laboratory have shown that 5-HT clearance parameters are not significantly altered when loss of 5-HTT is less than 80% (Montañez et al., 2003b). Thus, even when as few as 20% of 5-HTTs are spared; "normal" rates of 5-HT clearance are maintained. These data are analogous to those obtained when an irreversible antagonist is used to block some percentage of receptors and the unblocked or "spare" receptors are sufficient to maintain normal neurotransmission. Because loss of 5-HTT was greater than 90% in all cases, it is not likely that activity of "spared" 5-HTTs could account for these robust changes in kinetic parameters or drug effects observed in the present study, however this possibility cannot be excluded.

It is noteworthy to compare these data with our earlier findings in mice with a targeted disruption of the 5-HTT gene. Not surprisingly we found 5-HT clearance to be substantially slowed in the homozygote knockout, which express no 5-HTT (Montañez et al., 2003a). However, in heterozygote mutants, which express 50% fewer 5-HTTs than wild-type mice, the V_{max} for serotonin clearance was intermediate between that for the wild-type and KO mice. Interestingly, a clear divergence in serotonin clearance between heterozygote mutants and wild-type mice did not occur until extracellular fluid concentrations of 5-HT were around 2 μM (Montañez et al., 2003a). In this same study we showed that the density of NETs in the CA3 region of hippocampus was not different between the three genotypes. Together with the genotype-dependent differences in V_{max} values for 5-HT clearance it appears that the NET does not compensate for the partial or complete loss of 5-HTT in the genetically modified mice. These data are discrepant with our present results in rats treated with neurotoxin. This apparent discrepancy may be reconciled by genetic versus pharmacologic means to reach the same end (loss of transporters). Genetically modified mice have been so since conception and undoubtedly undergo a variety of developmental adaptations as a consequence. Treatment with a neurotoxin represents an acute pharmacologic insult. Although it is beyond the scope of this study to probe the underlying basis(es) for these differences our data underline potentially important differences between genetically and pharmacologically defined transporter expression.

The density of 5-HTTs and NETs in the corpus callosum is very low and reflected by low rates of 5-HT clearance. It is difficult to comment on the functional significance of transporters in this brain structure. Treatment with 5,7-DHT- or 6-OHDA-treatment did not significantly alter 5-HT clearance parameters and so it could be argued that 5-HT clearance in this structure is mediated primarily by diffusion. This interpretation is supported by earlier studies showing a lack of effect of fluvoxamine to inhibit 5-HT clearance in the corpus callosum (Daws et al., 1997). However, given that 5,7-DHT- and 6-OHDA-treatment did not produce >70% loss of 5-HTTs and NETs, respectively, it could also be argued that the remaining transporters could maintain normal rates of 5-HT clearance as we found in the CA3 region of hippocampus (Montañez et al., 2003b). A recent study by Reyes-Haro et al. (2003) reporting antidepressant-sensitive uptake of 5-HT in the corpus callosum provides support for the latter.

The data presented here raise important considerations for the use of SSRIs and other therapeutics that are used to treat a range of psychiatric disorders including depression, anxiety and alcoholism. For example, individuals who do not respond well to SSRIs may already have high 5-HT tone and therefore the effect of SSRIs to increase extracellular 5-HT further is dampened by activity of the NET. Alternatively, but not mutually exclusively, these individuals might have increased NET function in brain regions important in mediating such psychiatric illnesses. Combined with the growing body of evidence documenting interactions between genetically defined expression of the 5-HTT (e.g. 5-HTTLPR polymorphism) (e.g. Collier et al., 1996; Smits et al., 2004), environment (e.g. Caspi et al., 2003) and response to drug treatment (e.g. Eichhammer et al., 2003; Smits et al., 2004), the design of a biological system with built in functional redundancies make evolutionary sense in order to maintain homeostasis of serotonergic neurotransmission. The data presented here clearly demonstrate that the NET is capable of clearing 5-HT. How-

ever, it appears that this mechanism for 5-HT clearance is effected when the extracellular concentration of 5-HT exceeds the K_T of the 5-HTT for 5-HT and/or in brain regions where the density of NETs exceeds that of the 5-HTT.

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