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In vivo electrochemical measurements of serotonin clearance in rat striatum: effects of neonatal 6-hydroxydopamine-induced serotonin hyperinnervation and serotonin uptake inhibitors

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Summary. Diffusion and clearance of extracellular serotonin (5-HT) was examined using in vivo chronoamperometry with "delayed-pulse" recordings after pressure ejections of 1 to 60 picomoles 5-HT into rat striatum at a fixed distance from a Nafion-coated carbon fiber electrode. Signals obtained were identified based on the signal characteristics to consist of 5-HT. Clearance times of 5-HT decreased, while amplitudes and rise times increased with serotonergic hyperinnervation induced by neonatal 6-hydroxydopamine (6-OHDA) lesions of dopamine (DA) neurons. Local applications of the 5-HT uptake inhibitors zimelidine or fluoxetine, in conjunction with 5-HT ejections, produced increased clearance times in both normal and 6-OHDA-treated animals. Thus, direct in vivo evidence was obtained for the importance of high affinity nerve terminal uptake as a key mechanism for clearance of 5-HT from the extracellular space. Inhibitors of 5-HT uptake appear to prolong the extracellular presence of 5-HT by increasing its clearance time.

Keywords: In vivo electrochemistry, serotonin, uptake, diffusion, clearance, hyperinnervation.

Introduction

The central serotonin (5-hydroxytryptamine; 5-HT) system is composed of cell bodies located in the midline of the brainstem, which have extensive projections throughout the brain and spinal cord (Dahlström and Fuxe, 1964; Steinbusch, 1981). Following synaptic release and action on receptors, termination of 5-HT neurotransmission appears to depend primarily on clearance of the neurotransmitter by reuptake into the presynaptic serotoninergic terminals (see Ross, 1980). In vitro studies have shown that neuronal 5-HT uptake is a selective and saturable process obeying Michaelis-Menten kinet-

ics, which is temperature-, voltage- and sodium-dependent (Ross, 1980; Reith and O'Reilly, 1990). Uptake of 5-HT can be antagonized by agents binding to the transporter site, and such inhibitors have found clinical use as antidepressant and anxiolytic drugs (Coppen et al., 1979; Fuller et al., 1991). The existence of a specific neuronal reuptake system of 5-HT has been confirmed by the identification of a gene encoding a selective 5-HT membrane transporter which is expressed at high levels in 5-HT cell bodies (Blakely et al., 1991; Hoffman et al., 1991).

In vivo demonstrations of 5-HT uptake have historically been indirect, employing 5-HT depleting agents such as parachloroamphetamine and fenfluramine, that require uptake via the membrane transporter (Carlsson et al., 1969; Meek et al., 1971). Autoradiographic or histoflourescence techniques have also been used to visualize in vivo uptake of 5-HT (Fuxe et al., 1978; Parent et al., 1981), while in vivo binding of labeled uptake inhibitors can be used to quantitate 5-HT uptake sites (Scheffel and Hartig, 1989). Functional information concerning the regulation of extracellular 5-HT or its metabolite 5-hydroxyindoleacetic acid (5-HIAA) has been obtained by the use of push-pull cannula and in vivo microdialysis (Kalén et al., 1988; Sharp et al., 1989; see also Marsden, 1985). By the use of microdialysis it has been shown that consistent increases in extracellular 5-HT occur after local administration of 5-HT uptake inhibitors (Kalén et al., 1988; Auerbach et al., 1989). Unfortunately, microdialysis does not provide sufficient temporal resolution to study extracellular diffusion and clearance of neurotransmitters.

Electrochemistry can be used to measure monoamines in vivo, and new modifications of the technology, involving high-speed chronoamperometry (chronocoulometry) or fast cyclic voltammetry, allow sub-second measurement of monoamine overflow and clearance in restricted brain regions (Stamford et al., 1988; Wightman et al., 1988; Adams, 1990; Ng et al., 1991; Gonon et al., 1993). However, in vivo electrochemical studies on 5-HT involve particular problems compared with examination of catecholamines, such as dopamine (DA). First, selective detection of 5-HT is difficult to accomplish since the oxidation potentials of 5-HT and 5-HIAA are similar (Gonon et al., 1983). Second, 5-HT has a tendency to adsorb to the working electrode surface at high sampling rates, leading to rapid loss in sensitivity (Adams and Marsden, 1982; Verbiese-Genard et al., 1984; Justice, 1987; Stamford et al., 1990). The selectivity issue may be addressed by coating the recording electrodes with Nafion, a perfluorinated polymer with ion-exchange properties, which is selective towards cationic electroactive species (Gerhardt, 1984; Nagy, 1985). Furthermore, recordings of both oxidation and reduction current signals may yield chemical proof as to the identity of the detected electrochemical species (Gratton et al., 1989; Stamford et al., 1990; Su et al., 1990b; Luthman et al., 1993a).

In this paper, we have used in vivo chronoamperometry to directly evaluate 5-HT diffusion and clearance in the extracellular space of anesthetized rats after local injections of 5-HT into striatum. The rat striatum has traditionally been the target of choice for in vivo electrochemical studies on DA

neurotransmission. It receives a very large dopamine (DA) input, while the 5-HT innervation is relatively sparse (Steinbush, 1981). Nevertheless striatum is a suitable brain region to study the importance of the serotoninergic system for clearance of extracellular 5-HT since a striatal serotoninergic hyperinnervation develops after neonatal lesion of DA neurons with 6-hydroxydopamine (6-OHDA) (Stachowiak et al., 1984; Snyder et al., 1986; Luthman et al., 1987), concomitant with an increase in 5-HT uptake (Luthman et al., 1987) and density of transporter sites (Mrini et al., 1995). Initially, the chronoamperometry procedure was optimized to minimize loss of sensitivity. The reproducibility and reliability of the 5-HT measurements were thereafter determined in vitro, in comparison to DA, as well as in vivo. Subsequently, the dynamics of extracellular 5-HT were investigated in normal rat striatum and after neonatal 6-OHDA lesions as well as after local application of the 5-HT uptake inhibitors. The 6-OHDA lesion-induced 5-HT hyperinnervation was confirmed by studies on synaptosomal 5-HT uptake and HPLC assays of monoamine levels in the striatum.

Materials and methods

Neonatal 6-OHDA lesions

Newborn male rats (Sprague-Dawley, B&K Universal AB, Sweden) were randomly divided into 6-OHDA or sham treated groups. On day 1 after birth the pups were anesthetized by hypothermia and 6-OHDA (6-OHDA-Br, Sigma) was injected intracisternally (i.c.) in a dose of 75 μg (free base) in 10 μl 0.9% NaCl containing 0.1% ascorbic acid. Sham treated (control) animals received equal volumes of the vehicle alone (0.9% NaCl containing 0.1% ascorbic acid) i.c. All animals were pretreated with the noradrenaline (NA) uptake inhibitor desipramine (DMI; 25 mg/kg, Pertofran, Ciba-Geigy, Switzerland), which was injected subcutaneously (s.c.) 30 min prior to the 6-OHDA or vehicle i.c. administration in order to obtain a selective lesion of the DA system (see Luthman et al., 1987). All animals were kept in an air-conditioned room with controlled temperature and standardized day-night schedule. Following weaning at 3 weeks of age, animals were housed in groups of three or four with food and water supplied ad libitum. All rats were studied at an age of 60–80 days (350–400 g body weight). Eleven sham treated rats and eight 6-OHDA treated rats were used in the in vivo electrochemistry study. Six additional rats from each group were used for the synaptosomal 5-HT uptake experiment and six rats from each group were used for monoamine measurements by HPLC.

The animal experimental protocols performed in the present study were approved by the Swedish Committee for Ethical Experiments on Laboratory Animals (license S93/92 and S92/92, Stockholm, Sweden).

In vivo electrochemistry

Rats were anesthetized with urethane (1.25 g/kg i.p.), intubated and placed in a stereotaxic frame. Body temperature was maintained at 37°C with a heating pad. A portion of the skull and dura which extended from approximately 1 mm posterior to 4 mm anterior to bregma and from 1 to 4 mm lateral to the midline was removed bilaterally. Remote from this site, a miniature Ag/AgCl reference electrode was inserted into the brain and cemented in place with dental acrylic.

High-speed chronoamperometric measurements were performed with a microcomputer-controlled apparatus (IVEC-5; Medical Systems Corp., Greenvale, NY).

Working electrodes of the Nafion-coated (5% solution, Aldrich Chemical Co., Milwaukee WI) single carbon fiber/graphite epoxy glass capillary type with fiber diameter of 30 μm and an overall recording length of approximately 50 μm were used for all recordings (Su et al., 1990a). Electrodes were prepared with 7–10 dip-coatings of Nafion and dried between coatings at 85°C. An oxidation potential of +0.55 V versus a Ag/AgCl reference electrode was applied at a display rate of one Hz. There were two recording modes that were employed. The "delayed-pulse mode" involved a single square-wave pulse application for 100 ms and the resulting oxidation current was digitally integrated during the last 70 ms of the pulse. The reduction current response was digitized in the same manner when the potential was dropped back to its resting level of 0.0 V for 100 ms. For the remainder of the one sec measurement (800 ms), the electrode was maintained at 0.0 V. The "fast-slow mode" involved the same square-wave protocol of 100 ms duration for the oxidation phase and 100 ms for the reduction portion of the recording. However, the square wave was repeated five times in the one second epic (5 Hz) and the data were averaged to enhance the signal-to-noise of the recording. Thus, in the "delayed-pulse mode", the measurements were not recorded continuously, while in the "fast-slow mode" the measurements were continually made over the one sec period.

5-HT/ascorbic acid (AA) selectivity ratios for each electrode were determined prior to any experiments and only electrodes exhibiting 5-HT to AA selectivity ratios > 400:1 were used. The linearity and sensitivity of all electrodes used for the in vivo experiments were also determined before in vitro using solutions of increasing concentration of 5-HT (range 0.5 to 2.5 or 5.0 μM) as well as compared to DA (range 0.5 to 2.5 or 5.0 μM). Only electrodes exhibiting highly linear responses ($r > 0.98$) to 5-HT were used in the in vivo experiments. The signal-to-noise ratio for 5-HT was >3 in the 50–100 nM range for either recording mode.

The in vivo electrochemical experiments were performed using electrode assemblies consisting of a carbon fiber working electrode and a single glass micropipette (Gerhardt et al., 1986) or a double-barrel glass micropipette (5-HT and zimelidine/fluoxetine ejections) (Friedemann and Gerhardt, 1992). The outer diameter of the glass capillaries used for micropipettes was 1 mm, while the inner diameter was 0.58 mm. A long 7–10 mm thin tip was obtained by pulling the glass capillary. The tip was thereafter opened by gently bumping the end of the tapering capillary, leading to a pipette diameter of 10–15 μm (Gerhardt et al., 1986). The electrode and the micropipette(s) were mounted together with sticky wax with a tip separation of 300 ± 20 μm. The electrode/pipette assembly was lowered into striatum in 500 μm steps (4.0–7.0 mm from the cortical surface) at sites located 1.0 mm anterior to bregma and 2.5 mm lateral to the midline (Paxinos and Watson, 1986). In each animal 5–15 recordings were performed (bilaterally).

Local applications of 5-HT (Sigma Chemical Company, USA), zimelidine (Research Biochemical International, USA) or fluoxetine (Eli Lilly, USA) were performed by pressure ejection (Gerhardt and Palmer, 1987) using a pneumatic pump (PPM-2, Medical Systems Corp.). The volumes applied were recorded using a stereomicroscope to measure the movement of the meniscus of the liquid in the pipette (0.1 mm = 25 nl) (Friedemann and Gerhardt, 1992). 5-HT, in a barrel concentration of 100 μM, was ejected in doses of 1 to 60 pmol (10 to 600 nl using 5–30 psi pulses with durations of 2 to 5 seconds), while ± zimelidine or fluoxetine were applied at a barrel concentration of 400 μM in doses of 50–400 pmol.

All baseline signals recorded from the brain were considered to be the theoretical zero baseline response. The maximal amplitudes of all signals were expressed as μM 5-HT using the in vitro calibration curve for 5-HT determined for each electrode (see above). In addition, the rise times until maximal amplitude (T100%) were recorded, and the decay times, when 50% ($T_{1/2}$ or T50%) of the maximal signal remained, were measured. In the in vivo studies each measurement, or each paired measurement (pretreatment with uptake inhibitors), performed in one animal was regarded as a single observation. Statistical comparisons were performed using unpaired or paired (zimelidine or fluoxetine applications) Student's t-tests.

In vitro ^{3}H-5-HT uptake

In vitro striatal ^{3}H-5-HT uptake was performed essentially as described in Jonsson and Sachs (1976). Fresh tissue was obtained from the whole striatum, weighed in a humid chamber, homogenized in 30 volumes of cold 0.25 M sucrose, and centrifuged at 1,000 g for 10 min. Aliquots of the supernatant containing a crude synaptosomal preparation were preincubated for 5 min at 37°C in Krebs-Ringer buffer. [^{3}H]-5-HT (NEN, sp act; 24.7 Ci/mmol) was then added to give a final concentration of 50 nM [^{3}H]-5-HT and the incubation continued for another 5 min. The incubation was stopped by adding an excess of ice-cold Krebs-Ringer buffer. Vials containing synaptosomes were centrifuged (10,000 g × 10 min). The pellets were dissolved with Protosol®, scintillation phosphor was added, and radioactivity was determined by liquid scintillation spectrometry. The high-affinity 5-HT uptake was determined by subtracting from the total uptake values obtained from incubations carried out in the presence of 20 μM chlorimipramine in the incubation medium. The results were compared using an unpaired Student's t-test.

Tissue monoamine levels

The rats were sacrificed by decapitation. The brain was rapidly removed and chilled in saline (4°C) before the rostral striatum was dissected out from a 1 mm slice. The brain samples were immediately frozen on dry ice and stored at −80°C until analysis. Samples were processed for HPLC with electrochemical detection (Luthman et al., 1993b). Basically, all tissues were homogenized in a volume of 5 μl 0.1 M perchloric acid/mg tissue (wet weight) followed by a filtration step. The endogenous tissue levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT and 5-HIAA were determined. The separations were performed using a reverse phase column (Supelcosil LC-18, 75.0 × 4.6 mm ID, 3 μm particle diameter; Supelco Inc, Bellafonte, PA, USA). The mobile phase consisted of a 0.05 M sodium phosphate/0.03 M citric acid buffer containing 0.1 mM EDTA with 20% methanol and sodium-l-octane sulphonic acid, at a flow rate of 0.5 ml/min. The monoamines were detected using a glassy-carbon electrode detector set at +0.7 V vs. a Ag/AgCl reference electrode and the resulting peak heights were measured. All tissue level values were expressed as ng/g wet weight. Statistical comparisons were performed using an unpaired Student's t-test.

Results

In vitro measurements of 5-HT

Initially, a number of electrodes were tested in vitro using two different chronoamperometric recording modes; the "fast-slow mode" (5 Hz), with continuous 100 ms square wave pulses, and the "delayed-pulse mode", which provided oxidation and reduction measures in 200 ms followed by a 800 ms resting interval. The linearity of the oxidation signal and the sensitivity of the electrodes used were determined using solutions of increasing concentration of 5-HT. As a reference standard, the electrodes were also tested for linearity and sensitivity to DA.

With the "fast-slow mode", the calibration curve for 5-HT showed a downward deflection from a linear response with increasing concentrations, which was became evident at concentration <2.0 μM (calibrations between 0.5 to 5.0 μM: average correlation coefficient 0.877 ± 0.039; n = 6), while a linear response to DA was seen (calibrations between 0.5 to 5.0 μM: average correlation coefficient 0.994 ± 0.011; n = 6). The sensitivity for 5-HT was substantially lower (56%) compared to DA in the "fast-slow mode"; an oxida-

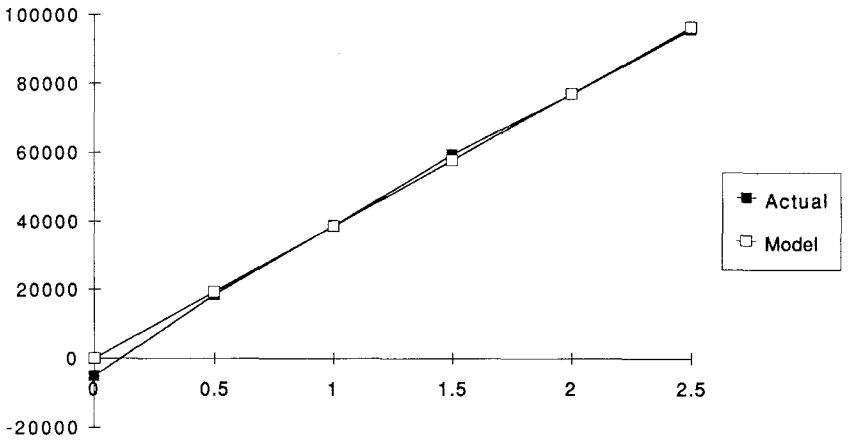

Fig. 1. Oxidation signal after in vitro calibration with increasing concentrations of 5-HT. The actual signal obtained is presented as well as a model of linear response. The scale on the x-axis is μM 5-HT concentration, while the scale on the y-axis is oxidation current (arbitrary)

tion slope of 37,293 ± 9,547 (SEM) arb. units (n = 6) was obtained for 5-HT and 66,552 ± 8,485 arb. units (n = 6) for DA.

In contrast, the "delayed-pulse mode" (1 Hz) provided an acceptable linearity in the response to 5-HT up to concentrations of 2.5 or 5 μM (average correlation coefficient 0.981 ± 0.006; n = 6; Fig. 1). Also a higher sensitivity (176%) was seen for 5-HT in the "delayed-pulse mode" as compared to the "fast-slow mode" (oxidation slopes of 65,656 ± 4,788 arb. units vs. 37,293 ± 9,547; n = 6). In the response to DA, an excellent linearity was obtained (average correlation coefficient 0.9992 ± 0.0005; n = 6). When comparing 5-HT and DA in the "delayed-pulse mode" for recordings, the sensitivity of the electrodes to 5-HT was somewhat lower than that for DA (76%) (oxidation slopes 48,506 ± 3,489 arb. units vs. 63,580 ± 5,558 arb. units; n = 7). The red/ox ratio for 5-HT in the "delayed-pulse mode" was 0.12 ± 0.03; n = 6, while DA showed a red/ox ratio of 0.48 ± 0.05; n = 7.

Repeated in vitro calibrations with 5-HT in the "delayed-pulse mode" were performed with five electrodes to determine changes in sensitivity by frequent exposure to 5-HT (Table 1). An initial decrease in the slope of the oxidation current was experienced following the first 5-HT calibration, while a less pronounced decrease in sensitivity to 5-HT was seen in the subsequent calibrations. The red/ox ratio did not differ between the various 5-HT calibrations.

Using the "delayed-pulse mode", in vitro studies were also performed adding increasing concentrations of 5-HIAA up to 50 μM. However, no oxidation signal could be recorded with 5-HIAA; in fact in several instances a slight decrease in the oxidation current was observed (mean oxidation slope −7,864; range −9,799 to +5,715; n = 5; calibration up to 10 μM). Consequently, the 5-HT/5-HIAA selectivity ratios of the oxidation current were at least >300:1 for any electrode tested.

Table 1. Effects of repeated in vitro calibrations with 5-HT on electrode sensitivity

1st calibration	2nd calibration	3rd calibration	4th calibration
100%	81.2% ± 10.0	77.8% ± 8.7	73.0% ± 2.7

The 5-HT calibrations were performed using increasing concentrations of 5-HT in 0.5 μM steps up to 2.5 μM. Data presented constitute the mean ± SEM (n = 5) oxidation slope in % of first calibration (the oxidation slope of the first calibration was 57,414 ± 1,078)

Table 2. In vitro electrode sensitivity for 5-HT before and after in vivo recordings

	Before in vivo recordings	After in vivo recordings
Oxidation slope	43,278 ± 2,972	17,277 ± 3,157 (60% decrease)
Red/Ox ratio	0.11 ± 0.01	0.10 ± 0.02

The oxidation slope is given in arbitrary units. Data presented constitute mean ± SEM (n = 33). Four to 15 in vivo recordings were performed for each electrode between the two different in vitro 5-HT calibrations

In vivo measurements of 5-HT

All electrodes were calibrated in vitro before being used in vivo with 5-HT (range 0.5–2.5 μM); after the electrodes had been used in brain tissue, an additional 5-HT calibration was performed (Table 2). A decrease in the 5-HT sensitivity was found after they had been used in vivo, ranging from 14% to 75% (average 60%; n = 33) loss in sensitivity compared to the 5-HT calibration performed before the in vivo measurements. The red/ox ratios for the recorded 5-HT currents did not differ between the two calibrations (Table 2).

Exogenous 5-HT was applied by pressure ejection into the extracellular space of the rat striatum in volumes of 10–600 nl, corresponding to 1 to 60 picomoles. These ejections induced oxidation signals of differing amplitudes. Generally, the injected amount of 5-HT was kept between 5 to 30 picomoles, and the amplitudes measured were below 2.5 μM (predominantly between 0.3 and 1.0 μM) as determined from precalibrations with 5-HT. The recorded oxidation signals consisted of a rapid increase in signal amplitude, followed by a slower decrease after 5-HT application (Fig. 2, Ox; upper trace). Both the rise time until maximal amplitude was achieved and the half-decay time (T50%) of the oxidation signals were determined. The reduction current responses were of much lower amplitudes and consisted generally of a slight decrease initially, followed by a small increase (Fig. 2, Red; lower trace). A slightly lower red/ox ratio was seen in the in vivo measurements of 5-HT (0.04 ± 0.01) when determined at T100%, as compared to the in vitro calibrations (see Table 2).

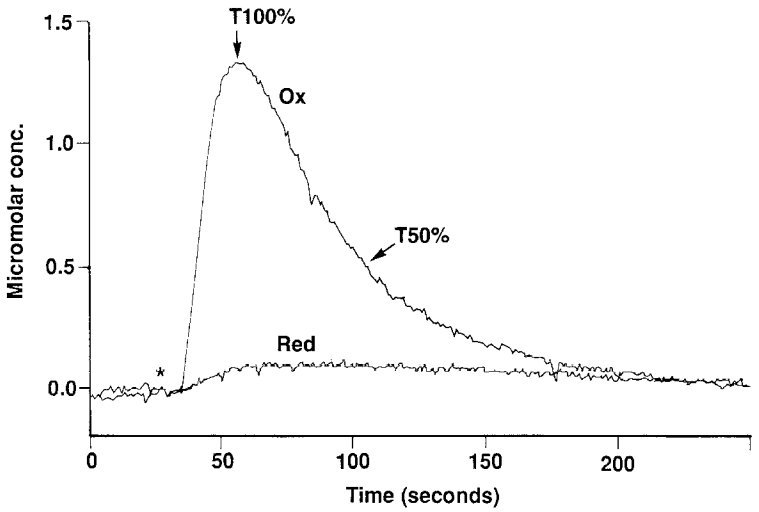

Fig. 2. Oxidation (Ox; upper trace) and reduction (Red; lower trace) currents after 5-HT ejection (37 pmol) in a normal rat. Time of ejection in this and all succeeding traces is denoted by an asterisk

Table 3. Effects of neonatal 6-OHDA lesion of the DA system on 5-HT signal characteristics

	μM/10 pmol	T100% (sec)	T50% (sec)
Control	1.61 ± 0.23; n = 106	22.0 ± 2.0; n = 106	50 ± 4; n = 104
6-OHDA	3.11 ± 0.11*; n = 95	16.1 ± 1.2*; n = 94	40 ± 3*; n = 94

Data presented constitute mean ± SEM. Eleven control animals and eight 6-OHDA-treated animals were studied. Statistical comparison was performed using an unpaired Student's t-test, *$p < 0.05$ compared to control; n = total number of recordings in each group

Effects of neonatal 6-OHDA lesions on serotonin diffusion

There were several differences between signals recorded from 6-OHDA-treated vs. control animals (Table 3). In the 6-OHDA animals the average amplitudes were significantly higher compared to the control animals (control 1.02 ± 0.23 μM, n = 106; 6-OHDA-treated 1.88 ± 0.30, n = 95; $p < 0.05$). Importantly, since the average amounts of serotonin ejected in 6-OHDA-treated and control groups were kept at the same magnitude over the entire experiment (averaging 15.5 ± 3.3. and 17.4 ± 1.6 pmol, respectively), measurements of the volumes of 5-HT applied allowed the expression of the amplitude data obtained as μM responses per 10 pmoles 5-HT applied. Still after correction of the data for the volume of 5-HT applied, the increase in signal amplitude after 6-OHDA-treatment was evident (Table 3). The rise time of the 5-HT signals was changed after the neonatal 6-OHDA-treatment; the T100% (rise time) was significantly faster in the 6-OHDA-treated group compared to the control group. Furthermore, the time course of 5-HT clearance, determined at the T50% (half decay time), was significantly shorter in

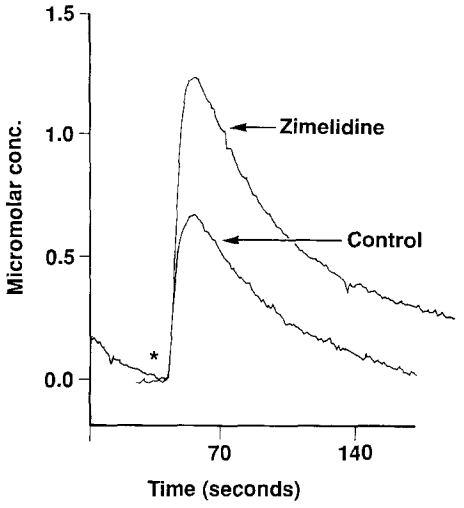

Fig. 3. Oxidation signals evoked by 5-HT (17/15 pmol) in a normal (unlesioned control) rat striatum 10 minutes before (control) and immediately (10–20 secs) after zimelidine administration (88 pmol). Note increased amplitudes of the signals evoked by 5-HT ejection after zimelidine

the 6-OHDA-treated rats in comparison to the controls. Thus, a faster clearance of 5-HT was seen in the 6-OHDA-treated striatum, in spite of the increased signal amplitude.

Effects of local application of zimelidine or fluoxetine

The effects of local administration of the 5-HT uptake inhibitor zimelidine (Ross et al., 1976) on the amplitude and clearance of locally applied 5-HT were studied in the striatum of both normal (control) (Fig. 3) and 6-OHDA-treated rats. In addition, the effects of fluoxetine, another 5-HT uptake inhibitor (Wong et al., 1974), were studied in the 6-OHDA-treated animals (Fig. 6). 5-HT was ejected two times at 10 min intervals, in the same site and using the same amount of 5-HT. The uptake inhibitors were given in doses of 50–400 pmol, 10–20 sec before the second 5-HT ejection. Application of either uptake inhibitor did not induce any detectable changes in the baseline currents. In control rats, there was a significant increase in the amplitude, expressed as μM response per 10 pmoles 5-HT applied, immediately after zimelidine (Fig. 4). In the 6-OHDA-treated animals, a trend for increased signal amplitude was seen immediately after zimelidine administration, although it did not reach significance ($p < 0.13$) (Fig. 4).

The clearance of 5-HT, as determined by the T50% time-point was also affected by zimelidine administration. Thus, the T50% of 5-HT clearance increased slightly, but significantly, in control striatum following zimelidine application (Figs. 3 and 5). In the 6-OHDA-lesioned animals, there was a more pronounced increase in T50% at the 5-HT ejection performed immediately after zimelidine administration (Fig. 5). The rise times did not differ significantly after zimelidine administration at any of the time-points studied in either group (pre-zimelidine T100% in the control group 27.7 ± 8.3 sec and

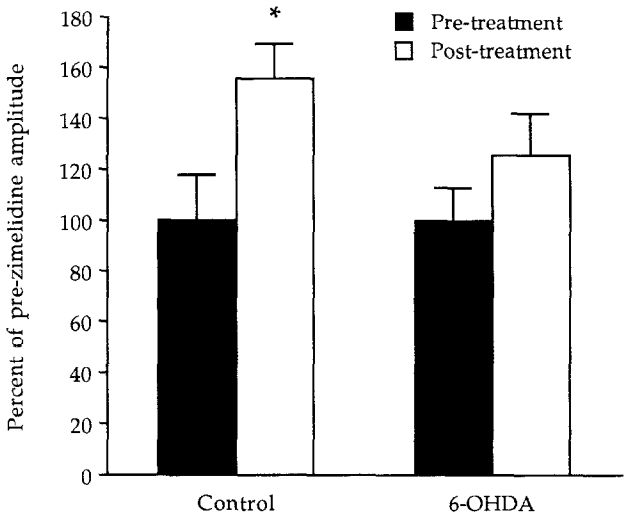

Fig. 4. Bar graph showing normalized changes in amplitude of the oxidation signal produced by 5-HT ejection before and after zimelidine administration, in normal (control) and neonatally 6-OHDA-treated rats. 5-HT was applied in doses of 1–40 pmol and approximately the same dose was used in each set of applications. Zimelidine was applied 10–20 sec before the Post-treatment in doses of 50–400 pmol. Data presented constitute mean ± SEM (Control n = 16; 6-OHDA n = 21). Statistical comparisons were performed using a paired Student's t-test, *$p < 0.05$ compared to Pre-treatment. Pre-zimelidine amplitudes were 1.4 ± 0.5 μM/10 pmol in the control group and 2.1 ± 0.7 μM/10 pmol in the 6-OHDA-treated group

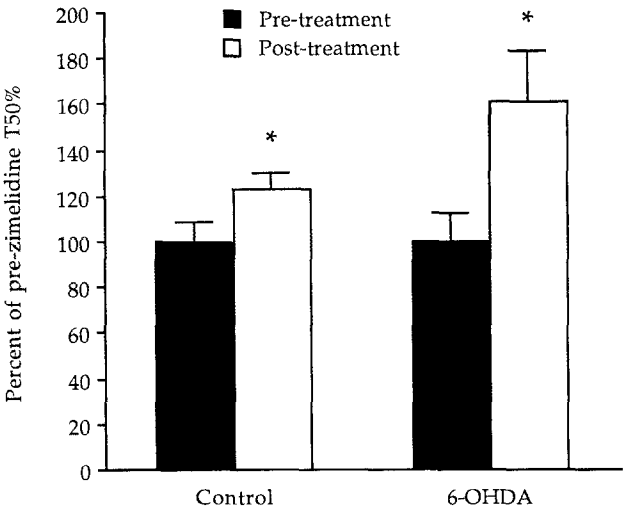

Fig. 5. Bar graph showing normalized changes in T50% of the oxidation signal produced by serotonin ejection before and after zimelidine administration in normal (control) and neonatally 6-OHDA-treated rats. 5-HT was applied in doses of 1–40 pmol and approximately the same dose was used in each set of applications. Zimelidine was applied 10–20 sec before the Post-treatment in doses of 50–400 pmol. Data presented constitute mean ± SEM (Control n = 16; 6-OHDA n = 21). Statistical comparisons were performed using a paired Student's t-test, *$p < 0.05$ compared to Pre-treatment. Pre-zimelidine T50% was 48.5 ± 9.4 sec in the control group and 28.0 ± 6.2 sec in the 6-OHDA-treated group

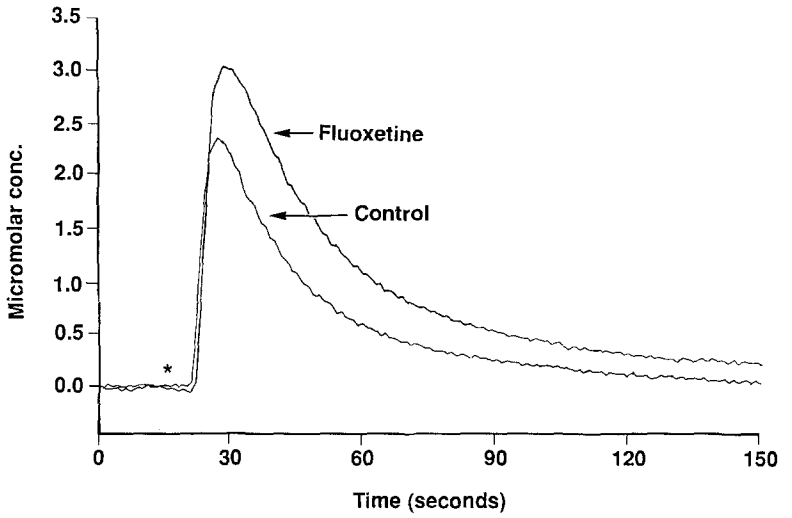

Fig. 6. Oxidation signals evoked by 5-HT (17/17 pmol) in a neonatally 6-OHDA-treated rat striatum 10 minutes before (control), and immediately (10–20 secs) after fluoxetine administration (98 pmol). Note increased amplitudes of the signals evoked by 5-HT ejection after fluoxetine

in the 6-OHDA-treated group 11.6 ± 2.4 sec; post-zimelidine in the control group 25.2 ± 8.8 sec and in the 6-OHDA-treated group 11.5 ± 2.2 sec).

The effects of fluoxetine were only studied in the 6-OHDA-treated animals (Fig. 6). An increase in the amplitude, expressed as μM responses per 10 pmoles 5-HT applied, was seen immediately after fluoxetine administration (Fig. 7). This increase was larger than the trend of an increment observed after zimelidine administration in 6-OHDA-treated striatum and reached significance. The increase in T50% following fluoxetine administration was similar to the increase seen after zimelidine in 6-OHDA-lesioned rats (Fig. 7).

Synaptosomal 5-HT uptake and endogenous monoamine levels in the striatum

In order to verify the 5-HT hyperinnervation produced by the neonatal 6-OHDA treatment, uptake of [^{3}H]-5-HT was studied in synaptosomes prepared from tissue obtained from the whole striatum of normal or 6-OHDA lesioned rats. The in vitro synaptosomal uptake of [^{3}H]-5-HT was increased by 67% in the 6-OHDA treated animals. In the control group the uptake was 59 ± 4 fmoles 5-HT/mg tissue/min (n = 5), while the uptake was 99 ± 20 fmoles 5-HT/mg tissue/min (n = 5) in the 6-OHDA-treated group ($p < 0.05$).

Endogenous tissue levels of monoamines were measured in punch biopsies of the rostral striatum. The neonatal 6-OHDA treatment produced a 91% decrease in the DA content in the striatum compared to control rats (807 ± 325 vs. 8,621 ± 380 ng/g tissue; $p < 0.001$; n = 7), while both DA metabolites, DOPAC and HVA, decreased by 86% (219 ± 73 vs. 1,612 ± 162 ng/g tissue; $p < 0.001$, and 130 ± 42 vs. 957 ± 45 ng/g tissue; $p < 0.001$, respectively). The 5-HT tissue content in the striatum increased by 107% in the 6-OHDA treated rats (655 ± 61 vs. 317 ± 24 ng/g tissue; $p < 0.001$), while the tissue

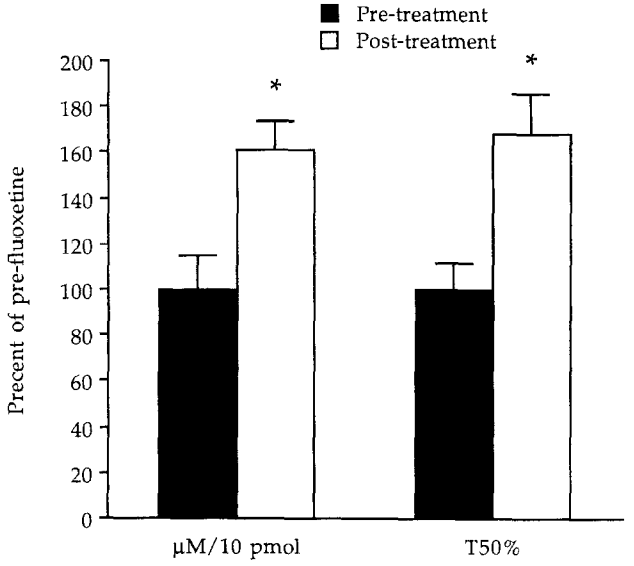

Fig. 7. Bar graph showing normalized changes in amplitude and T50% of the oxidation signal produced by 5-HT ejection before and after fluoxetine administration in neonatally 6-OHDA-treated rats. 5-HT was applied in doses of 1–40 pmol. Fluoxetine was applied 10–20 sec before the Post-treatment in doses of 50–400 pmol. Data presented constitute mean ± SEM (n = 9). Statistical comparisons were performed using a paired Student's t-test, *$p < 0.05$ compared to Pre-treatment. The pre-fluxetine amplitude was 2.2 ± 0.4 μM/10 pmol and the T50% 28.6 ± 3.1 sec

content of 5-HIAA increased by 86% (565 ± 62 vs. 353 ± 29 ng/g tissue; $p < 0.001$).

Discussion

The data presented here indicate that the dynamics of 5-HT diffusion and clearance in the CNS can be studied in vivo with "real-time" temporal resolution using in vivo chronoamperometry. Furthermore, the importance of serotoninergic innervation density and 5-HT uptake carrier mediated removal of extracellular 5-HT can be clearly documented. Thus, clearance times for 5-HT were reduced after neonatal 6-OHDA-treatment that induced hyperinnervation of striatal 5-HT nerve fibers. Moreover, local application of 5-HT inhibitors, in conjunction with 5-HT ejection, produced increased signal amplitudes and clearance times.

Over the past decade, electrochemical techniques have been developed to allow monitoring of extracellular monoamines and metabolites in the intact brain (see Adams, 1990). However, since there are significantly more problems related to selectivity and sensitivity when studying 5-HT with in vivo electrochemistry, compared with the examination of DA or NA, studies on the serotoninergic system have been more limited in scope. Conti et al. (1978) and Marsden et al. (1979) made the first attempts to detect 5-HT, but it was soon realized that selective detection of 5-HT is difficult to accomplish since the oxidation potentials of 5-HT and 5-HIAA as well as uric acid are similar

and the endogenous extracellular levels of 5-HT are low in comparison to 5-HIAA (Gonon et al., 1983; Adell et al., 1991). Because of this, studies on the serotoninergic system have measured primarily 5-HIAA and utilized relatively slow electrochemical recording techniques, like differential pulse voltammetry, with better separation possibilities (Gonon et al., 1983; Marsden, 1985; Crespi et al., 1988; Broderick, 1993). However, by using Nafion coated electrodes selectivity towards cationic electroactive species, like 5-HT, can be obtained, while interference from anionic electroactive species, such as 5-HIAA, are markedly reduced (Gerhardt, 1984; Nagy, 1985). When using differential pulse voltammetry, it has been shown that Nafion coating dramatically reduces sensitivity for 5-HIAA and uric acid, while the sensitivity for 5-HT is maintained (Crespi et al., 1988; Rivot et al., 1995). In the present study 5-HIAA was not detectable with Nafion-coated electrodes and high-speed chronoamperometry. Endogenous striatal 5-HIAA appear to be at a concentration of approximately 1–2 μM (see Kreiss and Lucki, 1995). Thus, it is unlikely that any significant part of the recorded oxidation signal following 5-HT application consisted of 5-HIAA, even though extracellular 5-HIAA levels substantially exceeds 5-HT (Adell et al., 1991).

Recordings of both oxidation and reduction current signals may provide further information concerning the identity of the measured electrochemical species (Gratton et al., 1989; Stamford et al., 1990; Su et al., 1990b; Friedemann and Gerhardt, 1992). In a series of studies, O'Connor and Kruk have measured changes in extracellular 5-HT in brain slices after electrical stimulation using fast cyclic voltammetry and oxidation/reduction current signal identification (see O'Connor and Kruk, 1994). This approach has also been used to detect 5-HT in vivo in striatum after 5-hydroxytryptophan loading (Stamford et al., 1990). Furthermore, we have previously applied in vivo high-speed chronoamperometry coupled with calculations of red/ox ratios to identify striatal 5-HT overflow and clearance following potassium stimulation (Luthman et al., 1993a). In the present study, the red/ox ratios obtained for DA and 5-HT in vitro were in line with previously reported values when using chronoamperometry with Nafion-coated electrodes; DA being the most reversible monoamine transmitter, with red/ox ratios of approximately 0.5–0.7, while 5-HT being the least reversible with a ratio of approximately 0.1 (Gratton et al., 1989; Su et al., 1990b; Luthman et al., 1993a). The recorded in vivo signals obtained after local 5-HT application had a similar red/ox ratio when studied at T100% as found for 5-HT in vitro. The slightly lower ratio found during in vivo recordings as compared to in vitro recordings may be related to that some oxidized 5-HT molecules are allowed to diffuse and are not reached by the reduction current. Coating of the electrodes with tissue debris may also slightly change the current characteristics. The temporal resolution of the present recording technology made it possible to follow both the oxidation and reduction currents in "real time" after the local application of 5-HT, establishing that no major changes occurred in the relationship between the oxidation and reduction currents during the entire course of the signal.

Another problem with electrochemical measurements of 5-HT is that it has a tendency to adsorb to the oxidation surface of the working electrode at high sampling rates, leading to rapid loss in sensitivity (Adams and Marsden, 1982; Verbiese-Genard et al., 1984; Justice, 1987; Stamford et al., 1990). Hence, the measuring procedures for 5-HT have to be adapted to minimize loss of sensitivity and at the same time to maintain the desired recording speed and selectivity. In the present study, the procedure for measuring 5-HT was altered. A recording mode with discontinuous ("delayed-pulse mode") application of the oxidation potential was selected instead of continuous cycling of the potential ("fast-slow mode"). Calibrations with the "delayed-pulse mode" did not show any major deflections in the linear response of the oxidation current obtained with increasing 5-HT concentrations up to 2.5 μM. Furthermore, repeated in vitro calibrations indicated that, despite an initial loss of inter-calibration sensitivity, an acceptable level of decline was observed in the subsequent calibrations. Therefore, the "delayed-pulse mode" provided the required sampling speed with an acceptable level of adsorption to the electrode. It is important to note that the most marked loss in sensitivity in vitro occurred during the first exposure to 5-HT, since repeated exposure to 5-HT occurred in the in vivo recordings. Generally, a more marked loss in sensitivity was seen following in vivo recordings. This might be due to that coating of the electrode surface with tissue debris contribute further to adsorption of 5-HT to the electrode. Still, despite a loss of electrode sensitivity, it appears possible to record robust in vivo signals.

In the present in vivo experiments, we selected to use local application of exogenous 5-HT. A potential drawback with this approach is that concentrations of 5-HT applied may exceed physiological levels, which could lead to unspecific uptake of 5-HT in e.g. DA terminals or astrocytes (Stamford et al., 1990; Fuller et al., 1991). However, at the same time this provided the possibility to obtain a clear 5-HT signal in vivo without the need to use stimulated overflow, with the occurrence of other electroactive species interferring with the measurement. Furthermore, with the use of the lesion paradigms, e.g. 6-OHDA treatment leading to hyperinnervation of 5-HT terminals, or the application of selective 5-HT uptake inhibitors the specific 5-HT terminal-dependent component of the clearance can be identified. It is also possible that the present technology would allow in vivo measurements of exogenous 5-HT under conditions when selective release can be obtained.

In vivo chronoamperometry has been used to study potassium evoked monoamine release as well as clearance of exogenously applied DA in the neonatally 6-OHDA-lesioned striatum (Luthman et al., 1993a,b). In these studies, the importance of DA innervation density for uptake of extracellular DA was verified. In the present study, altered 5-HT signal characteristics were observed in the neonatally 6-OHDA-treated rats as well as following administration of 5-HT uptake inhibitors. A number of the changes in the 5-HT signals seen in animals treated with 6-OHDA deserve further comment. The shortening of the half-life (T50%) of the signals is readily understandable, in terms of increased 5-HT uptake due to hyperinnervation of serotonergic afferents into the striatum (Stachowiak et al., 1984; Snyder et al., 1986;

Luthman et al., 1987). Studies have suggested that this hyperinnervation is functional with active 5-HT release and uptake (Jackson and Abercrombie, 1992; Luthman et al., 1993a) as also shown by the enhanced synaptosomal 5-HT uptake. Hence increased uptake of exogenously administered 5-HT would be expected, leading to a reduced clearance time in the 6-OHDA-treated animals. The faster rise times and increased signal amplitudes in the 6-OHDA-treated group are more difficult to explain. Since the lesion doubtless produces major anatomical changes in the striatum, an alteration in tortuosity and/or extracellular volume is certainly a possibility (Luthman et al., 1993b). In addition, the loss of DA afferents may be a factor insofar as these might be sites for a minor degree of 5-HT uptake at high concentrations. Interestingly, a similar decrease in rise times has been observed for DA following intrastriatal application in neonatally 6-OHDA-lesioned animals (see Luthman et al., 1993b). Thus, neonatal DA lesion may lead to more general changes that could influence diffusion characteristics in the extracellular compartment. Further experiments using tracer compounds that are not taken up or metabolized would shed some light on these questions (see Nicholson and Rice, 1991).

Several effects of local administration of 5-HT uptake inhibitors zimelidine (Ross et al., 1976) and fluoxetine (Wong et al., 1974; Fuller et al., 1991) were seen on 5-HT diffusion and clearance. These effects were most robustly seen as a prolongation of the T50% but also as an increased signal amplitude was observed. These changes are most readily explained as an enhanced presence of ejected 5-HT in the extracellular space as a function of diminished uptake into serotonergic terminals. The differences observed between control and 6-OHDA-treated rats following administration of zimelidine further substantiate the importance of the innervation density for clearance of extracellular 5-HT. Moreover, the present study provides direct evidence that at least acute local administration of 5-HT inhibitors may potentiate 5-HT neurotransmission by either enhancing the concentration of extracellular 5-HT or the duration that it is present in the extracellular space. An enhanced electrically stimulated 5-HT overflow, as studied ex vitro with fast cyclic voltammetry, has been decribed to occur following 21 day systemic administration of fluoxetine, while no effects on T50% was seen (O'Connor and Kruk, 1994). However, major differences in experimental design, such as the measurement of exogenous or endogenous 5-HT, chronic systemic treatment vs. local application of fluoxetine, in vitro vs. in vivo measurements etc., may account for the differences seen in the effects on T50%. At any rate, the present findings are in line with previous studies on DA diffusion and clearance in striatum. In these studies, a prolonged T50% of DA was seen after local administration of the catecholamine uptake inhibitor nomifensine (van Horne et al., 1992; Cass et al., 1993; Luthman et al., 1993b). Also, similar results have been obtained for NA clearance in the cerebellum after uptake inhibition by either nomifensine or ethanol exposure (Lin et al., 1993).

Hence, technical complexities notwithstanding, our results do indicate that extracellular diffusion and clearance of 5-HT can be studied using in vivo chronoamperometry with the present recording technology. This allows the

examination of 5-HT dynamics in real time and consequently provides the means to directly study the effects of innervation density, differences between various uptake inhibitors, and putative release potentiating drugs on extracellular 5-HT in different brain regions.

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