

Tryptophan Availability Modulates Serotonin Release from Rat Hypothalamic Slices

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Abstract: Application of a novel in vitro experimental system has allowed us to describe the relationship between tryptophan availability and serotonin release from rat hypothalamic slices. Superfusing hypothalamic slices with a physiologic medium containing *l*-tryptophan (1, 2, 5, or 10 μM) caused dose-dependent elevations in tissue tryptophan levels; the magnitude of the elevations produced by supplementing the medium with $<5 \mu M$ tryptophan was within the physiologic range for rat brain tryptophan levels. Slice serotonin levels rose biphasically as the tryptophan concentration in the medium was increased. Superfusing the slices with medium supplemented with a low tryptophan concentration (1 or 2 μM) caused proportionally greater incremental changes in serotonin levels than the increases caused by further elevating the tryptophan concentration (5 or 10 μM). The spontaneous release of serotonin from the slices exhibited a dose-dependent

relationship with the tryptophan concentration of the superfusion medium. Electrically evoked serotonin release, which was calcium-dependent and tetrodotoxin-sensitive, also increased in proportion to the medium tryptophan concentration. These data suggest that the rate at which serotonin is released from hypothalamic nerve terminals is coupled to brain tryptophan levels. Accelerations in hypothalamic serotonin synthesis, caused by elevating brain tryptophan levels, result in proportionate increases in the rates of serotonin release during rest and with membrane depolarization. **Key Words:** Serotonin release—Tryptophan availability—Hypothalamus—Superfused brain slices. **Schaechter J. D. and Wurtman R. J.** Tryptophan availability modulates serotonin release from rat hypothalamic slices. *J. Neurochem.* **53**, 1925–1933 (1989).

The rate at which the brain synthesizes serotonin (5-HT) in vivo varies with its tryptophan concentration (Eccleston et al., 1965; Weber and Horita, 1965; Moir and Eccleston, 1968). This relationship exists because the rate-limiting enzyme in 5-HT biosynthesis, tryptophan hydroxylase, is unsaturated with tryptophan. The concentration of tryptophan normally present in the brain (approximately 10–40 μM) (Curzon, 1986) is less than the estimated K_m of tryptophan hydroxylase for its amino acid substrate (50 μM) (Friedman et al., 1972), and thus much less than that needed for full saturation of the enzyme. Hence, treatments which raise brain tryptophan levels within their physiologic range (e.g., administration of a low dose of tryptophan or consumption of an insulin-releasing carbohydrate meal) cause proportionate increases in 5-HT synthesis (Fernstrom and Wurtman, 1971*a,b*; Colmenares et al., 1975). Manipulations which cause greater, unphysiologic increases in brain tryptophan levels (e.g., a phar-

macologic dose of tryptophan) cause tryptophan hydroxylase to approach full saturation, thus yielding successively smaller increments in 5-HT synthesis (Weber and Horita, 1965; Fernstrom and Wurtman, 1971*a*; Grahame-Smith, 1971).

Treatments that change brain tryptophan levels can modify behavior. Increased pain sensitivity (Harvey et al., 1975; Lytle et al., 1975), motor activity (Jacobs et al., 1975; Marsden and Curzon, 1976), aggression (Kantak et al., 1979; Chamberlain et al., 1987), and drug-induced stereotypy (Kozell et al., 1987) reportedly result from experimentally induced reductions in brain 5-HT levels, and all of these behavioral changes are reversed if brain tryptophan levels are elevated by administering the amino acid (Lytle et al., 1975; Marsden and Curzon, 1976) or by dietary manipulations (Lytle et al., 1975; Kantak et al., 1981; Kozell et al., 1987). Conversely, administering tryptophan can decrease locomotor activity in rats (Stewart et al., 1976; Taylor,

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Abbreviations used: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HICA, 5-hydroxyindolecarboxylic acid; 5-HT, serotonin; 5-HT-CH₃, 5-hydroxy-*N*-methyltryptamine oxalate; TTX, tetrodotoxin.

1976) and increase daytime drowsiness (Smith and Prockop, 1962; Greenwood et al., 1975) and decrease nighttime sleep latency (Hartman and Spinweber, 1979) in humans. Rats which raise their brain tryptophan levels by consuming a carbohydrate-rich meal (Fernstrom and Wurtman, 1971*b*) choose to eat less carbohydrate, relative to protein, at the subsequent meal (Wurtman et al., 1983). Oral tryptophan also reportedly reduces food intake in lean men (Leiter et al., 1987), facilitates weight loss in obese patients (Heraief et al., 1985), and elevates mood in depressed persons (Lapin and Oxenkrug, 1969).

These behavioral effects suggest that physiologic and pharmacologic treatments which increase brain tryptophan levels thereby enhance 5-HT synthesis and facilitate 5-HT-mediated neurotransmission. Evidence for a physiologic coupling between brain tryptophan levels and 5-HT release has also been provided by some experiments performed *in vivo*. Treatments which elevated brain tryptophan levels increased the amounts of 5-HT released into the media perfusing the rat lateral ventricle (Ternaux et al., 1976), the surface of the cat caudate nucleus (Ternaux et al., 1977), and the rat nucleus accumbens (Guan and McBride, 1987). *In vivo* voltammetric experiments, which are now interpreted as reflecting the summed extracellular levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) (Joseph and Kennett, 1981; Baumann and Waldmeier, 1984), indicate that increases in brain tryptophan accelerate 5-hydroxyindole release in the rat hippocampus during physiologic stress (Joseph and Kennett, 1983) and in the rat striatum with electrical stimulation of the dorsal raphe nucleus (De Simoni et al., 1987). Under basal conditions, tryptophan administration has been observed to produce elevations in extracellular 5-hydroxyindole levels in the rat hippocampus (Joseph and Kennett, 1981), but not in the rat striatum (Marsden et al., 1979; De Simoni et al., 1987). *In vitro* studies similarly have yielded conflicting results. A dependence of 5-HT release on brain tryptophan availability was observed in a study using incubated rat hippocampal slices from which neurotransmitter release was evoked by a high potassium medium (Auerbach and Lipton, 1985), but not in one using incubated whole-brain slices electrically stimulated at a high rate (100 Hz) (Elks et al., 1979*b*).

Interpretation of such studies sometimes has been confounded by the use of experimental approaches which may not reflect accurately the physiologic processes which control 5-HT release. For example, elevations in brain tryptophan levels commonly have been induced by giving a pharmacologic dose of tryptophan (i.e., 100 mg/kg), even though this dose elevates the level of brain tryptophan approximately ninefold (Moir and Eccleston, 1968; Grahame-Smith, 1971), an increase that is well beyond its physiologic range, and depresses the firing rate of dorsal raphe serotonergic neurons (Aghajanian, 1972; Gallager and Aghajanian, 1976). The characteristic slow firing rate of these neu-

rons (Aghajanian et al., 1968; McGinty and Harper, 1976) is not well simulated by the application of a high potassium medium or by the delivery of high-frequency electrical pulses, methods often used to evoke 5-HT release from brain tissue *in vivo* and *in vitro*.

We have designed a novel *in vitro* experimental system which has allowed us to reexamine the relationship between tryptophan availability and 5-HT release under experimental conditions perhaps more reflective of those occurring physiologically.

MATERIALS AND METHODS

Slice preparation

Male Sprague-Dawley rats (180–240 g) were killed by decapitation; two rats were used in each experiment. Each brain was rapidly removed from the skull and immersed in ice-chilled physiologic medium (previously gassed with 95% O₂/5% CO₂) of the following composition (in mM): NaCl, 130; KCl, 3.5; CaCl₂, 1.3; MgSO₄, 1.5; NaH₂PO₄, 1; NaHCO₃, 25; *d*-glucose, 10. The hypothalamus was dissected out in block (5 × 5 × 2 mm) and cut in half along the third ventricle. The demihypothalami were cut simultaneously into 300- μ m coronal slices using a Mellwain tissue slicer, and then quickly submerged in chilled medium. Each slice was isolated sequentially using a fine sable brush, and placed alternately into one of two glass tubes containing chilled medium. Some of the slices, approximately every fifth, were retained, frozen over dry ice, stored at -70°C, and used subsequently for biochemical assays. The hypothalamic slices in each of the tubes were transferred into parallel superfusion chambers; thus, each chamber contained two demihypothalami. The chambers were constructed as described by Milner and Wurtman (1984), though modified by the placement of a nylon disc on the bottom electrode on which the slices rested. The slices were equilibrated by superfusion (0.6 ml/min) for 50 min at 37°C with physiologic medium which was gassed continuously with 95% O₂/5% CO₂ to maintain pH at 7.4. This superfusion medium included the drug fluoxetine hydrochloride (2 μ M; provided by Eli Lilly Laboratories, Indianapolis, IN, U.S.A.) to block the reuptake of synaptic 5-HT (Wong et al., 1974). (In the absence of fluoxetine, 5-HT released spontaneously into the medium was not detected, and only about 20% of the 5-HT released with electrical field stimulation was recovered.)

Release experiments

In experiments designed to examine the relationships between tryptophan availability and 5-HT release, the medium superfusing the hypothalamic slices in one of the two chambers was supplemented with *l*-tryptophan (1–10 μ M; Sigma Chemical Co., St. Louis, MO, U.S.A.). Five-minute fractions were collected for 80 min, between 50 and 130 min, beyond the initial equilibration period. The slices were electrically stimulated for three periods of 4.7 min each, starting at 60, 85, and 110 min from the onset of superfusion. Electrical field stimulation was induced by delivery of bipolar square-wave pulses (5 Hz, 2 ms, 100 mA/cm²) using a stimulator (Grass Instruments Stimulator S88; Quincy, MA, U.S.A.) in series with a 12-V relay; pulses were monitored continuously by an oscilloscope (Hitachi V-212; Tokyo, Japan). At the end of each experiment the slices were removed from their chambers, quickly rinsed in distilled water, frozen over dry ice, and stored at -70°C for subsequent biochemical assays.

In experiments addressing the calcium dependence of 5-HT release, the slices in one of the two chambers were exposed to calcium-free medium, prepared without CaCl_2 and containing EGTA (1 mM; Sigma), from the onset of superfusion until 85 min; from 85 to 135 min, the slices were superfused with the usual physiologic medium (1.3 mM CaCl_2). Slices in the other chamber were superfused with the physiologic medium throughout. Fractions were collected every 5 min between 50 and 85 min and between 100 and 135 min. Electrical field stimulation (using the same parameters as above) was delivered starting at 65 min (S1) and again at 115 min (S2).

To test whether 5-HT release from the rat hypothalamic slices required the activation of voltage-sensitive sodium channels, we monitored the effect of the voltage-sensitive sodium channel inhibitor, tetrodotoxin (TTX; 1 μM ; Sigma), on basal and electrically evoked 5-HT release. Slices in the two chambers were superfused initially with physiologic medium; between 30 and 85 min, the slices in one of the chambers were superfused with medium to which TTX had been added. Slices in both chambers were then washed rapidly (1 ml/min) for 1 h (85–145 min) with physiologic medium, and then superfused at the usual rate for the remainder of the experiment. Fractions were collected at 5-min intervals between 50 and 85 min and between 145 and 180 min. The slices were electrically stimulated (parameters as above) beginning at 65 min (S1) and 160 min (S2).

Each fraction of superfused medium was collected in 100 μl of 7 mM ascorbate containing two internal standards: 5-hydroxy-*N*-methyltryptamine oxalate (5-HT- CH_3) and 5-hydroxy-2-indolecarboxylic acid (5-HICA) (both purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A.). Once collected, each fraction was mixed by vortex and stored in the dark, on ice, until undergoing further processing.

Biochemical analysis

Each fraction of superfused medium was passed through a preparative column to concentrate 5-HT and 5-HIAA; these columns were prepared by loading 100 mg of dry C_{18} reverse-phase sorbent (40 μm ; Analytichem International, Harbor City, CA, U.S.A.) into glass wool-plugged Pasteur pipettes (9 in). The columns were conditioned with 1.5 ml of methanol followed by 0.75 ml of 0.1 M NaH_2PO_4 (pH 3.0). Sample fractions and standard fractions, prepared with known amounts of 5-HT and 5-HIAA, were brought to pH 2.8–3.0 with 1.0 M HCl. After a sample passed through its column, the aqueous phase in the column was displaced by 125 μl of 70% methanol/30% acetic acid. The 5-hydroxyindoles were eluted into Eppendorf tubes with 300 μl of this organic solution, and the solvent in each tube was evaporated under a stream of nitrogen. The dried eluates were reconstituted with 50 μl of 0.15 M HCl containing 0.25 mM ascorbate. Recoveries of 5-HT and 5-HIAA were generally 80–90%, based on calculations using the internal standards.

The amounts of 5-HT and 5-HIAA in the reconstituted samples were assayed by HPLC with electrochemical detection. The samples (45 μl of 50 μl) were automatically injected (Waters Intelligent Systems Program; Milford, MA, U.S.A.) over a reverse-phase C_{18} column (5 μm , 25 cm; Beckman Instruments, San Ramon, CA, U.S.A.). The mobile phase was of the following composition (in mM): NaH_2PO_4 , 200; $\text{Na}_2\text{-EDTA}$, 0.1; octyl sodium sulfate, 0.17; with 13% methanol (vol/vol) and having a final pH of 4.3. The substances were detected electrochemically (Model LC-4A; Bioanalytical Systems Inc., West Lafayette, IN, U.S.A.) at 2 nA/V when

the potential of the glassy carbon electrode was set at 0.55 V against the Ag/AgCl reference electrode. Tissue levels of tryptophan, 5-HT, and 5-HIAA were assayed using this chromatographic system, although the applied potential was set at 0.85 V, in order to detect tryptophan in the samples, and the sensitivity at 5 nA/V. Frozen tissue samples were sonicated in 0.2 M HClO_4 (approximately 0.4 ml/mg of protein), containing 0.5 mM ascorbate and internal standards, and centrifuged (35,000 g, 10 min). Aliquots (50 μl in duplicate) of these tissue supernatants were injected over the reverse-phase column. The tissue pellets were assayed for their protein content by the method of Lowry et al. (1951) using bovine serum albumin as the standard protein.

Data analysis

Amounts of the indoles in each sample of superfusion medium and tissue supernatant were estimated by correcting the recorded peak height for its recovery, using the designated internal standard (5-HT- CH_3 for 5-HT and tryptophan; 5-HICA for 5-HIAA), followed by linear regression analysis based on standard curves run in parallel with each set of samples. These amounts were normalized to the amount of protein in the tissue pellet, allowing data to be expressed as picomoles of 5-HT or 5-HIAA per gram of protein per minute for the rates of release, or nanomoles of tryptophan, 5-HT, or 5-HIAA per gram of protein for levels within the slices. Values are reported here as means \pm SEM.

Calculations of the average rates of 5-HT release during the four rest periods (spontaneous 5-HT release) and the three periods of electrical stimulation (evoked 5-HT release) were made for each experiment in which the effect of tryptophan availability on 5-hydroxyindole release was to be examined. The rate of 5-HIAA efflux was taken as an average across the 80-min collection period. Effects of increasing the tryptophan concentration of the superfusing medium on slice indole levels and release were evaluated using the two-tailed Student's paired *t* test. Statistically significant differences were noted for these and all other analyses when the probability value was less than 0.05. Dose-dependent relationships between tryptophan availability and slice 5-HT levels and release were analyzed by one-way analyses of variance, followed by the Duncan's multiple range post hoc test.

The calcium dependence and TTX sensitivity of spontaneous 5-HT release were assessed by comparing the average rates of 5-HT release from slices superfused with calcium-free or TTX-containing medium versus release occurring in the presence of the physiologic medium. The calcium dependence and TTX sensitivity of evoked 5-HT release were evaluated by comparing the rates of 5-HT release due to delivery of S1, when the slices were being superfused with altered media, versus release in the presence of the usual physiologic medium. Restoration of evoked 5-HT release was determined by comparing the amounts of 5-HT released from slices in the two chambers due to S2. These data were evaluated by the *F* test for equality of two variances, and then by the two-tailed Student's *t* test with equal or unequal variances where appropriate.

RESULTS

The levels of tryptophan, 5-HT, and 5-HIAA in the hypothalamic slices prior to superfusion were 167.8 ± 4.8 , 73.1 ± 2.3 , and 46.0 ± 1.6 nmol/g of protein, respectively. Following 130 min of superfusion with tryptophan-free medium (including three periods of

electrical stimulation), these levels were 181.7 ± 6.0 , 61.1 ± 1.9 , and 4.6 ± 0.2 nmol/g of protein. The actual tryptophan concentration in the "tryptophan-free" medium 15 min after the start of superfusion was approximately $0.1 \mu\text{M}$, and this concentration declined steadily over the next 115 min to approximately $0.05 \mu\text{M}$ (data not shown). Endogenous 5-HT was released spontaneously from the slices at a rate of 41 ± 2 pmol/g of protein/min (Fig. 1A). This rate was increased to 213 ± 7 pmol/g of protein/min for 10 min with each of the three periods of electrical field stimulation, after which 5-HT release returned to its basal rate. The rate of 5-HIAA efflux was an average of 460 ± 23 pmol/g of protein/min across the 80-min collection period, which included transient poststimulation elevations (Fig. 1B). The molar ratio of 5-HT to 5-HIAA in the superfusion medium when the slices were at rest was approximately 0.09; with electrical stimulation, this ratio rose to approximately 0.40.

The electrically evoked release of 5-HT from the hypothalamic slices was dependent on the presence of calcium in the superfusion medium (Fig. 2). Slices superfused with calcium-free medium containing EGTA (1 mM) released only $18 \pm 2\%$ as much 5-HT with electrical field stimulation as control slices ($p < 0.01$). In contrast, basal 5-HT release was unaffected by the

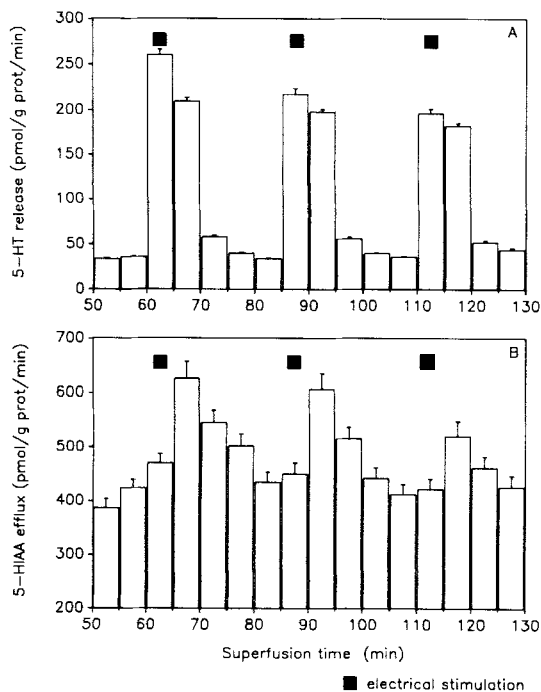


FIG. 1. Time course of 5-HT release (A) and 5-HIAA efflux (B) from rat hypothalamic slices superfused in tryptophan-free physiologic medium. Fractions of superfusion medium were collected every 5 min after an initial equilibration period, and the amounts of 5-HT and 5-HIAA released (pmol/g of protein/min) were monitored. The slices were electrically field stimulated for three periods. $n = 32$.

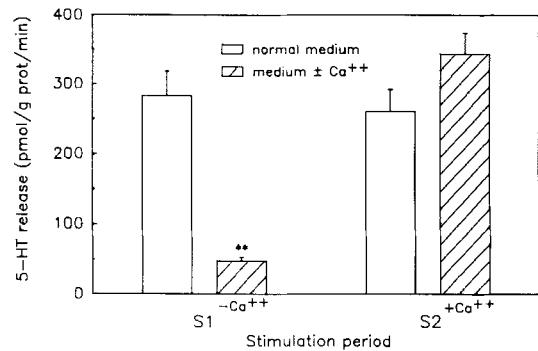


FIG. 2. Calcium dependence of electrically evoked 5-HT release. Rat hypothalamic slices were superfused initially with either physiologic medium or calcium-free medium containing 1 mM EGTA, and were delivered the first period of electrical field stimulation (S1) under these conditions. Physiologic medium replaced the calcium-free medium prior to the second period of electrical stimulation (S2). The amounts of 5-HT released (pmol/g of protein/min) were monitored. Values are group means \pm SEM for $n = 9$. ** $p < 0.01$, differs from control by the Student's t test with unequal variances.

lowered calcium concentration (43 ± 3 and 35 ± 4 pmol/g of protein/min with physiologic and calcium-free media, respectively). When slices which had been exposed to the calcium-free medium were superfused subsequently with physiologic medium (calcium concentration = 1.3 mM), the electrically evoked release of 5-HT returned to control values.

Evoked 5-HT release was sensitive to the activity of voltage-dependent sodium channels (Fig. 3). Inhibiting their activation with TTX ($1 \mu\text{M}$) caused a $60 \pm 4\%$ reduction ($p < 0.05$) in 5-HT release with electrical stimulation, again without changing basal 5-HT release

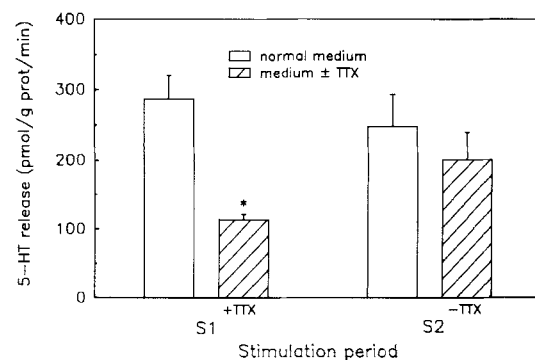


FIG. 3. TTX sensitivity of electrically evoked 5-HT release. Rat hypothalamic slices were superfused initially with physiologic medium; the medium superfusing one of the two parallel chambers was replaced with TTX-containing medium ($1 \mu\text{M}$) prior to delivery of the first period of electrical field stimulation (S1). Slices of both chambers were then superfused rapidly (1 ml/min) with physiologic medium before the second period of electrical stimulation (S2). Values are group means \pm SEM for $n = 3$. * $p < 0.05$, differs from control by the Student's t test with unequal variances.

(39 ± 2 and 38 ± 4 pmol/g of protein/min with physiologic and TTX-containing media, respectively). The rate of evoked 5-HT release was partially recovered, to $80 \pm 2\%$ of control, after the slices were rapidly superfused for 1 h with physiologic medium.

Tryptophan levels, measured in the slices at the end of each experiment, increased in a dose-dependent manner with the addition of tryptophan (1–10 μM) to the superfusion medium (Fig. 4A). Slice tryptophan levels increased by $40 \pm 4\%$ ($p < 0.01$), $105 \pm 8\%$ ($p < 0.01$), $223 \pm 25\%$ ($p < 0.01$), and $490 \pm 30\%$ ($p < 0.01$) when 1, 2, 5, or 10 μM tryptophan, respectively, was added to the superfusion medium. Final tissue levels of 5-HT rose biphasically when the medium tryp-

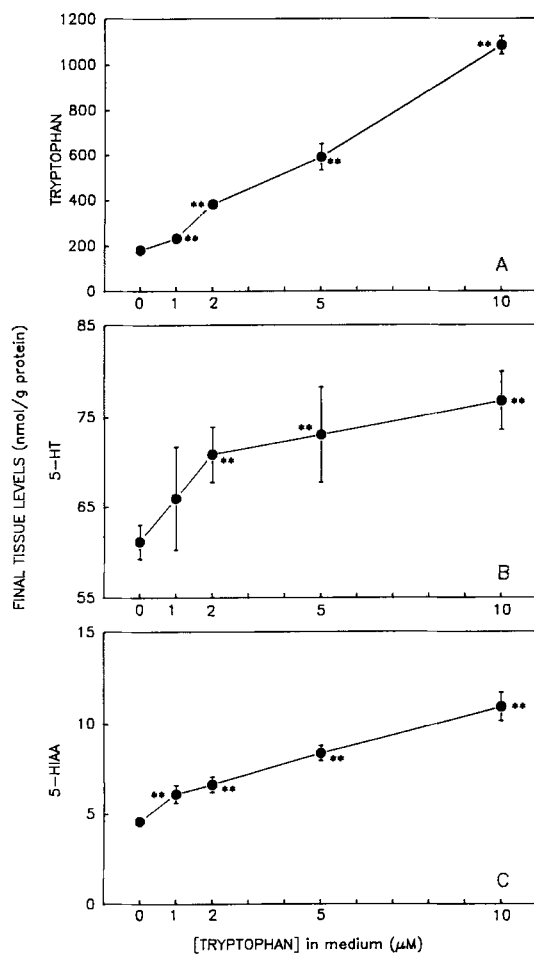


FIG. 4. Dose-dependent relationships between the tryptophan concentration of the superfusion medium and final slice levels of tryptophan (A), 5-HT (B), and 5-HIAA (C). Paired hypothalamic slices were superfused in physiologic medium which was either tryptophan-free or tryptophan-supplemented (1, 2, 5, or 10 μM). Slices were retrieved at the end of each experiment and assayed for their contents of these indoles. Data are expressed in nmol/g of protein and are given as group means \pm SEM for $n = 8$ –12 pairs. ** $p < 0.01$, differs from slices superfused in tryptophan-free medium by the Student's paired t test.

tophan concentration was increased (Fig. 4B): Low tryptophan concentrations caused proportionately greater incremental changes in tissue 5-HT levels [1 μM : $8 \pm 3\%$ over control; 2 μM : $13 \pm 3\%$ ($p < 0.01$) over control] than higher tryptophan concentrations [5 μM : $19 \pm 2\%$ ($p < 0.01$) over control; 10 μM : $34 \pm 4\%$ ($p < 0.01$) over control]. The amount of 5-HIAA remaining in the slices following superfusion also exhibited a dose-dependent relationship to the tryptophan concentration of the superfusion medium (Fig. 4C).

Spontaneous and electrically evoked release of 5-HT from the slices increased in a dose-dependent manner with the medium tryptophan concentration (Table 1): Addition of 1, 2, 5, or 10 μM tryptophan to the medium elevated spontaneous 5-HT release by $8 \pm 4\%$, $36 \pm 8\%$ ($p < 0.01$), $47 \pm 9\%$ ($p < 0.01$), and $67 \pm 13\%$ ($p < 0.01$), respectively. Electrically evoked release of 5-HT was elevated by $5 \pm 3\%$, $19 \pm 4\%$ ($p < 0.01$), $34 \pm 6\%$ ($p < 0.01$), and $59 \pm 10\%$ ($p < 0.01$) in the presence of these tryptophan concentrations. The efflux of 5-HIAA increased by $21 \pm 5\%$ ($p < 0.01$), $47 \pm 9\%$ ($p < 0.01$), $106 \pm 17\%$ ($p < 0.05$), and $138 \pm 19\%$ ($p < 0.01$), respectively. Slices superfused with physiologic medium containing a 10 μM concentration of a different amino acid, *l*-histidine, released 5-HT under basal and depolarizing conditions at rates not different ($101 \pm 5\%$ and $99 \pm 3\%$ of control, respectively) from those superfused with physiologic medium.

Superfusing rat hypothalamic slices with tryptophan-containing medium (1–10 μM) thus caused dose-dependent elevations in tissue 5-HT [$F(3, 38) = 10.37$, $p < 0.01$], spontaneous 5-HT release [$F(3, 38) = 8.35$, $p < 0.01$] (Fig. 5A), and electrically evoked 5-HT release [$F(3, 38) = 14.48$, $p < 0.01$] (Fig. 5B). Although each increment in the tryptophan concentration of the superfusion medium did not always produce a parallel significant increment in 5-HT levels or in 5-HT release (spontaneous and evoked), a clear dose dependence between tryptophan and 5-HT (levels and release) was observed, as assessed by the Duncan's multiple range test.

DISCUSSION

These data describe a reliable *in vitro* method for estimating the rates at which endogenous 5-HT is released spontaneously and with electrical depolarization from brain slices. This experimental system, which combines the techniques of brain slice superfusion, a novel extraction method, and an HPLC assay, grants the sensitivity necessary to detect small and/or transient changes in 5-HT release. The rates of 5-HT release measured in this study are similar to those presented by other authors working *in vivo* (Ternaux et al., 1977; Guan and McBride, 1987) and *in vitro* (Elks et al., 1979a; Auerbach and Lipton, 1985). We found that 5-HT release evoked by electrical field stimulation was more calcium-dependent than TTX-sensitive (Figs. 2

TABLE 1. Effect of tryptophan availability on release of 5-hydroxyindoles from rat hypothalamic slices

Tryptophan (μM)	5-HT release		5-HIAA efflux
	Basal	Evoked	
0	38 \pm 2	219 \pm 15	460 \pm 39
1	41 \pm 3 (108 \pm 4)	232 \pm 18 (105 \pm 3)	554 \pm 46 ^a (121 \pm 5)
0	44 \pm 4	221 \pm 13	510 \pm 33
2	60 \pm 6 ^a (136 \pm 8)	260 \pm 16 ^a (119 \pm 4)	740 \pm 47 ^a (147 \pm 9)
0	40 \pm 3	207 \pm 22	448 \pm 79
5	58 \pm 5 ^a (147 \pm 9)	274 \pm 31 ^a (134 \pm 6)	917 \pm 168 ^b (206 \pm 17)
0	44 \pm 4	202 \pm 12	413 \pm 50
10	72 \pm 6 ^a (167 \pm 13)	319 \pm 24 ^a (159 \pm 10)	985 \pm 138 ^a (238 \pm 19)

Rat hypothalamic slices were superfused in physiologic medium which either contained no exogenous tryptophan or was supplemented with tryptophan (1, 2, 5, or 10 μM). The amounts of 5-HT released, basally and with electrical field stimulation (5 Hz, 2 ms, 100 mA/cm²), and 5-HIAA effluxed from the slices are expressed in pmol/g of protein/min. Values in parentheses are the relative amounts, in percent, of the 5-hydroxyindole released from slices superfused with tryptophan-supplemented medium as compared to slices superfused with tryptophan-free medium. Data are given as group means \pm SEM for $n = 8-12$ pairs.

^a $p < 0.01$; ^b $p < 0.05$, differs from control group by Student's paired t test.

and 3). This suggests that the influx of calcium ions caused directly by stimulation-induced activation of voltage-sensitive calcium channels may be greater than that caused indirectly by sodium influx-induced membrane depolarization. Basal 5-HT release was not altered by the omission of calcium from the superfusion medium nor by the addition of TTX to the medium. This differential sensitivity of basal and evoked 5-HT release to calcium or to TTX has been observed previously by others (Elks et al., 1979a; Göthert, 1980; Schlicker et al., 1985), and suggests that spontaneous 5-HT release from brain nerve terminals is regulated by factors other than the influx of calcium ions through voltage-sensitive calcium channels.

Slice tryptophan levels were maintained over the superfusion period in the absence of exogenous tryptophan, probably reflecting the fact that only a relatively small proportion (approximately 2%) of brain tryptophan is converted to 5-HT in situ (Pardridge, 1977), and the turnover of tryptophan-containing protein was at steady state. Final 5-HIAA levels were markedly lower than those measured in presuperfusion tissue (46.0 ± 1.6 as compared to 4.6 ± 0.2 nmol/g of protein). The utilization of the 5-HT reuptake inhibitor, fluoxetine, in the superfusion medium presumably prevented synaptic 5-HT from being recaptured into serotonergic nerve terminals, and thereby diminished

the contribution to tissue 5-HIAA levels of metabolized 5-HT which had been released previously (Reinhard and Wurtman, 1977). Additionally, those 5-HT molecules which were degraded intraneuronally (i.e., a fraction of newly synthesized 5-HT molecules) were probably transported, via an acid transporter, across the neuronal membrane into the extracellular space and removed by the superfusing medium. The activity of this transport process most likely accounts for the relatively low levels of 5-HIAA that remained in the slices and the high concentrations of 5-HIAA detected in the superfusion medium.

Although the inclusion of a 5-HT reuptake inhibitor in the superfusion medium was necessary to detect released 5-HT reliably, the resulting elevation in synaptic 5-HT may have activated serotonergic autoreceptors on hypothalamic nerve terminals (Cerrito and Raiteri, 1979), and thus suppressed 5-HT release. Indeed, the addition of a nerve terminal autoreceptor antagonist, methiothepin (Göthert, 1980), to the medium (in the presence of fluoxetine) potentiated spontaneous and electrically evoked 5-HT release (data not shown). Activation of these autoreceptors may have caused an underestimation of the magnitude of the effect of tryptophan supplementation on 5-HT release.

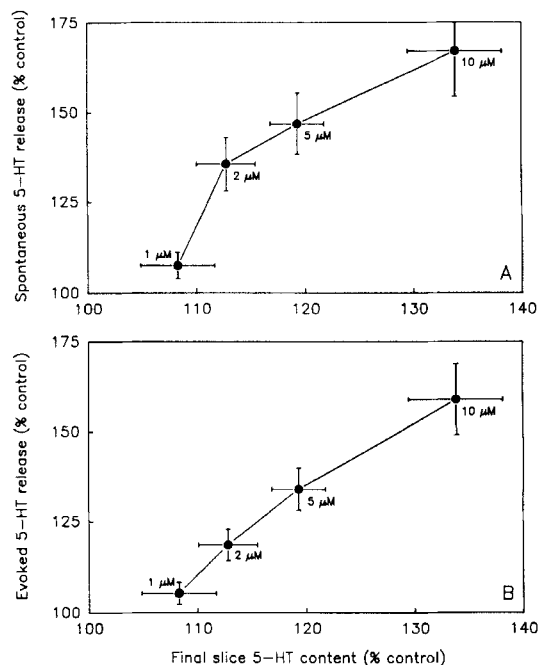


FIG. 5. Dose-dependent relationships between changes in final tissue 5-HT levels and in spontaneous (A) and electrically evoked (B) 5-HT release when the slices were superfused with tryptophan-supplemented medium (1, 2, 5, or 10 μM) as compared to tryptophan-free medium (control). These are replottings of data from Fig. 4B and Table 1. Data are group means \pm SEM for $n = 8-12$ pairs. Analyses of variance detected significantly different effects within levels of tryptophan supplementation for changes in 5-HT levels ($p < 0.01$), spontaneous 5-HT release ($p < 0.01$), and evoked 5-HT release ($p < 0.01$).

Electrically stimulating the slices transiently enhanced 5-HIAA efflux, but this effect was delayed compared with the immediate increase in 5-HT release (Fig. 1). The depolarization of serotonergic neurons has been shown previously to accelerate 5-HT synthesis *in vivo* (Shields and Eccleston, 1972; Herr et al., 1975) and *in vitro* (Elks et al., 1979b; Hamon et al., 1979). Conceivably, this newly synthesized 5-HT was released into the synapse during periods of electrical field stimulation, but was metabolized to 5-HIAA after stimulation ceased.

The "tryptophan-free" medium actually contained low concentrations of tryptophan which declined over the superfusion period, from approximately 0.1 to 0.05 μM . The presence of endogenous tryptophan in the medium possibly reflects the physiologic flux of amino acids across plasma membranes (Parfitt and Grahame-Smith, 1973) and an artifactual elevation in free amino acid levels due to protein breakdown associated with preparation of the slices. The time-dependent decrease in medium tryptophan levels probably resulted from the continual dilution of this "tryptophan-free" medium with fresh (truly tryptophan-free) medium. Adding exogenous tryptophan to the medium at concentrations within the range others have measured in cerebrospinal fluid of untreated rats (1–3 μM) (Sarna et al., 1983; Hutson et al., 1985; Anderson et al., 1987) caused elevations in tissue tryptophan levels quantitatively similar to those which occur physiologically in rat brain, e.g., with exercise (Chaouloff et al., 1985, 1986), certain stressors (Knott et al., 1973; Kennett and Joseph, 1981; Čulman et al., 1984; Dunn, 1988), ingestion of a carbohydrate-rich meal (Fernstrom and Wurtman, 1971b; Colmenares et al., 1975), or diurnal variation (Morgan et al., 1975; Hery et al., 1977).

The observed relationship between increased tryptophan availability and tissue 5-HT levels (Fig. 4B) is indicative of the particular kinetics of tryptophan hydroxylase, and probably reflects the sensitivity of 5-HT biosynthesis to changes in the degree of saturation of tryptophan hydroxylase with its amino acid substrate. When tissue tryptophan levels were elevated by superfusing the slices with media containing 1 or 2 μM tryptophan, 5-HT levels increased proportionately; however, superfusing the slices with 5 or 10 μM tryptophan caused smaller incremental rises in 5-HT levels. Notably, this inflection in the curve relating medium tryptophan concentration to 5-HT levels occurred when tissue tryptophan levels were between approximately 40 and 60 μM , concentrations which bound the K_m value of tryptophan hydroxylase for the amino acid [50 μM (Friedman et al., 1972)]. [These calculations assume that tryptophan is distributed uniformly throughout the tissue (Wurtman and Fernstrom, 1976).] Such levels were attained when the slices were superfused with 2 and 5 μM tryptophan, respectively.

Elevating slice tryptophan levels within their physiologic dynamic range caused proportionate increases

in 5-HT levels and in the spontaneous and electrically evoked release of the neurotransmitter. This observation supports the view that a physiologic coupling does exist between tryptophan levels, 5-HT synthesis, and 5-HT release in the rat hypothalamus. This precursor dependence of 5-HT release may allow the brain to sense metabolic processes which alter brain tryptophan levels. This property of serotonergic neurons may be utilized, for example, to determine the proportion of carbohydrate and protein in a meal (by the effects of the meal on brain tryptophan levels) and to decide about subsequent food choices.

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